Advances in Environmental Microbiology 8

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Editor

Microbes: The Foundation Stone of the Biosphere

Springer

Advances in Environmental Microbiology

Volume 8

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This book series addresses the questions of which microbes, microbial genes and gene products are present at particular places and times, as well as the environmental transport and survival capabilities of microbes. The authors define the ways in which microorganisms interact chemically as well as physically with their surroundings, including microbial actions that change our planet's geochemistry. *Advances in Environmental Microbiology* facilitates an understanding of how microbes have contributed towards coevolutionary processes and addresses microbial contributions to the successional colonization of environmental locations. The explorations of topics include a microbiological perspective of public health, animal husbandry and agricultural issues, including consideration of the fact that infectious diseases are often either acquired from environmental reservoirs or transmitted through the environment, plus an explanation of how microbial establishment either on or within a host results in transformation of the colonization site into a microbially modified environment. This series also will include both microbial pest control and microbial diversity, along with insights into industrial production processes that are connected to environmental microbiology.

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ISSN 2366-3324 ISSN 2366-3332 (electronic)
Advances in Environmental Microbiology
ISBN 978-3-030-63511-4 ISBN 978-3-030-63512-1 (eBook)
https://doi.org/10.1007/978-3-030-63512-1

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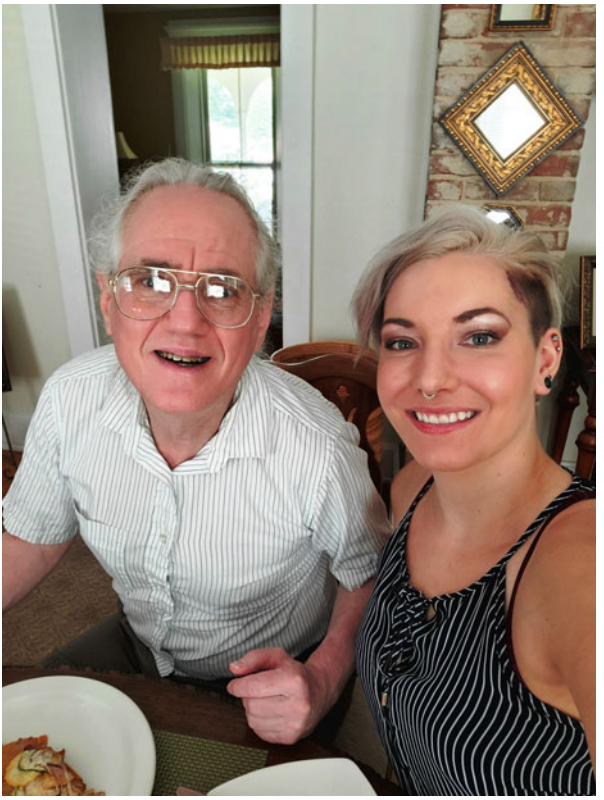
Families are assembled in two ways and one of those is by birth. The other way is by adding to our family those people who come into our lives and naturally seem to belong with us. Both my mother and father helped me to understand the idea of adopting others into our lives. My mother unofficially adopted Elizabeth Schnell as a sister. My mother and Elizabeth met when Elizabeth brought her children to the physician's office in Dayton, Ohio, where my mother worked as a nurse. My mother eventually added to her sense of family by unofficially adopting our neighbor, Terry Miller, as a father. Patricia Goodman, who worked with my mother, in essence became a daughter, and David Morgan who was a member of my Boy Scout troop came to be considered a son. My father's mother, Pearle Payne Hurst, came to consider her tenant Mehdi Shirazi as a son, and his eventual sons were like grandchildren for Pearle. When my father worked in South Vietnam he adopted Kohana, whose last name I do not remember, as if she were a daughter. I

similarly adopted William Holodnak as being my son, and that happened in September of 2012.

During the Fall Semester of 2014, Karrisa Martino entered my life when she enrolled as a student in the Ballroom Dance classes that I was teaching for Xavier University, in Cincinnati, Ohio. At some point during her second semester in my classes, Karrisa and I began sharing some heartfelt conversations. I then asked Karrisa if she would allow me to be her father, and she said "Yes." Karrisa completed all of the beginning and advanced dance classes that I taught. She then spent several more semesters as a volunteer helping me to teach the newer students. Karrisa has a biological mother and father, plus the entire range of biologically related family members. She also has me as her second father, and having her as a daughter brings a great sense of happiness to my life. I have tremendously enjoyed dancing with my daughter Karrisa. Our favorite thing to do as father and daughter is something called greeting the Sabbath on Friday evenings. We usually do that at a local restaurant where she begins our celebration by lighting candles, which starts the Sabbath. After the candles are lit, I bless her as my daughter, we have wine and bread, and then we share a few hours of conversation plus dinner. I normally eat my meals very quickly, but when greeting the Sabbath with Karrisa my plate of food slowly grows cold because I become absorbed into the conversation.

With a fatherly sense of appreciation and pride I dedicate my efforts on this book to Karrisa Maria Martino.



Christon J. Hurst and Karrisa M. Martino greeting the Sabbath on July 3, 2020

Series Preface

The light of natural philosophy illuminates many subject areas including an understanding that microorganisms represent the foundation stone of our biosphere by having been the origin of life on Earth. Microbes therefore comprise the basis of our biological legacy. Comprehending the role of microbes in this world which together all species must share, studying not only the survival of microorganisms but as well their involvement in environmental processes, and defining their role in the ecology of other species does represent for many of us the Mount Everest of science. Research in this area of biology dates to the original discovery of microorganisms by Antonie van Leeuwenhoek, when in 1675 and 1676 he used a microscope of his own creation to view what he termed "animalcula," or the "little animals" which lived and replicated in environmental samples of rainwater, well water, seawater, and water from snow melt. van Leeuwenhoek maintained those environmental samples in his house and observed that the types and relative concentrations of organisms present in his samples changed and fluctuated with respect to time. During the intervening centuries, we have expanded our collective knowledge of these subjects which we now term to be environmental microbiology, but easily still recognize that many of the individual topics we have come to better understand and characterize initially were described by van Leeuwenhoek. van Leeuwenhoek was a draper by profession, and fortunately for us his academic interests as a hobbyist went far beyond his professional challenges.

It is the goal of this series to present a broadly encompassing perspective regarding the principles of environmental microbiology and general microbial ecology. I am not sure whether Antonie van Leeuwenhoek could have foreseen where his discoveries have led, to the diversity of environmental microbiology subjects that we now study and the wealth of knowledge that we have accumulated. However, just as I always have enjoyed reading his account of environmental microorganisms, I feel that he would enjoy our efforts through this series to summarize what we have learned. I wonder, too, what the microbiologists of still future centuries would think of our efforts in comparison with those now unimaginable discoveries which they

will have achieved. While we study the many wonders of microbiology, we also further our recognition that the microbes are our biological critics, and in the end they undoubtedly will have the final word regarding life on this planet.

Indebted with gratitude, I wish to thank the numerous scientists whose collaborative efforts will be creating this series and those giants in microbiology upon whose shoulders we have stood, for we could not accomplish this goal without the advantage that those giants have afforded us. The confidence and very positive encouragement of the editorial staff at Springer DE has been appreciated tremendously, and it is through their help that my colleagues and I are able to present this book series to you, our audience.

Cincinnati, OH Christon J. Hurst

Volume Preface

There is a large piece of rock in Jerusalem which is called the Foundation Stone. Technically, it is karsted limestone from the Upper Turonian Stage, Late Cretaceous. It has been cut free from its surrounding rock and shaped to a roughly flat surface.

The Foundation Stone sits atop Mount Moriah. The ground around the Foundation Stone was raised with retaining walls, filled, and the top paved with stones to create the Temple Mount. It is called the Temple Mount because the Jewish Temples sat there. Those temples may in fact have had their altars sitting upon the Foundation Stone. I am Jewish and our religious buildings around the world all are arranged so that the congregations face that piece of rock. The Western Wall in Jerusalem, also called the Wailing Wall and Wall of Lamentations, is a part of the retaining walls that were used to create the Temple Mount. Muslims believe that Mohamed rose to heaven from that rock and the gold domed structure called "the Dome of the Rock" is an Islamic shrine which protects the Foundation Stone. The crusaders removed fragments of the Foundation Stone as souvenirs. As you may have guessed, the Foundation Stone possibly is the most contested piece of territory on earth, because the religions whose adherents have struggled to control Jerusalem often were fighting for possession of that rock.

The Foundation Stone atop Mount Moriah in Jerusalem. This photograph is titled "Temple area, Mosque of Omar (i.e., Dome of the Rock), etc. Rock Moriah, from the south LOC matpc.23162.jpg." It is a public domain image from the G. Eric and Edith Matson Photograph Collection, United States Library of Congress

I arrived at the theme for this book from knowing some of the legends about the Foundation Stone. The biblical legend of creation is that God first created the Foundation Stone and then God touched that rock which caused heaven to spring from one side and earth to spring from the other side. I do not believe that legend. Microbes are, however, the foundation stone of the biosphere. Life on this planet began with the microbes, and the microbes are essential for sustaining all of life.

My colleagues and I have created this volume of essays which considers different aspects of the concept that microbes are the foundation stone of the biosphere. Each of us had the same assignment for this volume, to share the personal understanding of microbiology which we have gained individually and collectively. Three of us, Ann Hirsch, Ron Oremland, and Aharon Oren, have entered the Dome of the Rock building and seen the Foundation Stone.

The contents of this volume are organized into seven sections.

The first section is titled "Recognizing the Role of Microorganisms in our World." It presents the fact that life is a constant and continuing struggle for habitat and niche. The first section also mentions our philosophical inheritance from Charles Darwin, continuing through considerations of more recent times, and it also presents an introduction to the ways in which microorganisms prepare the sediments and soils upon which many other life forms depend for a necessary physical and nutritive base of support.

The second section helps us to consider that "Microbial life persists within even the most extreme environments" and provides examples of how microbial life manages to exist in some amazing ways and places. Microbes are metabolically

active drifting in atmospheric clouds, and every time that one of us inhales a breath of fog we also are inhaling a countlessly high number of respiring microbes. Microbial persistence in the freezer-burned surface soils of Antarctica sometimes means obtaining liquid water by surviving hypersaline conditions within the soils' interstitial pores. Lithotrophic microbes may play the less charismatic roles in microbiology, but their reliance on alternative electron acceptors allows the lithotrophs to provide a foundation for the world's deep subsurface ecosystems.

The third section presents our "Understanding the core values of microbial metabolism." Life, as we know it on this planet, has a very critical need for nitrogen's presence in proteins. The occurrence of nitrogen in its different oxidation states also has an important role in numerous cycles which provide energy for microorganisms. Oxidation of methane provides energy in anaerobic environments when coupled with various terminal electron acceptors such as metals, nitrogen compounds, and sulfate. Within marine sediments, microbes can couple the oxidation of sulfide in anaerobic layers with a near simultaneous reduction of the oxygen present in overlying aerobic layers. Anoxygenic photosynthesis by bacteriochlorophyll can oxidize hydrogen sulfide to release sulfur, and evolutionarily that process may well have preceded the oxygenic photosynthesis which created our oxygen-rich atmosphere by oxidizing water molecules.

The fourth section examines some aspects of how "Microbes established and sustain life." There is a duality in the microbial world; its various processes include some that we perceive as being environmentally good and contributing toward long-term ecological stability, although other processes seem environmentally destructive. Very often, we notice only the large-scale results and seem to ignore that all large-scale microbial processes are the sum of microscale activity.

Human activities unintentionally enhance some of the environmentally destructive microbial processes that contribute to climate change, including the overproduction of greenhouse gasses such as carbon dioxide, methane, and nitrous oxide, which increase the warming of our atmosphere. The warming of our atmosphere does in turn destructively increase the warming of our oceans. We need to work on improving the environmentally beneficial side of that microbial duality. The future of our biosphere depends in part upon aquatic carbon cycles including those which occur in the ocean. Looking toward the goal of achieving environmentally supportive microbial activity on the land, cultivating beneficial microbial communities by means of regenerative farming systems may help to mitigate damage that is caused by agriculture's environmental footprint. It is possible that modification of agricultural processes can reduce greenhouse gas emissions and ultimately increase carbon sequestration. Changing human dietary practices can cultivate beneficial gut microbiomes. The functions of a forest, and the functions of agricultural fields, depend upon the microbial functions within their soil. The functions of aquatic ecosystems similarly depend upon the activities of their microbial communities.

The fifth section reveals our knowledge regarding "The basic aspects of microbial symbioses." Our efforts to understand the symbiotic nature of microbial life can be traced back to Mikhail Stepanovich Voronin's conclusion, published in 1866, that the nodular root growths on black alder (*Alnus glutinosa*), and the bulbous root

outgrowths of lupine (*Lupinus*), are in some ways identical phenomena and in both cases the appearance is caused by foreign organisms. Eventually, we learned that those foreign organisms are microbes which serve by providing fixed nitrogen to the plant in exchange for products of photosynthesis received from the plant. The fifth section of this book describes numerous examples, and their underlying mechanisms, of known microbial symbioses that have become established with other microorganisms, with plants, and with animals.

The sixth section explains the concept of "Microbial symbiosis as a driving force in evolution." Perhaps evolution is a game won by competitive cheating, with the microbes, including viruses, being both master and servant.

The seventh section of this book helps us to remember "The adventure of microbiology research," including some reminiscences and perspective on the life and career of being a microbe hunter.

Together, my colleagues and I have in total spent perhaps a thousand years studying and researching microbiology. Our journeys have included slogging through a lot of outdoor environments under conditions that sometimes were awe-inspiring and at other times nearly unbearable. All of that effort has allowed us to reach a time point where we could write the essays for this book. That thousand years is barely a fraction of the time period for which the Foundation Stone has sat upon Mount Moriah.

My colleagues and I hope that you, our readers, will enjoy the results of our collective effort as presented in this book. We also hope that you will add your own individual and collective perspectives to this fascinating story of microbiology.

Cincinnati, OH Christon J. Hurst

Contents

Part VII The Adventure of Microbiology Research

Part I
Recognizing the Role of Microorganisms in Our World

Chapter 1
Our Living World Rests upon a Foundation of Microorganisms: The Constant Struggle for Habitat and Niche

Christon J. Hurst

Abstract Microbes are the foundation upon which our living world rests. All of life evolved to use the activities and products of microorganisms as not only our foundation but also as the basis for our building blocks. The interactions between species often may seem cooperatively peaceful, but in fact they represent a competition for habitat and niche.

1.1 Introduction

The organic chemicals that may have begun with a spark of energy gave rise to an organized and interconnected biosphere.

1.1.1 Perhaps it Started with a Spark

Our living world presumably would have begun as a collection of water, minerals, simple chemical compounds, and sources of energy. During the 1950s, Harold Clayton Urey and his student Stanley Lloyd Miller tried to understand how the complex chemical compounds used to assemble life on our planet might have formed. The sources of energy which they considered to initially have been available for creating those organic compounds were: cosmic rays, which they believed would have had a negligible role; electric discharges; ultraviolet light; and thermal energy. Their initial experimental discovery was that amino acids could be generated when vapor from a boiling flask of water was recirculated through a sealed glass reactor. The sealed system in their experiments contained CH_4, NH_3 and H_2 in addition to the liquid water, and included tungsten electrodes that exposed the recirculating mixture to an electric discharge (Miller 1953). A more detailed evaluation of their

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_1

Table 1.1 Yields from sparking a mixture of CH_4, NH_3, H_20, and H_2; 710 mg of carbon was added as CH_4 (was Table 2 of Miller and Urey 1959)

Compound	Yield [moles (x 10^5)]
Glycine	63.
Glycolic acid	56.
Sarcosine	5.
Alanine	34.
Lactic acid	31.
N-Methylalanine	1.
a-amino-n-butyric acid	5.
a-Aminoisobutyric acid	0.1
a-Hydroxybutyric acid	5.
β-Alanine	15.
Succinic acid	4.
Aspartic acid	0.4
Glutamic acid	0.6
Iminodiacetic acid	5.5
Iminoacetic-propionic acid	1.5
Formic acid	233.
Acetic acid	15.
Propionic acid	13.
Urea	2.0
N-methyl urea	1.5

This was Table 2 in the publication by Miller and Urey 1959, presenting the analytical results of an experiment in which they tried to replicate production of complex organic compounds from simpler chemical precursors under primitive earth conditions. The Table 1.1 of their publication was a list of possible energy sources on primitive earth

experimental approach resulted in a 1959 publication (Miller and Urey 1959), the results of which are listed in Table 1.1.

1.1.2 Microbes Were the First to Form and they Are Everywhere

The question of how organic compounds led to formation of the first cell undoubtedly will be answered, but it seems as though that answer is not arriving soon. Presumably, it was the prokaryotes which appeared first. Prokaryotes lack a membraned nucleus, and those beings which today remain prokaryotes are the archaea and bacteria. At some point, a eukaryote, which is a term that refers to those beings which possess a nucleus, ingested something but failed to digest its meal. That event of indigestion eventually resulted in the meal and its predator coevolving, with the ingested meal become a mitochondria. A eukaryote also would have ingested, but failed to digest, something which contained chlorophyll and evolutionarily that event

of indigestion produced the chloroplasts that now are in the plants which surround us. The names prokaryote and eukaryote reportedly have come to us from a book by Édouard Chatton. Only a few copies of Chatton's book are known to still exist. Although the current coronavirus pandemic has prevented me from personally verifying its text, these words should be on approximately page 50 of Chatton's book (Chatton 1938).

> Les protistologues s'accordent, aujourd'hui, à considérer les Flagellés autotrophes, comme les plus primitifs des Protozoaires à noyau vrai, des Eucaryotes (ensemble qui embrasse aussi les Végétaux et les Métazoaires), parce qu'ils sont les seuls à pouvoir faire la synthèse totale de leur protoplasme à partir du milieu minéral. Les organismes hétérotrophes sont donc subordonnés à leur existence, ainsi qu'à celle des Procaryotes chimiotrophes et autotrophes (Bactéries nitrifiantes et sulfureuses, Cyanophycées). (Chatton 1938)

English translation:

> [The protistologists agree today to consider the autotrophic Flagellates as the most primitive of the Protozoa with a true nucleus, of the Eukaryotes (group which also embraces Vegetals and Metazoans), because they are the only ones able to make the total synthesis of their protoplasm from the mineral medium. Heterotrophic organisms are therefore subordinate to their existence, as well as to that of the chemotrophic and autotrophic prokaryotes (Nitrifying and sulfurous bacteria, Cyanophyceae).] I will add to that text a notation that Cyanophyceae refers to members of the phylum Cyanobacteria.

The possible routes that may have led to formation of mitochondria and chloroplasts have been summarized by William Martin and his colleagues (Zimorski et al. 2014).

Microbes successfully have filled our shared world with life.

1.1.3 Discovering the Metabolic Processes of Life

Some of lifes metabolic process were relatively easy for scientists to discern, such as the combustion of carbohydates to carbon dioxide. But, the revealing of microbes and their metabolic process has not always been easy and some of those processes do yet remain hidden.

Discovering the individual nature of the smallest members of life on this planet began with the skill of Antonie Philips van Leeuwenhoek and his initial invention of a microscope (van Leewenhoeck 1677). Although life would have begun relatively small and seemingly simple, the process of evolution has seen the creation of some very complex and large organisms. Indeed, life evolutionarily created Antonie. The mostly small size and seemingly simple nature of microoganisms matches them with the characteristics of their habitats and niches. And, although the organisms which have remained as microbes may look very simple, we need to remember that the microbes have had their metabolic and physiologic abilities honed by evolution to optimally fit the requirements of life in such challenging places as hypersaline environments (Plominsky et al. 2018).

van Leewenhoeck's publication (van Leewenhoeck 1677) mostly was about the microoganisms that could be seen by examining water droplets. Our planets simplest life forms do create some impressive, although not always favorably appreciated, communal aquatic displays that are know as freshwater microbial scums and slimes. Those particular displays are produced by algae including the diatoms, bacteria including the cyanobacteria, and the participation list progresses to the upwardly mobile protozoa and zooplankton. Aquatic displays do go upscale, to exhibitions of floating macroscopic plants including the duckweeds (*Lemna*, *Spirodela*), water meal (*Wolffia*), and water fern (*Azolla*) as photographically described by Kannan and Lenca (2013) and all of which have vital associations with microorganisms.

Perhaps one of the bigest advances in understanding microbial processes was made by Sergei Nikolaievich Winogradsky during a time period when Winogradsky was working with Heinrich Anton de Bary at the University of Strasbourg. There, Winogradsky discovered lithotrophy when he found that *Beggiatoa* can form intracellular sulfur deposits by oxidizing hydrogen sulfide *(*Winogradsky 1887). *Beggiatoa* are able to use reduction as a means of generating hydrogen sulfide from gypsum, which is calcium sulfate dihydrate. They then can form sulfur by using internally stored nitrate to oxidize the hydrogen sulfide. The sulfur is further oxidized and releasing as sulfuric acid into the surrounding water. Biogenic sulfuric acid corrosion unfortunately causes damage to sewerage and wastewater treatment facilities. *Beggiatoa* typically are considered to be aquatic microbes, although they also can use molecular oxygen to oxidize hydrogen sulfide in the rhizosphere of swamp plants, releasing water and elemental sulfur. In the presence of oxygen, *Beggiatoa* can heterotrophically gain energy by oxidizing organic compounds to carbon dioxide.

One of the most complex outcomes from microbial metabolism has been the creation of soil. Soil represents the foundation, both physically and figuratively, for terrestrial life. We also have needed to understand, and we should never forget, the importance of microorganisms in sustaining those characteristics of soil upon which much of evolution has relied.

Helen Cecilia De Silver Abbott explained to a general audience the state of knowledge in plant biochemistry (Abbott HC de 1887). I would summarize her presentation as being that plants combined four basic elements, which were carbon, hydrogen, oxygen, and nitrogen, grouping these elements with each other along with sulphur, phosphorus, and ash-elements derived from the mineral world. She mentioned the concept of plant evolution, and that the soil supplied what was needed by the plants. But, notably missing from her presentation was mention of microbiology. Indeed, at that time soil was considered by many as being almost a magical resource. We knew that soil could be healthy, and that repeated agricultural use often depleted soil of its healthiness, but we did not understand that it was the microbial players contained in soil and their roles which made the soil healthy. Soil is in fact a magical resource, but the magic in soil comes from the interactivity of its microbes and their macrobial symbionts.

Forty years later, our understanding of soil microbiology had advanced to the point that Selman Abraham Waksman was able to published what seems to have

been the initial version of his marvelous textbook on soil microbiology (Waksman 1927). I would say that perhaps his textbook was ground breaking. In that book, Waksman presented his instructions on the occurrence and chemical activities of the microorganisms that contribute to soil. He included mention that soil is also a habitat for many microorganisms that cause diseases of plants and animals.

1.1.4 Life Has Levels of Organization

There are formal names given to biological organizations and groupings of organisms, other than just calling them scums and slimes. What are the levels of biological organization? Some living things contain one cell that performs all needed functions. Multicellular organisms are made of many parts that are needed for survival. These parts of multicellular organisms are divided into five levels of organization from the simplest to the most complex: cells, tissue, organs, organ systems, and organisms. Above the organism level, there are some defined ecological levels. We consider a group of organisms belonging to the same species as representing a population of that species. A community, or biocoenosis, is an interacting population that includes more than one species. When groups of organisms from all biological domains act in conjunction with the abiotic (nonliving) physical materials in their environment, we call that interactive grouping an ecosystem. The adjoining and interconnecting ecosystems of geographically contiguous areas, perhaps on a continental scale or oceanic scale, are collectively referred to as being a biome. The biosphere, or ecosphere, is a level that includes all life on earth plus the abiotic aspects of those environments where life resides.

1.1.5 Life Is Interactive and Often Interdependent

Life evolves to use what is available, and it is far easier to use what already is available rather than to continuously reinvent the wheel. But, once life has evolved to use materials that already are available, there can be a dependency upon the supply of those materials. Depending upon materials supplied by others can in turn lead to symbiosis. Unfortunately, losing availability of those materials requires either that usable substitute materials must be found or else the evolved dependent life may become extinct.

Symbiosis is the necessary living together of two or more beings as first defined by Albert Bernhard Frank (Frank 1877). Symbiosis commonly is considered to be an attribute of progress. And yet, symbiosis can involve taking as well as giving, enticements and denials, loving and lying, cheating and sometimes eating, targeting both friend and foe.

Francisco Carrapiço, who very graciously joined us as an author for this book poject, has summarized the history of symbiosis versus symbiogenesis (Carrapiço 2015).

Symbiogenesis is a word used to describe cooperations between species which increase their mutual survival. Endosymbiotic theory is the idea that eukaryotic cells formed from prokaryotic organisms and that organelles such as mitochondria and chloroplasts formed from ingestion of other organisms. John Archibald has offered his opinion that endosymbiosis may not have played a role in the origin of cytosolic compartments other than mitochondria and the plastids (Archibald 2015). Chloroplasts are one group of plastids.

At this point I would like to return to a mentioning of Helen Abbott's presentation (Abbott HC de 1887). Abbott said that sugar is not a reserve material for plants and indeed for some few of the plants, with an example being sorghum-cane (genus *Sorghum*), "... it [sugar] must be regarded as a waste product attending euthanasia which marks decay of the plant after the maturity of growth." Helen Abbott was a very well noted plant chemist and later became a physician. She did not consider the knowledge that carbohydates sometimes are produced as an inducement for mutulistic symbiont animals to consume the plant in a way that supports dispersal and fertilization of the seeds. Sugar production prior to maturation of the seed would be counter productive, because the plant and its seed-bearing products might get eaten too early, before the seeds are ready for dispersal. Humans are in fact mutualistic symbionts of the plants that we grow agriculturally. Sugar cane and humans are just one example of symbiont enticements and consequent ingestion!

The microbial interactions that connect plants and soil have very nicely been discussed by Ahkami et al. (2017). One of the strongest areas of interest in agriculture is the symbiotic interactions of plants with the rhizospheric microbial communities, those microbes which live in the area of the plants roots. The interactions of plants and microbes cover a broad spectrum, from mutualistic and commensal to parasitic. Li et al. (2019) helpfully have mentioned our knowledge that colonization of plant roots by endophytic fungi may aid plant survival during periods of water stress caused by drought. Gage (2004) has presented a very nicely detailed review of the initial signalling process which occurs between many plant hosts and potentially symbiont *Rhizobium* bacteria, after which the rhizobia move from the root surface into the inner root tissue, and then the *Rhizobium* populate cells of the plants developing root nodules. The *Rhizobium* provide fixed nitrogen in exchange for carbohydates received from the plant (Moore 1905).

I would like to also mention for you the symbioses that involve protists. Protists are a diverse group of eukaryotic species considered as being neither animal, nor plant, nor fungi. Nowack and Melkonian (Nowack and Melkonian 2010) very nicely summarized information about endosymbionts of protists. The microbes known as flagellates are included among the protists. Both prokaryotes and eukaryotes are represented among the endosymbionts that are housed within protists. Those authors (Nowack and Melkonian 2010) also included mention of the tripartite symbiotic associations which exist between termites which house intestinal flagellates, and the bacteria that reside endosymbiotically within those flagellates. Nowack and Melkonian (2010) presumed that these tripartite symbioses might support the capability of termites to survive on recalcitrant plant matter as their sole nutrient source

and, thereby, contribute to the success of these insects. That tripartite interaction also contributes to the healthiness of soil.

Perhaps one of the more interesting stories in microbe and host symbiotic ineractions is that of the ambrosia beetle *Euwallacea validus*, which feeds on fungi that it cultivates inside galleries which the beetle digs within the tissue of its host plant (Aoki et al. 2018). That symbiosis is fascinatingly beneficial for the beetle, but not fascinatingly beneficial for the attacked plant.

1.2 Evolution Is Not Based upon Altruism, Life Is a Constant Struggle for Habitat and Niche

The cooperative and competitive nature of life represents an often tenuous balance, and a constant struggle to maintain evolutionary success.

1.2.1 Life Is Cooperative but it Also Is Competitive

When we look at microbial life we may perceive each species to be doing some collaborative part in an overall cooperative venture. In actuality, it can all be perceived as a competitive struggle for habitat and niche.

The collective existance of life, with each species contributing to at least one and perhaps several microbiomes, has health benefits but also potential perils. A microbiome is a competitive environment. The health of animals, as one example, depends in part upon the outcomes of numerous competitive and cooperative interactions that include the animals gut microbiota. Those relationships exist in part because they reduce the need for a host to reinvent wheels. If microbes can supply the needed materials, then the host will not need to make those materials for itself. Microbes will be found associated with all surfaces and openings that have exposure to the external environment As such, in addition to gut microbiota, there also are respiratory microbiota, skin microbiota, genital urinary microbiota, and even middle ear microbiota. Microbes whose presence is considered beneficial are solicited and maintained in those microbiota as a bulwark against more harmful microbes. We often describe those microbiota as representing ecosystems or microbiomes, with both of those terms signifying inclusive participation by the hosting species. We use the term ecosystem to represent living organisms as part of a community which interacts to achieve the flow of nutrients and energy.

Symbionts evolve to compete within the microbiomes which comprise the ecosystem of their host. All the while, hosts evolve to keep their ecosystem on a leash (Foster et al. 2017). One example of these concepts would be the fact that biofilms contribute to the heath associated with colonization of a hosts gastrointestinal mucosa by beneficial microorganisms (de Vos 2015). And yet, the establishment

of intestinal biofilms by pathogens can be very hazardous to health of the host. Those interactions are dynamic and vary across a range of conditions, through space and time (Coyte and Rakoff-Nahoum 2019). Infectious pathogens can even destructively target the mitochondria of their victim (Escoll et al. 2017).

Sir Alexander Flemings' discovery of the antimicrobial enzyme lysozyme (Fleming 1922) was a famously pioneering step towards the goal of understanding how chemical warfare occurs between animals and potentially harmful microorganims. Lysozyme is an N-acetylmuramide glycanhydrolase. It is produced by animals and is part of the innate immune system. Lysozyme attacks the 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine of peptidoglycan. That attack compromises bacterial cell wall integrity, resulting in lysis of the bacteria. A few years later, Sir Alexander also discovered Penicillin (Fleming 1929) which represents part of the chemical battle that occurs between microorganisms. Discovery of streptomycin by Waksman and his colleagues added another layer to our knowledge of warfare between micobes (Waksman et al. 1946).

1.2.2 And the Struggle Is for Habitat and Niche

Life and all of its interactions represents a constant struggle for possession of habitat and niche.

Each species will have a broadly defined potential habitat within which its members will be restricted to a more narrowly defined operational habitat. Each species also will have a broadly defined potential niche within which its members will be restricted to a more narowly defined operational niche. Those concepts of potential versus operational habitat and niche hold true at the genus level and on upward through the higher taxonomic levels. The restrictions, from potential to operational habitat and niche, occur because of competive exclusion caused by other biological groups.

The interesting question for me becomes not why symbiotic associations develop, but rather why those associations are not more widely present. Is it because they have been limited by competitors? My presumption is that a species, and even its symbiotic associations, will be found in all places except those from which it specifically has been excluded. For example, there is a broad diversity of animals in which *Chlorella* can establish itself as an ingested photosynthetic symbiont (Chap. 26: The Game of Evolution is Won by Competitive Cheating). However, there are many more animals which receive sunlight but do not form that interaction with *Chlorella*, and the question becomes one of "Why not?". If we could discover what is preventing the formation of rhizobial nodules in other plants, then might we be able to increase the range of crop plants in which rhizobial nodules can form, and thus help reduce our reliance upon synthetic nitrogen fertilizers?

Long ago, when I began creating ceramic sculpture, which is done by sculpting moist clay and eventually sintering that material in a kiln, I learned that the sculptor can do anything which their medium allows. Perhaps the host and symbiont can do

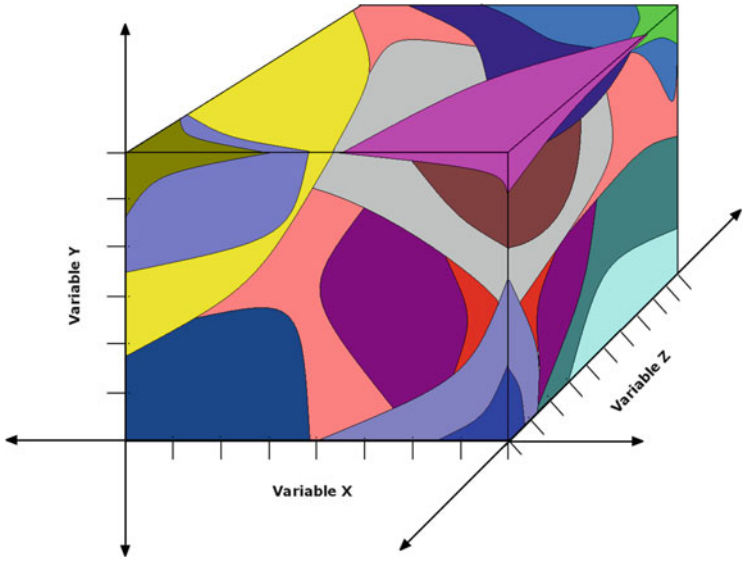

Fig. 1.1 Mutually exclusive forms in a three dimensional space (from Hurst 2016). This figure is being used to represent the result of competition between different species, depicted here as different colors, for living space described as habitat within a three-dimensionally defined location. This figure also could be used more basically as a representation of competition for niche space, although that type of usage needs to be accompanied by an understanding that truly depicting niche space would require incorporating a far greater number of dimensions as variables. The factor of time has not been included here for the sake of simplicity. However, the importance of time cannot be considered negligible because the competition for niche space and its accompanying partitioning of habitat space results in three-dimensionally defined spatial arrangements or patterns of species appearance that consequently change with time. The term biotope often is used to describe the location pattern which results when species partition available living space

anything which their partnering species allows, and anything which competitions with other species do not prevent.

I envision the limitating way in which habitats and also niches are restricted by competitions with other species as being a theoretical multidimentional space. Figure 1.1 shows mutually exclusive forms in a three dimensional space, with three variables. It is perhaps easier to percieve habitat as being dimensional if we consider only such attributes as external environment factors, among which could be altitude or aquatic depth. Figure 1.1 allows me to present only three dimensions. There are far more variables which define the habitat of a species and indeed of any taxonomic group. Each of those variables would have its own axis, and the sum would be a spacial orientation that cannot be defined in only three dimensions. The parameter values of those variables also would change with respect to time. I have defined niche as being a hypothetical volume with a mathematically complex surface, and the name which I have assigned to that concept is 'Niche space' (Hurst 2016). The surface complexity of a species niche space arises from intercalation at its surface, which is the interface where interaction occurs with other

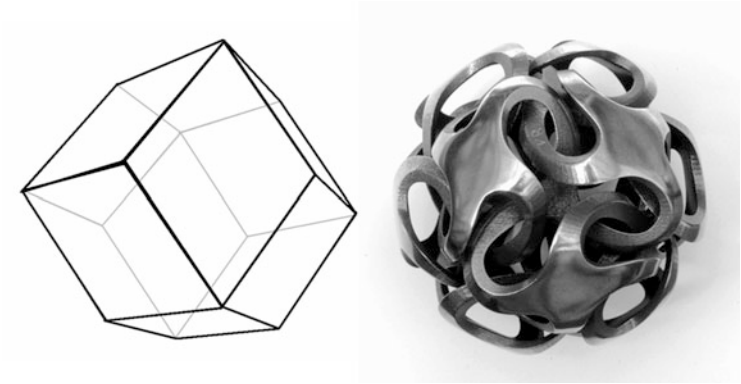

Fig. 1.2 Rhombic Dodecahedron as a line drawing and a sculpture (from Hurst 2016). The left image shows the edges that define a basic rhombic dodecahedron. The right image of a printed metal sculpture is titled "Rhombic Dodecahedron I" and appears courtesy of Vladimir Bulatov. This sculpture by Bulatov is being used here to suggestively depict a species niche as a hypothetical volume that mathematically represents those numerous variables and their parameter ranges which define the niche, with that mathematical volume visualized by its surface complexity

species. Interactions with other taxonomic groups that are more broadly or narrowly defined than species, also are represented by complexity at the surface of the niche. The niches of different groups that interact with one another can interlock. Perhaps an easy way to imagine this idea is by loosely interlocking the fingers of your hands, and them perceiving that the interlocking of your fingers represents the way in which niches interlock.

Each species, and indeed each higher taxonomic group, will try to occupy as much niche space as it can.

Figure 1.2 uses a metal sculpture as a visual representation, although the sculpture has a fairly limited surface complexity and it unfortunately is restricted to a presentation only in three dimensions, of how a given volume of niche space will have a complex surface and be shaped around voids. The voids in the sculpture by Bulatov, shown on the right side of that figure, would be occupied by the niche spaces of other groups whose niches interact with those occupants of this niche. Voids also would result from distortions caused by groups which have competitively restricted the niche of the species which is, for me, represented by Bulatovs sculpture. Figure 1.3 shows a casting of an ant nest which can be a suggestive example of the surface complexity which might arise as a taxonomic group tries to maximize its collective occupation of niche space.

Each species, each genus, and on upward through the taxonomic levels, will try to occupy as much niche space as possible. The pressure to achieve successful occupation will include evolution and speciation as either necessary or allowed. There are many ways to occupy the same approximate volume of niche space, as represented in Fig. 1.4 by castings of ant nests created by two different species. The volumes of

Fig. 1.3 Aluminum casting of a fire ant nest, genus Solenopsis (from Hurst 2016). This casting symbolically is being used to represent an aspect of evolution. Its purpose is illustrating the concept that creation of new niches and movement into existing but otherwise occupied niche space occurs through a combination of two forces, an outward evolutionary pressure and resistive external exclusion, acting against one another as forces in opposition. Biological groups attempt to occupy the greatest possible amount, depicted here as a volume, of niche space with that action including speciation as either necessary or possible and consequently biological groups compete with each other to achieve successful evolutionary expansion. In this example, initial formation of the nest volume and its structure by the ant colony can be perceived as representing an outward expansion force acting as a pressure applied against resistance from the surrounding soil and that expansion pressure was accompanied by removal of the surrounding soil. If the colony was alive when the casting was made, then the molten aluminum could be perceived as having represented a successful displacement of existing species (the ants) from their niches by overwhelming evolutionary pressure arising from some other biological group (represented here by the molten aluminum). Following mass extinctions, the surviving biological groups often expand their total niche space by movement into already available morphospace that had been populated differently. If this nest was vacant when the casting was made, then flow of molten aluminum into the existing but vacated space could represent the relatively rapid evolutionary expansion into unoccupied niche space which is effected by biological groups as they quickly undergo radiative evolution following a mass extinction. This casting weighs 25.3 lbs. and is 21.5 in. high. The image courteously was provided by David Gatlin of Anthill Art

those nests were approximately the same but the surface areas and surface complexities differed tremendously.

I was appreciative when I read Chap. 17, "Diversity-function relationships and the underlying ecological mechanisms in host-associated microbial communities", which Catalina Cuellar-Gempeler authored for this book. She mentioned the niche space of symbionts, and that mention was without any suggestion having been made on my part. Catalina, I thank you!

How can we imagine the way in which a niche evolutionarily changes with time? Perhaps one way is by noticing that the physical and physiogical attributes of a species give a representation of its niche. When we see a depiction of how the

Fig. 1.4 Aluminum castings of Aphaenogaster and Camponotus nests (from Hurst 2016). These two aluminum castings of ant nests are: top, Aphaenogaster treatae (cast weight 2 lbs., 6.5 in. high); and bottom, carpenter ants Camponotus castaneus (cast weight 2.5 lbs., 22 in. high). These two images are being used to symbolically represent the concept of there being many different ways of occupying an approximately similar volume of niche space. These images kindly were provided by David Gatlin of Anthill Art

physical characteristics of horses evolutionarily have changed, as shown on Fig. 1.5, we actually are visualizing the way in which the niche of horses has changed. Thus far, the equids have been able to evolve in ways that allowed them to adapt to changes in their available niche space. Failure to successfully adapt as a groups potential niche or operational niche changes, or failure to successfully adapt when confronted with a complete loss of operational niche, would result in extinction.

1.3 Can we Utilize our Knowledge of Microbes to Gain Ecological Benefits?

Perhaps we can!

One conceptual example of beneficially using symbiotic interactions between microbes and hosts is the idea that commercially applying plant growth promoting bacteria may help us to reduce the demand for synthetic fertilizers (Khan et al. 2020).

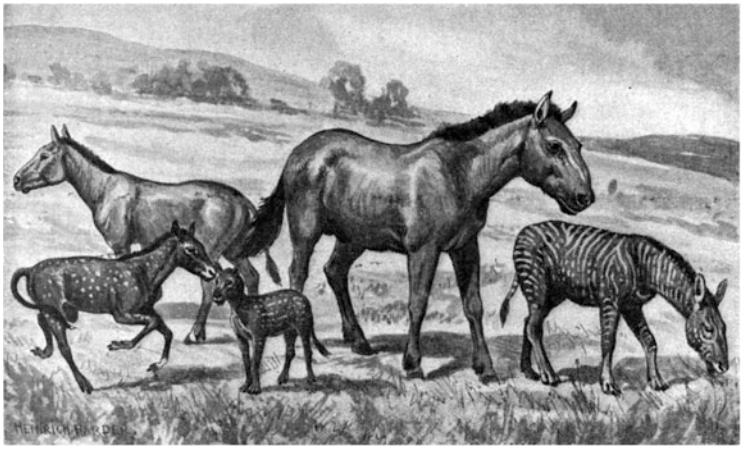

Fig. 1.5 A depiction of the evolutionary history of horses. I am using this image to visually represent how the changing physical form of horses has reflected their evoultionary adaptation to changes in their available niche. This is a public domain image titled "Extinct horses.jpg" by author Heinrich Harder (1858–1935)

There is a serious downside to continuously using synthetic fertilizers, because the nitrogen and phosphorus which they contain can decrease soil health, increase emissions of greenhouse gasses including N_20, and the surface runoff of artificial fertilizers leads to eutrophication of waterways (Khan et al. 2020). George T. Moore (Moore 1905) made one of the pioneering efforts in that subject area when he studied culturing and agriculturally applying root nodulation bacteria to assist productivity of legume crops. Historically, people had used soil transfer to provide appropriate microbes for achieving effective root nodulation in legumes. That nodulation enables legumes to have a source of usable nitrogen. However the inadvertent movement of pathogens associated with soil transfer can cause such plant diseases as wilts and blights.

Microbial treatments could help us to reduce the use of pesticides that we otherwise might apply for enhancing agricultural productivity. The use of pesticides can decrease overall health of an ecosystem including the health of our own species (Khan et al. 2020). Rachel Carsons book "Silent Spring" had a profound effect upon humanity by helping us to recognize the ecological damage which can result from usage of artificial pesticides (Carson 1962). One interesting possibility for reducing the use of pesticides, such as dichlorodiphenyltrichloroethane (DDT) which was a topic of Carson's book, comes from our knowledge that a microsporidian symbiont vertically transmitted in *Anopheles arabiensis* mosquitoes can impair the transmission of *Plasmodium falciparum* (Herren et al. 2020). *Plasmodium falciparum* is one of the parasitic unicellular protozoa that cause the disease malaria.

Acknowledgements I thank Vladimir Bulatov and David Gatlin for generously provided me with images to use in this chapter. I thank Aaron Margolin for a conversation during which he long ago helped me to understand how the requirements of a niche direct evolution. Aaron would like my

choice of horses to represent the concept which he explained to me. I thank Jerónimo Pan for kindly reviewing the information which I had assembled about the metabolism of *Beggiatoa*.

Christon J. Hurst with self sculpture

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Chapter 2
Darwin's Science's Impact on the Evolution of the Microbiological Sciences

Kenneth M. Noll

Abstract Charles Darwin is not known as a pioneer of the microbiological sciences, but examinations of his correspondence demonstrate that he was informed about and supportive of early investigations of the microbial world. His theories influenced the work of early microbiological investigators who were trained in late nineteenth-century natural history. However, those who tried to carry on this work into the twentieth century soon found that bacteria did not have characteristics necessary to examine evolutionary questions as had been done with plants and animals. Consequently, studies of evolution and efforts to create natural taxonomies of bacteria failed to create a solid evolutionary science for microorganisms. It was not until the advent of macromolecular sequencing, when characters that could be compared to reveal phylogenetic relationships appeared, that solid data could be used to test Darwin's theories. Subsequent full genome sequencing and reintroduction of laboratory evolution of bacteria both generated new discoveries of the mode, extent, and course of microbial evolution. Darwin's place as a pioneer in the microbiological sciences has finally been established.

2.1 Introduction

The name Darwin does not appear often in modern microbiological research publications. Only 47 publications were found in a search of PubMed using the text words "Darwin AND bacteria" (including NOT Australia and NOT Antarctica to exclude place names). By comparison, a search substituting his contemporaries, "Pasteur" and "Koch," yielded 158 and 161 hits, respectively. The impact of Darwin's studies, outside the general processes of evolution and natural selection, on the field of microbiology is generally thought to be rather limited. Many ascribe this to the idea that Darwin knew little to nothing about microorganisms, particularly bacteria, since his major work occurred just before the concepts of the germ theory of diseases

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_2

and pure cultures were formulated. The reality is very different. In this article I will attempt to show that Darwin's knowledge of microbes was very current for his time and he did a small amount of work with microbes and microscopic organisms. He also included them in his thinking about the evolution of life. Finally, I will show how his work greatly impacted the thinking of those who founded modern microbiology and how his work has been rediscovered in recent years and now underpins revolutionary new thinking in the field.

2.2 Darwin's Research Topics

Charles Darwin is best known, of course, for positing that species arise from variants of existing species, called the "transmutation of species" in his time (Fig. 2.1). It was only in later editions of *The Origin of Species* that he adopted the modern term, evolution, for this process. Though he used examples from the plant and animal world to support his theory, he knew the process began with a simpler form of life, likely microbial, and he noted this in the first edition of *Origin*, "Therefore I should infer from analogy that probably all the organic beings which have ever lived on this earth have descended from some one primordial form, into which life was first breathed" (Darwin 1959 p. 484). He is also known for discovering the means by which new species come to survive through the mechanism he called Natural Selection and through a second mechanism, sexual selection, as it pertained to sexually reproducing creatures. The impacts of his ideas about evolution and natural selection on the development and progress of the field of microbiology will be considered in detail below.

Many of the areas of study in which Darwin made contributions had little direct impact on microbiology. For example, his first studies were largely geological and

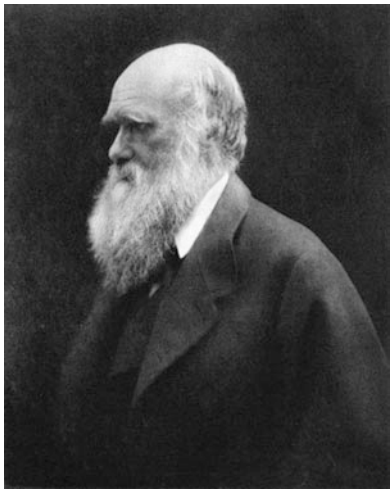


Fig. 2.1 Charles Darwin, 1868. Photographed by Julia Cameron. It reportedly was his favorite photograph (https://en.wikipedia.org/wiki/Charles_Darwin)

paleontological, and these were typically about very large-scale phenomena. Though he observed small fossils to the level of foraminifera (see discussion of this below), fossils below this scale were unknown in his time. Other non-microbiological areas of his studies, many pursued to support his larger theories, included coral reef formation, animal breeding, barnacles, orchids, honeybees, the effects of seawater on seeds, climbing plants, ants, expression of emotions, the evolution of man, insectivorous plants, flowers, the movement of plants (phototaxis and circumnutation), animal intelligence and instinct, and earthworms.

His interest in microbes was largely expressed through his correspondence about Pasteur's work and Koch's discoveries (see below). He was interested in the cause of the potato blight and supported work by Miles J. Berkeley in the USA and provided financial assistance to potato breeder James Torbitt who sought to introduce disease-resistant plants in England and Ireland (Ristaino and Pfister 2016). He even cultivated some potato varieties that he had sent back from Peru on the *Beagle* voyage in an effort to find disease-resistant varieties (Ristaino and Pfister 2016). There is no evidence that he ever cultured microbes, though he did observe a tube of sterile medium sent to him by Tyndall who asked him to open it and report whether microbes grew in it (Darwin Correspondence Project Letter no. 10207 2020c). They did of course, thus providing Tyndall with support for his contention that microbes were in the air everywhere and so providing evidence to discount the theory of spontaneous generation.

2.3 Darwin and Microbes

Darwin was a very good microscopist. He wrote a chapter entitled "On the Use of the Microscope on Board Ship" in the 1849 book *A Manual of Scientific Enquiry; Prepared for the Use of Her Majesty's Navy: and Adapted for Travelers in General* (Darwin 1849). Darwin in his later studies used a compound microscope that could achieve a magnification of 1300 X, though he apparently used it at lower power since it was prone to visual aberrations, as were all microscopes at that time (Jardine 2009). He could certainly see bacteria with this kind of microscope, but he did not comment on them in any of his writings.

Darwin lived until April 1882, so he overlapped with scientists known to have made contributions to bacteriology including Louis Pasteur, Ferdinand Cohn, Robert Koch, Martinus Beijerinck, and Sergei Vinogradskii (Fig. 2.2). Of these, there are only copies of correspondence with Cohn, though the contributions of Pasteur and Koch are mentioned in other letters.

During Darwin's lifetime, 1809–1882, the concept of microorganisms changed radically. Since their description in detail by Antonie van Leeuwenhoek in 1683, the nature of bacteria was little studied until the early nineteenth century. This may have been due to the fact that few could reproduce the lenses that van Leeuwenhoek constructed. Studies of the larger protozoa and yeast cells made progress, however, and the role of yeast cells in fermentations became the subject of heated debate in the

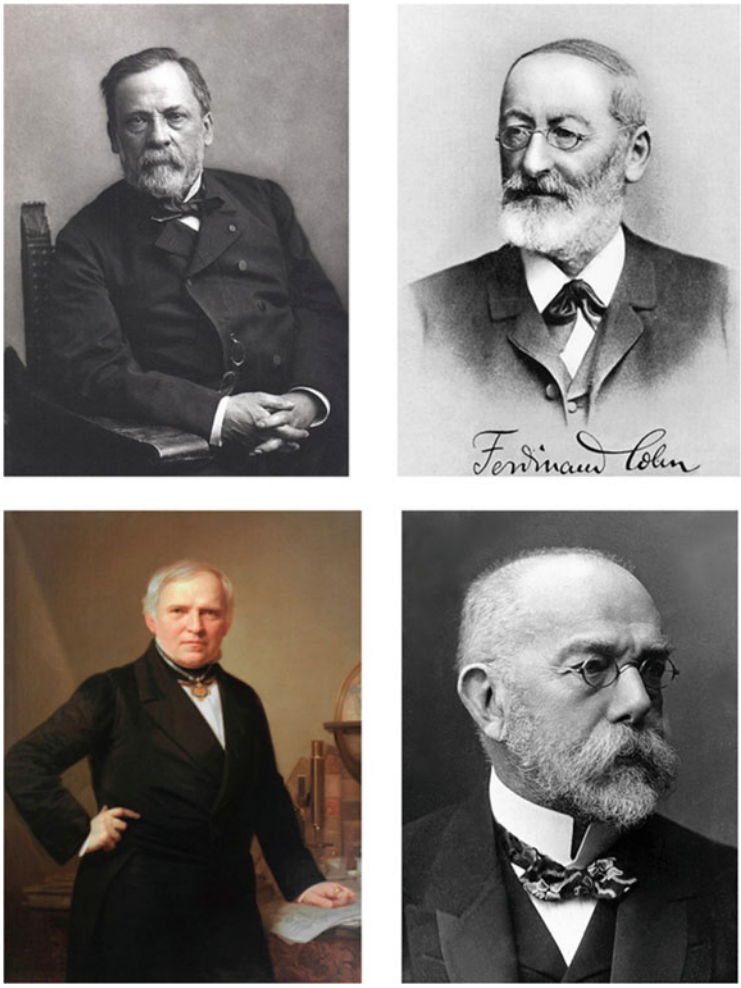


Fig. 2.2 (clockwise from upper l) Louis Pasteur; Ferdinand Cohn; Robert Koch, 1907; Christian Gottfried Ehrenberg, painting by Eduard Radke, 1855 (Pasteur: https://en.wikipedia.org/wiki/Louis_Pasteur; Cohn: https://en.wikipedia.org/wiki/Ferdinand_Cohn; Koch: https://en.wikipedia.org/wiki/Robert_Koch; Ehrenberg: https://en.wikipedia.org/wiki/Christian_Gottfried_Ehrenberg)

1830s (Barnett 2003). Organic chemistry was a new field at the time, and it held that the chemical transformation of the "ferments" (fermentation broths used to produce products) was caused by the vibrations of organic molecules or some catalytic process. Yeast cells seen in these ferments were thought to play some secondary role. Among those holding these views was Justus von Liebig, who argued against a causative role of yeast cells well into the late nineteenth century (Hein 1961). Louis Pasteur's experiments in the 1850s and 1860s conclusively demonstrated that living organisms were responsible for the alcoholic and lactic acid fermentations. With the

improvements in microscope lenses in the mid-nineteenth century, the role of bacteria in some of these fermentations was established.

At the same time, Pasteur was performing experiments to refute the claims of Félix Archimède Pouchet regarding spontaneous generation. It was this work that attracted Darwin's attention. He mentioned Pasteur's memoir (*Mémoir sur les corpuscules organisés qui existent dans l'atmosphère* (1861)) in letters to colleagues and remarked that he was impressed with his work on this subject. Darwin was interested in the subject because of his interest in the origin of life on Earth. He thought it possible that life arose from inorganic material at the beginning of life. In a letter to his friend, the botanist Joseph Hooker, in 1871, he wrote:

> It is often said that all the conditions for the first production of a living organism are now present, which could ever have been present.— But if (& oh what a big if) we could conceive in some warm little pond with all sorts of ammonia & phosphoric salts,—light, heat, electricity &c present, that a protein compound was chemically formed, ready to undergo still more complex changes, at the present day such matter wd be instantly devoured, or absorbed, which would not have been the case before living creatures were formed. (Darwin Correspondence Project Letter no. 7471 2019c)

However, Darwin rejected the idea of spontaneous generation, or the theory of "heterogeny" as it was then called, occurring in modern times (Darwin 1863). He knew that a spontaneous origin of life from nonliving matter was important for the completeness of his theory, but he also thought that we are a long way from knowing how this event came to occur, writing, "It is mere rubbish thinking, at present, of origin of life; one might as well think of origin of matter (Darwin Correspondence Project Letter no. 4065 2019b)."

Ferdinand Cohn corresponded with Darwin from 1874 to 1882, though he is mentioned in Darwin's letters from 1862. Cohn played an important role in the beginnings of microbiology and its relation to Darwin's work. Their correspondence was about Cohn's work with plant movements, a topic that Darwin studied for many years. Darwin was aware of his work regarding the question of spontaneous generation, and Cohn sent him details of Koch's work and Koch's first photographs of bacteria in 1877 (Darwin Correspondence Project Letter no. 11298 2019d). Darwin also commented to him on the discovery of the role of bacteria in diseases in 1878 (Darwin Correspondence Project Letter no. 11310 2019e). Cohn thought highly of Darwin's work, and he even visited him at Down in 1876 where Emma, Charles' wife, wrote in her diary, "Professor Cohn (quite deaf) and his wife (very pleasing) and a Professor R. came to lunch—anything like the noise they made I never heard" (Litchfield 1915).

Darwin used microbes to support his Natural Selection theory against criticisms that microbes were evidence against the theory (O'Malley 2009). Readers of *Origin of Species* generally thought that natural selection operated to bring about increasing complexity. Indeed, Darwin even wrote in the first edition, "And as natural selection works solely by and for the good of each being, all corporeal and mental endowments will tend to progress towards perfection (Darwin 1959 p. 489)." Critics pointed out that infusoria were no more complex now than they were in the past,

as evidenced by fossil foraminifera, for example. Darwin recognized this criticism and addressed it in the third edition of *Origin*:

> But it may be objected that if all organic beings thus tend to rise in the scale, how is it that throughout the world a multitude of the lowest forms still exist; and how is it that in each great class some forms are far more highly developed than others? Why have not the more highly developed forms everywhere supplanted and exterminated the lower? . . . On my theory the present existence of lowly organised productions offers no difficulty; for natural selection includes no necessary and universal law of advancement or development—it only takes advantage of such variations as arise and are beneficial to each creature under its complex relations of life. And it may be asked what advantage, as far as we can see, would it be to an infusorian animalcule—to an intestinal worm—or even to an earth-worm, to be highly organised? If it were no advantage, these forms would be left by natural selection unimproved or but little improved; and might remain for indefinite ages in their present little advanced condition. (Darwin 1861 pp. 134-5)

2.4 Biogeography of Infusoria

Biogeography was a major point in Darwin's formulation of his theories of Evolution and Natural Selection (Richardson 1981). While on the *Beagle* voyage and during the analysis of his samples afterward, he recognized that the distribution of animals and plants could not be explained by special creation of creatures that were designed for the habitat they occupied. Instead, their distribution could be better explained by migration of creatures between locations and the subsequent adaptation of the founding population to the local conditions in the new environments. He especially observed this in the animals of South America and the Galapagos Islands. Darwin said in his "Essay of 1844" (quoted in Richardson 1981):

> I think then I am justified in asserting that most of the above enumerated and often trivial points in the geographical distribution of past and present organisms (which points must be viewed by the creationists as so many ultimate facts) follow as a simple consequence of specific forms being mutable and of their being adapted by natural selection to diverse ends, conjoined with their powers of dispersal, and the geologico-geographical changes now in slow progress and which undoubtedly have taken place. This large class of facts being thus explained, far more than counterbalances many separate difficulties and apparent objections in convincing my mind of the truth of this theory of common descent.

In 1844 Darwin turned to the German naturalist Christian Gottfried Ehrenberg to include infusoria, or in modern terminology Protista, in his biogeographic studies (Fig. 2.2). This was perhaps the only time that he included microorganisms directly in his work. Darwin was alerted to Ehrenberg's interest in the geographic distribution of infusoria by his German publisher, Ernst Dieffenbach. In April 1844, Darwin wrote to Ehrenberg for the first time offering to send him samples he had collected from the Galapagos Islands and Tierra del Fuego, along with samples provided by his friend, the botanist Joseph Dalton Hooker who had sailed on an Antarctic expedition (Darwin Correspondence Project Letter no. 747 2019a). While on the *Beagle* voyage, he collected soil and rock samples from various locations as well as dust that settled on the ship while in the mid-Atlantic.

Following a trip with Alexander von Humboldt through eastern Russia and the Chinese frontier in 1829, Ehrenberg began a 30-year study of infusoria in soils, sediments, rocks, and dust samples that he and others had collected from around the world. He studied and named many species of protists and diatoms, in particular. He attempted to associate different species with their geographic distribution. It was these studies that attracted Darwin's attention. Darwin thought that his samples would help him extend his biogeographic studies to the microbial world. He also thought that the microbiology of some of his rock samples would help him prove how those sedimentary rocks formed. In particular, he was looking for proof of his theory that mineral samples he had collected in the Pampas had formed in an estuary and so should contain marine foraminifera (Darwin Correspondence Project Letter no. 870 2020a).

Darwin reported that Ehrenberg's examination of the dust that he had collected and dust that had been collected later by Lieutenant Robert Bastard James of the HMS *Spey* off the northwest coast of Africa were both composed in large part of infusoria, including over 67 different forms (Darwin 1846). His dust and that of Lieut. James contained 37 species in common. All but two species were freshwater organisms. Oddly, none of these had been found to be of Saharan African origin based on Ehrenberg's previous studies. Instead, they were South American or Senegalese species. This was contrary to where it appeared the dust came from, based on the wind direction at the time of its deposition. Darwin commented, "I think there can be no doubt that the dust which falls in the Atlantic does come from Africa. How to explain the enigma of the absence of characteristic African forms and of the presence of two species from S. America, I will not pretend to conjecture" (Darwin 1846).

A dispute over the origin of the Pampas region of South America was a subject that Darwin hoped Ehrenberg's examinations could help settle. The French geologist Alcide de′Orbigny posited that this raised geological formation had been formed through a catastrophic inundation of seawater. Darwin contended that it had formed through a gradual process involving formation of an estuary of freshwater or brackish water and mud (Jardine 2009). Darwin sent Ehrenberg soil samples collected there, and he reported, "...Professor Ehrenberg has had the kindness to examine for me a little of the red earth, taken from low down in the deposit, ... and he finds in it many infusoria, partly salt-water and partly fresh-water forms, with the latter rather preponderating; and therefore, as he remarks, the water must have been brackish.... this shows that just before the Pampas was slowly elevated into dry land, the water covering it was brackish" (Darwin 1845).

2.5 Natural Taxonomy: Phylogeny-Based Systematics

After his death, Darwin's greatest impact on the development of microbiology was the effect of his theory of evolution on the systematics of microorganisms. Taxonomy of plants and animals before Darwin's theory was largely based on the scheme of Carl Linnaeus, though other taxonomic schemes were attempted. In his work on

barnacles and plants, Darwin struggled with defining species and varieties under this system. Overall he believed that genealogy should be the basis of a taxonomic system (Padian 1999). He frequently expressed frustration when dealing with taxonomy. He found that the use of morphological characteristics to define species was necessary and recognized that such factors could help reveal genealogy. He wrote to his friend, zoologist Thomas Huxley, on September 26, 1875:

> In regard to Classification, & all the endless disputes about the 'Natural System' which no two authors define in same way, I believe it ought, in accordance to my heteredox notions, to be simply genealogical.—But as we have no written pedigrees, you will, perhaps, say this will not help much; but I think it ultimately will, whenever heteredoxy becomes orthodoxy, for it will clear away an immense amount of rubbish about the value of characters &—will make the difference between analogy & homology, clear.—The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature. (Darwin Correspondence Project Letter no. 2143 2020b)

The use of characters to demark taxonomic differences among microorganisms was hindered by the limited number and often indistinct nature of their characters. Early attempts to describe the characters of microbes were thwarted by defects in the optical properties of early microscopes. Ehrenberg, for example, stated, "The infusorian has the same sum of organization-systems as a man." This reflected his dislike of systems that place organisms in hierarchies of apparently "simple" organisms at the bottom and "higher" ones at the top (Churchill 1989). He claimed that protozoa ("Polygastrica") had circulatory, digestive, excretory, motor, sexual, and nervous systems. For example, he believed that the nucleus was their stomach. Contemporaries of his, however, recognized the nucleus as not an organ, but a structure somehow involved in cell reproduction. A homology between the nuclei of single-celled organisms and those of animal cells was increasingly recognized. This culminated, by the 1870s, to the acceptance of single-celled protozoans as a taxonomic group of organisms separate from multicellular organisms.

The place of bacteria in this taxonomy was, however, still problematic. In 1838, Ehrenberg placed them in with the protozoans as the groups Monadina and Vibrionia, in line with a 1786 classification by Otto Friedrich Müller (Drews 2000). Theodor von Siebold moved them to the plants in 1845 on the grounds that their movements were involuntary (Churchill 1989). There they stayed until Ernst Haeckel in 1866 divided the animal kingdom into the Protozoa and Metazoa and included the Monera as one of eight divisions of the Protozoa. Monera were described as the "most simple organisms, without structure, homogeneous pieces of Plasma," and bacteria known as Vibrio were given as an example (Kutschera 2016).

The introduction of solid culture media in the 1870s and especially the development of the method of streaking inocula to get single colonies by Robert Koch in 1877 lead to the concept of stable phenotypes in bacteria. At that time several investigators held to the idea that bacteria developed different shapes depending upon the conditions of their growth. Consequently, some taxonomies lumped all bacteria into a single genus or even species (Drews 2000). Ferdinand Cohn, a student of Ehrenberg, at that time began to characterize "form-genera" and "form-species" based on the stable morphologies provided by pure cultures. As mentioned previously, Cohn was not only aware of Darwin's work, but was a correspondent of his

and so appreciated Darwin's thoughts about taxonomy. Consequently, Cohn realized that bacteria, too, needed to be classified based upon their genealogy. He realized, though, that using their forms would be insufficient to this purpose and there was a need to examine their developmental processes and chemical features to determine their descent. Pasteur had even commented that differentiation of bacteria could only come through examinations of their physiological functions (Cohn 1875). Thus Cohn began a 20-year effort to find these features.

Cohn's work led him to believe that bacteria form a distinct group of life and that they transmit stable characters to subsequent generations, though variations sometimes appear, as they do in all life. He thought their group best fit in the plant kingdom with their closest relatives the Schizophyta (fission plants) or Cyanophyceae (cyanobacteria). The isolation of pure cultures also led Cohn to propose that bacteria could be classified into distinct species (Cohn 1875). His ideas did not take hold at first as the idea that they were morphologically pliable in nature was still strong.

Microbiology research in the mid-nineteenth century, with the exception of Pasteur, was largely being conducted in Germany (Matta 2007). Mattias Jakob Schleiden was a botanist at the University of Jena and wrote in 1838 that plants were made of cells and argued that botanists should focus on the functions of plant cells, rather than their gross parts, to understand the nature of plants (Schleiden 1838). Cohn accepted this call, though, in doing so, his work disputed some of the work of Schleiden (Drews 1999). Schleiden was early to accept Darwin's theory of evolution. Schleiden advocated for the study of cryptogams, plants without true seeds and flowers like mosses, algae, and fungi, since they would allow easier investigations of the basic processes of plants. This approach naturally included examinations of microbes, including bacteria. Thus microbiological studies developed among botanists. A separate line of microbiological investigations arose among medical practitioners, particularly after the work of the physician Koch. It was, however, the work of botanical microbiology that focused on the functions of microbes that led to interest in their place in the world and their evolution. Evolutionary questions were a natural part of botanical studies, but not those of investigators interested in medical aspects of microbes. Consequently, as the latter gained greater influence over the science of microbiology after the turn of the twentieth century, microbial evolution studies entered their "Dark Ages" as described below.

2.6 Early Attempts at a Natural Taxonomy of Bacteria

Cohn had also developed the idea of the cycle of life as applied to bacteria. In 1872 he described the "entire arrangement of nature" in which the decomposition of dead material by microbes provided the materials for new life (Ackert Jr 2007). This, along with Pasteur's examinations of microbial ferments, began examinations of the biochemical physiology of microorganisms and their contributions to the functioning of the planet.

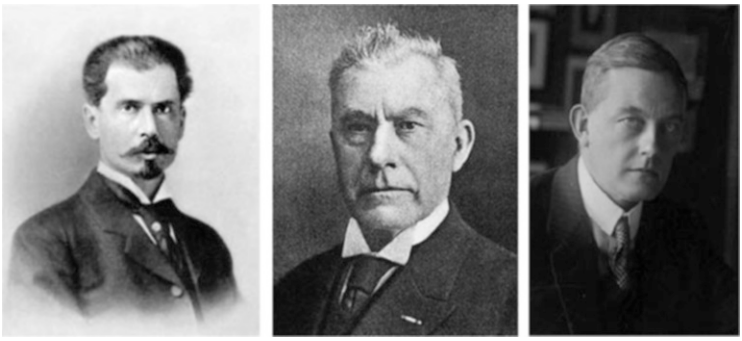


Fig. 2.3 (l to r) Sergei Vinogradskii, 1890s; Martinus Beijerinck; Sigurd Orla-Jensen (Vinogradskii: https://en.wikipedia.org/wiki/Sergei_Winogradsky; Beijerinck: https://en.wikipedia.org/wiki/Martinus_Beijerinck; Sigurd Orla-Jensen: Olsen 1950)

The idea of cycles of life was picked up by plant physiologist Andrei Famintsyn who began an examination of the physiology of fungi. This project was taken on by his new student, Sergei Vinogradskii (Fig. 2.3). Interestingly, Famintsyn said in 1860, "that animals and plants share a common fundamental beginning of life and that a deeper and more attentive study of their most central vital functions will present much that is analogous." This would have pleased Darwin who, in his own work, tried to connect animals and plants in an effort to support his idea that these groups arose from a common ancestor.

Vinogradskii employed a device called a Geissler chamber to study the nutritional needs of the fungus *Mycoderma vini* (now *Candida vini*) (Fig. 2.4). His device was essentially a continuous culture chamber, placed on a microscope to allow him to observe "characteristics of form and manner of growth in various nutritive liquids with the presence or absence of one kind of material in the liquid" (Vinogradskii 1883). This allowed him to examine the nutritional needs of the organism as well as observe its developmental cycles (Ackert Jr 2007).

In 1885 Vinogradskii went next to the laboratory of Anton de Bary at the University of Strasbourg where he turned his attention to *Beggiatoa*. De Bary thought that microscopic fungi (like *Beggiatoa* was thought to be) had constant life cycles, which appealed to Vinogradskii's interests in studying the stability of microbial species (Ackert Jr 2006). Unlike *Mycoderma*, *Beggiatoa* at that time could not be grown in the laboratory using the newly devised culture methods, so he had to continually collect samples from nature. His Geissler chambers, however, let him recreate natural conditions in the laboratory so he could study *Beggiatoa* cells as they grew. He modified his methods to use microscope slide microcultures and, in so doing, was able to examine many variables of nutrition, particularly the role of elemental sulfur in *Beggiatoa* metabolism. This led to his discovery of chemolithotrophic metabolism, in this case, the oxidation of sulfide to sulfur. He called this "chemosynthesis" (Dworkin 2012).

Vinogradskii's efforts to replicate natural conditions in the laboratory to cultivate otherwise unculturable organisms lead him to successfully cultivate many different

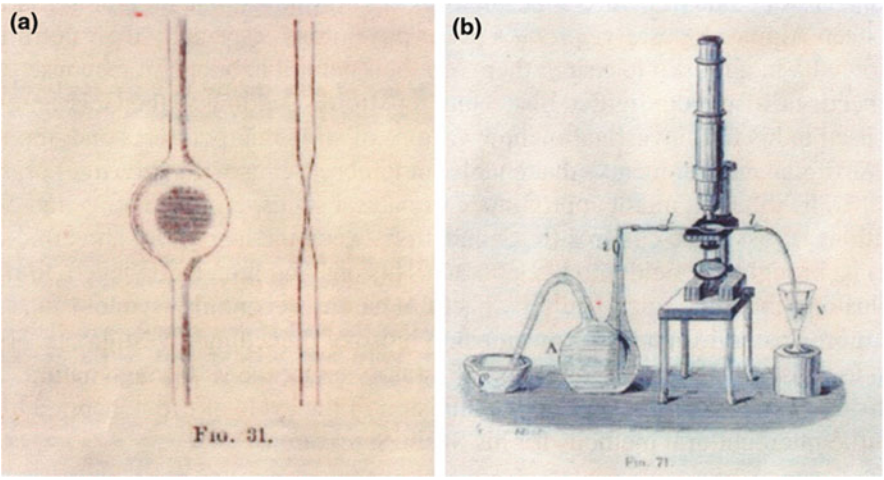


Fig. 2.4 (**a**) Geissler chamber showing a flattened glass tubing that allowed a culture to pass through slowly for examination under a microscope. (**b**) The Geissler chamber on a microscope showing its connection with a culture vessel. (https://www.semanticscholar.org/paper/Sergei-Winogradsky%3A-a-founder-of-modern-and-the-Dworkin/fa815c1146bb6a9d50ef346db6fb6c3ef3f482d9; Reproduced from Dworkin 2012 and by permission of Oxford University Press on behalf of the Federation of European Microbiological Societies)

kinds of organisms and opened new worlds to microbiology. This, then, presented many new species that further challenged taxonomic efforts. Vinogradskii did not explicitly study bacterial taxonomy, but his later work with native cultures cultivated in their native material, principally soils, gave rise to the concept of ecologically defined species (Stanier 1951). This concept has arisen again in recent years (Cohan 2002).

Cohn's work developed out of the botanical microbiology tradition espoused by Schleiden. He was influenced by Schleiden's cell theories and began studies on cryptogamic plants. During the course of his investigations, he became aware that bacteria are unlike the other cryptograms and that they should be considered as belonging to a separate taxonomic group among the plants. He was bothered by the loose terminology that authors used when referring to bacteria and even took Pasteur to task when he wrote in 1872 (quoted in Matta 2007):

> . . .great confusion has been introduced by the vague use of names and the introduction of new terms. Especially Pasteur, whose researches are greatly lessened in value by want of knowledge, has, on his part, made confusion of the terminology and methods of biology.

Cohn attempted to develop a consistent nomenclature and taxonomy for bacteria. He credited Ehrenberg with attempting the first classification scheme in the 1830s. In 1872 he published his taxonomy and named six genera: *Micrococcus*, *Bacterium*, *Bacillus*, *Vibrio*, *Spirillum*, and *Spirochaeta* (Cohn 1872). He emphasized that having observed the development of bacteria, one could show that species were stable and so could be subject to taxonomic arrangement. He also posited that

bacteria belonged to separate species that had heritable traits that could be passed on stably generation after generation. To differentiate genera and species, he used their size and shape as the primary criteria. To further distinguish them, he used their association with, among other characters, fermentations and diseases; colony form; pigments; and motility (Matta 2007). For the first time, physiological characters were used in a taxonomic scheme.

Martinus Beijerinck became a student at the Landbouwschool (agricultural school) in Wageningen, the Netherlands, and received a Ph.D. there in 1877 (Theunissen 1996) (Fig. 2.3). He studied plant galls there under the guidance of botanist and systematist Willem F. R. Suringar. Beijerinck was attracted to the subject by Darwin's discussion of galls as modifications of the plant's structure by external agents. Darwin was interested in the possibility that the environment of an organism could cause variations and saw galls as one possible manifestation of this phenomenon that could be examined in detail. Beijerinck stated, "as soon as more light is thrown on the causes of the changes which parasites bring about in the cells that nurture them, we shall have come a step closer to the explanation of the small, stepwise changes which, fixed by inheritance and natural selection, bring new varieties, species *etc* into being."

Beijerinck eventually became dissatisfied with the descriptive approach to botany and took up experimental studies of plant physiology, an approach that was growing in popularity at the time. He also took up the study of plant diseases, and this led to his first forays into microbiology. He determined that fungal infections, like the insect infestation with galls, activated cell growth that was normally latent in the plant. This activation, he concluded, was the result of the action of a parasite-produced enzyme (a newly coined term replacing "ferment"). This conclusion, though, made him realize that this was not a process that would help answer Darwin's question about the cause of variability as this activation by an enzyme would not be a heritable trait. He continued to examine variation, however, by crossing plants. Those studies were short-lived and, in later years, he regretted abandoning them as they might have led him to be one of the people who rediscovered Mendel's experiments (Theunissen 1996).

In 1885, when Beijerinck moved to Delft to work at the Nederlandsche Gisten Spiritus Fabriek (Dutch Yeast and Spirits Factory), he embarked on serious microbiological studies. These works, though, continued to have growth and variability themes (Theunissen 1996). The best example of this is his 1888 proof that symbiotic microorganisms are responsible for the formation of nodules on the roots of leguminous plants and that fixed nitrogen that was provided to the plant. Again, a substance, likely an enzyme, was produced by the parasite that activated the growth of plant tissue. He came to believe that microbiology could provide insights into growth, variability, and other biological phenomena that could not be made in plants or animals because microbes were experimentally tractable. He said in his opening lecture at the Delft Polytechnical School in 1895:

> There can be no doubt that in some bacteria changes in the external conditions of life bring about deeper changes in the hereditary characteristics than has been observed to occur in higher organisms, and that therefore it will be the bacteria which will provide the building

> blocks for the erection of a theory of variability, which until now has mainly been supported by creations of the imagination. (Beijerinck 1922 quoted in Theunissen 1996)

Beijerinck began studies on different kinds of microbes. He observed variations occurring in *Photobacterium fischeri*, *Lactobacillus fermentum*, *Chlorella*, *Bacillus prodigiosus*, and yeast. He thought microbes were not different from metazoans in principle and so could provide insights into variations, and what were now called mutations. Like Darwin's thoughts on the influence of environments on variation, it seemed clear to Beijerinck that the origin of the variants must in one way or another be related to the condition of the medium in which the bacteria were grown. He found it likely that nutritional factors or the bacteria's own secretion products played a role in the process (Theunissen 1996). He sought to determine the frequency of variant formation during growth and so developed what came to be one of his major contributions to microbiological research, selective media. He developed the technique not to isolate a single variety of a single species as we use it today. He knew that Koch's method of streaking plates would do that. He developed it instead to select for all the individual variants in a species that would grow under the conditions he created. He called this his "accumulation method." A "perfect" accumulation experiment, he said, would result in a culture of a single species together with all its varieties.

Beijerinck's view of variation was closer to that of Darwin and his gemmule theory, though Beijerinck did not believe in gemmules, per se. The alternative Mendelian view of his time posited that variation arose through random mutations independent of the conditions of the organism. Beijerinck believed that the growth conditions of microbes, as well as conditions present during the ontological development of plants and animals, somehow impacted heritable changes. He believed that the study of microbes would reveal the laws guiding these processes. In his 1877 thesis, Beijerinck quoted Darwin: "If it were possible to expose all the individuals of a species during many generations to absolutely uniform conditions of life, there would be no variability" (O'Malley 2007).

Biochemical physiological traits of bacteria were examined near the turn of the twentieth century by many investigators, but most were limited to analysis of end products of fermentations. The chemical techniques necessary to measure cellular reactions and intracellular contents were only then being developed and so were not widely used. The lactic acid bacteria, because of their commercial and agricultural importance, were among the best-studied bacteria. Sigurd Orla-Jensen was among the leaders in these studies and proposed a "natural" classification of bacteria based upon physiological characters in 1908 (Olsen 1950) (Fig. 2.3). He published a proposal for a more comprehensive taxonomic scheme in 1921 in which he kept morphological characters as a part of the system along with physiological characters (Orla-Jensen 1921). It was more "natural" in the sense that, in his opinion, it was more logical than that proposed by the Committee of the Society of American Bacteriologists. His background in chemical engineering is apparent in his system as he seems to want to approach the logic of the nomenclature system adopted by chemists for chemicals. It was not a "natural" system as usually expressed by

Fig. 2.5 (l to r) Israel J. Kligler; Albert J. Kluyver, 1921. (Kligler: https://en.wikipedia.org/wiki/Israel_Jacob_Kligler; Kluyver: https://en.wikipedia.org/wiki/Albert_Kluyver)

biologists in being a reflection of the relations among organisms based on their evolutionary descent.

Other systems, like that of microbiologist Israel J. Kligler, made an honest effort to involve bacterial evolution in their schemes (Fig. 2.5). Kligler's 1917 proposal tried to arrange organisms in the order in which he posited that they had appeared based on ideas from the time about the origin and early history of Earth (Kligler 1917) (Fig. 2.6). He proposed that the gases available at that time would serve as the simplest food sources for the first organisms, methanomonas, carboxymonas, and oxybacteria, living on methane, carbon dioxide, and oxygen, respectively. Next, organisms using more complex molecules would have appeared. He reasoned that since carbon is the most important nutritional element for modern microbes, then organisms that use organic carbon sources were the next to appear. Since many current bacteria can use sugars, he thought those bacteria would be saccharolytic. Next, bacteria using amino acids would arise, as inorganic nitrogen sources would become limiting through their use by earlier bacteria. He continued his speculations using other criteria to develop an evolutionary scheme for all known bacteria. His scheme came under criticism, but the criticisms had no more experimental basis than his did (Kligler 1918).

In 1905, Albert J. Kluyver enrolled as a student at the Polytechnical School in Delft, then renamed the Technological University, where Beijerinck had established a laboratory which later was incorporated into the chemistry department (Fig. 2.5). Kluyver studied and received a degree in chemical engineering and took a position with Gerrit van Iterson Jr., a former student of Beijerinck, who now supervised a second biology division in chemistry. Here Kluyver worked on yeast fermentations leading to a D.Sc. degree in 1914. When Beijerinck was forced to retire in 1922 due to his age, Iterson recommended his student Kluyver to assume Beijerinck's professorship in general and applied microbiology.

Kluyver's interests were not in areas of biology relevant to evolution, not surprising given his chemical engineering background. Instead he focused on the potential of microbes to contribute to the production of chemicals resulting from their catabolic activities. He realized, even then, the limiting supply of fossil fuels

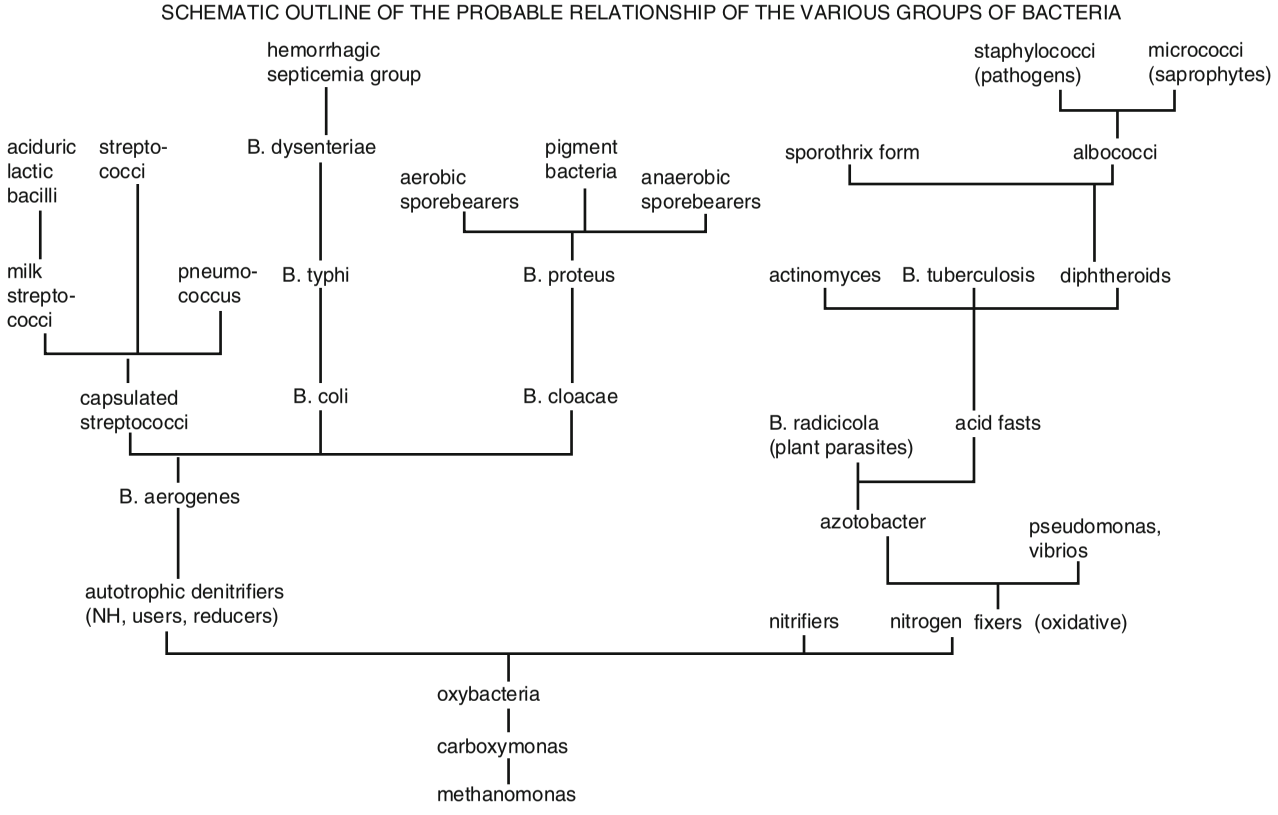


Fig. 2.6 Kligler's 1917 suggestion of the relations among some of the bacteria known at the time. The bacteria started with organisms living on gases thought to be available on the early Earth and continue with increasing use of organic carbon and nitrogen sources. Later radiations are based on increasing morphological complexity. (Reproduced from Kligler 1917)

and the need to seek other sources of industrial chemicals, becoming, perhaps, the first "green chemist" (Van Niel 1957). This interest led to studies of the comparative biochemistry of catabolism in many bacteria and an appreciation for not only the diversity of catabolisms but also the unity of biochemistry throughout the biological world.

Though he did not entertain thoughts about evolution, per se, he noted in his 1922 inaugural address the unsatisfactory state of bacteria classification. He later realized that classifications had been developed in a haphazard state with different investigators having different goals and no guiding principles. He decided that perhaps by judiciously choosing morphological and biochemical characteristics, groups above the species level could be discerned. He formulated some general principles of catabolic properties among the organisms that he studied and tried to create a more rational classification scheme. He published an outline of such a system in 1936 along with his student Cornelis B. van Niel (Kluyver and Niel 1936).

Kluyver famously wrote, "From elephant to butyric acid bacterium — it is all the same." The unity of biochemistry that he recognized is in line with Darwin's idea that all life came from a common ancestor. In their 1936 scheme, van Niel and Kluyver attempted to create a "natural" system for taxonomy of bacteria as opposed to the developing "practical" (i.e., Bergey's) taxonomies that were made by those who thought "idealist" taxonomies were impossible, so "realistic" ones were needed (Singleton Jr and Singleton 2017; Stanier and van Niel 1941). They wrote:

> . . .the only truly scientific foundation of a classification is to be found in an appreciation of the available facts from a phylogenetic point of view. Only in this way can the natural interrelationships of the various bacteria be properly understood. It has to be admitted that, inasmuch as the course of phylogeny will always remain unknown, the basis of a true phylogenetic system of classification will be very unstable indeed. On the other hand it cannot be denied that the studies in comparative morphology made by botanists and zoologists have made phylogeny a reality. Under these circumstances it seems appropriate to accept the phylogenetic principle also in bacteriological classification. (Kluyver and van Niel 1936)

They believed that the way organisms meet their energy needs, catabolism, should rank as the first physiological trait. However, they also held that morphology is the evolutionarily defining trait and so should be the premier trait to keep the whole scheme phylogenetic. Morphological traits included spore formation, mode of reproduction, flagella, and Gram stain. Physiology was secondary to distinguish groupings within the morphological units. Physiological traits were energy source, use of oxygen, catabolic substrates, and mode of their decomposition (products). The use of different oligosaccharides was not a trait to be considered as this property was thought to be devoid of energetic significance. They thought it could be used to distinguish species, but not higher units.

Van Niel went to the Hopkins Marine Station of Stanford University in 1938 and soon established a summer course in general microbiology that became a highly influential training ground for microbiologists. A new University of California, Berkeley undergraduate, Roger Stanier, enrolled in its first class and found his calling (Fig. 2.7). He enrolled in the following year, too, choosing the first part of

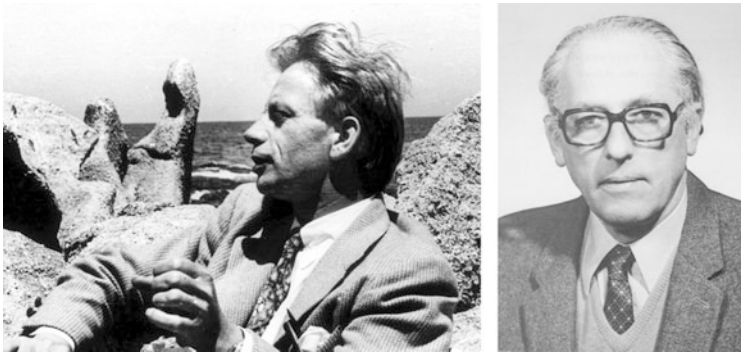


Fig. 2.7 (l to r) Cornelis B. van Niel at a summer course at the Hopkins Marine Station; Roger Stanier (van Niel: Image appears with the permission of the Delft School of Microbiology; Stanier: https://en.wikipedia.org/wiki/Roger_Stanier)

the summer program on morphology and taxonomy (Stanier 1980). He spent a third year at the Station and, using his new-found interest in taxonomy and his relationship with van Niel, he co-authored a paper on taxonomy with him in 1941.

The use of Kluyver's comparative biochemistry findings to gain insight into Darwin's early ancestors through a "natural" system of taxonomy soon failed. In 1941 Stanier and van Niel made a classification exclusively using morphological criteria and abandoned the use of biochemical traits as criteria. They did so because a physiology-based system necessitates making many highly speculative assumptions as to what constitute primitive and advanced metabolic types as well as unsupported speculations about the state of early Earth conditions. This problem haunted such systems for decades to come causing evolutionary studies to be largely dismissed by most microbiologists, thus beginning the "Dark Age" (below).

By 1946 van Niel gave up on creating a phylogenetic scheme altogether (van Niel 1946). He capitulated to the "realist" doctrine and created a determinative taxonomic scheme. He recognized that morphology in plants and animals is associated with reproduction and so their taxonomies based on morphology carry phylogenetic value. This is not so in bacteria where their morphology is unrelated to their mode of passing traits on to progeny and so cannot carry evolutionary information (Cohan 2002). He knew that groupings based on morphology sometimes create undesirable polyphyletic groupings, but in the absence of true evolutionary characters, true bacterial phylogenies would be very hard to determine. Nevertheless, he did not dispair of the need for a bacterial phylogeny and stated, "However, . . . the search for a basis upon which a 'natural system' can be constructed must continue."

In 1962 Stanier also abandoned the earlier effort at a natural system of taxonomy for bacteria, and he published, along with van Niel, a paper stating that they could no longer defend their 1941 effort (Stanier and van Niel 1962). They argued that, as a practical matter, microbiology needed a definition of a bacterium. Stanier noted later, "One of the intellectual scandals of general microbiology was an absence of a clear definition of the bacteria. . . .I was deeply puzzled about this" (Stanier 1980). They

acknowledged that most of the defining characters of bacteria were negative and so were of no phylogenetic value. The new technique of electron microscopy had, however, clearly shown clear differences between bacterial cells and those of protists and yeasts. This solved the long-standing dispute about the true relationship of blue-green bacteria (later cyanobacteria) to nonphotosynthetic bacteria and algae. These authors argued that the terms prokaryote and eukaryote, adopted from Édouard Chatton, should be used to distinguish these two types of cellular life. Although they did not seem to ascribe any evolutionary significance to this grouping, they did mention that the two groups likely had separate evolutionary histories, implying an evolutionary coherence within each lineage, a notion later proven false.

Stanier later confessed that he was not proud of his 1941 paper (Stanier 1980). He had persuaded van Niel to put his name on it, which was unlike van Niel to do. Stanier considered that work to be "the last gasp (at least in bacteriology) of speculative 'phylogenetic' system-building before molecular biology took over and brought some solid facts to the assessment of evolutionary relationships." Stanier was a bit harsh on himself in this remembrance. In 1941 he was a 25-year-old student, only a few years into the study of microbiology, so the speculations of an excited, naive student can be forgiven.

Others continued the challenge of puzzling out the early evolution of life, using sometimes creative reasoning. Geneticist Norman Horowitz, who had shown that biochemical processes occurred in pathways of enzymes each encoded by single genes, proposed in 1945 that pathways had evolved in the reverse direction of their current activity (Horowitz 1945). He did not, though, link this evolutionary process to any organisms and so did not contribute to the search for bacterial evolutionary relationships. In the same year, forensic chemist Erik M. P. Widmark said he did not believe that chemicals could have come together to form living matter, what he considered the disproven notion of spontaneous generation (Widmark 1945). Instead, reviving the spirit of Lamarck fully (though not acknowledging it), he said that life arose through a manifestation of a "vital principle." He went on to state that, "Evolution, if such exists, has for its sole object the creation of the ideal vital substance." Microbiologist Darryl C. Reanny wrote an unusual treatise on bacterial evolution that posited that bacteria arose from a eukaryote-like ancestor that lost genes through genetic streamlining (Reanney 1974).

2.7 The "Dark Ages" of Bacterial Evolution Studies

Carl Woese named the state of mid-twentieth-century microbiological science as the "Dark Ages" of bacterial evolution studies (Woese 1994). Although this is an exaggerated characterization, it is true that this was a time that serious study of bacterial evolution diminished to short musings in the Discussion sections of research articles about how the biochemical phenomenon of the article might have arisen during evolution. There were some who still wrote longer treatises on the evolution of specific microbial traits or process. Engelbert Broda was prolific in this,

writing articles about the appearance and evolution of photosynthesis, nitrogen fixation, and respiration (Broda 1970, 1975a, b; Broda and Peschek 1979).

In parallel with traditional microbial physiology and biochemistry research, the field of molecular biology was born and flourished along with the "Golden Age" of bacterial genetics. Neither of these fields seemed to bother with examining how evolution played any role in their respective research topics, but this is perhaps understandable given the state of efforts to determine natural relations among bacteria. In their training, these researchers would not have been exposed to any bacterial evolution research since it was floundering at that time. Contrary to Woese's suggestion (Woese 1994), those studying microbial physiology still did take an organismal view of their research and sought to relate the biochemical physiology of their research subjects with their roles in nature. Relating their findings to the evolution of their subjects had been shown to be a fruitless effort and not amenable to experimental verification. In regard to those who attempted to study bacterial evolution, to paraphrase Ralph Wolfe who studied the physiology of methanogens, "the heat of the [evolutionary] arguments was inversely proportional to the soundness of the data," and so not worth pursuing.

During these years, two apparently unrelated streams of research were appearing that would go on to have a major impact on the resurgence of interest in microbial evolution later. First, technological advances allowed for the sequencing of the amino acids in proteins. These studies were done as part of efforts to examine the structure and function of proteins. Sequences of hemoglobins, insulin, and cytochromes c appeared in the literature.

Cytochrome c was of particular use in recognizing evolutionary relationships since it was the most widely distributed of these proteins, found from vertebrates to bacteria. At first, only the amino acid composition of horse heart and yeast cytochromes c was determined and comparison of those compositions was found to be very similar, and this was thought to perhaps have evolutionary significance (Nunnikhoven 1958). Later full amino acid sequences of cytochromes c were determined, and the first of such publications to suggest that this information could be of use to address evolutionary questions was published by Emanuel Margoliash (Margoliash 1963). He used sequences of cytochromes c from horse, man, pig, rabbit, chicken, tuna, and yeast, the most extensive collection yet assembled. He realized that the sequence similarities of these proteins must be the result of evolution from a common primordial cytochrome c and that convergent evolution was improbable. He stated that they were homologous in the evolutionary sense. Sounding like Darwin, he wrote, "If, as seems likely, this conclusion can be extended to a large variety of vertebrate and invertebrate species, as well as to the plant kingdom, strong support would be obtained for the speculation that living matter was effectively formed only once, within the confines of our planet, all living forms deriving from a common precursor." Although he did not attempt to draw any sort of evolutionary tree, he was aware that such relationships could be derived from these data. He said, "It should be noted that the present results are compatible only with the commonly accepted scheme of evolution represented by series of branching lines, and are not consistent with a simultaneous formation of all species, which then

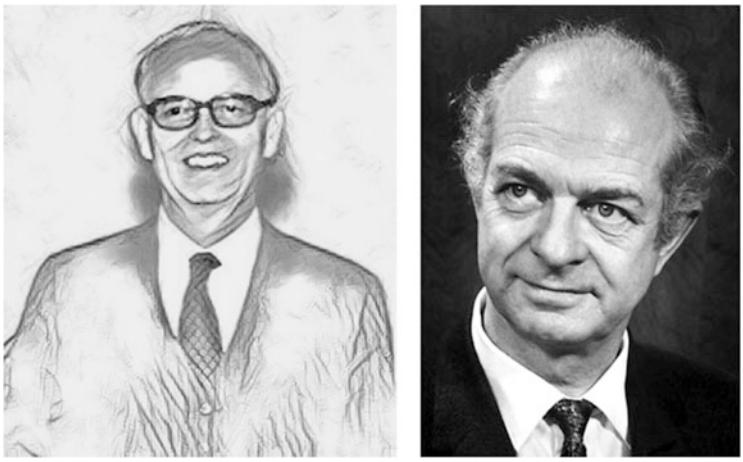


Fig. 2.8 (l to r) Émile Zuckerkandl, 1986; Linus Pauling, 1962 (Zuckerkandl: https://en.wikipedia.org/wiki/Emile_Zuckerkandl; Pauling: https://en.wikipedia.org/wiki/Linus_Pauling)

proceed to accumulate mutations independently. In the latter case all the cytochromes c should be equally different from all others."

Cytochrome c played an interesting role later in one of the first suggestions of horizontal acquisition of genes. A 1979 report by Richard Ambler touched on the issues of interspecies gene sharing and the evolutionary deficiency of determinative bacterial taxonomies. Ambler was first author on a study that indicated that genes encoding cytochromes c in the purple nonsulfur photosynthetic bacteria had been shared among different lineages so that their relations to one another did not match the relations among the organisms that encoded them as determined by Bergey's Manual (Ambler et al. 1979). This conclusion was later refuted by both Richard Dickerson and Woese in contiguous articles in Nature in 1980 (Dickerson 1980; Woese et al. 1980). They found that grouping these cytochromes c by their structural features did align with the grouping by their corresponding organism's 16S rRNA sequences, a better phylogenetic marker, and so interspecies gene transfer was not evident.

In 1965 Émile Zuckerkandl and Linus Pauling published an article that expanded on an earlier publication of theirs (Zuckerkandl and Pauling 1962) by defining "semantides," molecules that carry the information of the genes or a transcript thereof (Zuckerkandl and Pauling 1965) (Fig. 2.8). The genes themselves were the primary semantides, and proteins were tertiary semantides. Although they did not state it, perhaps because the role of rRNA was only becoming known, rRNAs would also be primary semantides since they are direct copies of genes, just using slightly different nucleotides. Semantides contain information relevant not only to biological function but also to the history of the genes themselves. They wrote "...the most rational, universal, and informative molecular phylogeny will be built on semantophoretic molecules alone." The article was primarily written about how protein sequences could be used to discern phylogenetic relationships. The authors

note the deficiency of using protein sequences since, due to the degenerate code, they do not directly contain the whole evolutionary history of their genes. They may not have seriously considered nucleic acid sequences because, at that time, such sequences could not be obtained.

That all changed the same year. Frederick Sanger published a report in 1965 of a method to sequence "fingerprints" of rRNAs from *Escherichia coli* and yeast (Sanger et al. 1965). Stable RNAs extracted from cells were digested with ribonuclease T1 and the resulting oligonucleotides resolved by paper electrophoresis. These oligonucleotides could then be extracted from the paper and digested with ribonuclease and phosphodiesterases and the digests again resolved by paper electrophoresis. From the migrations of these products, the sequences of the oligonucleotides could be determined. Although a sequence of the entire rRNA molecules could not be determined, it did provide a "fingerprint" of the molecule that allowed comparisons between molecules to be made. The method also provided the basis for further technological development of sequencing methods. Despite the fact that DNA would contain the information most useful to evolution studies, DNA sequencing was out of the question given the size of chromosomal DNA molecules.

Interest in using protein sequences for phylogenetic analyses diminished once nucleic acid sequencing began and proved superior for discerning evolutionary relationships. Margaret Dayhoff began her sequence comparison work using protein sequences, including those of cytochrome c. She also included ferredoxin sequences that allowed her to include members of bacterial and eukaryotic lineages. When nucleic acid sequencing became possible, she took up sequencing rRNAs and tRNAs to extend her analyses and was among the first to do so (McLaughlin and Dayhoff 1970; Schwartz and Dayhoff 1981). Dayhoff was the first to compile protein sequences in a single place, at first a printed version of the *Atlas of Protein Sequence and Structure* and later as a computer database stored on magnetic tape.

Another development necessary for future phylogenetic analyses was the creation of algorithms to construct phylogenetic trees from sequence information. Walter M. Fitch and Emanuel Margoliash devised such a method in 1967 (Fitch and Margoliash 1967). Using cytochrome c sequences, they constructed a phylogenetic tree that largely agreed with eukaryotic phylogenies. The only microbes included in their analysis were yeast species and those diverged from one another in a lineage at the base of the eukaryotic tree, as was expected. No bacteria were analyzed. This development was important in providing an objective, quantitative way to compare characters, in this case semantide sequences, replacing the examination by eye of characters (morphologies or biochemical characters) of earlier phylogenetic efforts.

Dayhoff was among the first to publish phylogenetic trees constructed using molecular sequences (Dayhoff 1969). Using composite trees that combined comparisons of the sequences of several different kinds of macromolecules, she was able to confirm the evolutionary relationships among a small group of bacteria and the relationship of chloroplasts and mitochondria to those bacterial lineages (Schwartz and Dayhoff 1978). Later, after the discovery of archaebacteria, these could also be included in her analyses. Her analysis of those indicated that the two archaebacteria that she included, *Halobacterium* and *Thermoplasma*, were more closely related to the cytoplasmic line of eukaryotic sequences and so did not define a separate

phylogenetic lineage, as Woese had recently proposed (Barnabas et al. 1982). However, later genome sequencing showed that these two lineages share many closely related genes separate from their bacterial analogs, suggesting the cytoplasmic aspect of eukaryotes might have arisen from an archaeal ancestor (Spang et al. 2015). It is sad to realize that Dayhoff passed away shortly after that article was published. Had she lived to pursue this apparent discrepancy in her and Woese's findings, perhaps she might have spearheaded analyses that would have discovered the link between archaeal and eukaryotic evolution earlier and so have led to greater insights about this important question than we now have. She was already showing an interest in this question as evidenced by a posthumous article about bacterial and eukaryotic evolution (Hunt et al. 1984).

Those interested in microbial evolution now had a method, nucleic acid sequence comparisons, to read Darwin's "written pedigrees." Consequently, Darwin's dream had been realized:

> The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature. (Darwin Correspondence Project Letter no. 2143 2020b)

2.8 Macromolecular Sequencing Revives Darwinian Influences

The stage was now set to bring Darwinian ideas and principles back to the study of bacterial evolution. Except for a scattering of efforts using various protein sequences and the speculative articles about the evolution of microbial processes mentioned above, major advancement in the field at this time was largely through the efforts of Carl Woese (Fig. 2.9).

Woese was by training a biophysicist. While at Yale and the General Electric Research Laboratory, he became interested in the nature of the genetic code (Woese 1962) and, after moving to the Department of Microbiology at the University of Illinois, the evolution of the code (Woese 1965). This naturally led to interest in its decoding by translation, both prebiotic (Woese 1968a) and biotic (Woese 1968b).

His interest in the evolution of the translation apparatus led him to examine 5S rRNA sequences, basing this on the work of Zukerkandl and Pauling and Fitch and Margoliash cited above, among others (Sogin et al. 1972). Complete sequencing of rRNAs was impossible, but he realized that oligomer cataloging, as described above, might suffice. He was familiar with this technique as his departmental colleague, Saul Spiegelman, in 1968 set up Sanger's system of RNA fingerprinting for use in his studies of RNA viruses (Sapp 1979). Woese found that this method allowed him to detect relationships at least to the level of intra-family relations. This study already challenged existing phylogenetic schemes, suggesting that the Pseudomonadaceae family was misplaced. Interestingly, the article concludes by saying that this method is simple and could be used by any laboratory interested in studying bacterial phylogeny. It does not seem to have convinced anyone as virtually no labs did

Fig. 2.9 Carl Woese, 1976, Woese examining isopleths of 16S rRNA; in-set, Woese examining DNA sequences of 16S rRNA genes in the early 1980s (Woese, 1976 photo: https://distributedmuseum.illinois.edu/exhibit/carl-woese/ with permission of Kenneth Luehrsen; in-set photo: https://archives.library.illinois.edu/gallery/index.php?album = cs/FacultyPortraits&image = Woese_Carl_7.jpg. Courtesy of the University of Illinois Archives)

so. Woese unfairly complained later (Woese 1994) that such sequencing methods were largely overlooked by the microbiology community, but he does not consider that few were interested in the subject at that time for the reasons described above. Whole generations of researchers were not exposed to evolutionary thinking in their work with bacteria. Those who perhaps were still interested, like Stanier, were in the later stages of their careers and had moved on to other research topics and so were unlikely to adopt a new, unfamiliar method at that point in their careers.

Woese later adapted RNA cataloging to the 16S rRNA molecule since it provided more reliable classifications than the 5S molecule and was more manageable than the 23S molecule. Sequences of oligonucleotides could be determined by examining oligonucleotide "fingerprints" on orthogonal electrophoresis papers and then those sequences compared to show evolutionary relationships (Fig. 2.9). In a 1977 study, he showed the technique could provide a taxonomy for selected *Bacillus* species (Fox et al. 1977).

In a study published later in 1977, Woese reported results that would soon place microbial evolution studies more firmly on a Darwinian path. He applied his cataloging method to the 16S rRNAs of two methanogen species and found, unsurprisingly, that they were closely related to one another (Balch et al. 1977). However, he also discovered that they were only distantly related to enteric bacteria, *Bacillus* species, and cyanobacteria species. He suggested they diverged from these

bacteria very long ago, though he could not place a time on the divergence. He had, to that point, cataloged about 40 species of "bacteria," and these methanogens were the only representatives that showed such a divergence. Hori had examined the sequences of 5S rRNA from many bacteria a year before, but methanogens were not among those he examined and so he did not observe any deep divergences among the bacteria he examined (Hori 1976). Woese was fortunate in this respect in having the laboratory of Ralph Wolfe nearby in his department. Woese had little experience in growing microbes, and his lab was not set up to cultivate any exotic organisms. It was only because one of Wolfe's graduate students, William E. Balch, was interested in working with Woese that methanogens were among the organisms that he examined, yet another example of the sometimes serendipitous nature of scientific discoveries.

Shortly thereafter, George Fox and Woese included two more methanogens in their analysis, along with yeast, duckweed, and human 18S rRNAs, to show that life was comprised of three major lineages, or urkingdoms (primary kingdoms), that they named eubacteria (true bacteria), archaebacteria (suggesting an ancient form of life, perhaps predating bacteria), and urkaryotes (ancestor of modern eukaryotes) (Woese 1977). This finding upended the commonly held notion that prokaryotes were a monophylogenetic unit. However, even those, like Stanier, who had thought seriously about it were aware that this notion might not be true.

Later extremely halophilic and thermoacidophilic microbes were found to be archaebacteria, too (Woese et al. 1978; Magrum et al. 1978). As more sequences were analyzed, it was found that the archaebacterial lineage was comprised of two major subdivisions, the thermoacidophiles and a group including the halophiles and methanogens (Fox et al. 1980). The relationship among the three urkingdoms was not clear until the sequences of duplicated genes were compared and the root of the small subunit rRNA tree was found to lie between the bacteria and the combined archaebacteria and eukaryote lineages (Gogarten et al. 1989; Iwabe et al. 1989). The two lineages within the archaebacteria were later named the Crenarchaeota (suggesting the ancestral type of archaebacteria) and Euryarchaeota (denoting their broad spectrum of ecotypes), respectively (Woese et al. 1990). The three urkingdoms were now called domains (a taxon above kingdom) and renamed Eucarya (now Eukarya), Bacteria, and Archaea.

Woese's work appeared to have finally provided a "natural" classification for not only microbes but all life. Ribosomal RNA trees have been used as the basis for a universal tree of life (Maddison et al. 2007; http://tolweb.org/tree/). The tree has allowed investigators to begin examining the evolution of metabolic and genetic processes, using it as the basic framework on which to place evolutionary events. Elucidation of a more solid phylogeny certainly brought the study of microbial evolution back to being a respectable science.

The validity of the rRNA phylogeny was questioned from the start. Not including objections to the tree based largely on earlier notions of evolutionary history (Woese 1994; Woese and Goldenfeld 2009; Sapp 2005), the phylogeny was not congruent with those based on RNA polymerase sequences (Klenk et al. 1993) nor indel analyses (Lake et al. 2008). Indels are short DNA sequence polymorphisms that correspond to either the addition or removal of a small number of nucleotide bases.

Structural elements of ribosomes were used to argue that the crenarchaea are most closely related to eukarya and so constitute a kingdom, to be called the Eocyta, separate from the euryarchaeotes (Lake et al. 1984). None of these alternative trees has been as well supported by subsequent research as the original three-domain tree of Archaea, Bacteria, and Eukarya.

Ironically, the suggestion by Ambler of interspecies gene transfer in 1979 came to be accepted, if not for the particular instance cited in that work. When complete genome sequences of bacteria and archaea became available, evidence of horizontal or lateral gene transfer was obvious. There had been evidence for this prior to genome sequencing, but it was largely found in scattered publications about single genes. Certainly horizontal acquisition was known from studies of gene transfer processes like conjugation, transduction, and natural DNA uptake, but the evolutionary impact of those processes was not revealed by those studies. Widespread DNA sharing among distant relatives complicated simple depictions of evolutionary history as phylogenetic trees. Other depictions were proposed that attempted to incorporate gene sharing, and these include the New Rings of Life (Lake and Sinsheimer 2013) and the coral of life (Fournier et al. 2009). The ability to even make phylogenetic trees is frequently called into question given the impact of gene sharing (Doolittle and Brunet 2016). Darwin's hope that "...ramifying branches may well represent the classification of all extinct and living species in groups subordinate to groups" (Darwin 1959) may ultimately prove fruitless in the case of microbes. Complicating the effort further, recent discoveries of new organisms through metagenomic sequencing have revealed new, uncultivated lineages including members of the Archaea that contain genes that make them even more closely related to those of Eukarya. Consequently, the three-domain view of life may change to a two-domain view (Doolittle 2020) as Dayhoff's final studies had suggested earlier.

2.9 Laboratory Evolution Studies Directly Test Darwinian Principles

On the Origin of Species was published at the very birth of microbiology as a discipline. Pasteur was demonstrating that bacteria were the agents of ferments and not chemical processes. He was also showing that microorganisms did not spontaneously generate in broths and infusions. He showed the importance of microbes in the fermentation industries which led the way to the organization of institutes, companies, and, eventually, academic departments formed to support these industries. Those applied efforts did not directly utilize Darwin's insights. Nor did work directed to the medical and public health aspects of microbiology, though Darwin was familiar with Koch's work. It was left to those interested in microorganisms as living organisms to seek evidence of Darwinian attributes manifested in the microbial world.

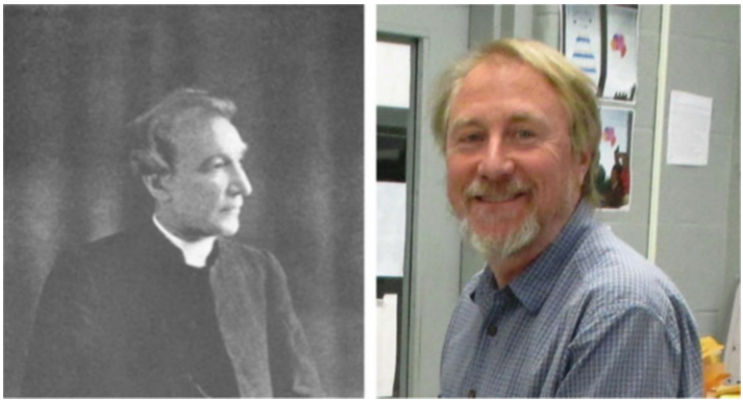


Fig. 2.10 (l to r) William Henry Dallinger; Richard Lenski (Dallinger photo by Edgar Herbert Thomas, https://en.wikipedia.org/wiki/William_Dallinger from Transactions of the American Microscopical Society 29:183-186. 1910; Lenski: https://en.wikipedia.org/wiki/Richard_Lenski)

One of the earliest of these pioneers was William Henry Dallinger, a Methodist minister and naturalist (Fig. 2.10). He was fascinated by microscopy since childhood and developed this interest more in his later life (Haas Jr 2000). At the time of Pasteur's work, he took note of the question of spontaneous generation. He described his work on this topic in 1869 at a meeting of the Liverpool Microscopical Society at which he said that this question could only be settled by "a careful examination of the lowest and minutest forms of life (Report 1869)." A debate about spontaneous generation soon emerged between medical microbiologists and biologists studying microbes. The former were addressing the problem of apparent different forms that a species of microbe could assume depending upon its cultivation conditions and its growth in a host. Some thought these forms resulted from the formation of the organisms from inorganic matter (Bastian 1872). Biologists did not accept this explanation and demanded solid evidence for this claim (Haas Jr 2000).

Dallinger was one who sought to test this proposal and reported that his studies of the monad *Bodo saltans* (now the euglenoid protozoan *Pleuromonas jaculans*) passed through seven metamorphoses in its life cycle. This study demonstrated that one must follow the entire life cycle of microbes before drawing conclusions about their origin. Bastian, like other proponents of spontaneous generation, failed to do such detailed studies. The spontaneous generation question frequently arose in criticisms of Darwin's theory because he claimed that all life arose from inorganic matter at its origin. Darwin's supporters sought to distance this unique event in the history of life, and, consequently, Darwin's theory, from the increasingly discredited claims that spontaneous generation proponents continued to put forward. Though Dallinger did not believe that spontaneous generation occurred now, he said that "as a minister of the Gospel, he had no fear of spontaneous generation . . . [but] the facts at hand were not sufficient to argue that life came from the non-living (Report 1869)." Dallinger agreed with Darwin that the question of how life originated was beyond the purview of science.

Dallinger engaged in long experiments watching various microbes through all their developmental stages. Like Ferdinand Cohn, his contemporary, Dallinger believed that one should observe the developmental cycle of an organism to demonstrate that species were stable. He was assisted in this work by his friend from the Liverpool Microscopical Club, the physician John James Drysdale. Dallinger corresponded with Darwin about his work, and Darwin wrote back saying:

> Allow me to add that I have read all your [Dallinger] & Dr. Drysdale's papers, & they seem to me to possess higher value than anything that has been published on such subjects, though I am too ignorant to have any right to express such an opinion. But I have a full right to say that they are extremely interesting. (Darwin Correspondence Project Letter no. 10354 2020d)

In 1878 Dallinger began preliminary studies to address the question whether it is possible to "superinduce" adaptive changes in a microbe by increasing the temperature of its environment. His efforts predated slightly later studies, described above, by Beijerinck. Dallinger did not believe it was necessary to provide a direct demonstration of Darwin's "great law," but he felt that, "if it be possible to look upon the progress of changes in minute living organisms, superinduced by elected changes of environment, however, simple, and which results in morphological and physiological adaptations and survivals, it cannot be other than a gain both to philosophical and practical biology" (Dallinger 1887a). He wrote to Darwin describing the results of this preliminary study and Darwin replied:

> I did not know that you were attending to the mutation of the lower organisms under changed conditions of life; and your results, I have no doubt, will be extremely curious and valuable. The fact which you mentioned about their being adapted to certain temperatures, but becoming gradually accustomed to much higher ones, is very remarkable. It explains the existence of algae in hot springs. How extremely interesting an examination under high powers on the spot, of the mud of such springs would be. (Dallinger 1887a citing a Darwin letter dated July 2, 1878)

Encouraged by Darwin, Dallinger then undertook a 7-year study of the adaptation of the flagellated algae *Tetramitus rostratus*, the protozoan *Monas dallingeri*, and the protozoan *Dallingeria drysdali* to increasing temperatures in a specially constructed thermal bath (Fig. 2.11). He found he could increase their temperature of growth from 69 °F (20.5 °C) to 158 °F (70 °C) by small increases in temperature over several years. Tragically, the experiment ended through an accident that occurred while he was away. He was not convinced that he had achieved the highest temperature that he could and, following an understandable period of depression over the incident, he finally decided to repeat the work (Dallinger 1887a). His initial results proved Darwin's contention that organisms could adapt to changing conditions. Further, since the organisms that had adapted to the highest temperature could no longer grow at the original temperature, he showed that they had undergone a permanent change, much like Darwin predicted should happen due to natural selection. Dillinger had, for the first time, demonstrated Darwin's theory of Natural Selection.

Unfortunately, it seems that he did not carry the second effort to completion. He apparently abandoned the effort and then took up work on technical aspects of

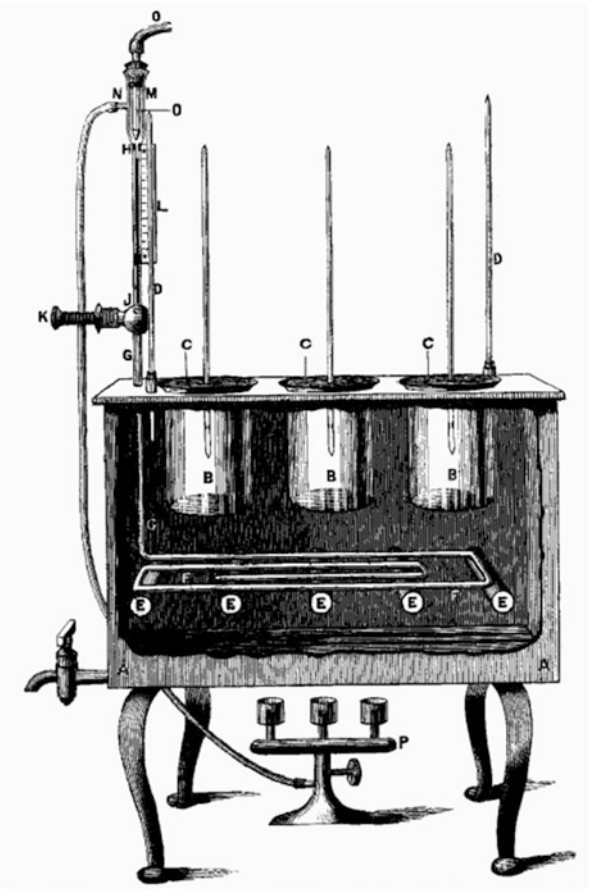


Fig. 2.11 Dallinger's temperature-controlled incubator for his 7-year cultivation of microbes at increasingly higher temperatures (https://en.wikipedia.org/wiki/William_Dallinger)

microscopy, his first love (Haas Jr 2000). He concluded his 1887 President's Address to the Royal Microscopical Society by saying about his 7-year thermal adaptation experiment:

> I can only claim for this fragment its suggestiveness, and its possible value as an incentive to others to treat the lower and minuter forms of life in corresponding manners, and as showing that such work cannot be without value. (Dallinger 1887b)

No one seems to have been incentivized by his work, however, and this kind of prolonged laboratory evolution study to address aspects of Darwin's work was not taken up again for almost 100 years.

Lamarck's view of the inheritance of acquired characters continued to be held by many biologists at the end of the nineteenth century. It was not until the rediscovery of Gregory Mendel's work that those ideas were largely abandoned. But not so among those interested in bacterial evolution. Dallinger's study and those of others showed that bacteria could adapt to new conditions and display new characteristics as a result. It was not clear whether this resulted from the appearance of a variant in the population

of microbes (a mutation) or if the conditions caused the new characters to appear in the cells of the population (the Lamarckian explanation). In 1934 I. M. Lewis summarized reports of experiments on *Bacillus coli mutabile* and concluded:

> The subject of bacterial variation and heredity has reached an almost hopeless state of confusion. Almost every possible view has been set forth and there seems no reason to hope that any uniform consensus of opinion may be reached in the immediate future. There are many advocates of a Lamarckian mode of bacterial inheritance while others hold to the view that it is essentially Darwinian. The early workers regarded variation in so-called mutabile strains as mutation in the sense of DeVries. Some more recent workers have explained this behavior as due to Mendellian segregation, while others have regarded it as evidence of a cyclogenic life history. (Lewis 1934)

Salvador Luria and Max Delbrück solved the dilemma in 1943 by publishing a study that showed that mutations occurred in populations of *Escherichia coli* before selection was applied to the cultures, thus demonstrating that Lamarckian evolution was not occurring. This work spurred a modest increase in interest in examining the genetic aspects of bacterial evolution through laboratory culturing (Atwood et al. 1951; Novick and Szilard 1950). This ironically occurred at nearly the same time that those who had long-standing interests in bacterial evolution were giving up hope of ever finding characters that would allow one to trace evolutionary events.

Lab evolution of bacteria was used for many years to evolve strains to improve or create enzyme activities or to enhance the ability of cells to catabolize new substrates or detoxify environmental pollutants (Hegeman and Rosenberg 1970; Liu and Suflita 1993; Campbell et al. 1973). Those studies, however, did not attempt to test or apply Darwinian principles.

Dallinger's challenge was finally taken up by Richard Lenski (Fig. 2.10). Lenski conducted his postdoctoral research examining genetic diversity of aphids (Service and Lenski 1982). Later, he looked for an experimental system that would allow more direct examination of evolutionary principles, and he hit upon using bacteria, specifically *E. coli*. He published several papers about the genetic changes necessary to elicit the appearance of phage-resistant mutants in a population, evolution in response to thermal stress, and gene stability. He developed an interest in the course of evolution in a population. How quickly do traits change, are the patterns of change repeatable, what is the effect of contingency on the mode of evolution? He realized that by maintaining cultures of *E. coli* for many generations, he could perhaps observe changes in the population and, so, address these questions (Lenski 2011). In February 1988, he started 12 cultures growing in a glucose-limited defined medium that he transferred daily after each had exhausted its glucose. Each daily cultivation provided six to seven generations, and he originally planned to grow the cultures for about 2000 generations, or less than a year. This Long-Term Evolution Experiment (LTEE) has now been running for over 32 years and has produced over 70,000 generations (Good et al. 2017; Lamrabet et al. 2019).

Cultures in the LTEE have unexpectedly shown great similarity with one another in their increases in fitness (defined as ability to outgrow the parent culture) and have generally improved quickly and then slowed in later generations (Lenski 2011). The size and shape of cells changed along with their preference for carbon sources.

Similar genes had mutated in the different cultures, but not always by the same mutations (Blount et al. 2018). Six populations even developed mutator phenotypes, which, in some cases, later reverted (Good et al. 2017). The later invention of genome sequencing allowed more thorough examinations of the extent and course of mutation pathways in the populations. Examining the frozen stocks of past generations allowed his group to delineate the course of evolution to a degree never before seen. He compared this to having a complete fossil record of a lineage, a dream of Darwin's that had now come true (Lenski and Travisano 1994).

One of the most important developments of the LTEE was the evolution of a strain that could grow on citrate, a carbon source that *E. coli* strains typically do not use because they lack the ability to transport it into their cells. Citrate was being produced by the cells in the cultures. This strain showed a diminished ability to use glucose, so it could evolve independently of its parent strain (Blount et al. 2008). Some have contended that this gave the first example of seeing a new species evolve.

Shorter cultivations can also yield important insights into evolutionary processes. In 1998 Paul Rainey and Michael Travisano examined the phenomenon of adaptive radiation to determine what mutational events underlay sudden adaptations to fill new niches (Rainey and Travisano 1998). Using an extraordinarily simple system to create different niches, they inoculated a culture of *Pseudomonas fluorescens* into small flasks of liquid medium and simply left them unshaken. The cells rapidly consumed the dissolved oxygen in the medium setting up an oxygen gradient in each flask. Cells growing dispersed in the medium differed from those growing in mats on the surface. *P. fluorescens* was chosen for these experiments because it shows differences in colony morphology that are correlated with their niche preference, specifically based on oxygen availability (Rainey et al. 1993). Their work showed that the heterogenous environment provided competition that drove adaptive radiation.

The spectrum of mutations available to a population to allow evolutionary divergence has been shown to be a function of contingency, evolutionary history, and the environment (Blount et al. 2018; Maharjan and Ferenci 2017). All of these influences had been envisioned by Darwin as important factors in evolution.

Darwin thought that the question about how life started was beyond the purview of science and was the basis of his contention that he could not call himself an atheist, but, instead, an agnostic, a term coined by his friend Thomas Henry Huxley (Barlow 1958). Using new sequencing technologies, investigators are examining biological events very near that origin of life, so perhaps it is a question amenable to scientific investigation after all. New metagenomes have found Archaea that have several molecular traits in common with eukaryotes supporting the idea that eukaryotes arose through a fusion of archaeal and bacterial cells (Fournier and Poole 2018; Spang et al. 2015), though this theory is highly controversial (Nasir et al. 2015; Imachi et al. 2020). The application of Darwinian principles of evolution to cells as they evolved to form multicellular entities is also being examined using microbes (see review in Rainey et al. 2017).

Darwin's Natural Selection can also be examined in detail using microbial cultures. The mode of cultivation can be used to examine the effects of different

selective regimes (Rabbers et al. 2015). An example of this can be found in studies of the influence of environments that select for growth rate vs growth yield, an old area of study in microbiology, that use the new cultivation technique of oil emulsions coupled with genomic analyses (Bachmann et al. 2013).

Gene and genomic sequence comparisons have demonstrated the important role of horizontal gene transfer (HGT) in the evolution of bacteria and archaea. Despite this, there have been relatively few examinations of the impact of HGT of chromosomal genes on population evolution during laboratory evolution experiments. Experiments conducted with *E. coli* by mixing cells capable of transferring chromosomal genes by conjugation with recipient cells that were sterile showed that the latter strains showed mixed benefits from recombination of donated genes. In some situations recombination allowed more rapid acquisition of beneficial traits, but in others no benefit or even deleterious effects were noted upon incorporation of foreign DNA (Chu et al. 2018; Maddamsetti and Lenski 2018). Mixed cultures of donors of chromosomal genes and recipients naturally competent for DNA uptake need to be cultivated for long periods to examine the potential impact of HGT on the evolution of strains. It is not known if this time frame is short enough to allow laboratory examinations of this phenomenon. This area is largely unexplored at this time.

Bacteria provide the means to examine principles of evolution, some of which Darwin anticipated, at a level of detail that cannot be achieved with macroorganisms. Their short generation times, the ability to control their cultivation conditions, the availability of an extensive database of genome sequences, and the ability to preserve in a viable state ancestral generations of cells all provide the potential to gain undreamed of insights into the processes behind the evolution of life. A recent review provides useful links to past studies as well as speculations about future directions for this research (Rainey et al. 2017). In general, laboratory evolution provides a robust means of examining many aspects of Darwin's central theory, Natural Selection (Cooper 2018; Lenski 2017).

2.10 Concluding Thoughts

As microbiology evolved as a discipline in the middle to late nineteenth century, only a limited number of practitioners were trained to address evolutionary questions. Traditional evolution studies were done by those with training in zoology or botany, largely in academic settings or by natural historians with sufficient independent means to support their studies. Microbiological studies arose along two separate lines. Pasteur's work gave rise to studies of the application of microbes in fermentation industries, and this later branched into agricultural applications. Those who developed these lines of investigation usually had a background in botany and so brought some knowledge of evolutionary theories to their work. Interest in disease transmission at that time led to the elucidation of the "germ theory" of disease and medical microbiology. Scientists in this line of investigation usually came without

formal education in zoology or botany since many were physicians whose training at the time was through internships and brief exposure at universities to human anatomy and medical practices. Evolution concepts were generally not a part of their education or interest. Consequently, though Darwin appears to have kept abreast of developments in microbiology and encouraged some of its practitioners, his impact on the development of this new field of science was limited. As the field grew, it slowly abandoned him and largely ignored the principles of evolution that he had laid out (O'Malley 2009).

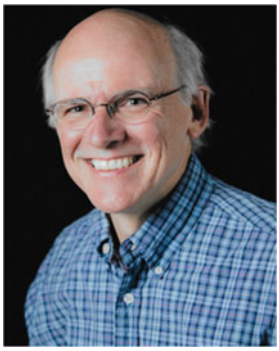
As microbiology developed in the twentieth century, the fermentation and agricultural applied aspects took a back seat to the growing interest in research in medical microbiology. Those interested in bacterial evolution lacked the ability to use characters that easily showed evolutionary changes through related lineages. They also lacked the ability to rationally identify the evolutionary relationships among bacteria. Both of these features of their subjects were available to those interested in the evolution of plants and animals. Without these, early bacterial evolutionists were left without the means to base their work on generally accepted principles and so hard data were impossible to generate. Interest in evolutionary microbiology began to falter.

In mid-century, those who founded the area of molecular biology often started their studies using bacteria, but those investigators were trained in a microbiological tradition in which evolution studies were being dismissed as futile. Some came from the biochemical sciences, typically seated in chemistry, and so had no background in evolution science. Similarly, some of the molecular biology pioneers were physicists, disillusioned with the course of atomic sciences, who came to the new field with no background in evolution. All these factors prevented development of a solid examination of evolutionary questions for many decades and led to the "Dark Age" of microbial evolution science.

Later, the ability to sequence macromolecules finally provided characters that could be compared to generate phylogenies and show the course of evolution. Genome sequences, both of cultured and non-cultured organisms, opened the door much wider and now allowed a more comprehensive picture of microbial evolution to be seen. These have also allowed detailed examinations of cultures undergoing evolution in the laboratory and, to a lesser extent, so far, to populations evolving in nature.

Theodosius Dobzhansky famously entitled a 1973 article, "Nothing in biology makes sense except in the light of evolution" (Dobzhansky 1973). One would be correct to rephrase this as "Nothing in microbiology makes sense except in the light of evolution." Many early microbiologists realized this and made their best efforts to provide this basis for their new science. As Dobzhansky wrote in that article, "Without that light it [biology] becomes a pile of sundry facts—some of them interesting or curious but making no meaningful picture as a whole." For nearly a century, microbiology was just such a pile of sundry facts. Not until the work of molecular evolutionists, Woese being the most important, was the light shone on the microbial world. Since then we have found Darwin's theories to be profoundly in evidence there. Even more, we have found evolutionary processes that Darwin could

not have envisioned, opening several new areas of investigation. Darwin has finally gained his deserved place in microbiological research and thinking.



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Chapter 3
Microbes and Marine Sediments: A Lifelong Relationship on Earth's Biosphere

Jerónimo Pan

Abstract The relationship between microbes and marine sediments probably dates back to the very origin of life on Earth. This intimate association is exemplified by how the physical and geochemical environment within sediments determines which microbes dominate a certain *consortium* and, in turn, how microorganisms have profound effects on sediment properties through their metabolic activities at various (spatial and temporal) scales. Microbial mats, the oldest ecosystems on Earth with a fossil record dating back to 3.4 Ga, are biosedimentary structures in which microbes exploit all environmental niches that arise in relation to strong gradients in light, redox potential, the concentration of viable substrates for energy acquisition, as well as toxic compounds. In fact, microbial mats have been thought to be the environment where several metabolic pathways evolved, establishing microbial lineages and biogeochemical processes that last to this day. This chapter provides a concise summary of the evolution of those early microbial lineages, to then revisit modern, hypersaline microbial mats, and some of the microbially induced sedimentary structures (MISS) that arise from mats colonizing siliciclastic sediments. Salient ecological features and the metabolism of their microbes are briefly discussed, while emphasis is made on what can be learned from their study through the actualistic perspective of GeoBiology. Emergent properties of microbe-sediment interactions essential for the development, establishment, and preservation of microbial mats in modern and fossil settings are discussed.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_3

3.1 Introduction

If you happen to be an outdoorsy type of person, chances are that you have stepped into a river or lake and found the rocks of its bottom to be slippery. Or maybe visiting a mudflat at low tide, you saw thousands of crabs and periwinkle snails coming out to the mud surface and shorebirds probing the mud to pick up worms with their tube-like bills. Have you ever wondered what the crabs and worms feed upon? What fuels these ecosystems? Let me break the news to you: it is myriads of microscopic life forms that harvest the energy from the sun and convert it into different forms of organic matter. Simply put, *sediments are not inert*, but rather thriving with millions of microbes per cm^2. Multiple and complex interactions between microbes and sediments have taken place for millions of years, a relationship that probably is as old as the existence of microbial life itself. Throughout this time, the rocks and sediment particles that cover the surface of the Earth have provided a viable substrate for these opportunistic critters, as is testified by a continuous rock record dating back to 3500 million years. Yet, to many inattentive dwellers like you or me, these vegetated sediments in well-illuminated shallow waters may have remained a "secret garden" (MacIntyre et al. 1996).

This essay is an account of the intricate relationships that microbes have developed with sediments over millions of years of evolution on Earth. It starts by providing a concise summary of the evolution of the early microbial lineages, the key metabolic pathways they devised, and how the association with sedimentary habitats has been a constant feature throughout microbial evolution. Microbial mats from hypersaline environments and their associated microbially induced sedimentary structures (MISS) are treated to some further extent as being the archetypical kind of biosedimentary ecosystems that have transcended since the Archaean eon and as such deserve a special mention in a volume dedicated to microbes as the *foundation stone* of the biosphere. Salient ecological features and the metabolism of their integrating microbes are briefly discussed. Then the emergent properties of microbial-sediment interactions are outlined and illustrated by present-day examples that prove how these same processes have played a significant role in past times.

GeoBiology is the discipline that explores the interactions between the physical Earth and the biosphere often with an interdisciplinary approach grounded in modern-day processes (i.e., an actualistic approach). Hopefully this essay will illustrate how seemingly disparate disciplines such as sedimentology, paleoenvironmental reconstruction, microbial ecology, microbiology, and geochemistry can reconcile at disciplinary crossroads, when it comes to studying the lifelong relationships of microbes and marine sediments. Let us now start by the very beginning.

3.2 Early Life Evolution on Earth and Microbe-Sediment Interactions

The Earth is 4.5 Ga old (Ga = Giga-annum, 10^9 yr. = billion years), and the most firm hypotheses about early life signal the primitive oceans as having been the environment where life first became established; yet, due to the constant recycling of seafloor by the process of plate tectonics, the oldest sediments and rocks in modern oceans are 180 Ma (Ma = 10^6 yr. = million years). This makes reconstruction attempts of ocean history a difficult task, and evidences of past biological processes that took place on marine settings need to be looked for in continental rocks. The detection and understanding of the early fossil record, the biogenicity of structures, and the interpretation of paleoenvironmental processes and patterns is by no means an easy task. The authentication of early Archaean fossils calls for rigorous criteria (Walter and Allwood 2005) that not only rely upon morphological premises, as these may sometimes be misleading (García Ruiz et al. 2002). In that sense, the contribution of actualistic perspectives (Bartley 1996) and geomicrobiological experiments carried out with modern analogues (e.g., Cuadrado and Pan 2018; Hickman-Lewis et al. 2019) contributes a valuable alternative to other traditional paleontological approaches. Accordingly, the disciplinary approach of GeoBiology is predominantly actualistic.

To put things in perspective, let us start this essay with a brief history of some major events that have had a tremendous impact on Earth's physical and living systems. How about delving into the primitive ocean for a start? There is consensus among scientists that primitive oceans formed around 4.0 Ga ago (Fig. 3.1), through a series of planetary-scale processes that implied general cooling and condensation of atmospheric water vapor into torrential rains that accumulated into arcane ocean basins. This was followed by the absorbance of large amounts of atmospheric CO_2 into the primitive ocean, the formation of limestone deposits on the seafloor, and an increase in the alkalinity of seawater in the form of bicarbonate (HCO^{-3}) and carbonate ($CO_3{}^{-2}$) ions. On the other hand, with a thinning atmosphere, the penetration of incoming solar radiation progressively increased.

How life came to be on this planet is a subject for discussion elsewhere, and indeed much has been written in that respect since the hypotheses of Oparin (1924) and Haldane (1932) and Miller and Urey's 1950s' experiments (Miller 1953; Miller and Urey 1959) that demonstrated the synthesis of prebiotic organic compounds. Let us just mention briefly that very early in Earth's history, a variety of different metabolic pathways would have evolved and permitted various ways of life (Oschmann et al. 2002) (Fig. 3.1). The general consensus favors a heterotrophic metabolism for early life forms over an autotrophic one on the basis that a heterotrophic organism is simpler than an autotrophic one and that the synthesis of prebiotic (non-biologically produced) substances under reducing conditions has been successfully demonstrated by controlled experiments (Lazcano and Miller 1996).

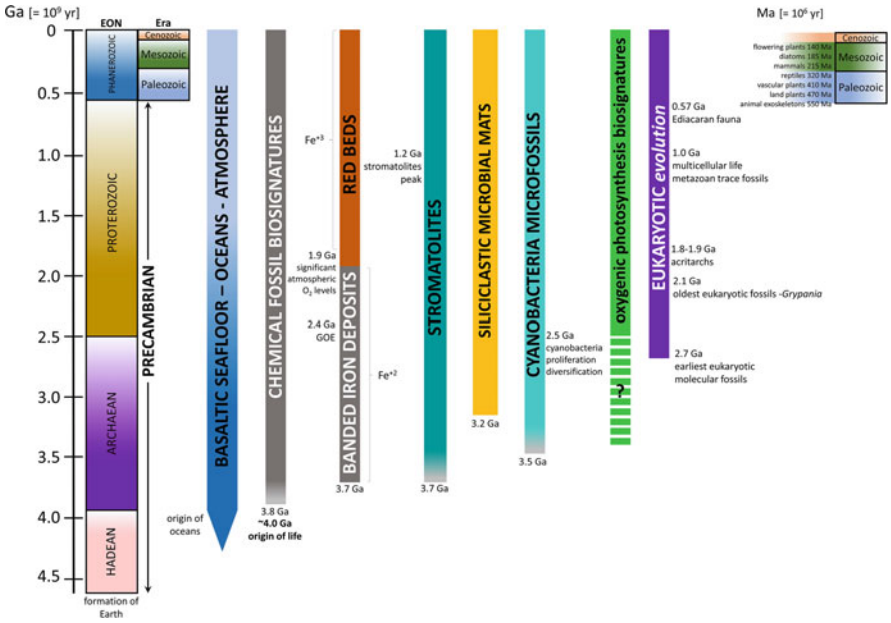

Fig. 3.1 Geologic time scale and chronology of some of the most significant Precambrian life events; see text for further details (compiled from various sources; based on original concepts from Margulis and Dolan 2002; Carrión 2003)

The geochemical evidence of biotic activity (what may arguably be considered the oldest *fossil biosignatures*) dates back to 3.8 Ga (Fig. 3.1) and comes from carbonaceous inclusions in sedimentary sequences from Greenland (Mojzsis et al. 1996); the isotopically light carbon from these rocks is indicative of biological fixation. On the other hand, direct evidence (i.e., *microfossils*) indicates that around 3.5 Ga ago, there were living organisms feeding upon organic molecules that had some sort of "community" organization, so as to leave traces of their life. Although not uncontestable (see Brasier et al. 2002; Brasier et al. 2004), 3.5-Ga-old carbonaceous cherts (microcrystalline quartz in silicified clay stones and mudstones) from northwestern Australia provide evidence of microbial colonization by filamentous bacteria of evaporitic and shallow lagoonal and peritidal environments of an ancient ocean (Awramik et al. 1983). Experimental evidence of silicification and fossilization of cells and biosignatures of archaeal strains suggest that different lineages made up the microbial composition of the original communities (Orange et al. 2009). The terrain for tracing possible evolutionary lines among early microbes and how novel metabolic pathways might have been devised to solve elementary problems is no doubt fascinating, and microbial associations in the form of biofilms or mats are firm contenders as the most plausible candidates for the earliest ecosystems. For instance, it has been hypothesized that microbial mats of coexisting bacteria and archaea may have been formed as simple biofilms in which microbes with different metabolisms cooperated in the exploitation of diversifying niches (Nisbet and Fowler 1999).

The association of early life and shallow ocean sediments seems to have been very plausible, as further evidence in the form of 3.2-Ga-old spheroidal, organic-walled microfossils, for which cellular morphology and ultrastructure can be discerned, has been described from South African siliciclastic tidal deposits (Javaux et al. 2010) (Fig. 3.1). Without an aqueous environment to buffer the effects of short-wavelength solar UV radiation, the existence of life on land was not only improbable but most likely impossible. Nucleic acids and proteins strongly absorb UV light, which ruptures chemical bonds, causes cellular damage, and eventually leads to cell death (Margulis and Dolan 2002).

With the progressive depletion of organic compounds by heterotrophs (a topic that is still under debate whether it happened relatively fast or later among early life forms), and despite the invention of simple metabolic pathways such as fermentation and glycolysis, as forms of obtaining energy from simple organic molecules (Margulis and Dolan 2002), the evolution of autotrophy may seem like the next necessary evolutionary step. Some scientists argue that it might have been possible for microbes to explore the chemoautotrophic pathway, as it occurs to this day among hydrothermal vent archaea and bacteria (Nisbet and Sleep 2001; Rollinson 2007); some others even hypothesize that photosynthesis arose from chemotrophy (Nisbet et al. 1995). On the other hand, the first photosynthesizing bacteria likely were anaerobic and putatively devised a form of photosynthesis that did not generate O_2, nor did they consume it in any way (Margulis and Dolan 2002). But what has been firmly established is that by 3.5 Ga ago, a certain clade of bacteria (cyanobacteria) began producing a lithologic record attesting to their metabolic activity (Brasier et al. 2004).

Cyanobacteria are photoautotrophs that use the sun's radiation to fix inorganic CO_2 into organic compounds, producing O_2 as a by-product of photosynthesis, hence termed *oxygenic* photosynthesis (Nisbet and Fowler 1999). The gradual accumulation of photosynthetic residual O_2 in the atmosphere over time led to what geoscientists refer to as the Paleoproterozoic Great Oxygenation Event (GOE) when some ~2.45–2.20 Ga, atmospheric oxygen levels rose to >1% of modern levels (Hazen et al. 2008; Schopf 2012; Farmer and Cook 2013). The GOE coincides with a peak in cyanobacterial diversity (Margulis and Dolan 2002; Noffke and Awramik 2013), providing an unequivocal clue as to which microbes were responsible for the O_2 accumulation in the atmosphere. The transition from a reducing atmosphere to an oxidizing one not only produced a shift in chemical reactions that impacted the lithosphere and the biosphere, but also the newly formed stratospheric ozone layer resulted in a significant cutoff of most UV radiation. Furthermore, no further synthesis of prebiotic compounds was possible in an oxidizing atmosphere, indirectly benefiting photoautotrophs over the more primitive heterotrophs (Margulis and Dolan 2002).

The transition from a non-oxidizing atmosphere to one characterized by free oxygen has been recorded in sedimentary sequences from South Africa (Eriksson and Cheney 1992), which document the disappearance of banded-iron formations (BIFs, Fig. 3.1) which had presented a continuous record dating back to at least 3.9–3.8 Ga (Koehler et al. 2010). The BIFs are controlled by the availability of

hydrothermal ferrous Fe^{+2} in seawater, which may have precipitated following the oxidation of Fe(II), either by biotic oxidation by chemolithotrophic bacteria or by abiotic oxidation by cyanobacteria-produced O_2 (Koehler et al. 2010). After BIFs became discontinuous, red beds and hematitic coatings on grains (forms of ferric oxides, with oxidized Fe^{+3}) began to occur in 1.9-Ga-old sequences (Fig. 3.1).

Oxygenic photosynthesis is arguably the most significant bioenergetic process on past and modern Earth, a biochemical pathway by virtue of which the radiative energy from the sun is transformed into chemical energy within cells. The biochemical innovation that made oxygenic photosynthesis possible was the ability to split the water molecule and use the produced H^+ as the electron acceptor in the electron transport chain of the light reactions (Margulis and Dolan 2002). Evidences from several lines point that this pathway evolved within the *cyanobacteria* clade and paved the way for this group to be ecologically successful and almost ubiquitous to this day, and having left their mark on the substrates they have used for living (Noffke et al. 2008). Still, it has been challenging to find undisputable fossil evidences of cyanobacteria oxygenic photosynthesis in the rock record. One of the oldest is the record of a biosignature lipid that occurs in high proportion in modern cyanobacterial mats and was found in 2.5-Ga-old bitumens (Summons et al. 1999).

One of the first undisputable life forms that left conspicuous fossils throughout the Precambrian are *stromatolites* (Grotzinger and Knoll 1999). These lithified biogenic structures have a fossil record dating back to 3.5 Ga (Walter and Allwood 2005) (Fig. 3.1) and showed a peak in diversity of forms in the Mesoproterozoic, 1.6 to 1.0 Ga ago (Noffke and Awramik 2013). The precursors of stromatolites are *microbial mats*, as they are composed of microbial consortia dominated by photoautotrophic cyanobacteria and other types of bacteria (with aerobic and anaerobic tolerances) that secrete copious amounts of exopolymers as part of their normal metabolism, trapping sediment particles and secreting in situ sheets of carbonate. These microbial mat laminae are sequentially stacked up in layers (*stroma* = mattress) that grow in height by accretion, towards well-illuminated surface water, creating a three-dimensional structure. Modern stromatolites occur in shallow seas and have recently been found on high-altitude volcanic lakes (Farías et al. 2013). The prevalence of well-illuminated, warm, alkaline (carbonatic), hypersaline waters seems to be a condition for the occurrence of stromatolites, and in situ microbial aragonite precipitation is a major feature.

With prokaryote clades well established and exploiting diverse ecological niches, the course of microbial evolution then saw a series of successful associations between prokaryotes or *serial endosymbiosis*, through which microbial cells acquired genomes and metabolic pathways from one another and set up a novel kind of cell with a distinct, compartmentalized cytoplasmic arrangement (Kutschera and Niklas 2005; Keeling 2010). Biological lipids preserved in shales from Pilbara Craton (Australia) suggest this happened around 2.7 Ga ago (Brocks et al. 1999), although the earliest fossil eukaryotes are 2.1 Ga old (Han and Runnegar 1992). A new kind of cellular division (meiosis) implying genetic recombination and the invention of sex would follow, making genomes ever more complex and leading to higher levels of biological complexity; by around 1.0 Ga ago, organisms evolved

multicellularity (Margulis and Dolan 2002). But all that falls way beyond the scope of this essay. Those readers wishing to go beyond this very brief account of early microbial life and learn more about the fossil record and how it provides clues as to clarify the emergence of the main evolutionary lines should refer to publications that provide a more in-depth summary (e.g., Schopf 2012; Tomescu et al. 2016). For now, let us go back to microbial colonization of marine siliciclastic sediments, to then focus on Earth's oldest ecosystems, microbial mats.

3.3 Microbial Colonization of Siliciclastic Marine Sediments

Coastal marine environments may fall into either of two broad groups with respect to their geochemical signature, *carbonatic* or *siliciclastic*. In the former ones, chemical precipitation of minerals and Ca^{+2} ions has a preeminent role in biogeochemical processes (Kazmierczak et al. 2013) producing carbonatic sediments and rocks, whereas siliciclastic settings are characterized by quartz sediments, and the chemical precipitation of minerals, while possible, plays a less significant role than physical processes (Cuadrado 2017). Thus, siliciclastic sediments have imprinted an organizing signature on microbial communities since erosion and weathering of crustal rocks produced vast amounts of sediments for colonization (Noffke et al. 2002). As previously mentioned, stromatolites have been the dominant biosedimentary structure in carbonatic settings, whereas in siliciclastic depositional environments, another type of biogenic structures different from stromatolites has predominated. These have been termed microbially induced sedimentary structures (MISS) and result from the interaction of epibenthic cyanobacteria (*epi* = growing on top of; *benthic* = making reference to a sedimentary substrate) with physical agents of erosion, deposition, transportation, or deformation (Noffke et al. 2001). The remainder of this essay will deal mostly with MISS and their emergent properties.

Similarities and differences between stromatolites and MISS have been critically analyzed (Noffke and Awramik 2013), on the comparative premise that both biosedimentary structures have a microbial mat as the principal constructional unit. Besides the abovementioned contrasting geochemical and sedimentary environments in which either one develops, one of the most striking differences is that MISS are generally surface phenomena (two-dimensional, only a few mm thick) and fail to develop into substantial three-dimensional stacked layers of mats, as stromatolites do. The mode of growth of microbial mats will be discussed in depth later.

The association between marine siliciclastic sediments and microbes has been revisited many times, focusing on fossilized and living MISS in modern settings, by either applying paleontological or actualistic approaches. For example, 2.9-Ga-old microbial mat facies have been recognized from South African sandstones, and the biogenicity of their microscopic textures has been confirmed by comparative microscopic analyses with modern ones, as well as by the light isotopic signature of

organic matter (Noffke et al. 2008). Likewise, other studies (Bouougri and Porada 2002) provide a detailed description of microbial mats and associated structures from Neoproterozoic (780 Ma) peritidal siliciclastic sediments and relate them to the energy environment for their formation and preservation in intertidal and supratidal zones. Moreover, fossilized sequences of siliciclastic *biolaminites* that describe the cyclic interplay between mat growth and sediment trapping have been described from Ediacaran (~ 545 Ma) fossils (Bouougri and Porada 2007). Often, the fossil MISS have modern analogues which they may seem easily relatable to (Cuadrado et al. 2015); yet this comparison needs to be made with caution, as morphological similarity alone does not constitute scientific proof of a common origin, and the likelihood of conclusively identifying microbial structures from visual appearance alone is slim (García Ruiz et al. 2002; Davies et al. 2016). The evidence gathered with experimental approaches using modern mats and MISS is of paramount importance for such inferences. For instance, studies have experimentally quantified the forces that microbial mats can withstand when subject to viscoelastic and plastic deformation (Thomas et al. 2013; Pan et al. 2019) and how they translate to the establishment of MISS.

Microbial colonization of intertidal sediments may have been favored over other coastal sedimentary environments such as the supratidal band that is not regularly inundated by seawater or subtidal deposits permanently covered by seawater for several reasons. Firstly, it is an absolute truth that most (if not all) microbes rely on liquid water for their development (Walter and Allwood 2005). In the case of supratidal environments, the supply of water is not as reliable or steady as it is in intertidal and subtidal zones, even though there are modern microbial mats that abound in the supratidal region (e.g., Bolhuis and Stal 2011). Moreover, nutrients play a significant role in the distribution of autotrophic microbes in intertidal sediments. Studies on the rate of nutrient transport through tidal advection and diffusive fluxes between sediment and porewater have shown that the sediment-water exchange of nutrients attributed to tidal flooding in intertidal microbial ecosystems is several orders of magnitude larger than any other physical exchange process such as diffusive fluxes through porewater (Ospina-Alvarez et al. 2014). Hence autotrophic microbes developing in the intertidal band might exploit this regular resupply of inorganic nutrients to their advantage.

As stated, the archetypical biogenic structure falling under the MISS classification is the *epibenthic microbial mat*. Some 3.5 Ga ago, microbial mats covered most of well-illuminated shallow seas, and their constituent microbes exploited all ecological niches available within a layered biosedimentary structure a few mm thick (Des Marais 1990). Fossil microbial mats and associated MISS from tidal deposits in the Barberton belt of South Africa differ fundamentally in appearance and genesis from early Archean stromatolites and bacterial cell fossils preserved in chert (Noffke et al. 2006). With a dating of 3.2 Ga, these Barberton fossils are the oldest microbial mats and MISS recorded from restricted coastal habitats. As the remainder of this essay will largely deal with microbial mats and MISS from siliciclastic settings, it is fitting to introduce them with certain depth.

3.4 Hypersaline Epibenthic Microbial Mats

Microbial mats may be considered as a type of biofilm (Costerton and Stoodley 2003; Tomescu et al. 2016), and in fact, some authors go as far as stating that microbial mats "must, by definition, *begin* as biofilms" (Castenholz 2009). As it will be further developed in this section, modern epibenthic microbial mats are photosynthetic, *stratified consortia of prokaryotes* (bacteria and archaea) *and microeukaryotes* developed at sediment-water interfaces in shallow, intertidal, and lower-supratidal marine sediments (Des Marais 2003), and this stratification is an emergent property of microbial mats that distances them from mere two-dimensional biofilms. Microbial adhesion and the tendency to form biofilms will be discussed further in Sect. 3.5.1.

Furthermore, epibenthic microbial mats are complete, self-sufficient ecosystems that participate in nutrient uptake and recycling and play a paramount role in sediment biostabilization in present-day shallow sand- and mudflats. As discussed in the previous section, they have a long fossil record, dating back to at least 3.4 Ga; in fact, some of the oldest fossils correspond to filamentous cyanobacteria colonizing coastal siliciclastic sediments in the form of biofilms and microbial mats (Schopf and Walter 1982; Tice and Lowe 2004; Noffke et al. 2006; Noffke et al. 2013). Modern representatives of these remarkable micro-ecosystems may well be considered as present-day models for the study of ancient relationships between the three domains of life on Earth (bacteria, archaea, and eukaryotes) that have been established since the Archaean (Bartley 1996; Nisbet and Fowler 1999; Oschmann et al. 2002).

The *hypersaline* conditions under which most modern coastal microbial mats develop exclude the presence of metazoan predators; thus, complete biolaminite sequences are preserved in sediments without major disruptions. Hypersaline conditions also set the scenario for the dominance and spread of microbial mats and stromatolites throughout most of the Precambrian, when mats were ubiquitous biosedimentary features in shallow ocean sediments in the absence of metazoan grazers, predators, and bioturbators (Seilacher 1999; Stal 2012).

A typical microbial mat, unaltered by mechanical disruptions or hydrodynamic deformation, presents a laminar structure in which different groups of microorganisms alternate their dominance in vertical section, in accordance with factors governing redox potential, illumination, and sediment physicochemistry (Fig. 3.2). The description that follows is based on the vertical profile of undisturbed, modern, hypersaline microbial mats from Paso Seco (Argentina), with which I am most familiar. Paso Seco is an obliterated tidal channel with an intermittent connection to the coastal ocean. The dominant sediment type corresponds to fine sand, with feldspars and quartz grains (Cuadrado et al. 2015).

As it is appreciated in Fig. 3.2b and schematized in Fig. 3.2c, a thin *diatom biofilm* (~ 300 μm-thick) colonizes the uppermost sediment layer, underneath which the epibenthic microbial mat *proper*, dominated in biomass by *filamentous cyanobacteria*, develops. This layer varies in thickness ~ 0.3–1.5 mm. These two topmost layers are characterized by oxygenic photosynthesis. Below the

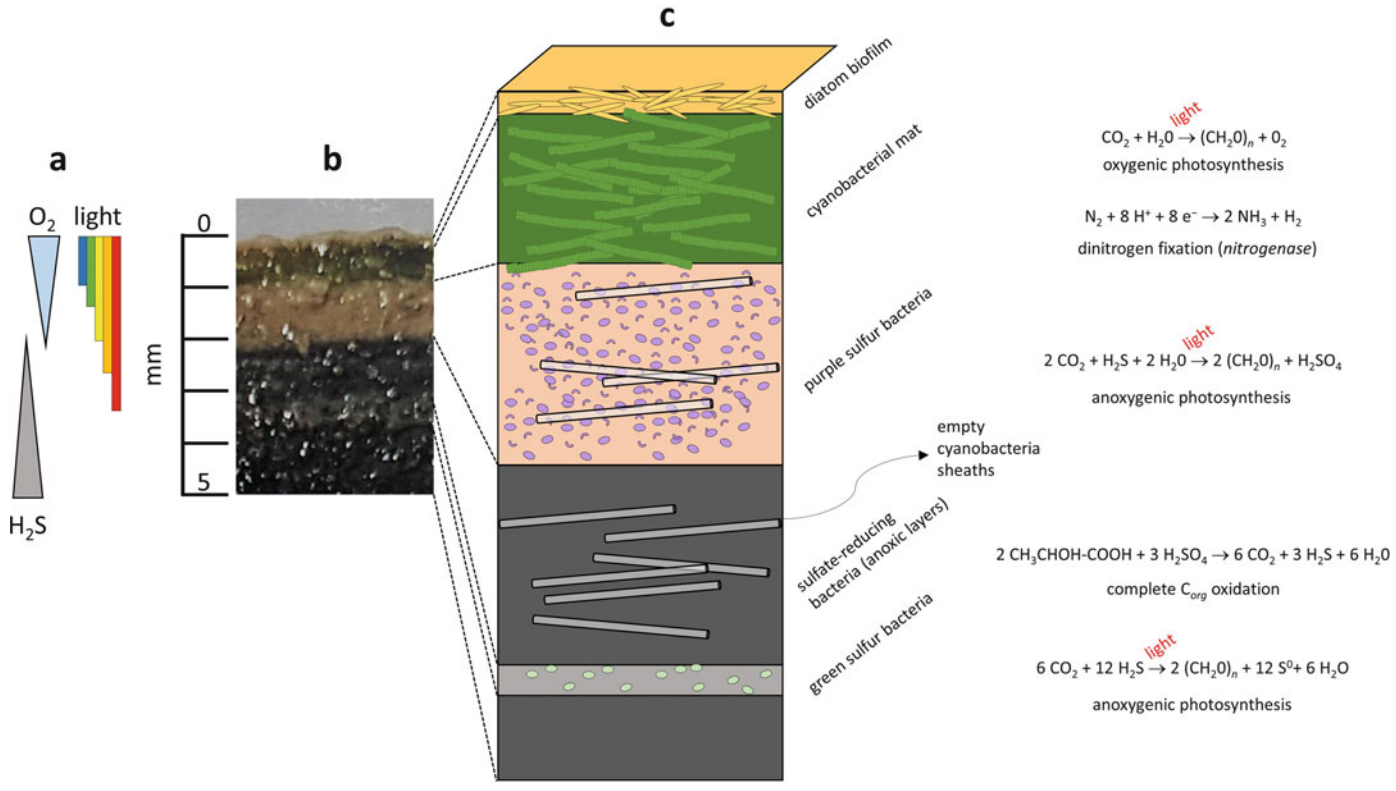

Fig. 3.2 A typical modern microbial mat with characteristic layering. (**a**) Steep gradients in physicochemical parameters (O_2 and H_2S concentration gradients and light penetration) that govern the distribution of microorganisms. (**b**) Photograph of the layered structure of microbial sediments. (**c**) Schematic representation of a typical microbial mat dominated by cyanobacteria. The layer of green sulfur (anoxygenic) bacteria is not always present and marks the maximum penetration of the far-red light wavelengths. Microbes in (c) not drawn to scale

cyanobacteria layer, there is a light-brownish layer (~ 1.5–2.5 mm) with an abundance of *purple sulfur bacteria* and *Beggiatoa*; oxic conditions prevail up to this layer. Purple bacteria are Proteobacteria that contain bacteriochlorophyll *a* or *b*, by virtue of which some are photoautotrophs using CO_2 as the source of carbon; others may be photoheterotrophs using cyanobacteria-derived organic carbon (Castenholz 2009), but still using light as the source of energy. As it can be seen in Fig. 3.2a, orange and red wavelengths penetrate down to these layers (Stal et al. 1985). Purple bacteria have been pointed to as the plausible candidates for early bacteriochlorophyll-based photosynthetic mats (Nisbet and Fowler 1999).

From 2.5–3.0 mm down to deeper layers, anoxic conditions set the scenario for the dominance of *sulfate-reducing bacteria*, whose metabolism is based on SO_4^{2-} (or elemental S) replacing O_2 in the oxidation of organic matter derived from cyanobacterial production, such as accumulated extracellular polymeric substances (EPS; Section 3.5.2) (Castenholz 2009; Stal 2010). This anoxic layer is appreciated as a thick, black stratum that may reach several cm in depth. In deeper layers of this anoxic stratum, a band of *green sulfur bacteria* may occur, marking the maximum penetration of the far-red light wavelengths (Fig. 3.2a). Green bacteria have a high sulfide tolerance and can grow in conditions which are toxic to purple bacteria (Nisbet and Fowler 1999). They are obligate anaerobic photoautotrophs with bacteriochlorophylls in a single photosystem that use H_2S as the electron donor (Castenholz 2009). Finally, methane-producing *archaea* (not shown) are common throughout the anoxic zone (Robertson et al. 2009; Cardoso et al. 2019).

Cyanobacteria are dominant from a bioenergetic point of view, as they produce most of the reduced organic matter, and this opens a niche for recycling anaerobes. The dominance of cyanobacteria also applies, in ecological terms, to the biomass. A compilation of bacterial 16S rRNA gene clone libraries generated from modern hypersaline microbial mats (Des Marais 2010) found that the upper 0–5 mm of these biosedimentary structures produce as many as 949 clones, with cyanobacteria and plastids comprising up to 20% of the library. Filamentous cyanobacteria (e.g., the pioneering *Oscillatoria* sp. and the dominant *Coleofasciculus* (*Microcoleus*) *chthonoplastes* occurring in well-established mats; Stal et al. 1985) usually make up the larger portion of the total biomass of the microbial consortium, even if diatoms and other prokaryotic groups may contribute a significant proportion (see Ley et al. 2006); this is due to the trichomous nature of the dominant cyanobacteria (i.e., organized in linear chains of cells that may be several 100s of μm long). These filamentous cyanobacteria grow conspicuously in the upper mm in order to find the optimal light intensity for photosynthesis. A highly coherent structure is produced by the interwoven cyanobacterial filaments (Fig. 3.3a), providing a dense and coherent fabric for the binding of sediment particles and giving a remarkable external leathery appearance to the microbial mat (Figs. 3.4e, 3.5, 3.6c). A striking feature of these thick mats is their elastic deformation, partly resulting from the cohesiveness of this cellular lattice (Pan et al. 2019).

Microbial consortia are complex biochemically and biologically diverse systems, in which the microbial constituents cooperatively exploit every niche (Nisbet and Fowler 1999; Noffke et al. 2013). As it becomes apparent from the previous

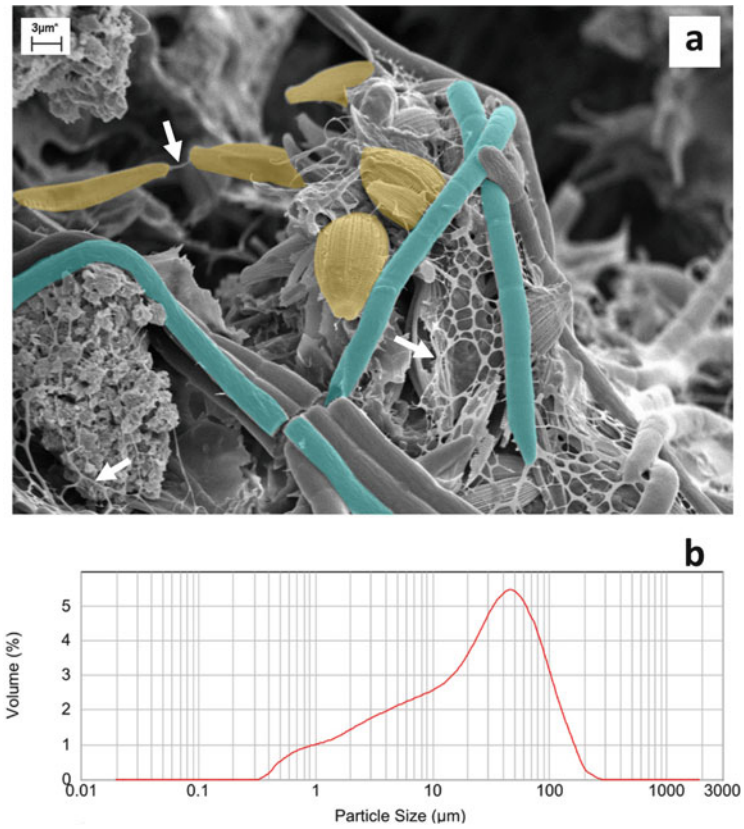

Fig. 3.3 Biosedimentary components within a microbial mat lattice. (**a**) Artificially colored scanning electron microscopy (SEM) photomicrograph depicting the arrangement of cyanobacteria filaments (green) and diatom cells (golden) and the EPS threads (arrows) (modified from Cuadrado et al. 2015). (**b**) Particle size distributions of siliciclastic sediments for the topmost 10 mm of a modern microbial mat, estimated through laser diffraction with an automated particle size analyzer; the pattern represents a bimodal distribution with a peak abundance for silt, a right tail of very fine to fine sand, and less proportion of clays

description of strata, a diversity of microbial functional groups alternate within a few mm of depth, adapting to steep physicochemical gradients (in light penetration, O_2 and H_2S; Fig. 3.2a) and exploiting every possible ecological niche generated within the mat (van Gemerden 1993). The same taxa (even species) are often found in similar arrangements and niches, which is an indication of the cosmopolitanism of these microbes.

It is very important to bear in mind that these gradients vary on a diel basis, with O_2 supersaturation during daytime when photoautotrophs are active and accumulation of H_2S at night, when oxygenic photosynthesis is halted. Therefore, the O_2/H_2S interface schematized in Fig. 3.2a is highly dynamic and critical to the functioning of the microbial mat consortium. Furthermore, a rather radical hypothesis has proposed

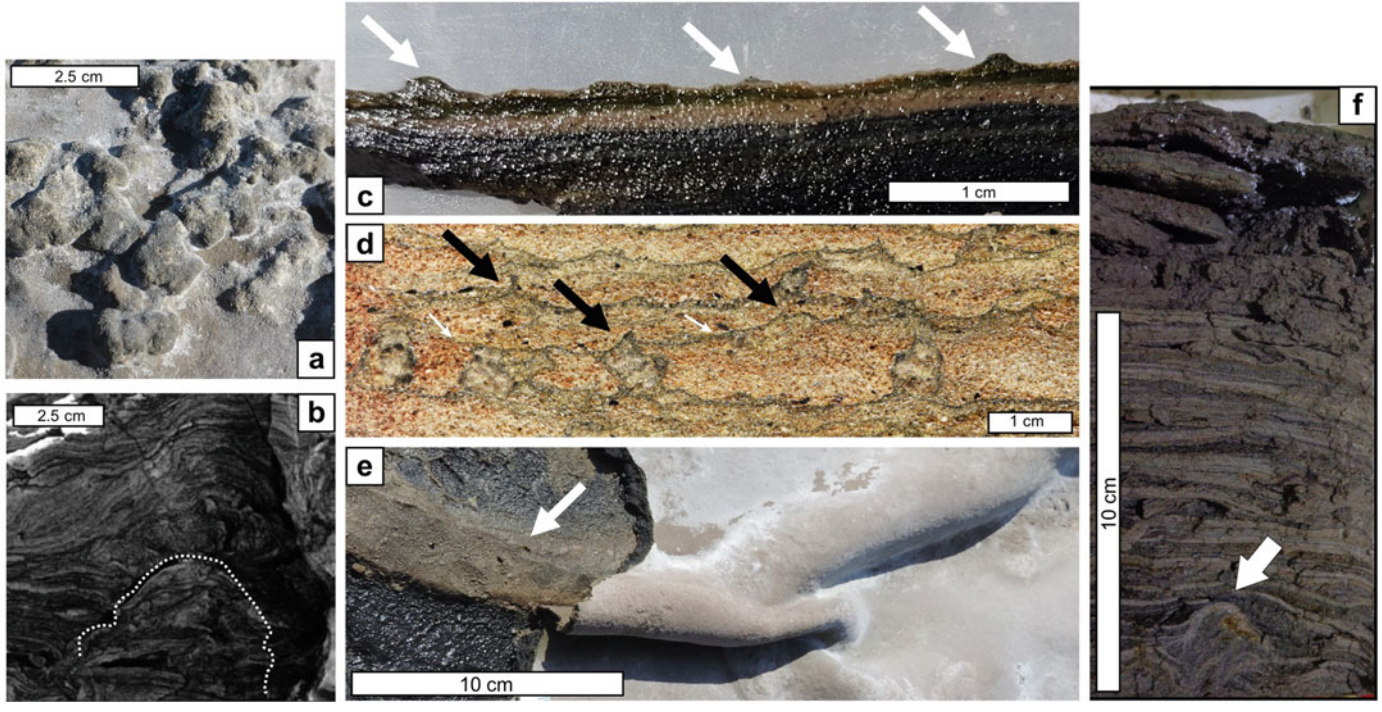

Fig. 3.4 Modern microbially induced sedimentary structures (MISS) from a coastal siliciclastic supratidal flat (Paso Seco, Argentina; 40°38' S, 62°13' W) and their counterparts in sedimentary sequences and fossils. (**a**) Modern pustular structures in plane view derived from gas domes. (**b**) Neoproterozoic pustular structures in section embedded within a biolaminite sequence (modified from Bouougri and Porada 2002). (**c**) Modern "pinnacles" (white arrows) in cross section of a microbial mat. (**d**) Fossilized "pinnacles" (black arrows) from Archaean coastal deposits (modified from Homann et al. 2015). (**e**) Modern microbial mat fold in plane view; note the fluidized and oxidized sediment (arrow) that lies in the cavity created by the mat fold with stark contrast to the anoxic sediment underlying the mat. (**f**) A fold preserved in a Holocene biolaminite sequence (modified from Cuadrado et al. 2015)

Fig. 3.5 Modern MISS from the Paso Seco (Argentina) coastal supratidal flat. Big fragments of detached microbial mats transported by the unidirectional incoming tidal current at the location (direction indicated by white arrow on top). The seawater penetrating through a mat tear (schematized by wavy white arrows) liquefies the underlying sandy sediment, making the microbial mat prone to detachment. Current-generated transport creates other mat deformation structures such as flipped-over edges in the direction of the current (red arrows) and folds (yellow arrows). See text for further details. Metric tape in background = 50 cm

this steep and diel-fluctuating redox boundary as a hotspot where cooperative symbioses between microbes with O_2-tolerant and anoxic metabolisms may have given origin to the eukaryotic cell in the late Archaean (Nisbet and Fowler 1999).

Figure 3.3b shows the particle size distributions of siliciclastic sediments for the topmost 10 mm of the microbial mat depicted on top. The pattern represents a bimodal distribution with a peak abundance for silt (40–50 μm) and a right tail of very fine to fine sand (60–200 μm). Cyanobacteria prefer fine sandy sediment as substrates for the formation of microbial mats (Watermann et al. 1999; Stal 2003). On the other hand, fine silt sediments with adsorbed nutrients are preferentially colonized by diatoms (Stal 2003; Stal 2010), which present elevated growth rates and outcompete cyanobacteria when nutrients are high. Despite having low nutrient demands, cyanobacteria-dominated microbial mats can fix atmospheric N_2 independent of having heterocystous (e.g., *Calothrix* sp.) or non-heterocystous representatives among the cyanobacterial assemblage (Stal 2003; Stal 2012), by virtue of genes acquired through horizontal gene transfer processes (Bolhuis et al. 2010).

Microbial mats grow by accumulation of mat-derived organic matter buried in anoxic layers (Gerdes 2007) and by sediment accretion. A large proportion of the organic matter derives from the accumulation of colloidal exopolymers and empty cyanobacterial sheaths, the latter being recalcitrant to chemical and microbial

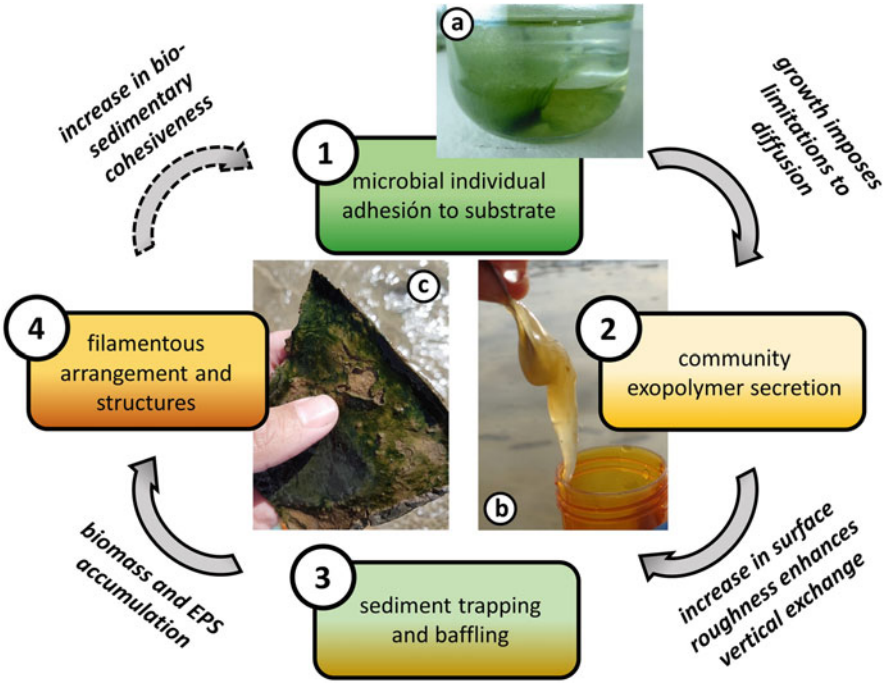

Fig. 3.6 Schematic flowchart depicting emergent properties of microbial communities which determine biosedimentary "architecture" in siliciclastic settings. (1) Microbial adhesion to a substrate is illustrated in (**a**) by an 8-day-old cyanobacteria biofilm growing under culture. (2) Exopolymer excretion is illustrated in (**b**) by mucous diatom-cyanobacteria aggregates over an inundated (stagnant) mudflat. (3) Sediment trapping and baffling, and (4) the formation of cohesive, filamentous biosedimentary structures, is illustrated in **(c)** a portion of mature modern microbial mat. (Fig. 3.6b taken from Cuadrado and Pan 2018)

degradation (Fenchel and Kühl 2000; de los Ríos et al. 2004; Stal 2010). On the other hand, sediment accretion may occur by processes such as baffling, trapping, and binding of sediment grains (Noffke et al. 2001; Walter and Allwood 2005). Baffling is done by perpendicularly oriented filaments which act like obstacles in a current carrying sediment particles; trapping of grains takes place within the organic lattice created by interwoven cyanobacteria filaments, and binding is achieved by the organic lattice and EPS (Fig. 3.3a; see also Sect. 3.5.4). Moreover, even in siliciclastic settings, the deposition of very thin (μm-scale) micritic calcareous sheaths, ooids and peloids, may take place within hypersaline microbial mats (Gerdes et al. 1994; Kaźmierczak et al. 2015; Maisano et al. 2020).

3.4.1 GeoBiology: Examples of Modern Microbially Induced Sedimentary Structures (MISS) and Their Fossil Counterparts

It has been previously stated that GeoBiology has largely benefited from actualistic approaches, interpreting ancient processes and fossil structures in tidal sandstones of all Earth ages, from the study of modern, analogous MISS (Noffke et al. 2008). In this section, a few examples of modern MISS derived from microbial activity will be presented comparatively with their fossil and sedimentary sequence counterparts. All three examples are drawn from the siliciclastic basin at Paso Seco, for which the structure of a hypersaline microbial mat has been described in the previous section.

Gas domes arise from post-burial decay of buried mats and the accumulation of archaea-produced methane in deep anoxic layers, which diffuses upward and gets trapped underneath a levelled, cohesive, plastic mat (Gerdes 2007). Eventually, the mechanical stress created by the accumulation of gas acting upon a biostabilized surface leads to the formation of gas domes that may or may not become encrusted by gypsum (Gerdes et al. 2000). A gas dome is a somehow labile, transient structure that evolves into another type of MISS termed *pustular structure* (Bohacs and Junium 2007) or *tepees* (Gerdes et al. 2000; Gerdes 2007). This is achieved through cycles of desiccation and re-wetting, gypsum encrusting, and ecological succession of the microbial community, and it may happen in a relatively short period of time (months). Pustular structures known as tepees have been recognized in modern (Fig. 3.4a) (Horodyski et al. 1977; Gerdes 2007) and fossil environments (Schieber 2004; Bohacs and Junium 2007). Figure 3.4b illustrates Neoproterozoic pustular structures in section, embedded within a biolaminite sequence (Bouougri and Porada 2002).

The modern "pinnacles" and protruding tufts shown in cross section in Fig. 3.4c are three-dimensional MISS that form from regular reticulate patterns created by highly motile cyanobacteria, at their junctional encounter points over a levelled plane. The process implies the secretion of copious amounts of EPS by the microbial community when submerged under stagnant seawater and subjected to repeated cycles of desiccation and rehydration (Cuadrado and Pan 2018). Ultimately, these "pinnacles" become a perennial feature with an active role in sediment baffling and trapping that can withstand high-energy hydrodynamic regimes. Their fossil counterparts replicating their morphology and morphometrics (Fig. 3.4d) have been described for 3.22-Ga-old Archaean coastal deposits from the Barberton belt in South Africa (Homann et al. 2015).

Mat folds are a type of MISS that are formed by a series of hydrodynamic process acting on a levelled microbially colonized sediment. First, microbial mats subject to pulsating inundation get thickened and hardened by cycles of desiccation and rehydration, as the underlying sandy sediment is liquefied (Cuadrado et al. 2014). Subsequently a tear (that acts as a weakness line) may be formed due to solar radiation, and then, under hydrodynamic shear stress acting on the flexible and re-wetted mat surface, folds are formed and detached from the liquefied sandy

substrate. The fluidized and oxidized underlying sandy sediment (pointed by an arrow in Fig. 3.4e) lies in the cavity created by the mat fold in stark contrast to the otherwise anoxic sediment underlying an undisturbed mat. As shown by Fig. 3.4f, mat folds may become embedded and preserved in biolaminite sequences and have been cited for paleoenvironments (Hagadorn and McDowell 2012).

Finally, the MISS shown in Fig. 3.5 corresponds to an unusually big fragment of microbial mat that has been detached, transported, and flipped on its edges by a unidirectional tidal current. These remarkable MISS (that make the person in the picture look like as if he were deploying "canvasses" over the sedimentary plain) are a type of mat deformation structures that most likely originated from a tear in the mat and its interaction with strong hydrodynamic forces. The high shear stress acting on the wet rims of a tear in the mat channels the incoming tidal water through the tear generating an ever bigger rip; on the other hand, the penetrating water liquefies the underlying sand making the surficial mat prone to lose its anchorage to the substrate. Once detached, the mat gets transported a few meters over as a piece of rug. As a by-product of the strong hydrodynamic forces, other MISS such as folds and flipped-over edges may also be formed, in what probably represents the first step of the formation of roll-up structures (Cuadrado et al. 2015).

3.4.2 Microbial Mats in Other Environments

The previous exposition referred to microbial mats in hypersaline environments, which occur in modern settings and also correspond to most fossil representatives. However, it is worth pointing out that present-day microbial mats occur in an array of environments and conditions. I will summarize these for you later in this section of my essay. The microbial diversity is not as high as in hypersaline microbial mats, nor do they always present the characteristic layered structure previously described. A common feature of these environments is the occurrence (at least seasonally) of extreme conditions in either one or several environmental parameters.

Coastal and estuarine sandflats with large tidal fluctuations provide an excellent habitat for the development of cyanobacterial mats (Stal 2012) under environmental conditions that preclude the existence of abundant predators, without being as extreme as in hypersaline microbial mats. Coastal mats have a large number of microeukaryotic representatives, primarily diatoms (Prieto-Barajas et al. 2018).

Geothermal alkaline spring mats develop at high temperature ($> 70\ ^{\circ}C$) in combination with abundant H_2S. These mats share similar features with the hypersaline mats, such as biolaminations (Castenholz 2009) and the presence of oxygenic (cyanobacteria) and anoxygenic photoautotrophs. The anoxygenic phototroph *Chloroflexus* is a dominant member of the microbial community, often forming a reddish to orange layer that overlays a cyanobacterial mat, as anoxygenic photosynthetic activity scavenges the sulfide that otherwise would be toxic to most cyanobacteria (Stal 2012). Species of *Synechococcus* are particularly abundant among the cyanobacteria and usually form conical microbialites (Bosak et al.

2012). At peripheral areas where temperatures drop below 45 °C, eukaryotic microalgae may occur. These mats are usually soft, although deposition of SiO_2 and $CaCO_3$ may harden them.

Acidic hot springs support mats of eukaryotic microalgae of the order Cyanidiales (a basal clade of Rhodophyta) as all photosynthetic bacteria, including cyanobacteria, are excluded at pH < 4.0 (Castenholz 2009; Stal 2012). These acidic conditions are common in hot springs (40–56 °C) linked to volcanic activity.

Microbial mats develop in *Antarctic and Arctic meltwater ponds and lakes* at low temperatures (4–10 °C). These are perennial, slowly accreting cyanobacteria-dominated mats that develop in the absence of efficient grazers and lacking potential eukaryotic microalgal competitors (Castenholz 2009); however, the secretion of copious amounts of polysaccharides provides protection to less thermotolerant organisms such as diatoms, flagellates, and ciliates that only thrive seasonally (Prieto-Barajas et al. 2018). Cyanobacteria in these mats effectively undergo a dormant phase during winter conditions, and heterotrophic bacteria play a major role in nutrient cycling.

Terrestrial cyanobacterial mats and crusts can be found in a variety of different environments, from hot and cold deserts (Castenholz 2009), sand dunes, to the bottom of drying oxbow lakes (Fayó et al. 2020). A common feature is the occurrence of thickly sheathed cyanobacteria to attenuate the effects of desiccation and unreliable water supply (Stal 2012).

3.5 Emergent Properties of Sedimentary Microbial Mat Communities

The morphology of microbialites (stromatolites or MISS) is the resultant of an overlap of two factors: (1) the intrinsic control owing to the microbial community (genotype and phenotype) that forms the structure and (2) extrinsic physical factors such as sedimentology and the effect of hydraulic and sediment dynamics (Noffke and Awramik 2013). As a resultant microbial communities colonizing siliciclastic sediments share some common and emergent properties which on the one hand determine the microbial mat "architecture", and on the other hand apply to mat development. According to a model drawing from the study of Archaean examples (Tice et al. 2011), biosedimentary structures owe their characteristics to the interplay between *cohesion* among their components and *mat-surface roughness*.

The steps leading to the formation and consolidation of a microbial mat are schematically illustrated in Fig. 3.6. A first step necessary for the development of microbial communities is (1) the *individual adhesion* of microbes to substrate surfaces. As it will be discussed later, this results in the development of microbial biofilms. As the surface colonization progresses, (2) *community-secreted exopolymers* accumulate, contributing to the development of a more cohesive mat that already departs from the structure formerly presented by a laminar biofilm.

Eventually, small focalized increases in mat surface elevation translate into an increase in the local effective mat surface area available for exchange and decrease the thickness of the laminar sublayer; this ultimately results in an incremental surface roughness (as proposed by Tice et al. 2011). A microbial mat's surface roughness sets its maximum potential rate of diffusive exchange with the overlying water column, and one of the components that are exchanged are sediment particles. With an increase in surface roughness, (3) *accretion of sediment particles* (through baffling, trapping, and binding) becomes an emergent property of the biosedimentary structure (Noffke et al. 2001; Bouougri and Porada 2007). This is a crucial point at which the sedimentary signature of mats becomes more complex, beyond that of the underlying sediment. Finally, through the continuous accumulation of microbial EPS and biomass and the development of filamentous structures (that may include living, interwoven cyanobacteria filaments and the recalcitrant sheaths enveloping them), mat cohesion increases over time. As a whole, the resulting mesh of interweaving cyanobacterial filaments together with the microbially secreted EPS entangle sediment grains more efficiently than does a diatom biofilm (de Winder et al. 1999; Pan et al. 2013) which translates into cyanobacterial biofilms displaying a significant *increment in the cohesiveness of sediments* (4).

There are salient features of what has been considered "biology-like behavior" exhibited by microbes which ultimately are indicative of biogenicity in fossils. These features that are common to microbe-sediment associations include their organization into biofilm-like structures (Hall-Stoodley et al. 2004), a preference for certain substrates, and a tendency to form clusters and "mats" (Brasier and Wacey 2012). Such observation is congruent with the abovementioned evolutionary model proposed for Archaean mats (Tice et al. 2011), which translates well to modern microbial mats, as it has been well-documented in the field. For instance, the in situ microbial succession, formation, and consolidation of mm-size microbial reticulate structures with specific geometries has been described in Sect. 3.4.1 (Cuadrado and Pan 2018). Under certain environmental cues even under varying hydrodynamic energy regimes, these regular microbial arrangements ultimately yield tufts or "pinnacles" that become regular and stable biosedimentary features over extended periods of time (months). Their fossil analogues have been described for 3.22-Ga-old coastal habitats (Homann et al. 2015). Thus, even if calling these "ontogenetic" stages of mat and MISS development may be far-fetched until more actualistic evidence is gathered on this respect, the idea is nonetheless tempting as the regular observation of the genesis of such structures provides linking references to laboratory and rock record interpretations of MISS.

What follows is an expanded treatment of the four emergent properties (or steps) that seem to be common features in the formation and consolidation of microbial mats and MISS.

3.5.1 Microbial Adhesion and Biofilms

Microbes rely on *surface adherence* processes, a feature that becomes important when it comes to the colonization of substrates and eventually the creation of biofilms (Hall-Stoodley et al. 2004). Through surface tension, water molecules create a hydrosphere around sediment grains which is a prerequisite for all microbial life, thus creating a dependency of microbes on *moistened surfaces* (Stoodley 2016). Extremely small *epipsammic* diatoms that grow attached to the surfaces of sand grains (sometimes at densities of ~100 cells *per* individual grain) know this all too well (Mann et al. 2017).

On evolutionary terms, surfaces may have provided a protective niche in which attached cells could create a localized homeostatic environment (Stoodley et al. 2002). Thus, the stage for what may be regarded as the two-dimensional world of *biofilms* was set. In fact, not only did biofilms appear early in the fossil record (reviewed by Hall-Stoodley et al. 2004), but concomitantly, it has been hypothesized that complex interactions within prokaryotic communities evolved in surface-associated biofilms (Stoodley et al. 2002).

The term "biofilm" was coined more than 30 years ago (Costerton et al. 1987) and refers to aggregated microbial cells that are *adhered to a biological or non-biological surface* and have secreted a gelatinous matrix of EPS. Cells in biofilms grow in matrix-enclosed microcolonies separated by a network of open water channels (Stoodley et al. 2002). Prokaryotic biofilms are highly structured multispecies communities that may take over 10 days to reach structural maturity and in which metabolic activities are integrated.

This coordination in ecophysiological responses among biofilm microbes calls for *signalling* or communication among its members. The mechanism has recently been discovered, and it implies a cluster of organisms pertaining to a microbial biofilm population that produces diffusible chemical signals in a coordinated manner; then, the signal concentration builds up until the microbial population reaches a critical "quorum" level from which the signals act as a "switch" to synchronize the behavior of other individuals in the population; these switches are used to regulate various functions (Stoodley 2016). The EPS matrix favors this intercellular communication within biofilms by keeping clusters of organisms within relative proximity, more effectively allowing their *quorum sensing* to occur (Flemming and Wuertz 2019). Quorum sensing has been observed even in hard rock interfaces, making viable effective microbial communication, which would otherwise be impossible in a liquid-only world.

Biofilm anchorage to the sedimentary substrate may seem relatively weak when extrapolations are made to coastal marine environments. In intertidal mudflats where the microphytobenthos is dominated by diatoms, patches of biofilm of significant size can be resuspended in the water when water flows at high tide (Saint-Béat et al. 2014). This may be regarded as a drawback of microbial association into larger biofilms, although it may also represent an opportunity to disperse and colonize new niches. In sum, there is consensus that the aggregation into biofilms (1) confers a

degree of stability in the growth environment, (2) affords protection from a wide range of environmental challenges, and (3) might have catalytic functions through localizing cells in close proximity (Hall-Stoodley et al. 2004).

3.5.2 EPS Secretion and Interaction with Sediments

Extracellular polymeric substances (EPS) are a set of compounds of various chemical identities that are purposefully produced by microbes (a) as secretions of biofilms that secure attachment and enhance their local microenvironments; (b) as metabolic-excess waste products (Decho and Gutierrez 2017); or (c) as the result of *unbalanced growth*, when the supply of nutrients lags behind photosynthetic CO_2 fixation (Staats et al. 2000; Staats et al. 2000; Stal 2010). It is important to point out that EPS are not an essential component to microbial life, implying that individual cells can survive and grow without them.

Chemically, EPS consists of complex molecules of which highly hydrated polysaccharides make up the larger fraction. Other minor components include sugars, amino sugars, amino acids, proteins, lipids and lipopolysaccharides, phosphate and sulfate groups, and uronic acids which confer EPS their acidic nature (Stal and Brouwer 2005; Stal 2010; Decho and Gutierrez 2017). Depending on the extractive method applied, diatom EPS can be separated into different (operational) fractions. Hence, there are *soluble* and *bound* EPS. Also, as some microorganisms produce exopolymers as part of their natural organic coatings (e.g., cyanobacterial sheaths), there is also *capsular* EPS in sediments. Finally, some EPS chelate Ca^{2+} and Mg^{2+} ions through which the EPS matrix gets bound to the sediment by cation bridges with charged silt and clay particles (Decho 1990).

On the one hand, EPS facilitate microbial attachment to surfaces that leads to the formation of biofilms; on the other, sediment-EPS interactions have important sedimentological implications, of which the most immediate is the role of EPS in *sediment stabilization*. Sediment stabilization or *biostabilization* (de Boer 1981; Stal 2010) is one of the most important emergent properties of microbial activity over the substrates they colonize (Noffke 2000; Noffke et al. 2001; Noffke and Paterson 2008). Sediment stabilization is a direct consequence of the cohesiveness conferred by microbial EPS *and* the microbial cells themselves (Decho and Gutierrez 2017). Individual microorganisms may exert small-scale effects on substrate stability, but whole microbial communities and their metabolic EPS create scale-dependent positive and negative feedbacks that have consequences for the functioning of whole ecosystems (Stal 2010).

A variety of sediment microbes produce a variety of EPS. For example, experimental studies have demonstrated that sediment bacteria may produce exopolymeric capsules as an induced protective mechanism from enzymatic digestion (Plante 2000). Other studies have focused on the role of bacterial EPS with high uronic acid content in increasing the erosion threshold of intertidal fine-sand beds (Dade et al. 1990); this EDTA-extractable EPS is tightly bound to the sediment and also

plays a major role in early diagenesis (Stal 2003). Cyanobacteria produce EPS as a structural cell component, in the form of the external sheaths that encase trichomes (hence called *filaments*) (Hoiczyk and Baumeister 1995; Li et al. 2001). On the other hand, EPS secretion by epipelic (those that live freely on sediment surfaces) diatoms is associated with their gliding motility through sediment grains (Edgar and Pickett-Heaps 1984; Hoagland et al. 1993; Poulsen et al. 1999) as a means of structural protection from predation and potentially offers protection against toxic contaminants (Lawrence et al. 1998). This motility-associated secretion of exopolysaccharides in diatoms clearly has an energy cost to the cell, but recent estimates conclude that the cost is almost negligible, representing on average only 0.0001% of the daily net photosynthetic production of a diatom cell (Marques da Silva et al. 2020).

EPS in sediments also contribute to the precipitation of authigenic minerals (those minerals which are formed in situ) (Sutherland 2001; Decho 2010; Stal 2010). This is a crucial first step of early diagenesis, as authigenic minerals conserve the morphology of tidal flats creating rigid structures, in turn leading to the preservation of primary sedimentary structures in the geological record (Winsborough 2000).

The development of microbial mats in intertidal and lower-supratidal environments implies regular exposure to air where diel variations in temperature are much larger than in aquatic environments. Desiccation is a key environmental factor associated with temperature fluctuations and that elicits a number of ecophysiological responses in sediment microbes, among them being EPS secretion by epipelic diatoms (McKew et al. 2011). Behavioral responses linked to desiccation such as vertical migration through sediments also imply the secretion of EPS by epipelic diatoms (Hoagland et al. 1993; Stal and Brouwer 2005; Perkins et al. 2010).

On the other hand, shallow intertidal environments offer a functional water depth for sunlight penetration, needed for photosynthesis, but some wavelengths of the solar radiation spectrum (UV) might be detrimental, and excessive photosynthetically active radiation (PAR) may cause photoinhibition to mat-forming cyanobacteria (Cartaxana et al. 2013), which typically are adapted to low light intensities (Stal 2012). Accordingly, microbes in intertidal laminated mats have developed responses related to the synthesis of photoprotective compounds. For example, the cyanobacterium *Lyngbya aestuarii* (Fig. 3.7b) is responsible for synthesis of the passive sunscreening pigment scytonemin (Abed et al. 2008; Balskus et al. 2011) in response to UV-A radiation. Scytonemin accumulates within the extracellular sheath or slime (Garcia-Pichel and Castenholz 1991; Decho and Gutierrez 2017) and is capable of absorbing up to 90% of the radiation. As scytonemin is only found in epibenthic cyanobacteria (i.e., it is absent from planktonic representatives), it is plausible that its synthetic pathway had evolved in microbial mat communities (Gao and Garcia-Pichel 2011).

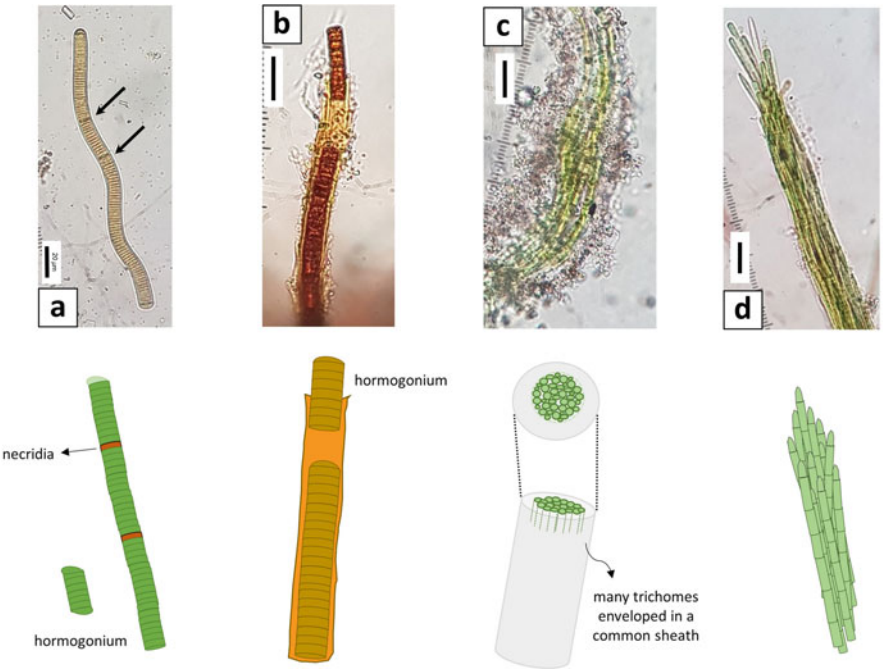

Fig. 3.7 Schematic representation of the morphologies of some cyanobacteria trichomes, common in modern hypersaline epibenthic microbial mats. (**a**) *Oscillatoria* sp., presenting simple filaments of discoid cells, without sheaths; necridia (pointed by black arrows) separate sections of the trichome that become detached as hormogonia. (**b**) *Lyngbya aestuarii*, presenting thick filaments of short discoid cells with firm sheaths containing scytonemin. (**c**) *Coleofasciculus* (*Microcoleus*) *chthonoplastes*, presenting bundles of parallel-arranged trichomes encased in a common gelatinous, colorless, and homogeneous sheath. Sediment particles adhere to the external sheath. (**d**) *Symploca* sp. presents specifically coiled and parallel-oriented filaments; each trichome is enveloped by thin sheaths. Scale bar in all microphotographs = 20 μm

3.5.3 Microbe-Mediated Sedimentary Processes

As mentioned in the preceding subsection, the exudation of EPS is a mechanism of paramount importance by which microbial mats and biofilms render stability to the colonized sediments (Stal 2010). On the other hand, the various metabolic pathways that grosso modo characterize the roles of microbial guilds in an epibenthic mat also have a remarkable impact on sediment biogeochemistry by driving redox boundaries and vice versa. Furthermore, microbial mat biogeochemistry creates favorable microenvironments for authigenic mineralization, for example, by concentrating phosphorus from seawater (Martin 1999) and by promoting the deposition of fine carbonate laminae within the mat lattice (Maisano et al. 2020).

But perhaps the most significant microbe-mediated sedimentary processes taking place in mats have to do with accretion (i.e., trapping, baffling, binding), and these necessarily are determined by scale. As shown in Fig. 3.3b, the sediment particles

that make up biolaminites range in size from fine silts to fine sands (~ 20–200 μm). On the other hand, most sedimentary marine bacteria are roughly 1 μm in diameter (Jumars 1993), and marine archaea are even smaller (Decho and Gutierrez 2017). Also, although the dominant microeukaryotic phototrophs in mats, which are the diatoms, have a large size range but rarely exceed 100 μm, it still has been shown that diatom biofilms have the capacity to retain sediment particles corresponding in size to clays and very fine silts (Garwood et al. 2015). In stark contrast to these microbial groups, trichomous cyanobacteria in microbial mats are 100s of μm long (Pan et al. 2019) and due to their photoautotrophic metabolism regulate their position within microbial mats to have a preeminent role in light harvesting and other surficial processes, such as trapping and baffling sediment particles (Noffke 1998; Noffke et al. 2001). Therefore, while bacteria and archaea may play active roles in surface adhesion processes and agglutination of minute sediment particles by secretion of sticky EPS (Dade et al. 1990; Flemming and Wuertz 2019), it is the trichomous cyanobacteria that exert a more important role in sediment accretion.

In addition to size-related issues, there are architectural features to be considered, such as the entanglement of cyanobacteria filaments which contribute physically to the stabilization of tidal flats (Margulis et al. 1980). The distinct trichomous nature of mat cyanobacteria also has a major impact on sediment accretion and, therefore, the creation of biosedimentary structures. Figure 3.7 illustrates the morphologies of some cyanobacteria trichomes, common in modern hypersaline epibenthic microbial mats. For example, due to its trichome being devoid of enveloping sheaths, *Oscillatoria* sp. does not provide mat consistency (García de Lomas et al. 2005), while the thick filaments of *Lyngbya aestuarii* present firm, thick sheaths that also bind sediment particles to the external walls. On the other hand, when it is a dominant member of the microbial community, *Coleofasciculus chthonoplastes*, contributes considerably to mat consistency and forms compact mats (García de Lomas et al. 2005), owing to the arrangement of the filament, consisting of several interwoven trichomes encased in a single, thick mucilaginous sheath. This filament "architecture" allows the free movement of individual trichomes while maintaining a firm anchorage of the bundle (Stal et al. 1985). On the other hand, *Symploca* sp. is made up of a thread of specifically coiled and parallel-oriented filaments, each one encased in a single, thin sheath; the coiling of filaments provides traction to the mat.

Finally, not to be dismissed is the gliding motility of cyanobacteria, responsible for trichome aggregation and reticulate formation, as it has been demonstrated in the early stages of regular reticular patterns that ultimately yield permanent MISS (Shepard and Sumner 2010; Cuadrado and Pan 2018).

3.5.4 Consolidation and Preservation of Multilayered Biosedimentary Structures

To close this essay, it is now fit to recapitulate how sediment microbes have gone from simple, two-dimensional biofilms to multilayered biosedimentary structures. It has been hypothesized (Nisbet and Fowler 1999) that modern microbial mats reflect metabolic developments and pathways acquired over the course of microbial evolution. Hence, the layered structure and the niche segregation among different microbial guilds with anoxic levels occupied by archaea and bacterial respirers, fermenters, and green sulfur bacteria and oxic levels harboring aerobic purple sulfur bacteria and cyanobacteria may reflect how the exposed sediments on Earth transitioned from being subject to reducing conditions to oxidizing ones. This necessarily called for a complexation in structure, from simple two-dimensional, mostly organic biofilms to multilayered biosedimentary structures that become *functional units by themselves* and in which the dominant microbes in each layer exploit specific niches and marked O_2, H_2S, and light gradients.

Surface processes are of paramount importance in photosynthetic microbial communities, particularly with respect to light harvesting and nutrient exchange. As much as this calls for a disposition of photoautotrophs in the uppermost layers, the cohesiveness created by trichomes likened to "cyanobacterial sealing" (sensu Seilacher et al. 1985), the accumulation of organic matter through EPS secretion, and the accumulation of empty sheaths also set limits for vertical diffusion of O_2 and create a boundary for obligate prokaryote anaerobes. On top of this metabolic segregation of microbes, there is an active physical role played by cyanobacterial trichomes in the accretion of sediment particles ultimately promoting the generation and consolidation of the three-dimensional biolaminites this essay has focused upon. Periodic or sporadic physical disruption in the form of tidal or storm currents may spatially affect surface microbial mats and generate deformation MISS. Hydrodynamic processes may also resupply or redistribute sediment, thus providing new substrates for colonization. Through the above-described steps which roughly match the properties of emergent microbial mats, three-dimensional "biolaminites" are generated over time (Gerdes et al. 1991), which may accumulate as they become buried, keeping record of microbial textures and MISS. Figure 3.3b provides a striking graphic synthesis of how the repetition of these processes over time yields biosedimentary sequences which aid in paleoenvironmental reconstruction.

Due to their biosedimentary nature, MISS are delicate structures that get preserved under exceptional circumstances and are known as *Lagerstätten* (Martin 1999). One such preservation process is cyanobacterial sealing (Seilacher et al. 1985). Microbial biofilms at the sediment-water interface facilitate the preservation, inhibit organic decay, and reduce the erosion threshold of sediments. Even while epibenthic microbial mats are currently restricted to a relatively few coastal environments such as hypersaline siliciclastic basins, their study with the actualistic, interdisciplinary approach of GeoBiology can shed light into the processes that led

these remarkable first ecosystems to be so successful in the early colonization of Earth's biosphere.

Acknowledgments I would like to extend my thanks to Dr. C.J. Hurst for inviting me to be part of this exciting book project and providing valuable and constructive comments on an early draft.

Jerónimo Pan

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Chapter 4
The Democracy of Dirt: Relating Micro-Scale Dynamics to Macro-Scale Ecosystem Function

Joshua Schimel

Abstract Soil can be analogized as a "democracy" in which there are three "political parties" (microbes, minerals, and organic molecules) that sometimes compete and sometimes cooperate in controlling biogeochemical process in the soil system. These interactions are in turn regulated by the media that regulate the flow of material and information through the system; in soil the key medium is moisture, which regulates substrate solubility and transport. Modern soil science is increasingly sophisticated in our tools to analyze these phenomena with high resolution at fine scales— 'omics identifies thousands of microbial taxa, tomography maps the 3-D pore structure of a single aggregate, and chromatography-mass spectrometry systems identify molecules in soil. Yet, we are still challenged to assimilate the masses of data these methods produce to explain the overall functioning of the soil system. For example, why do molecules that microbes can metabolize in minutes still persist for centuries or millennia? How we assimilate these new data into the models (conceptual and mathematical) that we use to understand and characterize the soil system is fundamental to developing integrated perspectives on soil and its roles in biogeochemical cycles. In this chapter, I discuss ideas and approaches for integrating data to better bridge from the micron-scale processes that occur on mineral surfaces or in individual soil pores to meter- and kilometer-scale phenomena within soil profiles and landscapes and which drive the functioning of terrestrial ecosystems.

4.1 Introduction

Democracy is the worst form of government, except for all those other forms that have been tried. . .

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_4

Winston Churchill[1]

Democracy is messy—parties battle for control, and who wins shifts over time and location. Different parties may dominate at local, regional, and national scales and on different issues. Yet, coherent patterns can sometimes emerge from the chaos. Democracy, then, is a lot like soil.

In soil, coherent patterns emerge from the interchange among the "parties," but soil doesn't have two parties, but *three*: microbes, minerals, and organic molecules; these battle for control over the biogeochemical functioning of the overall system. Each will dominate in some situations, be irrelevant in others, and work in concert with one or both of the others in yet other situations and on particular processes.

The final element of the "political" system that drives our *Democracy of Dirt* is the media,[2] which regulate interactions among the components—the flows of material and information. In soil, the essential medium, however, is not newspapers or the Internet, but *moisture* (Kleber et al. 2015). Water carries materials as it flows through soil and, even when static, allows dissolved materials to diffuse. As a solvent, water regulates dissolution and precipitation and so controls mineral weathering and soil development (e.g., Slessarev et al. 2016). Water is also an essential resource that controls the activity of microbes (Schimel 2018). Additionally, while metabolic waste products are small and often volatile (e.g., CO_2, CH_4, N_2O, NH_3, ethanol, etc.), most substrate molecules are larger and often more complex; they are usually nonvolatile and rely on water to make them accessible to microbes—solubility is a key control over a chemical's availability (Schimel and Schaeffer 2012).

Soil science, as an academic discipline, is about understanding these complex, intertwined, dynamics. Yet, just as political scientists may focus their attention on only one aspect of the overall political system, becoming expert in local politics or the history of a single political party, so too, is it with soil scientists. Although our ultimate goal may be to understand the integrated system that is *soil*, most scholars specialize, focusing on specific components (e.g., soil biology or chemistry). As a result, we struggle to understand the integrated system and how components shift in their role at particular levels of organization and scales of time. For example, microbial community composition may drive the short-term decomposition of litter and its conversion into soil organic matter (SOM), but over millennia the fate of that SOM is largely a function of mineral interactions that stabilize the molecules by making it inaccessible to microbial attack (Grandy and Neff 2008; Falconer et al. 2015; Schmidt et al. 2011).

The final thing to consider in developing an integrated understanding of soil function is the tools we use to represent, integrate, and portray that understanding—how do we *model* soils? The Oxford English Dictionary includes this definition of a model: "A simplified or idealized description or conception of a particular system."

[1]Churchill offered this famous quote in the House of Commons in 1947, but he ascribed to others: "It has been said. . ."

[2]OED: II. A person or thing which acts as an intermediary. a. An intermediate agency, instrument, or channel; a means; *esp.* a means or channel of communication or expression.

That a scientific model is an idealized vision of something is well reflected by another use of the word "model"—I don't think I've ever seen a real person who looks like the pictures on the covers of fashion magazines! We may be surrounded by such images, but they are distinctly *idealized*. Models of soil function are equally idealized (though hopefully not photoshopped to hide flaws), as well as being simplified. I can't comprehend the full complexity of soils, so a model that captured every aspect of soil function would be equally beyond my comprehension and so would not be useful. Instead, we represent our conceptual framing of processes and mechanisms in simplified forms, first as concepts and then in equations that we can incorporate into a mathematical architecture that allows us to put pieces together and quantitatively explore their interactions and outcomes (Blankinship et al. 2018).

4.2 Microbes and Metabolism

"Is soil alive?" Hans Jenny asked me that when I was a new Ph.D. student and I'm still not sure I have a good answer. But, unquestionably, soil is structured *by* life. Plants create organic matter, microbes rework it into new forms, and soil animals tunnel and mix. Without life, there's just rock and dust, not *soil*.

Soil is the most complex habitat on Earth, contributing to the vast diversity of microorganisms and microfauna that exist within the soil. Analyses of the diversity of microorganisms in soil typically identify on the order of ~10,000 phylotypes of microorganisms. But even before DNA sequence-based methods showed the full sweep of life in soil, researchers had recognized that soil contained vast biodiversity; for example, nearly a century ago, Waksman had noted "It is almost impossible at present to make a complete study of the various types of bacteria occurring in the soil, due both to the great variety of forms and to the lack of sufficient knowledge concerning many of them" (Waksman 1927).

Since that time, our ability to analyze the composition of the microbial community in soil has been vastly expanded by the development of DNA- and RNA-based molecular tools. 'Omic analyses have identified new microbial lineages and new biochemical pathways that had not been observed using previous culture-based approaches; they also have allowed exploration of how microbial communities are structured in complex environments and how communities respond to environmental perturbations (Jansson and Hofmockel 2020). The depth of information and understanding these tools give us about microbial communities is phenomenal. Yet, the significance of that diversity in the functioning of the soil system remains a subject of debate (Schimel and Schaeffer 2012; Sokol et al. 2019; Jansson and Hofmockel 2020; Reed and Martiny 2007; Fierer 2017). There also remain challenges in applying purely biological tools to understanding the rates and pathways of soil processes (Baveye et al. 2016). Challenges come from both chemical kinetic theory and biogeochemical modeling.

Even when only a limited group of organisms carry out a process, it can be difficult to link population sizes to process rates. This is harder for processes that are

broadly distributed across microbial groups (Schimel 2001) and when organisms have overlapping metabolic capacities; in this case much of the biodiversity can be seen as "redundancy" (Allison and Martiny 2008).

Chemical kinetic theory states that the overall rate of a reaction sequence is regulated by its slowest step—the "rate-limiting" or "rate-determining" step. That slowest step defines a reaction's kinetic rate equation.

Chemistry in soil occurs even in the absence of life, but life is the great "force multiplier" for chemistry; it organizes enzyme systems and enables cells to link energy-generating processes with energy-demanding ones to overcome activation-energy constrained or endergonic reactions.

Hence, the rate-limiting step in decomposition is rarely the physiological capacity to process a molecule once it is within a cell's membrane—most naturally occurring molecules, once brought inside a cell, can be rapidly metabolized. Thus, to model the kinetics of microbial processes, actual catabolism may be almost irrelevant in defining the rate of the overall process. Rather, the challenge, more commonly, is to mobilize molecules into a form a microbe can get across its membrane (Schimel and Schaeffer 2012); hence, the critical, rate-controlling step is likely the process that enables microbes to access a substrate molecule.

If, for example, substrate desorption from a mineral surface is substantially slower than microbial uptake and metabolism, the approximate rate expression would reflect the desorption rather than biochemistry (Scow 1993). An analogous situation exists with polymer biodegradation. Microbes must excrete enzymes to fragment plant polymers, which are generally too big for microbes to take up directly (cellulose fibrils can be >10 μm long—longer than a bacterial cell; Taiz et al. 2015). In such cases, the kinetic expression will reflect exoenzyme-driven depolymerization (Schimel and Weintraub 2003) or enzyme diffusion to substrates rather than microbial uptake and metabolism (Manzoni et al. 2016).

More important for the long-term dynamics of organic substrates is what microbes convert a substrate to—do they produce necromass or other constituents that are readily stabilized? The importance of how microbes allocate organic substrates is reflected in the growing interest in carbon use efficiency (CUE), and the closely related concept of microbial growth efficiency (MGE) which are vital in carbon cycling models (Wieder et al. 2013; Todd-Brown et al. 2011; Hagerty et al. 2018), and in a growing focus on microbial *anabolism*—how microbes grow and what they produce (Liang et al. 2017).

More sophisticated approaches to chemical reaction kinetics in complex systems, such as flow-through reactor systems like soil, recognize that both the actual reaction *and* the physical transport processes can be important. Hence approaches such as reaction-diffusion models (Von Fischer et al. 2009) and reactive transport models (Li et al. 2017) are becoming more common in ecological systems. Such models integrate physical movement with chemical reactions and use transport to constrain substrate and product concentrations at the point of reaction.

The second major challenge in using 'omics tools to drive models of soil biogeochemistry is the "simultaneous equation" problem: we may identify on the order of 10,000 phylotypes of microorganisms in a soil sample, but the models we

use for describing C and N cycling typically have at most 10 or 20 chemical flows. It is not mathematically possible to write solvable equations that link 10,000 independent driver variables to 10 or even 100 response variables. We must either collapse down the 'omics data set or increase the number of processes expressed in the model, or some combination of both, to match the numbers of independent and dependent variables. Here, too, approaches are developing to solve this problem (Treseder et al. 2011). Identifying functional guilds (e.g., Moorhead and Sinsabaugh 2006) possibly by using big data techniques such as network analysis to identify clusters of organisms that respond in similar ways to environmental drivers (moisture, substrates, etc.) offers great potential (Barberán et al. 2012; Lennon et al. 2012). Such tools provide a more empirical definition of "functional group" than using phylogeny as the only guide, or by classifying organisms purely functionally—i.e., "nitrifiers," a group that now includes bacteria, archaea, and even fungi, but different groups of ammonia oxidizers respond to environmental drivers quite differently. In the carbon cycle, of course, "heterotroph" encompasses everything from *Escherichia coli* to *Homo sapiens*, not a useful functional group definition!

The next challenge is model parameterization. To develop and run a model, you must know the values of the parameters that go into the model. If specific microbial populations are to drive processes in a model, we need to know the size and dynamics of those populations. But, we don't have a time machine—we can't know what those populations will be at some future time when climate has changed. Equally, we can't sample DNA from every hectare on Earth to map populations spatially. Requiring measured data over both future time and space to parameterize a large-scale model would therefore be impossible-*squared*. Including actual population sizes as drivers in a process model limits its scope to a fine-resolution, short-time-scale, mechanistic exploration of how processes function. That isn't a criticism, but it is a constraint.

We can get past that constraint to some degree by understanding what drives those microbial communities well enough to reasonably predict what they will be in the future, or in other locales, and so include them as explicit terms in an equation describing a process. But, if we can do that, then we also understand their behavior well enough to collapse them out of the equation!

Instead, approaches to capturing microbial processes in models—to count the votes of the "microbe party"—have "modelled past" the microbes by assuming that microbial communities are in equilibrium with their environment. This allows microbial dynamics to be collapsed into model equations *implicitly* as rate constants and response functions that relate the environmental drivers to process rates (Schimel 2001). But what do we do when conditions are changing and we cannot assume that communities are in equilibrium with their environment and resources?

If the goal is to better describe biogeochemical processes at large scales, and under changing environmental conditions, we need the insights into process dynamics and linkages that only fine-scale analyses and models can provide, but then we must figure out how to distill out the essence of those phenomena that make a difference at the macro-scale.

There has been progress in bridging the gaps, for example, using "trait-based" approaches to define and aggregate microbial functional groups (Lennon et al. 2012; Krause et al. 2014; Malik et al. 2019). This approach was developed in plant ecology, where to describe plant function at large scales, modelers have moved away from using specific taxa in a model; rather plants are represented by traits which can be quantified and modeled (e.g., Bonan et al. 2012). Those traits can then be mapped onto either individual species of plants or onto growth forms or other aggregated groups (Shipley et al. 2016). This approach may not be necessary in low-diversity ecosystems where there are only a few key plant species (e.g., conifer forests; Butler et al. 2017), but is key in more diverse communities (Asner et al. 2015) where taxa may share functional traits. Such a high-diversity condition certainly describes soil communities.

4.3 Minerals

In the *Democracy of Dirt*, minerals comprise the "Silent Majority." They create the physical structure of the soil and regulate the accessibility of substrates to microbial attack. It has become generally accepted that mineral-associated organic matter (MAOM) comprises the more persistent components of SOM (Kleber et al. 2015; Sokol et al. 2019), but this idea is not new. Concepts of "physical protection" date back decades (e.g., Russel and McRuer 1927) and have remained a dominant theory to explain how organic molecules that are readily metabolizable may persist for centuries or even millennia in soil.

Soil texture regulates a soil's organic matter content, with clay being seen as the key to soil texture effects on SOM (Rasmussen et al. 2018). Clay's high surface area and charged surfaces (ion exchange capacity) can sorb organic molecules, while its small particles allow clay to pack so tightly that pores may exclude microorganisms from accessing trapped resources. This phenomenon led to "habitable pore space" theory to explain soil foodweb structure (Elliott et al. 1980). Biogeochemical models such as CENTURY (which simulates the long-term dynamics of carbon and nutrients in soil-plant systems) or its modern iteration DAYCENT (a more sophisticated version that runs on a daily time step to model ecosystem fluxes and exchanges with the atmosphere) may not require microbial community data as input variables, but they do require knowledge of soil texture (Parton et al. 1987). Clay content is critical to describe how active C partitions into slow and passive pools, and such models predict ca. 50% higher C stocks in clay loams than in sandy soils.

Recent studies, however, have illustrated that "clay" on its own—encompassing all mineral particles smaller than 2 μm—is inadequate to describe organic matter stabilization. Rather, in neutral pH soils, where bulk clay mineralogy tends toward phyllosilicates, SOM is sorbed primarily by short-range-order (SRO) mineral phases—these comprise but a small, albeit chemically active, portion of the total clays (Rasmussen et al. 2018). In contrast, alkaline soils are rich in base cations, and so in these soils, SOM is stabilized by Ca-bridging where the divalent cations link

between clay surfaces and organic anions. In acidic soils, on the other hand, Fe and Al complexes are dominant in stabilizing SOM. Thus, Rasmussen et al. (2018) argue that we need to look "beyond clay" as a predictive variable to explain organic matter dynamics—we need to capture more than merely particle size but also actual mineralogy and the mechanisms by which the different mineral phases interact with organic molecules (Kleber et al. 2007). Despite that, it may still be easier to predict the "vote" of the silent mineral majority than of microbes because minerals are more geographically constrained and defined. The factors that control overall clay mineralogy are understood well enough to map them on geographic scales that readily feed into biogeochemical models.

4.4 Organic Molecules: Molecular Structure

The amount of organic matter in soil or subsoil has long been seen as an important characteristic of a soil and of an ecosystem (e.g., Cameron and Breazeale 1904). However, although we often discuss soil organic matter (SOM) as if it were a single aggregate entity, this is of course not true. In fact, SOM is an enormously complex mix of materials. The ways in which SOM behaves chemically, structures soil physically, and alters the surfaces of soil minerals make SOM a dominant party in the *Democracy of Dirt*. The chemical nature and availability of organic molecules for extracellular reactions or microbial uptake often regulate the "voting" of the soil microbes.

Older conceptual models had characterized SOM as "humic materials"; these were thought to be large poly-condensed molecules whose stability was a function of their chemistry. Individual humics were described as large (>5 kDa; Perminova et al. 2003) and so complex that almost no two might be identical (Stevenson 1982), analogous to snowflakes. In this concept, SOM molecules were viewed as being chemically recalcitrant: too large for a cell to take up, too complex to be susceptible to targeted enzymatic attack, and too aromatic to be easy to metabolize (Schnitzer et al. 1991). Hence they were thought to decompose slowly and to be inefficiently assimilated into biomass. However, the humic "snowflake" model is no longer broadly accepted. Rather, humics are now thought to either result from condensation reactions in the extraction process or to comprise conglomerates of small molecules that produce artificially inflated estimates of molecular weight (Lehmann and Kleber 2015).

Problems with describing soil organic matter dynamics based on a humic/fulvic/humin fractionation had long been recognized; thus, mathematical models such as CENTURY and Roth-C (the Rothamsted Carbon Model, which is structurally similar to CENTURY, describing C-turnover in non-waterlogged soil) that were developed as far back as the 1980s did not describe SOM by its chemical composition, but rather assigned OM to discrete pools based on turnover times, for example, Parton et al. (1987) assigned the turnover times of the "active," "slow," and "passive" C as 1.5 y, 25 y, and 1000 y.

Newer models have mapped SOM into more readily measurable and identifiable materials rather than relying upon "conceptual pools." An example of this approach is the "Millenial" model (which defines C and nutrients as low-molecular-weight (i.e., soluble), microbial biomass, particulate, mineral-associated, and aggregate-protected; Abramoff et al. 2017). Developing such models recognizes that, ultimately, our theories need to link more concretely with actual physical materials and the real chemistry and physics through which they interact. Such models recognize that microbes don't, in fact, metabolize "organic matter"; rather, they metabolize specific organic *molecules* that have to move to a microbial cell.

For a microbe to access a substrate, either the substrate must already be small enough to diffuse to the cell, or the microbe must fragment the substrate extracellularly by excreting an enzyme that can break the polymer into monomers or oligomers—molecules small enough that they can diffuse to the cell (Schimel and Schaeffer 2012).

Outside of a living cell's controlled environment, enzymes are limited in the chemistry they are capable of carrying out. Many extracellular enzymes catalyze simple hydrolytic reactions (e.g., glucosidase, cellobiohydrolase, N-acetylglucosaminidase, etc.). Others (e.g., peroxidase, phenol oxidase) oxidize substrates by what I describe as "shotgun metabolism"; these enzymes are non-specific in how they transfer an electron from a substrate to a metal ion, either in the enzyme itself or as a free intermediate (e.g., Mn^{3+} in the case of manganese peroxidase; Conesa et al. 2002). This process can almost randomly knock fragments off macromolecules, fragments that can then recondense via abiotic chemistry or that can be taken up and metabolized by microbes. Such shotgun metabolism allows oxidative enzymes to be very non-specific (Ruiz-Dueñas and Martínez 2009).

However, once a microbe takes up a molecule, it requires specific enzymes to convert that molecule into an intermediate of the central metabolic pathways that cells use to generate energy and provide C-skeletons for biosynthesis (e.g., glycolysis and the tricarboxylic acid cycle). Some enzymes are produced constitutively; hence, the substrates for those enzymes can always be metabolized. For other substrates, however, inducing their synthesis may invoke a high investment in C, N, and energy—possibly expenditures that can never be recouped.

Kaleta et al. (2013) calculated that to synthesize a protein costs 4.2 ATP per residue to polymerize amino acids. That calculation, however, assumes an average of 30 protein copies are made from each mRNA. For the *first* copy of a protein (hence, the minimum possible cost to induce a metabolic capability), the cost is 10 ATP per residue (6 ATP for each coding triplet in mRNA plus 4 to charge the tRNA and attach the amino acid to the forming polymer). For a small protein with 250 amino acids, the cost just to transcribe and translate a gene to produce one copy of a new protein (from existing amino acids) would therefore be 2500 ATP.

When a bacterium completely respires a glucose molecule, it can generate 38 ATP, so it would have to respire >65 glucose molecules to provide the energy to synthesize the needed ATP to assemble that new protein. There are additional costs associated with taking up the glucose and NH_4^+, plus those associated with synthesizing the needed amino acids. Accounting for all the costs means that to

induce even the simplest of metabolic capabilities would cost a cell an absolute minimum of the equivalent of 60–70 glucose molecules if the needed amino acids are available, to well over 300 if those amino acids are not. And, of course, the costs rise proportionally if several different enzymes are required. That investment must pay off.

That idea that synthesizing enzymes is energetically expensive has been incorporated into models of exoenzyme production and kinetics, because those enzymes are excreted and so don't contribute to cell biomass (Schimel and Weintraub 2003). Investment energetics has only recently begun to be explored in thinking about SOM dynamics more broadly or why simple molecules may persist for extended periods (Lehmann et al. 2020).

For a cell living in an environment where there is a lot of just a few dominant substrate types, those costs are not a problem. Consider a litter degrader on the soil surface which attacks primarily cellulose and hemicellulose. These are polymers composed of simple sugars such as glucose, arabinose, xylose, mannose, and galactose that are readily assimilated into core metabolic pathways (via glycolysis, pentose shunt, TCA, etc.) and hence readily catabolized to generate energy or used as carbon skeletons in anabolism.

Consider, instead, a microbe living deeper in the soil profile in a micropore or on the surface of a clay mineral. There is no fresh plant material; instead, the mineral-associated OM is a diverse mix of molecules, some of which may be plant-derived, but many may be from dead soil microbes (Kleber et al. 2007; Liang et al. 2017). No single molecule type is likely to be available in large quantities. Yet to metabolize any one of them, the acquired molecule must be shunted into core metabolism, which may require synthesizing new enzymes. If there is not enough of a potential substrate molecule to pay the energetic costs to make the enzyme(s) needed to metabolize it, then it would be detrimental for a cell to use that substrate—the investment might never pay off; there would be a negative energy yield. Essentially by "eating" this compound, a microbe would only starve faster. I might call this the "celery effect" after the well-known myth that celery has *negative* calories; i.e., it takes more energy to digest than you get from eating it.

Given enough time, it is possible that a microbe might eventually get enough of a substrate to pay off the costs of synthesizing the needed enzymes, but enzymes are not "immortal" in microbial cells—turning over proteins is a substantial fraction of overall cell maintenance costs (Kempes et al. 2017). A cell must take up enough of a particular substrate molecule over the life of the required enzymes to make using the substrate energetically and "economically" viable. A microbe might well have the genes for the necessary enzymes and even have access to some of that substrate, yet still leave it untouched.

This "celery effect" would become more likely to limit microbial activity with increased depth in a soil, as total organic matter levels decline, molecules are more tightly tied up with the mineral phase, and the diversity of organic structures that microbes have access to may increase (Lehmann et al. 2020). Despite having the genetic potential to use every substrate present, a microbe might starve.

Such an effect would not reflect chemical recalcitrance of a substrate, but rather "economic recalcitrance" that results from a negative return on investment. Hence, "chemo-diversity" may be important in regulating overall soil organic matter dynamics, particularly deeper in a soil profile, reflecting an overall decrease in SOM levels and an increasing likelihood that there is less of any single substrate that can be used constitutively to support microbial activity.

Disturbances, such as mixing or rewetting, that redistribute chemicals and make them available to microbes can overcome access limitations to increase microbial activity and growth. But such disturbances could also work to overcome energetic economic constraints on microbial use of soil substrates and may explain why disturbances cause larger relative increases in respiration and microbial growth in deeper soils than they do in surface soils (Xiang et al. 2008; Slessarev et al. 2020).

As far back as 1918, Morrow (1918) argued the challenge of characterizing the chemical composition of SOM:

> Our present knowledge leads us to believe that it is possible to isolate an almost infinite variety of chemical compounds from a soil, the number and variety reaching a limit only when we have isolated all of the compounds which are present in the plants which grew upon the soil, plus those compounds contained in the bodies of bacteria, protozoa, and fungi, plus all of the compounds which may be derived from these compounds under the peculiar soil conditions of decay, oxidation, bacterial action, and the secretions of fungi and living plant roots.

Luckily, Morrow's presumption of "almost infinite" is a bit of an overstatement, despite the diversity of molecules found in soil. The molecules that likely fuel microbial activity are water soluble, which limits their number and diversity. Untargeted metabolomic analyses of dissolved metabolites provide a limited set of molecules that are identifiable. For example, in a sandy loam of mixed mineralogy from a meadow within a forested area of Mendocino, California, only 55 metabolites were identified (Swenson et al. 2015). A similar number (50) was found in microdialysis samples from a Swedish Scots pine forest on a soil described as a "podzolized sediment of sandy silt" (Randewig et al. 2019). In each study, the compounds were dominated by simple compounds that should readily be channeled into core metabolism: amino acids, sugars, etc. This could explain why other soil organic constituents may persist.

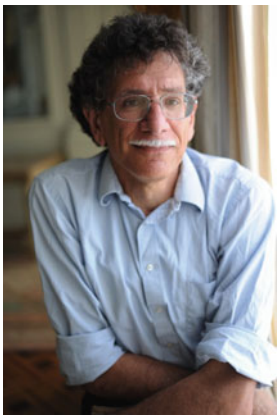
Understanding the fine-scale dynamics of SOM—which molecular types are readily available, how these fuel microbial survival and growth, which are more vulnerable to being metabolized vs. stabilized—is important if we are to understand how these molecular processes regulate soil and ecosystem function. Being aware that the "economics" of microbial substrate use may be a control on overall SOM dynamics is just a start; the next steps are to sort out when and how these cross-scale interactions operate.

4.5 Synthesis

How, then, do we resolve these complex interactions to advance our understanding of how soils function? It is certainly still not clear. In each of the core areas of analysis, microbial populations, mineralogy, and organic matter chemistry, advances are occurring rapidly. There is also a growing recognition that we need to understand how these components of soil interact with each other. How are we to determine which "party" is currently "in power"? How do the different aspects of chemistry, physics, and biology interact with each other to create the patterns we observe at the soil profile to landscape scales? These have been core foci in soil science since its inception—each generation of scholars develops new tools, which lead to new insights, and new theories to explain the dynamics of soil systems.

We have reached a point in the technologies we use in soils that we can analyze the individual component parts of a soil to an amazing degree—'omics allow us to analyze not only which taxa of microorganisms are present in a soil, but which are growing and which genes they are currently transcribing. We can analyze specific molecules that exist in soil (via instruments that couple chromatography and mass spectrometry), and we can use tomography to map the structure of the pore network within individual aggregates. However, we still lack theory to link such fine-scale measurements with larger-scale process dynamics. Such theory must consider the complex "politics" and the interplay among the parties, to understand how microbes interact with organic molecules and how those interactions are mediated, facilitated, or blocked by the interactions with mineral surfaces.

Reintegrating our approaches to studying fine-scale soil processes, bringing together our understanding of physical, chemical, and biological phenomena, is essential to develop more effective cross-scale theories and tools to understand and predict how changing environmental conditions will alter the functioning of soil systems. In concert with this reductionist push at the fine scale, we equally require more effective cross-scale theories and models to identify how the fine-scale dynamics may be most effectively captured to improve our understanding of larger-scale soil dynamics—it's not just pore-to-core, but pore-to-core-to-profile-to-landscape! How do we capture the "local politics" to understand, and better manage, the national scale of soil biogeochemistry?



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Part II
Microbial Life Persists Within Even the Most Extreme Environments

Chapter 5
The Concept of Evanescent Microbial Ecosystems in Earth's Atmosphere

Dale Warren Griffin

Abstract This essay presents the hypothesis that short-lived or evanescent microbial ecosystems exist in Earth's lower troposphere (~ < 4 km). This hypothesis is supported by culture- and molecular-based studies that have shown diverse, viable, and metabolically active microbial communities within Earth's atmospheric boundary layer. Surprisingly, microorganisms are routinely recovered in samples collected at extreme altitudes including those within the stratosphere (> 18 km). Volcanic eruptions, dust storms, fires, and sea spray are known to seed the atmosphere with microorganisms and to serve as potential nutrient sources while in the atmosphere and upon deposition. Recent research has demonstrated that microorganisms are metabolically active in clouds; for example, archaea capable of utilizing gases such as methane and hydrogen-nitrogen have been identified in clouds and in the atmosphere over natural and anthropogenic gas seeps. The only difference between this hypothesized ecosystem to more traditionally defined ecosystems is its evanescent characteristics where clouds or gas plumes eventually dissipate as they reside over and traverse Earth's terrestrial and/or aquatic environments. The life cycle of these hypothesized evanescent airborne ecosystems would be short-lived relative to the classically defined biomes or ecosystems.

5.1 Essay

Similar to the many definitions of an ecosystem that can be found online, Merriam-Webster's definition is simply "the complex of a community of organisms and its environment functioning as an ecological unit" (Merriam-Webster 2020). National Geographic defines ecosystem as "An ecosystem is a geographic area where plants, animals and other organisms, as well as weather and landscapes, work together to form a bubble of life" (National-Geographic 2020). While many definitions of

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ecosystems do not address time or the life cycle of an ecosystem, the influence of time on ecosystem variability and organism-specific viability was presented in Henry C. Cowles' concept of ecological succession (Cowles 1899). Ecological succession studies have just recently been applied to the micro-scale of microorganisms, but historically, most have occurred at the macro-scale of plants and animals, and on temporal scales of decades to millions of years (Ortiz-Alvarez et al. 2018; Walker et al. 2010).

I recall walking into a meeting room for a graduate-level course on molecular paleontology, being taught by a geology faculty member at the University of South Florida, and marveling at the geological timeline tape that extended around the ceiling trim. The segment of that tape that represents the existence of the only literate organism to have ever existed on the planet is just a tick (the last ~200,000 years) at the end of the very long ~4.5 billion-year timeline. The few other students in the class had backgrounds in geology versus my background in microbiology. What struck me as the course progressed was the differences in how we thought about time. The geologists commonly thought in terms of millions to billions of years whether it was referencing subjects such as evolution (humans and our atmosphere), extinction events, or continental drift. As a microbiologist I was keenly aware of theories on the evolution of life, like Cairns-Smith's "clay hypothesis" (Cairns-Smith and Hartman 1986), but I spend the majority of my time in the realm of public health microbiology where microbial replication and pathogenic outbreaks occur over periods of minutes, hours, and days. There is no doubt that both inorganics and organics contribute to the definition of an ecosystem, but all ecosystems as we know of them have one thing in common; they are evanescent in nature. It is only the stability of an ecosystem in relation to time that defines that system's degree of evanescence.

Micro-aerobiology or the study of microbial life in the atmosphere was pioneered by Louis Pasteur. In Pasteur's efforts to dispel the theory of "Spontaneous Generation," he demonstrated the presence of microbial life in atmospheric samples collected in caves, on mountain tops, and within the built environment (Pasteur 1861). Irish scientist John Tyndall, a supporter of Pasteur, later described the results of nutrient broth tray experiments utilized to detect viable microbial life in the atmosphere: "*Reflecting on the whole of this, I conclude that the germs float through the atmosphere in groups or clouds, and that now and then a cloud specifically different from the prevalent ones is wafted through the air. The touching of a nutritive fluid by a Bacterial cloud would naturally have a different effect from the touching of it by the sterile air between two clouds. But, as in the case of the mottled sky, the various portions of the landscape are successively visited by shade, so, in the long run, are the various tubes of our tray touched by the Bacterial clouds, the final fertilization or infection of them all being the consequence.*" (Tyndall 1882). This early research that discovered that microorganisms can routinely be found in the atmosphere and that their distributions are not universal, but patchy, was key to understanding variance in their concentrations due to such factors as location and altitude constraints. More recent culture- and molecular-based studies have demonstrated that very diverse microbial communities routinely occur in Earth's

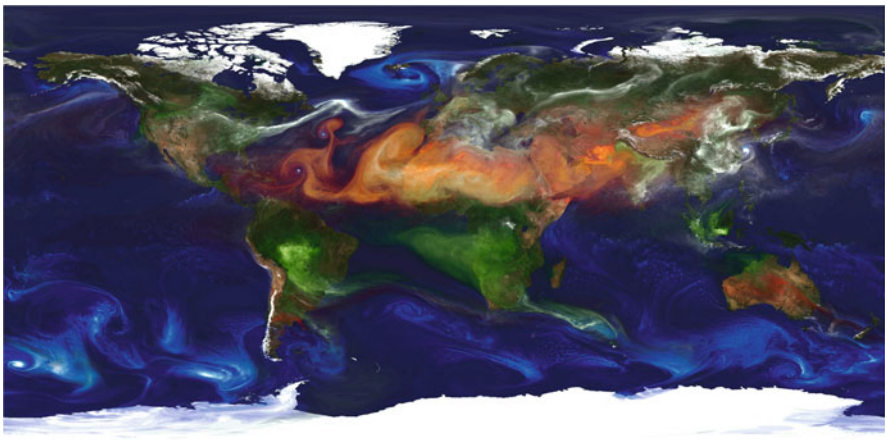


Fig. 5.1 Goddard Earth Observing System Model, Version 5. Different aerosol types modeled from 2005–2007 satellite data. Desert dust (red), sea salt swirls in cyclones (blue), fire smoke (green), and sulfates from volcanoes and fossil fuel burning (white). Image credit: William Putman, NASA/Goddard www.nasa.gov/multimedia/imagegallery/image_feature_2393.html

atmosphere over both terrestrial and aquatic environments, including extreme altitudes (upper troposphere, tropopause, and stratosphere) (Smith et al. 2018; Amato et al. 2017; Schuerger et al. 2018). In regard to the concentrations of atmospheric microorganisms that may originate from various terrestrial or aquatic sources, research conducted at several sites in Spain reported atmospheric deposition rates of bacteria and viruses that ranged from 3.0×10^6 to $>8 \times 10^7$ m^{-2} and 2.6×10^8 to $>7 \times 10^9$ m^{-2} per day, respectively (Reche et al. 2018). Over the last few years, scientific investigations have demonstrated active microbial metabolism in atmospheric environments, and these atmospheric and microbial interactions may occur on time scales of hours to weeks (Amato et al. 2017; Klein et al. 2016).

Some of the primary factors that affect microbial viability in the atmosphere are UV exposure, water availability, nutrient availability, temperature, and genetic capability to resist stresses caused by these (e.g., like the cold and radiation-resistant extremophile *Deinococcus radiodurans*) (Smith et al. 2011b; Smith et al. 2011a). Aerosols such as those depicted in Fig. 5.1 (fire smoke, volcanic ash, sea spray, and desert dust) can load microorganisms into the atmosphere on a global scale, and organics (detritus, cells, etc.) and inorganics within these aerosol sources can serve as nutrient sources for suspended microorganisms or for organisms in downwind ecosystems (Griffin and Kellogg 2004; Westrich et al. 2016; Lenes et al. 2008; Aller et al. 2005; Mims and Mims 2004; Griffin 2004; Van Eaton et al. 2013). Lightweight organic detritus with large surface areas (Fig. 5.2) can facilitate the long-range dispersion and increased suspension times through "rafting" of aerosolized microbial communities. Some of these events can result in continuous aerosol loading (sea salts) or episodic infusions (dust storms, fires, and eruptions) over periods of days to months. Large volcanic eruptions can load significant quantities of particulates into

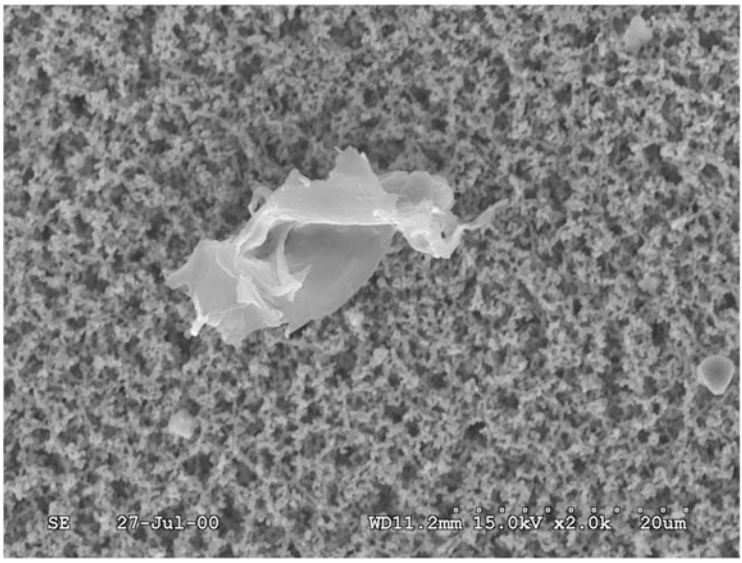


Fig. 5.2 A scanning electron microscope image of a piece of organic detritus that was collected in the Caribbean (27 July 2000) when Saharan/Sahel desert dust was visibly present. Lightweight detritus like this can provide UV shielding and result in increased residence time in the atmosphere through "rafting" of associated microorganisms

the stratosphere and can affect Earth's climate. Similar to the ability of dust storms to attenuate UV light (~50%), the other aerosol sources can similarly attenuate UV and thus provide some degree of UV shielding to associated microbial communities (Herman et al. 1999).

One of the most visibly obvious locations for a microbial ecosystem in the atmosphere is clouds. Clouds are an oasis-like environment where UV is attenuated and water is plentiful and so are potential microbial nutrients (organic detritus, nitrogen, methane, etc.) (Amato 2012; Hill et al. 2007). Temperature can also be a limiting factor which is dependent on a cloud's altitude. Clouds occur near surface to ~18 km in altitude, and temperature typically drops ~6 °C km^{-1} up to ~10 km and then remains stable up to ~20 km (Alpers et al. 2004). This constitutes a temperature range of near ground or ambient down to ~-60 °C. These temperatures can vary in mountainous regions where ambient temperatures at summits may reach typical lower altitude temperatures. The ~2.7 km summit at Mt. Bachelor, Bend, Oregon, USA, can reach ~32 °C in the summer, while the average July maximum at the ~4.3 km summit at Pikes Peak, Colorado, is ~0.9 °C. This temperature range difference between Mt. Bachelor and Pikes Peak indicates that significant rates of metabolism in clouds in the western United States are probably limited to an altitude of under 4 km. Metabolism at higher altitudes may occur at reduced rates or by specialized or yet to be identified adapted genera. Clouds typically have lifespans of minutes to hours and may morph from one cloud type to another or dissipate. Cloud propagation could influence the lifespan of cloud conditions within a given airmass

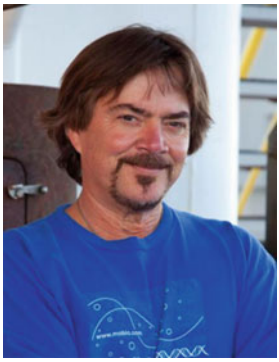
beyond the typical lifespan of an individual cloud. Cloud propagation is known to occur along storm fronts or under conditions where a cumulus cloud could potentially induce updrafts (Fovell and Kim 2003). Satellite imagery has tracked a propagating cloud cluster crossing between ~85°W and 165°E within a 5–10°N band between 22 July and 4 August 1967 (13 days) (Chang 1970). Airmasses that reside within propagating clouds at lower altitudes can provide conditions that favor the establishment of a microbial ecosystem in the troposphere, provided there are aerosolized microorganisms capable of metabolic activity in these types of environments (Schuerger et al. 2013; Amato et al. 2017; Aalismail et al. 2019). Once resident in the atmosphere, long-range dispersion of these microorganisms may occur at planetary scales. Recent work conducted atop Puy de Dôme in France (peak altitude ~1.5 km) found many cloud-associated microorganisms were metabolically active when spiked with nutrient sources (Vaitilingom et al. 2012; Vaitilingom et al. 2013). More recent experiments conducted at this location where the microorganisms were immediately fixed upon sample collection and evaluated using DNA and RNA extracts demonstrated in situ metabolic activity and a diverse community consisting of over 28,000 bacterial species (Amato et al. 2017). A study conducted at Mt. Bachelor Observatory (Bend, OR, peak altitude ~2.7 km) using a comparative RNA/DNA technique noted "Our observations suggest that metabolically active bacteria exist in the atmosphere and that these communities may be involved in the cycling of organic compounds in the atmosphere" (Klein et al. 2016). A number of different authors have presented the hypothesis of the existence of life in the atmosphere and clouds of other planetary bodies (Joseph 2019; Limaye et al. 2018).

Another location other than clouds where microbial atmospheric ecosystems may occur is aquatic or terrestrial gas seeps. The lifespan of these types of environments may exist on the order of hours to days depending on the stability of the gas plume, and this may be affected by emission rate, wind speeds (low and high energy), topography, and atmospheric phenomena such as inversions. Methane emissions or seeps occur naturally and from anthropogenic sources. Natural sources include wetlands, wildlife, volcanoes, and hydrocarbon deposits. Anthropogenic sources (~60% of the global emissions) include landfills, agriculture (rice paddies), livestock, and hydrocarbon extraction operations and their use (Saunois et al. 2016). The estimated global methane flux has been reported at ~500 Tg $yr.^{-1}$(~500 million metric tons), with landfills, rice paddies, wetlands, and soil contributing 40, 60, 110, and 140 Tg $yr.^{-1}$, respectively (Boeckx and Van Cleemput 1996). Estimates of methane emissions from livestock have ranged from 60 to 120 Tg $yr.^{-1}$ (Johnson and Ward 1996). Methane-oxidizing microorganisms at seeps that occur on the seafloor are known to function as the foundation of the foodweb in those island-like ecosystems and show genetic similarities at a global scale (Ruff et al. 2015). Near terrestrial seeps, children have been shown to harbor elevated concentrations of the methanogen *Methanobrevibacter smithii* (common human gut flora) in their intestines (de Araujo Filho et al. 2014). Within the atmosphere of swine confinement buildings, methanogenic sequences dominated archaeal sequences in aerosol samples (Nehme et al. 2009). In regard to the presence of methanotrophic bacteria in

outdoor atmospheric samples, researchers were able to demonstrate that isolates collected from the atmosphere over a landfill were able to oxidize methane in spiked experiments conducted under cloud-like conditions (Santl-Temkiv et al. 2013). Other types of seeps such as hydrogen-nitrogen seeps may be able to produce atmospheric plumes capable of supporting microbial metabolism (Sano et al. 1993). Research of cloud water collected by aircraft at an altitude between ~1.4 and 3.1 km (cloud temperatures greater than 6 °C) identified concentrations of ammonia, nitrate, and dissolved organic nitrogen at ~43, ~39, and ~ 18%, respectively (Hill et al. 2007). This research noted that concentrations of bacteria in the cloud samples averaged ~2.9×10^5 m^{-3} of cloudy air, that ~70% were metabolically active, and that ammonia-oxidizing bacteria were identified in these samples (Hill et al. 2007; Kourtev et al. 2011).

The evidence of metabolically active bacteria isolated from clouds and atmospheres associated with natural and anthropogenic gas seeps indicates the possibility of the existence of microbial evanescent ecosystems in Earth's atmosphere. Sources that may load the atmosphere with terrestrial and aquatic microorganisms and nutrients and also support these types of ecosystems include plumes originating from volcanic eruptions, dust storms, fires, and sea spray. The optimal location for these types of ecosystems is in the lower atmosphere where warmer temperatures would allow metabolic rates that would be expected for a wide range of microorganisms. It may be possible that similar ecosystems exist at higher altitudes at lower metabolic rates for known microorganisms or yet undiscovered groups of specially adapted microorganisms. Genomic technology now exists to advance our understanding of atmospheric microbial metabolic activity and how aerosol sources affect microbial densities and interactions by longitude, latitude, and altitude. However, funding constraints have hindered the acquisition and analyses of high-altitude atmospheric samples, and this has limited our ability to study and understand the extent and constraints of microbial life in Earth's atmosphere and those of other planetary bodies.

Acknowledgments/Disclaimer The USGS Environmental Health Mission Area supported the production of this article. Thanks to Tony Greco of the University of South Florida's College of Marine Science Electron Microscope Laboratory for assistance in acquiring the image in Fig. 5.2. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.



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Chapter 6
When the Vital Signs of Microbial Life Go Cold, Does That Mean the Pulse Is Gone? Microbial Life Persists at the Limits of Cryoenvironments on Earth

Jacqueline Marie Goordial

Abstract Cryoenvironments on Earth represent a natural laboratory in which we can observe the natural constraints to microbial activity and survival at low temperature. Microorganisms in sub-zero environments must contend with numerous stressors, and though microorganisms are nearly ubiquitous on this planet, the question of their activity under extremes of temperature is an open one. This essay describes soils in University Valley, located in the McMurdo Dry Valleys of Antarctica—a rare site in which microbial activity cannot be measured. Though the signs of active life were absent, the region is by no means dead. Microbial life persists in this valley, as in other cryoenvironments, and potentially on other planetary bodies within our solar system.

6.1 Introduction: Microorganisms Are the Foundation of Ecosystem Function in Cryoenvironments

What are the limits of microbial life? What are the most extreme conditions (temperature, pH, pressure nutrient deprivation, etc.) that a microorganism can survive in? And what is the difference between merely surviving and thriving? These questions have fascinated scientists for many years, ranging in fields from biology to planetary science. This essay will focus on the nature of life at extremes of cold temperature, in cryoenvironments. Cryoenvironments are generally defined as environments that exist either continuously or predominately at sub-zero temperatures. They occur in polar and alpine regions and include visually stunning large-scale features such as glaciers, sea ice, permanently ice-covered lakes and permafrost. Studies from both cryoenvironments and laboratories on Earth have demonstrated that microbial life is widespread in cold environments, can be metabolically active,

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_6

and can replicate at low, sub-zero temperatures if liquid water is present (Goordial et al. 2013; Mykytczuk et al. 2013). Some have even hypothesized that due to the slowing down of metabolic activity at cold temperatures, there is possibly no low-temperature limit to life on Earth (Price and Sowers 2004). In many cryoenvironments, microorganisms are the foundational life forms driving ecosystem function and, in many cases, are the only life present at all. These microbial-based ecosystems include microorganisms occupying multiple trophic levels and niches (e.g. autotrophy, heterotrophy) and those in varying physiological states, for example, metabolically active, replicating, dormant, or dead. Each of these physiological states plays an important role in ecosystem function.

This essay will discuss evidence of microbial "vital signs" in cryoenvironments—evidence for both activity and cellular replication at sub-zero temperatures. In particular, it will focus on one extreme site in the Antarctic McMurdo Dry Valleys, where microorganisms are thought to be on the verge of the cold-arid limit of life. It is an example of how microorganisms in different physiological states play a role in cryoenvironmental ecosystem function, even when vital signs of active life or a "pulse" is not detected. Finally, I will discuss what studying microbial life in cryoenvironments can tell us about the limits of life on Earth and potentially beyond.

6.1.1 Microbial Replication and Activity at Sub-zero Temperatures

Sub-zero temperatures affect microbial cells by causing lipids and proteins to become rigid and inflexible, affecting membrane and protein conformation and thus basic cellular function. Psychrophilic (cold-loving) and psychrotolerant (cold-tolerant) microorganisms have evolved multiple mechanisms to adapt to these conditions (Bakermans et al. 2009; Bakermans et al. 2011; Feller and Gerday 2003). For example, cold-adapted proteins have altered amino acid compositions, favouring residues that result in higher protein flexibility via less tertiary bonding and less densely packed hydrophobic cores (Raymond-Bouchard et al. 2018; Goordial et al. 2016). Psychrophilic organisms also have increased copy numbers of cold shock proteins and helicases for DNA and RNA processes such as replication and transcription (Mykytczuk et al. 2013). Cell membranes may be enriched with saturated and branched fatty acids to increase fluidity (Siliakus et al. 2017). In addition, as a result of thermodynamics, kinetic energies are lower at cold temperatures—lowering reaction rates and transport of molecules. However, the primary constraint to life at sub-zero temperatures may not be temperature itself, but the availability of liquid water, necessary for all of life as we know it. At low temperatures water freezes, and thus water availability for microorganisms in these environments decreases sharply. During the freezing process, any solutes present are squeezed and concentrated into thin brines within the frozen substrate, depressing the freezing point of water and permitting small pockets of liquid, though salty,

water for microbial life. Microorganisms are also squeezed into these salty veins, along with nutrients, for example, as seen in sea ice (Junge et al. 2004). Indeed, halotolerance seems to be a phenotypic trait shared by all psychrophilic organisms isolated to date. At sufficiently low temperatures, even highly concentrated brines may freeze to a mere film of water adsorbed to mineral particles. This is a condition where life is likely operating at the limits and activity is questionable. Additionally, the nature of the salts present also plays a role in microbial survival, as differing salts have different freezing points as well as different disordering properties which may be disruptive to cells (Hallsworth et al. 2007; Pontefract et al. 2017). As a result of understanding the added physiological stresses of sub-zero temperatures, the term cryophile is increasingly being used to denote organisms that grow at sub-zero temperatures, as opposed to psychrophiles, defined as having growth optima at temperatures between 5 °C and 15 °C (Raymond-Bouchard et al. 2018; Cavicchioli 2006; Seckbach 2013).

Sub-zero growth has been observed in microorganisms with broad phylogenic distribution and in all three domains of life. The current record for bacterial cellular division at low temperature is −15 °C by *Planococcus halocryophilus* OR1, a bacterium isolated from Arctic active-layer soils (the top layer above permafrost that thaws seasonally in the summer) (Mykytczuk et al. 2013; Raymond-Bouchard and Whyte 2017). Other examples include *Methanococcoides burtonii*, an Archaeal methanogen isolated from Ace Lake, Antarctica, and that can divide at −2.5 °C (Williams et al. 2011), and *Rhodotorula glutinis* FMT157, a yeast isolated from spoiled commercial frozen peas and that can grow at −18 °C (Collins and Buick 1989). This latter frozen pea isolate hints to a potentially overlooked aspect of low-temperature microbiology—that psychrophilic microorganisms may be more abundant and ubiquitous than expected. While most psychrophilic organisms have been isolated from polar environments considered by humans to be relatively "extreme", much of the northern hemisphere that is densely populated by humans (e.g. the cities of Toronto, or Montreal) undergoes a deep freeze regularly, encountering sub-zero temperatures for 4–6 months. It is quite probable that psychrotolerant or possibly even psychrophilic organisms exist in the many northern environmental niches that humans would not consider "extreme." These examples of sub-zero replication are known from isolated microbial cultures grown in laboratories. However as the vast majority of microorganisms cannot yet be cultivated, there may be much we are missing out on regarding the psychrophilic life cycle.

As illustrated, evidence for the activity and replication of microorganisms at sub-zero temperatures is now increasingly abundant. The question is not "Are microorganisms active at sub-zero temperatures?"; the unknowns are "Is there a low temperature limit of life?", "What abiotic mechanisms govern microbial life cycles at sub-zero temperatures?" and "What impact do cryoactive microbiota have on ecosystem function and global scale biogeochemical cycling?". Cryoenvironments at the edge of known habitability, where microorganisms are the only organisms present, are ideal natural laboratories to approach these questions.

6.2 University Valley, Antarctica: A Unique Example of a Solely "Microbial" Environment

The McMurdo Dry Valleys in Antarctica are the coldest and driest place on Earth and consist of over a 4500 km^2 (Levy 2013) ice-free region, which can be subdivided based on environmental conditions. Temperatures and aridity get colder and drier, respectively, from the lower-elevation and coastal Dry Valleys to the high-elevation inland Dry Valleys. Mean air temperatures in the coastal valleys are −5 °C, and valleys may experience annual precipitation (in the form of snow) of up to 100 mm per year. The stable upland zone (SUZ) microclimatic zone (elevations >1000 m.a.s.l., meters above sea level) (Marchant and Head III 2007) experiences average air temperatures of −10 °C and less than 10 mm snowfall annually. As a result of the cold and arid conditions, the high elevations of the dry valleys are one of the rare places on Earth where there is the presence of dry permafrost, soil that remains below 0 °C and has negligible frozen water content to bind soil particles together (Fig. 6.1b). Underneath the dry permafrost is ice-cemented permafrost more akin to the permafrost commonly thought of in alpine and polar settings, hard and frozen in place due to water-ice. Dry permafrost overlying ice-rich ground with sublimation processes as the dominant form of water exchange is rare on Earth, but widespread on Mars, making this area one of the best Mars analog sites on Earth. Up until recently, the only known analogous sites on Earth with dry permafrost were in the McMurdo Dry Valleys; however, dry permafrost has been recently discovered ~1000 km away, in Ellsworth Land, Antarctica (McKay et al. 2019; Schaefer et al. 2017), and is possibly present in high-elevation cold desert soils such as the Atacama desert in Chile, the driest place on Earth (Nagy et al. 2019).

University Valley (1700 m.a.s.l) is a small high-elevation valley, 1.5 km long and 0.5 km wide in the SUZ. In contrast to other regions in the McMurdo Dry Valleys, average daily air temperatures never rise above 0 °C (Marinova et al. 2013; Denis

Fig. 6.1 (**a**) University Valley (1800 m.a.s.l) contains a small glacier at its head. (**b**) Dry permafrost can be scooped easily, because it lacks sufficient moisture to bind the mineral particles together even at sub-zero temperatures

Lacelle et al. 2012), resulting in unique cryogenic processes. Parts of the valley have no active layer whatsoever. Here sublimation processes dominate. Isotopic measurements indicate that the water-ice content of the ice-cemented permafrost is condensation-diffusion in origin, from the past 100 Ka years to present, as opposed to liquid water deposited prior to freezing (Lacelle et al. 2013). As a result of the harsh conditions, life in University Valley is purely microbial; there are no plants or animals in the valley, and birds cannot be seen flying overhead. The presence of microbial life can be detected using standard microbiological techniques, and the soils are extremely low in biomass. Direct microscopy indicates the soils contain 1000's of cells per gram of soil (Goordial et al. 2016), orders of magnitude lower than lower-elevation Dry Valleys, or permafrost of similar latitude in the Arctic (Goordial et al. 2013; Goordial and Whyte 2014). The presence of microorganisms in the soils is also detected via molecular techniques (DNA based), confirming the presence of diverse bacterial and fungal organisms. Like many other lower-elevation Dry Valleys, Archaea do not seem to be abundant or diverse in this system.

A number of studies have established the ubiquitous presence of microbial life in Dry Valley permafrost soils via microscopy or DNA sequencing (Bakermans et al. 2014; Lee et al. 2012; Monteiro et al. 2020). However, such cell counts and DNA molecular approaches cannot distinguish between active microbial life, dormant life, or the DNA of life that has been preserved in the arid and sub-zero conditions, similar to a freezer where molecular extracts and glycerol stocks of microbial cultures are commonly kept for long-term storage. Though microorganisms are unambiguously present in the University Valley soils, unlike all other permafrost examined to date from other locations on Earth, metabolically active microbial life cannot be detected via in situ gas flux measurements, nor in the laboratory at in situ relevant sub-zero temperatures (Goordial et al. 2016). Past efforts to detect life in these soils include highly sensitive radiolabelled substrate mineralization assays (radiorespiration assays) carried out over 2 years, similar to the detection assays employed on Mars in the Viking lander mission to detect alien life (Horowitz et al. 1976). The absence of observable activity is not altered by the addition of nitrogen and phosphorus sources to soils; thus, nutrient limitation is not a likely factor. The addition of a known psychrophile to University Valley soil microcosms does result in measurable respiration, and thus soil toxicity can be ruled out. In the past, efforts to extract RNA which would indicate active microbiota have not been successful. Similar cold temperatures are present in many other cryoenvironments, and it is not likely that temperature itself is prohibitive to life in University Valley, so much as the corresponding unavailability of liquid water. Due to the location of University Valley, inland and high elevation, the influence of salts from the ocean is negligible, and solute concentrations in the soil are too low to facilitate the formation of veins of briny water in the ice. Combining soil temperature data over 3 years with soil geochemistry indicates that the conditions which would permit liquid water to be present, even as a thin film of water, are not met in University Valley for more than 74 cumulative hours per year (Lacelle et al. 2013; Goordial et al. 2016).

Microbial activity measurements are rare in dry permafrost-affected sites on Earth (Horowitz et al. 1972; Goordial et al. 2016; Gilichinsky et al. 2007; Bakermans et al.

2014), and thus the observations in University Valley remain to be tested against other sites to determine if these results are unique or whether such conditions represent a true cold-arid limit to active life. Crucially, though a "pulse" or evidence of in situ activity cannot be directly measured in University Valley soils, there is substantial evidence that microbial life persists nonetheless.

6.2.1 *Dormancy: The Foundation for Future Microbial Structure and Function*

Though no activity can be measured at sub-zero temperatures in University Valley soils, using the same radiolabelled substrate mineralization assay, microbial respiration can be detected when the incubation temperatures are raised to 5 °C, temperatures which would not have been experienced in this region for at least 150,000 years (Goordial et al. 2016; Lacelle et al. 2013). Classic cultivation techniques on solid agar media have yielded no isolated microorganisms; however, after soils were held at 5 °C for 1 month before cultivation attempts, four bacterial and two fungal isolates were obtained from University Valley soils. Two of the isolates, a bacterial *Rhodococcus* sp. and a yeast *Rhodotorula* sp., are capable of sub-zero growth relevant to the conditions they were isolated from (Goordial et al. 2016; Goordial et al. 2015; Goordial et al. 2016). This confirms that viable microbiota persist in University Valley, evident when clement conditions are present.

Metagenomic analysis suggests that much of the DNA in University Valley soils are from dead or merely surviving dormant cells (Goordial et al. 2017). The overall functional potential of the soils shares the most similarity with permafrost soils in the Arctic, not the nearby, lower-elevation Dry Valley surface soils. These functional similarities are driven primarily by a relative enrichment in genes associated with dormancy and sporulation and spore DNA protection. Dormancy is a reversible state of reduced metabolic activity, typically in response to adverse environmental conditions. Dormant microbial cells can act as seedbanks, prolonging the persistence of genotypic functions and specific populations of cells, and thus have important consequences for community- and ecosystem-level processes once conditions become clement again (Jones and Lennon 2010; Lennon and Jones 2011).

While dormancy is clearly an advantageous strategy for longevity and seeding future microbial populations, it cannot go on indefinitely and has several disadvantages. Dormant microorganisms are less able to quickly respond to signals of favourable conditions and changing environments, especially if the favourable conditions are only short lived. The transition out of a dormant state requires energy, and for the appropriate cellular machinery to respond to the new conditions (Lennon and Jones 2011). While in a dormant state, cells are also subject to agents of genomic decay, for example, background radiation which can cause mutations to accumulate, inhibiting a cell's ability to replicate and carry out cellular functions (effectively, the death of a cell). There is evidence that on "short timescales", dormancy may be an

effective strategy; however, low levels of microbial activity, sufficient to carry out survival functions such as DNA repair, may be a superior strategy for microbial viability on long timescales (Price and Sowers 2004; Johnson et al. 2007).

For example, in a relatively young Alaskan chronosequence aged 19 to 33 Ka, the relative abundance of spore-forming bacteria increased from 13% to 79% (Mackelprang et al. 2017). In contrast, in an Antarctic chronosequence, spore-forming *Clostridia* were observed to increase in soils from present day to 30 Ka; however, the *Clostridia* had accumulated DNA damage (as measured by nicks in the DNA structure), as opposed to non-spore-forming *Actinobacteria* from the same samples. In soils dated 400–600 Ka, the *Clostridia* could no longer be detected via molecular methods, and the *Actinobacteria* were still present, sustaining relatively little DNA damage (Johnson et al. 2007).

6.2.2 *Microbial Vital Signs?: Evidence for Activity and Replication at Sub-zero Temperatures*

Though no microbial activity can be measured in University Valley soils in situ or in the lab, and there is evidence for enriched dormancy traits in the soils, hints that active microbiota, or at the very least cells in a maintenance or survival state can be found. In addition to genes associated with dormancy, there were genes associated with cold adaptation at sub-zero temperatures (e.g. cold shock proteins, stress response, reactive oxygen species tolerance) (Goordial et al. 2017). Though conditions for laboratory cultivation of microbial isolates are significantly different from those experienced in the environment, the sub-zero growth demonstrated by the isolated (non-sporulating) *Rhodococcus* sp. JG3 and *Rhodotorula* sp. JG1b raises the question of whether such microbial life may be active at similar temperatures in the permafrost environment but remain undetected due to low biomass or slow metabolic rates.

The genome of *Rhodococcus* sp. JG3 demonstrates traits consistent with cold adaptation which would allow activity at cold temperatures. The genome encodes a higher copy number of genes associated with stress response and cold shock, compared to closely related mesophilic relatives (Goordial et al. 2016). At the amino acid level, protein-coding genes have residue substitutions which likely confer increased flexibility and fluidity to those proteins, advantageous in sub-zero environments. In many cases, numerous isozymes (enzymes encoding for the same function) are present with slightly different amino acid compositions. Numerous isozymes which are "hot" or "cold" adapted have been identified and are thought to be advantageous for carrying out the same function at different temperatures.

The temperature limit for cellular replication of *Rhodococcus* sp. JG3 is −5 °C, with a doubling time of 14 days. When cultured *Rhodococcus* sp. JG3 cells in exponential growth were rinsed and added without nutrients to sterilized University Valley permafrost in high abundance (final load 10^6 cells/g), microbial respiration

could be observed down to −15 °C (Goordial et al. 2016). This experiment utilized the exact same radiorespiration carbon mineralization methodology that could not detect activity at sub-zero temperatures in the University Valley soils that contained three orders of magnitude less biomass. These experiments draw a crude line, between −5 °C and − 15 °C where cellular replication stops, but non-growth activity persists. What the mechanisms allowing these physiological shifts are, and how levels of activity may change on geological timescales remains an intriguing outstanding question.

6.2.3 The Rocks of Life in University Valley: Cryptoendoliths

Though active life in the dry and underlying ice-cemented permafrost may be difficult to detect, the valley itself is not void of thriving microbial communities, if one knows where to look. The valley walls are composed of porous Beacon sandstone, trapping and holding what little moisture is available within pore spaces inside the rock. In the summer, the sun warms the rocks above ambient air temperatures. In comparison to the 74 h per year where moist conditions can be present in University Valley soils, boulders in a valley with similar environmental conditions (1650 m.a.s.l) are found to have moisture for over 700 h per year (Friedmann et al. 1993). As a result, hidden mere millimetres beneath the rock surface, in these warmer and wetter conditions, is a dense and colourful microbial community that cannot be seen without breaking open the rock (Fig. 6.2).

Cryptoendolithic ("hidden within rock") microbial communities are trophically simple communities, commonly visible as coloured bands beneath the surface of sandstone, gypsum and other siliceous rocks (Makhalanyane et al. 2014). They are composed of lichenized or free-living fungi (black or dark green band), photoautotrophic algae or cyanobacteria (green band) and heterotrophic bacteria (Friedmann et al. 1988; Friedmann et al. 1993; Selbmann et al. 2005; Coleine et al. 2018; Cary et al. 2010). Colonized Beacon sandstone, such as what composes the walls of University Valley, is widely colonized by cryptoendoliths. The translucent rock substrate allows light to penetrate into the pore spaces, providing energy for the photoautotrophs, which in turn fix carbon for the heterotrophic communities. In University Valley, a black band is found at the surface of the rock (Fig. 6.2b) composed of black yeast fungi with high contents of melanin and lichenized algae (Goordial et al. 2017). The high melanin content of the fungi protects the photoautotrophic algae and heterotrophic organisms deeper within the rock from harmful UV radiation found in high amounts in the Antarctic at high elevation. The narrow bands of colour correspond to a narrow zone of habitability where light conditions are "just right", and moisture and sun can permit increased metabolic activity. Though the rocks are warmed in the summer, sub-zero temperatures still persist in the rocks throughout the year. In stark contrast to the permafrost soils, metabolic activity at sub-zero temperatures, by both heterotrophic and photoautotrophic communities, is easily identified. Using the same radiorespiration assays as on the soils,

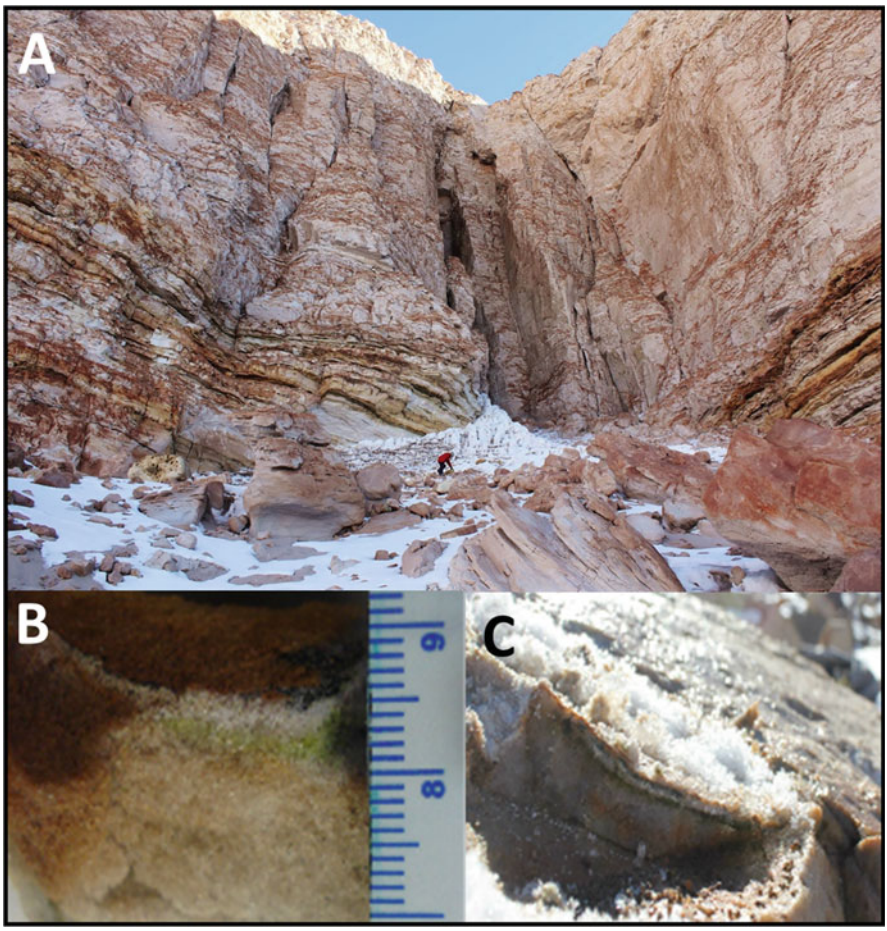

Fig. 6.2 (**a**) Sandstone valley wall in University Valley teeming with hidden life. (**b**) Cryptoendolith with ruler (showing cm scale). Black melanized fungi can be seen just beneath the surface of the rock. (**c**) Opened cryptoendolithic rocks, with visible life (coloured bands) within the first few mm of the surface of the rock facing the sun

heterotrophic activity can be detected at −20 °C within a few days, and photosystem activity required for photosynthesis can be detected at −20 °C as well. Several sub-zero-growing bacterial, fungal and algal isolates capable of growth at −5 °C can be readily isolated from the sandstone, without the enrichment steps needed for the University Valley permafrost soils (Goordial et al. 2017).

Cryptoendolithic microbial communities promote bioweathering of the rock and in some cases cause telltale exfoliation patterns to indicate where life once was abundant. Oxalic acid produced by the communities dissolves the cementing materials between mineral grains—causing more area to colonize but also overtime resulting in exfoliation and loss at once of the majority of biomass (Friedmann

1982; Sun et al. 2010). What microorganisms remain begin the colonization and biomass building process again, in a cycle estimated to take 10,000 years (Sun and Friedmann 1999). There is evidence that the endolithic microbial communities recycle carbon within the rock on similar timescales, mixing "modern" fixed carbon into the community at variable rates (Brady et al. 2018). The exfoliation process contributes to the mineral soils which make up the valley floor, delivering carbon, and microorganisms to the permafrost environment. Though the microorganisms delivered to the valley floors are adapted to growth at sub-zero temperatures, the conditions on the valley floor do not seem to be amenable to the same level of metabolic activity, and cells must shift to other means of persistence. The context of the rock habitat itself in University Valley is what lends itself to abundant and active life.

6.3 Prospects for Life Beyond Earth in Our Solar System

Within our solar system, there are multiple promising planetary bodies being explored for potential microbial life; these include the planet Mars and Enceladus, Titan and Europa, the moons of Saturn and Jupiter. What these planetary bodies share in common is the potential presence of a liquid solvent and cold temperatures. Average surface temperatures are −60 °C on Mars, −190 °C on Enceladus, −160 °C on Europa and − 180 °C on Titan. As a result of these frigid temperatures, any potential life would be constrained by the presence of a liquid solvent, similar to how life is constrained on Earth. Mars may have had vast oceans in its geologic history, and sinuous channels indicative of prolific water flow are globally distributed (Zuber 2018; Head et al. 1998; Dohm et al. 2001; Davis et al. 2016). As recently as 5 Mya, the conditions to melt ice-cemented permafrost would have been met in the Martian North pole, where vast quantities of water-ice are currently located (McKay et al. 2013). If life once was present on Mars during these more clement conditions, could it have survived in the subsurface permafrost in a state of dormancy or with low levels of microbial activity? On present-day Mars, transient dark streaks are observed on equatorial slopes in the Martian summer; these recurring slope linneae (RSL) are potentially formed by liquid brines flowing through permafrost, though a dry origin is also being considered (McEwen et al. 2011; Sun et al. 2010). Both Enceladus and Europa, moons of Saturn and Jupiter, respectively, are ocean worlds, with large bodies of salty water beneath km's thick ice. On Titan, no liquid water is known to be present, but lakes of liquid hydrocarbon can be found (Stofan et al. 2007). Could life at cold temperatures utilize other liquids as a solvent for life-sustaining metabolic processes? Cryoenvironments on Earth are the best analogs to inform where, and how, we search for life on other cold planetary bodies.

6.4 Conclusion

Microorganisms, in various physiological states, play important, foundational roles for the existence and persistence of life in cryoenvironments. Active microbiota play the most obvious role in ecosystem function through the actions of their metabolism—converting and consuming carbon and electron donors and producing waste products, all of which feedback into the environment. Replicating microorganisms exert a similar effect—increasing the number of microorganisms and copy numbers of key genes to carrying out a given function. Even dormant and dead cells play a foundational role (Blazewicz et al. 2013). Dormant cells act as reservoirs for future microbial community structure and function when environmental conditions may change, and dead microorganisms can become cannibalized—becoming fuel for other microorganisms to persist, in some environments for tens of thousands of years (Bradley et al. 2019). Though cryoenvironments are primarily discussed here, these physiological states and principles for ecosystem function apply in virtually every environment.

University Valley permafrost shows an example of microbial activity that cannot be measured using even highly sensitive methodologies—however, at one time the soils of the valley were considered to be completely sterile, as no microorganisms could be cultivated from them (Horowitz et al. 1972). Since then, the advent of molecular techniques has resulted in an appreciation for how much diversity and biomass these soils contain, as well as yielded insight into novel metabolic processes. More recently, studies examining the microbes in other cold soils have demonstrated the ability to derive energy from trace, atmospheric amounts of carbon monoxide and hydrogen to provide energy for the survival of microbial cells. These exciting observations may prove to be a widespread capability, possibly occurring at sub-zero temperatures in nutrient-poor settings (Ji et al. 2017; Greening et al. 2019; Greening et al. 2016; Lynch et al. 2012; Lynch et al. 2014).

Like the ancient foundation stone that inspired this essay, sandstone rocks in this University Valley are where life springs forth from and are the source of many of the microorganisms that fall to the valley floor as the walls erode. Though not obviously metabolically active, these populations will become the seeds for future microbial communities, when the permafrost thaws on geologic timescales or potentially as a result of wetting and thaw due to human-made climate change. There is also the tantalizing possibility that these microorganisms may in fact be active, even at small levels only sufficient for maintenance and survival, just not on scales that can be detected easily by our current methods.

The impression I hope the reader can walk away with is that we are continually learning more about the capabilities and extent of microbial life on Earth. It is entirely possible (likely!) that our methods of detection of activity will become more sensitive in the future, enabling detection of metabolic activity even when "slow". Continued cultivation efforts will surely yield novel psychrophiles in culture, as well as increased knowledge of their mechanisms for survival and activity. What we learn about cryophilic life will inform how we think about the limits of life

on Earth, as well as for how and where we look for life on other planetary bodies beyond our own. At least within our solar system, microbial life is undoubtedly the foundation upon which our planetary life functions.

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Chapter 7
Lithotrophic ("Stone-Eating") Microbes Provide the Foundation for Deep Subsurface Ecosystems

Thomas L. Kieft

Abstract Those of us who are microbiologists have no trouble thinking of microbes as the most important and indeed the most fascinating of all organisms. They're the center of our world. They're more numerous (~10^{30} individuals) than any other organisms on Earth ([Whitman WB, Coleman DC, Wiebe WJ, Proc Natl Acad Sci USA 95:6578-6583, 1998]; [Kallmeyer J, Pockalny R, Adhikari RR, Smith DC, D' Hondt, Proc Natl Acad Sci USA 109:16213-16216, 2012]), and they're vastly more diverse than the (some say) more charismatic macroorganisms (Locey KJ, Lennon JT, Proc Natl Acad Sci USA 113:5970-5975, 2016). Anthropocentric types may be surprised to learn that a human adult holobiome (a holistic view of a person as a consortium) is comprised of a trillion or more human cells and that an even greater number of microbes are living in and on each of us, providing myriad biochemical and protective advantages. They outnumber us on almost any scale and in every environment, whether we're looking at a gram of soil, a ml of seawater, our own bodies, or the entire biosphere. However, among all the possible environments for considering the supremacy of microbes, my own favorite and my choice for demonstrating that microbes are indeed the "foundation stone" is the deep terrestrial subsurface, i.e., very deep groundwater, the deepest, least investigated, and largest biome on Earth.

7.1 Foundation

We can start with "foundation" as a descriptor. One meaning of this term is the beginning or origin. The first life on Earth was unquestionable microbial, but where did this life arise? Multiple candidate environments have been posited, e.g., Darwin's "warm little pond," Yellowstone-like thermal springs, and deep-sea hydrothermal vents but also the terrestrial deep subsurface (Trevors 2002). The subterranean world has a number of attractive features for origin of life theories: it has water

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_7

that's warm or even hot; it has readily available and utilizable geochemically generated energy sources, notably hydrogen (H_2) and methane, and it has abundant mineral surfaces that may have catalyzed and formed a template for the formation of more complex organic molecules.

Hydrogen is a potent energy source that could one day fuel our cars, but it may have fueled early life, as well. It's produced abiotically and thus was also produced prebiotically in the subsurface. A variety of water-rock interactions generate H_2, including serpentinization of peridotite minerals (Schrenk et al. 2013), oxidation of ferrous silicate in basalts and other minerals (Stevens and McKinley 2000), and radiolysis of water (Lin et al. 2006). In the last of these, the energy of radioactive decay splits water into H_2, O_2, and reactive oxygen species (H_2O_2). Carbon monoxide (toxic to us but an energy source for microbes) is also present in deep fractures and is thought to have a geochemical origin. Further sources of energetic reduced gases include outgassing of mantle rocks (Nealson et al. 2005) and shearing of silicate minerals during seismic activity (Sugisaki et al. 1983). These are generally sluggish reactions, but may be accelerated when the rocks and water are jostled. Lippmann-Pipke et al. (2011) detected spikes of H_2 production coinciding with seismic events; thus, earthquakes may stimulate microbial activity. H_2 can accumulate in the subsurface to concentrations that support life, e.g., 2–3 mM (Kieft et al. 2005; Lin et al. 2006). When dissolved groundwater gases are exsolved, they can reach 30% by volume and more (Sherwood Lollar et al. 2014). H_2 is also relatively easily taken up and metabolized, requiring minimal biochemical machinery, mostly hydrogenase enzymes. Due to its presence on early Earth and its ease of metabolic use, H_2 is one of the prime candidates to have been the original electron donor for microbial life. Hydrogen can also react with CO_2 and CO to form methane and short-chain hydrocarbons (ethane, propane, butane) via Fischer-Tropsch-type syntheses. These abiotic hydrocarbons can also provide energy for microbial metabolism. Pedersen (2000) coined the term "geogas" for these geochemically generated energy-rich gaseous subsurface compounds; Stevens and McKinley (1995) described metabolism of H_2 by the methanogens in basaltic aquifers as part of what they termed "subsurface lithoautotrophic microbial ecosystems," or SLiMEs. Besides being common members of SLiMEs, methanogens have also been proposed to be among the earliest if not the first form of life on Earth.

A further advantage that may have been conferred by the subsurface onto early cells was protection from radiation and from obliteration during the Late Heavy Bombardment in the Hadean period, when massive asteroids slammed into early Earth, some with energy that may have been sufficient to vaporize the oceans. The timing and intensity of these impacts are not fully constrained, but any nascent life exposed at Earth's surface would have suffered setbacks if not outright extinction. Most scenarios place the most intense impact period in the 3.5–3.7 to 4.1 billion-years-ago time frame, which is close to (in the grand scheme of geologic time) the window of time associated with the origin and early evolution of life, ~3.5–4.0 billion years. Incidentally, the emergence of earthly life occurred at a time when Mars appears to have had conditions that were habitable to microbial life as we know it. If life appeared there and if it arose in or invaded Mars' nether regions, then it

could be there still, metabolizing the energetic products of water-rock interactions. Tullis Onstott and colleagues (Onstott et al. 2018) make a case for the deep subsurface of Mars remaining habitable ever since those early days (or "sols," as they're called on the Red Planet), and they further propose to search for life in fluid-filled fractures of deep Martian rocks. I suggest that we explore the depths of our own planet more thoroughly first, but then I'm all for seeking rock-hosted life elsewhere.

Another meaning of the term "foundation" is as the physical underpinning or base of a structure. One way of considering this with reference to subsurface microbes is to consider a trophic dynamic pyramid, for which the primary producers, i.e., the autotrophs, serve as the base, the compartment having the greatest biomass. This autotrophic base is overlain by successive layers, primary consumers, secondary consumers, etc., culminating in the lowest total biomass group, the top predators, e.g., wolves, sharks. These various levels of consumers are all heterotrophs. In terrestrial biomes, the autotrophic base is primarily plants. In fact, multicellular plants have been estimated to make up 450 gigatons of biomass carbon (Gt C) out of an estimated total biomass on the planet of 550 Gt C (Bar-On et al. 2018). The sources of organic carbon, i.e., the primary producers, in the oceans are primarily microbes and are dominated by a few genera of photosynthetic cyanobacteria, e.g., *Prochlorococcus* and *Synechocystis*. The sources of organic carbon in the continental subsurface vary with depth. In soils and at depths to a few tens or hundreds of meters, organic carbon is nearly all photosynthate, in other words, plant-derived organic carbon that has been buried underground or has been transported to the subsurface. The microbes in the shallow subsurface are therefore mostly heterotrophs. Deeper subsurface localities are removed both in distance and in time from the surface world, and therefore photosynthetically generated organic carbon is scarce to nonexistent (Kieft et al. 2018), except in isolated petroleum deposits, which actually make up only a small fraction of the volume of the deep subsurface. One might expect that the biomass of microbes would decline to zero in the absence of surface-derived organic C, but this is not the case. Even at great depth, 3 km and more, bacteria and archaea are found, albeit in lower numbers, ~1000–10,000 cells per ml of fracture water; and these microbes are dominantly chemoautotrophs functioning in SLiMEs. In other words, the *foundation* layer of the trophic pyramid in the deep subsurface is comprised of chemoautotrophs, a.k.a. chemolithotrophs. They make their living by combining H_2 and other geogas components as electron donors (fuel) with a variety of electron acceptors (oxidants) that include CO_2, sulfate, and nitrate.

As primary producers, these subsurface chemoautotrophs fix CO_2 into organic C. There are approximately six different biochemical pathways for CO_2 fixation (Fuchs 2011). The majority of photoautotrophs use the Calvin-Benson-Basham cycle, which relies on ribulose bisphosphate carboxylase oxidase, possibly the most abundant enzyme on Earth. However, many subsurface autotrophs including dissimilatory sulfate reducers and methanogens use the more ancient and also more efficient Wood-Ljungdahl pathway, also known as the reductive acetyl CoA pathway (Cotton et al. 2018). This pathway occurs only in anaerobes, which goes along

with the generally anoxic conditions deep underground. The anoxic nature of these habitats and the anaerobic lifestyles of the inhabitant microbes are also part and parcel of these rock-hosted ecosystems that function totally independently from photosynthesis; they not only don't require photosynthetic carbon; they have no need for and would be damaged by oxygen from photosynthesis. The widely distributed and recently isolated (Karnachuk et al. 2019) sulfate-reducing autotrophic bacterium *Desulforudis audaxviator* utilizes the Wood-Ljungdahl pathway (Chivian et al. 2008). In one case, *D. audaxviator* was found to be not just the foundation of the ecosystem, but the sole organism in the community (Lin et al. 2006). Methanogenic archaea are also common primary producers in deep SLiMEs. They can use H_2 as electron acceptor while fixing CO_2 into methane and organic C.

One might be tempted to view these chemoautotrophically based microbial ecosystems as rare and remote oddities (the living world is liberally peppered with quaint and improbable forms), but subsurface SLiMEs are globally widespread and likely form a huge fraction of the Earth's prokaryotic cells. The minerals required for H_2-forming water-rock interactions are commonplace. Barbara Sherwood Lollar et al. (2014) have estimated that Precambrian crust, which comprises 70% of the Earth's continental area, produces ~10^{10}–10^{11} moles/year of H_2. Basalt flows, with the potential for oxidation of Fe to release H_2, occur in large areas of the continents and also the oceans. Rocks bearing uranium, thorium, and other radioisotopes that drive radiolysis of water are common; one of the mechanisms for concentration of uranium occurs during granite formation. Put this altogether and you can envision SLiMEs as a dominant life form.

Astronomer Tommy Gold (1992) considered the possibility of H_2- and methane-fueled deep life almost 30 years ago and posited a vast "deep hot biosphere," essentially SLiMEs growing on geogas on a grand scale. While parts of his controversial hypothesis have not been supported (e.g., mantle-derived petroleum hydrocarbons), the basic premise of a vast underground biosphere powered by geochemically generated energy-rich substrates has now been borne out in many reports (Colman et al. 2017). Gold estimated the vastness of the subsurface biosphere based in part on the upper temperature limit for life, using a somewhat speculative value from the time, 150 °C. He was certainly correct that temperature controls the depth limit, but 122 °C is more currently accepted. The geothermal gradient varies across the Earth's continents from ~8 to ~30 °C/km, which corresponds to a depth limit for life ranging up to 12 km or even more in isolated areas. However, while a few extremophiles may be able to function at temperatures as high as 122 °C in especially energy-rich environs such as deep-sea hydrothermal vents where H_2 and H_2S spew out of the seafloor, life at high temperatures comes with a high energetic cost for repair and replacement of macromolecules, a cost that most subsurface microbes can't pay. Racemization of L-amino acids in proteins (Onstott et al. 2014) and DNA damage are accelerated at high temperatures and thus pose a special problem for thermophiles. Most subsurface microbes experience relatively low energy fluxes, probably too low to handle expensive maintenance costs. The actual upper temperature for most subsurface life might be in the neighborhood of 85 °C. Magnabosco et al. (2018) used this information combined with cell

abundance data for varied crustal lithologies to estimate the total abundance of continental microbes. Their estimate is 2 to 6 x 10^{29} cells, or 22–31 gigatons of C. That's a big chunk of Earth's total inventory of ~10^{30} prokaryotic cells (Whitman et al. 1998; Kallmeyer et al. 2012) and ~ 550 gigatons of biomass C (Bar-On et al. 2018). Since a large proportion of these subsurface microbes are chemolithoautotrophs, the primary producer base or foundation of the deep biomass pyramid is indeed vast.

So, the deep biosphere has been in place for a very long time, possibly the entire 3.5 to 4 billion-year span of life on Earth; it's widespread and deeply penetrating; and it contains myriad individual organisms. Given these characteristics and also the physical as well as chemical diversity of its potential microhabitats, it is perhaps not surprising that subsurface microbes have evolved into many different forms. Molecular sequencing analyses have revealed a diversity of bacteria and archaea, with many taxa being first discovered in the subsurface. Examples of these are the archaeal phyla first discovered by Ken Takai and others that have now been proposed as novel phyla such as the Bathyarchaeota, Hadesarchaea, and Aigarchaeota (Colman et al. 2017). Some of these subsurface microbes may be indigenous, i.e., unique to their subsurface habits, such as *D. audaxviator*, which has been detected exclusively deep underground; others appear to inhabit the surface world, as well. All told, the diversity is huge. How huge? Magnabosco et al. (2018) borrowed a scaling model from Locey and Lennon (2016), applying it to a sequence dataset compiled from a large number of subsurface studies, and from that model estimated that the total bacterial and archaeal species richness for the Earth's continental subsurface could be as high as one trillion OTUs (operational taxonomic units, the molecular microbial ecologists' stand-in for the concept of species, which strictly speaking doesn't apply to prokaryotes). Considering that this is approximately the same as Locey's and Lennon's own estimate for worldwide species richness, we see again that life underground deserves vastly more study (and funding, of course!).

The deep subsurface habitats beneath the oceans, i.e., deep-sea sediments and rock, harbor microbes, too, but there are some fundamental differences between the marine and continental habitats. Many, if not most, microbes in ocean sediment could be considered essentially accidental tourists that settled out from the water column above, along with dissolved and particulate organics, and now they're slowly metabolizing that organic matter and also slowly depleting their own cellular reserves. H_2-utilizing chemolithotrophs occur in the marine subsurface, too, but these marine SLiMEs are less prevalent than in continental systems. The continental SLiMEs are not only actively metabolizing geochemically generated inorganic substrates; they're interacting in complex communities of chemoautotrophs, with the products of one group forming essential metabolites for another. Maggie Lau et al. (2016) used a full suite of "-omics" (metagenomics (DNA), transcriptomics (RNA), and proteomics (protein)) analyses to characterize complex trophic interactions among chemolithoautotrophs in fluid-filled fractures 1.3 km deep in South Africa. There, methanogens form the base of the trophic pyramid, although somewhat oddly, it's an inverted pyramid wherein the methanogens are the primary producers,

but do not constitute the largest group in terms of biomass. Evidently, they're working very hard to support all of the other trophic groups. The next tier in the pyramid is made up of methanogens, as well, but these are methanogens that in simple terms run their biochemistry backwards as anaerobic methane oxidizers (ANMEs). ANME metabolism by itself is thermodynamically unfavorable, but the ANMEs are functioning mutualistically with sulfate-reducing bacteria in a tight consortium first discovered in marine sediments. The sulfide product of the sulfate reducers in turn serves as energy source for sulfur-oxidizing bacteria. There's no oxygen, so the sulfur oxidizers use nitrate as their electron acceptor. Thus, this chemolithoautotrophic pyramid nicely links the carbon, sulfur, and nitrogen cycles to form an isolated but self-sustaining ecosystem.

7.2 Stone

Life underground, once one descends beyond surficial sedimentary layers, consists of rock, and the microbes existing in the fluid-filled fractures in that rock exist in rock-hosted communities (Onstott et al. 2018). If we consider "stone" to have a similar meaning to "rock" and to "litho-," then continental subsurface microbes live in a stony world and get their energy from stone. In that realm, the chemo*litho*trophs truly dominate.

The frequently detected subsurface bacterium *D. audaxviator* has a special relationship to "stone." It's a sulfate reducer that gets its electron donor and electron acceptor from water-rock (-stone) interactions, as do most subsurface chemoautotrophs. The electron donor, H_2, is created via water-rock interactions, as described above. In deep South African rock-hosted habitats, the electron acceptor is produced indirectly by water-rock interactions when reactive oxygen species from radiolysis of water oxidize 2.9-billion-year-old pyrite to produce sulfate (Chivian et al. 2008). *D. audaxviator* has another "stony" connection, too. Dr. Esta van Heerden, biochemist/microbiologist and co-author of the original papers on this intriguing little organism, proposed that it be named after a mythical sprite in Zulu and Xhosa folklore called a "tokoloshe" (or "tikoloshe"). Tokoloshes are thought to be mischievous little gnome-like creatures. Among their special powers is the ability to become invisible, which comes about when they eat a stone, or in some versions, when they drink water. Either way, tokoloshe is an appropriate species moniker for a deep-dwelling bacterium that obtains its power from water-stone interactions. Incidentally, some say that tokoloshes are especially well endowed, such that they sling their members over a shoulder. I'm not sure how that fits our sulfate reducer, except that *D. audaxviator* is endowed with one or more flagella, long appendages that extend away from the body of the bacterium for locomotion. The attribute of locomotion does fit the species name that was actually selected; "*audaxviator*" means "bold traveler," which is appropriate to a motile microbe that has found its way to the deep subsurface of multiple continents.

7.3 Summary

To wrap up, while microbes are important everywhere and not just to microbiologists, they're everything in the subsurface. They make up nearly 100% of life underground. They're self-sufficient, needing no help from plants or other photosynthetic organisms for their organic carbon or for oxygen, since they rely on alternative electron acceptors. Life may have even started underground and only later emerged into the light. They form an enormous and varied component of the Earth's biosphere, and yet they haven't received scientific attention commensurate with their biomass and diversity. My hope in writing this little review is that one or two early career scientists looking for their niches will take up the mantle of subsurface geomicrobiology and dive (well drill, actually) deep into the Earth's crust to find new treasure.

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Part III
Understanding the Core Values of Microbial Metabolism

Chapter 8
Miraculous Fixation of Molecular Nitrogen from the Atmosphere

Vladimír Klaban

Abstract The fact that microorganisms are able to collect molecular nitrogen from the atmosphere and convert that into a more biologically usable form is a key factor in the production of plant life and that nitrogen eventually gets incorporated into proteins up through the food chain. This is one miraculous example of the way in which microorganisms serve as the foundation of our biosphere. This essay summarizes the involved biological processes and the history of our related discoveries.

8.1 Introduction

In the past, these bacteria used to be called "nitrogenic bacteria." The term nitrogenic is derived from words "Nitrogenium" for nitrogen and "gennao" meaning creation. That would suggest nitrogenic bacteria could "create or synthesize nitrogen" which, logically, is not true. Diazotrophic bacteria form a part of nitrogen circulation in nature, more specifically of the so-called biochemical nitrogen cycle. Let's begin our examination of nitrogen fixation from the atmosphere from a viewpoint of bioenergy. Such fixation is essential for soil fertility and therefore plant production.

8.2 Fixation of Nitrogen from the Bioenergy Point of View

Gaseous nitrogen is diatomic, meaning that it is a two-atom molecule. That form of nitrogen has the two atoms held together by a triple bond. For that reason, N_2 represents a very stable molecule, and splitting it requires a considerable amount of energy (946 kJ/mol). It is this splitting of a strong bond which is a condition for nitrogen fixation. The overall reaction can be expressed by this simple equation:

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$$N_2 + 3\,H_2 = 2\,NH_3 \tag{8.1}$$

At room temperature, nitrogen is chemically inert, and under standard conditions it does not bond even with very reactive elements. Gaseous nitrogen assumes different chemical properties when atomized by an electrical discharge or if its molecule is brought into an excited electronic state. In such situations, atomic nitrogen is especially reactive, and it reacts spontaneously with many elements and compounds. Theoretically it takes 16 molecules of ATP, and 4 molecules out of those 16 (25%) will be used to generate H_2. Some diazotrophic microorganisms contain hydrogenase, which reoxidizes H_2 and that regenerates 25% of the energy lost during the nitrogen fixation. Nitrogen fixation is described by the following reaction:

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP \tag{8.2}$$

It is outright fascinating to compare biological fixation of atmospheric N_2 with the Haber-Bosch process. During this industrial production of NH_3, the bonding of nitrogen and hydrogen takes place at a temperature of around 500 °C and a pressure of 30 MP, with the use of catalysts based on iron oxides. The chemical system of synthesis of these elements requires the above conditions, while the diazotrophic microorganism are capable of producing the final product at the room temperature and regular atmospheric pressure by the function of an enzymatic complex (nitrogenase) resulting in a formation of several byproducts. This is one of the illustrative examples of the incredible efficacy of nitrogenase formed by diazotrophic microorganisms.

8.3 Nitrogen Fixation by the Haber-Bosch Process

The molecular nitrogen used for this industrial synthesis of ammonia is not extracted directly from the air, but it is obtained by fractional distillation of liquid nitrogen. This production of NH_3 involves a direct synthesis from the respective elements at an increased temperature and pressure in the presence of inorganic catalysts, as stated earlier. The nature of the reaction was thoroughly studied in the years 1908–1912 by the German chemist Fritz Haber (1868–1934), who won the 1918 Nobel prize in Chemistry " . . . for the synthesis of ammonia from its elements." This reaction proceeds in an identical manner in the case of nitrogen fixation according to the earlier quoted equation. Hydrogen for the industrial production of ammonia is supplied from water gas containing approximately 40 volume percent of carbon monooxide, 50% hydrogen, 5% carbon dioxide, and 4% nitrogen. Water gas is generated by conducting water vapor through a layer of red hot coal.

It should be mentioned that Fritz Haber was director of the Kaiser Wilhelm Institute in Germany. This institute later developed a cyanide based pesticide gas

called Zyklon B. Unfortunately, this gas was later used in the genocide of Jewish prisoners in extermination camps during the Second World War. It is paradoxical that Haber himself was Jewish. He left Germany in 1933 to avoid Nazi persecution.

The method for large-scale synthesis of ammonia was developed by the German technologist Carl Bosch (1874–1940). Carl Bosch shared the 1931 Nobel Prize in Chemistry jointly with Friedrich Bergius for contributions to invention and development of high pressure chemical technology. This direct synthesis of ammonia is something that no living organism can perform. Plants as well as all microorganisms receive nitrogen in the form of nitrate ions NO_3^-. Prior to their incorporation into the organisms' organic compounds, these ions need to be reduced to ammonia cations NH_4^+.

8.4 Fixation of Nitrogen by the Genus *Azotobacter*, History, and Description

Azotobacter is one genera of the *Azotobacter* group which belongs to the family *Pseudomonadaceae*, and the resting stage of *Azotobacter* is a cyst. Another genus in the *Azotobacter* group is *Azomonas*, which does not form cysts. Here we deal with the *Azotobacter* only. Its name is derived from the Greek word azoton, meaning nitrogen. Literally "nitrogen bacterium." The genus *Azotobacter* contains gram-negative chemoorganotrophic bacteria, which are obligatory aerobic, ovoid, or in the shape of roundish rods, size of 2–3 × 3–5 μm. They do not form spores and belong to the class *Gammaproteobacteria*. They display pleomorphism (existence in a variety of shapes) depending on the living conditions. These bacteria move by the use of flagella. Only two species, *Azotobacter beijerinckii* and *A. nigricans*, are immobile. These bacteria live freely in the soil, and they can fix gaseous N_2 from the atmosphere since they contain an enzyme nitrogenase, which is very sensitive to the action of molecular oxygen. *Azotobacter chroococcum* represents a typical species of the genus.

Azotobacter belong to symbiotic fixers of nitrogen and are most often found in soils which are either pH neutral or slightly alkaline. In soils of pH lower than 6.0, *Azotobacter* is almost not present. It also requires soil which is well aerated and containing a certain degree of moisture. There is a relationship between *Azotobacter* and some species of the genus *Pseudomonas*. There exists a certain type of symbiosis between them, which appears to be quite strong so much so that it is very difficult to isolate *Azotobacter* from the soil in the form of a pure culture. In the symbiotic form, *Azotobacter* fixes considerably more molecular nitrogen.

Although *Azotobacter* does not form spores, as mentioned above, it is capable of developing cysts, which are rigid corpuscules resistant to dryness as well as being resistant to other adverse conditions of the outside environment. *Azotobacter* was discovered in 1901 by a Dutch microbiologist Martinus Willem Beijerinck. For the isolation of *Azotobacter*, he used a medium composed of inorganic salts and

containing sugar as a source of carbon. Although at the very beginning the bacterium was quite rare in the medium, it started to fix nitrogen from the atmosphere and used the nitrogen to synthetize cell proteins, while other microbes did not possess this ability. That is why *Azotobacter* multiplied considerably so that it eventually became a dominant microorganism in the culture. Another species, *Azotobacter vinelandii*, can be used for the production of alginate. Alginate is a polysaccharide containing D-mannuronic acid, which is derived from mannose, and D-guluronic acid which is a derivative of aldohexose gulose. It also synthetizes pigment called azotochelin. It belongs to the group of bacteria called *Gammaproteobacteria*. Research indicated that siderophores of this bacterium can bind metals other than iron. Overall, the genus *Azotobacter* contains seven species, two of which were presented here. The other four are as follows: *Azotobacter armeniacus* (isolated in 1984), *A. beijerinckii* (isolated in 1904), *A. nigricans* (isolated in 1949), and *A. salinestris* (isolated in 1991). There also is a related organism named *Azorhizophilus paspali* discovered in 1966. The species name "paspali" is derived from the genus *Paspalum*, a tropical plant. *Azorhizophilus paspali* colonizes the rhizosphere and forms elongated rods.

Almost all species of the genus *Azotobacter* live freely in the soil, and during the stationary phase of their growth, they display an ovoid shape. They differ among themselves in their morphology, mobility, and their tolerance to NaCl (so-called halotolerance), as well as in colony pigmentation. Bacteria of the species *Azotobacter* fix approximately 10 mg of molecular nitrogen N_2 per 1 g of the carbon source used. In addition to *Azotobacter*, genera *Beijerinckia* and *Derxia* also fix N_2 under aerobic conditions. These species are more common in the soils of subtropical and tropical regions.

8.4.1 Ecology and Development Cycle of the Genus **Azotobacter**

In the exponential phase of their growth, all the species of the genus *Azotobacter* display a rod-like morphology, and only after their transition into the stationary phase do they assume an ovoid or a cocoid shape. Often they exist in pairs or in short chains. They have glutinous colonies, dark-brown in color. Among their characteristics is a formation of exopolysaccharides. They require the trace elements molybdenum or vanadium which function as cofactors of the enzyme nitrogenase. *Azotobacter* is common in mild climate regions and can be found in both terrestrial and aquatic environments. In the polar regions, *Azotobacter* is very sparse although it was isolated even in a tundra and tolerates a soil temperature of 0 °C. *Azotobacter chroococcum* tolerates salts in the soil, while other species do not, and therefore the other *Azotobacter* species do not colonize salty soils. Species of the genus *Azotobacter* are known for their simple form of differentiation, because their vegetative cells only form cysts. Encystation mostly occurs in older cells at the end of the exponential phase of growth, when carbon sources in the surrounding environment

are depleted. The cysts are not considered to be spores because their interiors resemble vegetative cells although their metabolic activity is greatly reduced. Also, they are not thermoresistant; however they are resistant to the influence of chemical factors. In a dry soil, they remain viable for more than 10 years. In the 1960s, there were reports claiming the cysts can remain viable up to 2300 years.

Morphologically, the cyst contains the central body which resembles a vegetative cell. Then there is a cytoplasmic membrane and a cellular wall composed of muramic acid. In addition, there are two layers of polysaccharides and lipids called intina and exina, both of which contain a highly hydrated alginate, which prevents drying of the cyst. The biosynthesis of alginate is essential for development of the cyst. Mutants unable to form alginate are also unable to differentiate into cysts. The factor AlgU is responsible for the production of alginate. In the cysts, the lipid content is twice as high compared to vegetative cells. Seventy percent of the cyst lipid content is localized in the central body and 25 percent in the exina. The cyst formation can also be induced either by growth of the vegetative cells in medium containing n-butanol or by a transfer of the culture into medium that contains polyhydroxybutyrate.

8.4.2 Nitrogenase

Bacteria-fixing atmospheric nitrogen from the air must contain the enzyme nitrogenase with iron as a prosthetic group which activates nitrogen. Originally it was thought that nitrogenase is just one enzyme. However it has been later established that it is what we now call a "nitrogenase complex."

Nitrogenase is composed of two parts, neither of which has any function by itself. One of the two components has a molecular mass of 2.4×10^5 g/mol. Its molecule contains two atoms of molybdenum and 28–32 atoms of non-heme iron which is bound to sulfur. This component is called Mo-Fe protein, or molybdoferredoxin (also dinitrogenase or component 1). It reduces gaseous nitrogen to ammonia. In some cases, molybdenum can be replaced by vanadium. The other component is more acidic and has a lower molecular mass of 6×10^4 g/ml. It contains two atoms of iron, as well as two SH groups. It is called Fe protein, or azoferredoxin (component 2). It reduces dinitrogenase. Apart from nitrogen and nitrogenase, a source of hydrogen as well as a source of energy in the form of ATP is also essential for the fixation of nitrogen. Intermediate products of the microbial synthesis of ammonia include compounds called hydrazine N_2H_4 and hydroxylamine NH_2OH. Nitrogenase is sensitive to molecular oxygen, which inactivates it. Therefore a certain protection from oxygen is necessary.

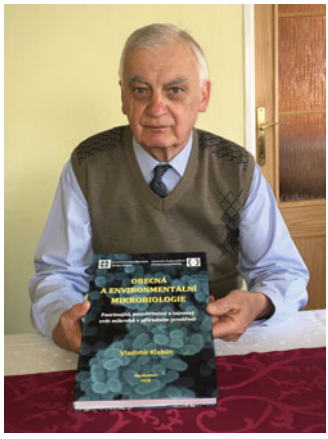
In the case of cyanobacteria, for example, this protection is achieved such that the nitrogen fixation takes place in specialized non-photosynthesizing cells called heterocysts. These are present during the whole vegetative period. They do not contain any assimilatory pigments. In the bacteria of legumes, oxygen protection is taken care of by the symbiotic synthesis of so-called leghemoglobin. The globin part of

this compound binds oxygen, and it is synthetized by the plant. The heme is formed by bacteria of the genus *Rhizobium*.

According to Cyril A. Appleby (1984), symbiotic oxygen fixation is considered as an "oxygen paradox." That means components necessary for the enzymatic reduction of nitrogen to ammonia are oxygen-labile (sensitive to the action of oxygen), while the respective microorganisms are obligatorily aerobic and dependent on respiration using oxygen as a terminal acceptor of hydrogen and of electrons for accomplishing the production of ATP.

8.4.3 Nonsymbiotic Nitrogen Fixation

The amount of nitrogen bound by non-symbiotic fixation in the soil is very small in comparison with the nitrogen volume fixed by microorganisms living in symbiosis with leguminous plants. However, non-symbiotic nitrogen fixation is of a great importance in aqueous environments. Atmospheric nitrogen can also be fixed without symbiosis by some species of the phylum *Cyanobacteria* (blue-green bacteria) and also by the sporogenic genus *Clostridium* and the genus *Beijerinckia*. The Dutch soil microbiologist Martinus Willem Beijerinck (1851–1931) isolated in 1893 an anaerobic spore-producing microorganism and named it *Granulobacter butylicum*; its present name is *Clostridium butyricum*. Two years later, a famous Russian microbiologist Sergei Winogradsky (1856–1953) described *Clostridium pasteurianum* as another nonsymbiotic fixer of atmospheric nitrogen. Yet another bacterium, *Azotobacter chroococcum*, one of the most common nitrogen fixers, was discovered by Beijerinck in 1901. There are some other atmospheric nitrogen-fixing bacteria, although less efficient ones, for example, bacteria of the genera *Bacillus*, *Enterobacter*, *Chromatium*, *Chlorobium*, and *Klebsiella*.



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Chapter 9
Mutagens, Radicals, Rocket Fuel, and Laughing Gas: Stringing Metabolic Modules to Survive on Nitrogenous Poisons

Martin G. Klotz and Lisa Y. Stein

Abstract Nitrogen is a key element, enabling life on planet Earth as we know it. In addition to being essential as a major component of biomass, its unsurpassed redox reactivity and versatility makes it an exceptional actor in abiotic nutrient cycling and cellular metabolism. While nitrogen compounds have been essential parts in the processes that led to the incredible diversity of life, from humble molecular beginnings throughout its evolution before and after the oxygenation of planet Earth, their impact has emerged and will persist into the future as instruments to the metabolic creativity of microorganisms. Whether this plays out in natural harmony, providing support for extant diversity, or eventually leads to abyss and an inhabitable planet to multicellular creatures is in the hands of humankind.

Nitrogen is a peculiar element. Like carbon and boron, nitrogen can form a triple bond with itself, one of the strongest covalent bonds known; however, dinitrogen (N_2) is the only triple-bonded molecule that exists as an inert gas. Its high molecular stability is the reason why N_2 gas is virtually unreactive and permits life to exist in an atmosphere that consists of about 78% dinitrogen. In contrast to the nonpolar N_2 molecule, triple-bonded carbons, the alkynes, are reduced hydrocarbons and thus polar and reactive gases, liquids, or even solids. Uniquely, nitrogen can also form a triple bond with carbon giving rise to the salt-forming (iso)cyanides, R-CN, which are rapidly interactive, inhibitory to many metabolic reactions and thus highly toxic to cells. Nevertheless, a sizeable number of microbes utilize cyanide derivatives such as cyanate as a source of energy and reductant (Fig. 9.1) while being able to protect sensitive intracellular targets from cyanide activity.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_9

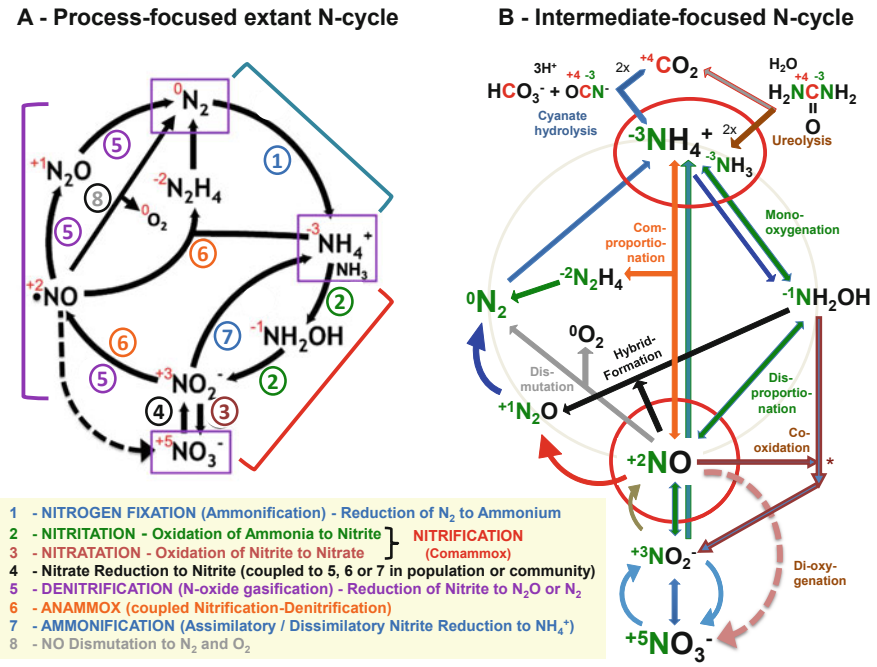


Fig. 9.1 Major processes and intermediates of the nitrogen cycle. (**a**) The numbered circles indicate the eight major processes and pertinent reactions in the nitrogen cycle as indicated in the embedded legend. Process 2, the oxidation of ammonia to nitrite or nitritation, can be linked to processes 3, 5, or 6 within (2 + 3, comammox; 2 + 5, nitrifier denitrification) or between populations leading to nitrification, N-oxide gasification or anammox. Process 4, the reduction of nitrate to nitrite, can be linked to processes 5, 6, or 7 within or between populations leading to denitrification, anammox, or ammonification. The colored brackets identify processes historically known as N-fixation (teal; process 1), nitrification (red), and classic denitrification (magenta). Process 8 represents the dismutation of nitric oxide (NO) leading to the intracellular formation of N_2 and O_2 gases in an anoxic environment. (**b**) Signature reactions between intermediates in the nitrogen cycle organized by flow of electrons between (the oxidation state of) nitrogen cycle intermediates including inorganic (cyanate) and organic (urea) complex sources of reduced nitrogen. The intermediate-focused rendition of the N-cycle documents the central position of NO in the cycle and highlights the immense capacity in redox power utilization for a diverse array of cellular metabolic lifestyles

Like carbon and oxygen, nitrogen can form double bonds with itself, yielding polar reactive molecules. When double bonded with carbon or oxygen, the result is reactive imines or nitrogen oxides. The most common bonds with nitrogen are single bonds formed with carbon, hydrogen, and oxygen. While the nitrogen-carbon single bond forms key linkages between amino acids in proteins, bonds with hydrogen and oxygen result in reduced nitrogen compounds in four different oxidation states or oxidized nitrogen compounds in five different oxidation states. The resulting ten different possible oxidation states of nitrogen molecules provide unmatched redox-reactive versatility and, together with the structural stability conferred by carbon

compounds, contribute to the foundation of the magic called "Life" on planet Earth, if not in the entire universe.

Nitrogen is an essential element in two of the four macromolecules constituting both the blueprint (nucleic acids) and the toolkit (Proteins) that dynamically executes (*the blueprint instructions into a*) functioning metabolism, thereby critically contributing to the hallmarks of life as we know it (evolution, emancipation, and communication of cells). However, it is actually the incredible versatility of redox reactivity of simple nitrogen molecules that steered life to its present diversity. Ironically, this incredible versatility in redox reactivity of nitrogen molecules represents also the greatest danger to a continued existence of metazoan life on the planet. The cause for unleashing this destructive potential is not the aforementioned central place of nitrogen in life's processes, but rather the ill-natured, albeit often unintended, far-reaching activities of human ingenuity and industriousness. The platform for this disastrous outcome is the connectedness of nitrogen compounds at different oxidation states through spontaneous and facilitated electron transfer reactions, individually and organized in pathways, which constitute the global *nitrogen cycle* (Fig. 9.1a). This biogeochemical cycle together with others cycling carbon, sulfur, iron, and other redox-active elemental compounds as well as plate tectonics keep the planet eligible for life. Interestingly, life itself is the active driver of these biogeochemical cycles: *microorganisms*. While this might read like a *circulus vitiosus*, it does so only in the absence of clues about how microorganisms came to be (*origin of life*). Once the single-celled archaea and bacteria came into being on an initially bare giant rock that eventually became our blue planet, they diversified, driven by horizontal gene transfers and viral transductions and quickly emerged as the drivers of biogeochemical nutrient cycles.

The "secret" to the evolved capability of single cell microbes to push all the biogeochemical cycles is the organization of their metabolisms in functional *modules* (Fig. 9.1), correlating with a modular organization of its encoding genetic information. Modular organization of genes and polygenic transcriptional units in the genomes of archaea and bacteria provides two advantages: (1) modular sections and even entire modules can be exchanged between microbes even across large taxonomic distances by lateral transfer, and (2) the co-location of genes and polygenic transcriptional units provides for a variety of regulatory opportunities suited for coordinating cellular emancipation and communication activities. The modular structural and functional organization of metabolism enabled the evolution of acclimation and adaptation of microbes at the levels of cells, populations, and communities. This was particularly useful during and after the shift from an anoxic Earth to increasingly oxic hydro- and atmospheres. Existing modules supporting electron flow using terminal acceptors other than oxygen could simply be modified by extension or substitution with those suited to reduce oxygen, which was accomplished, in part, by the emergence of a diverse complement of *quinone-reactive protein (QRP)* complexes that used copper as redox-active transition metal: the heme copper oxidases. This emerging diverse array of high-throughput, oxygen-reducing, terminal oxidases was the basis for an expansion of catabolic pathways with modular units capable of high-throughput oxidation of reduced substrates.

In the case of the nitrogen cycle, high-throughput oxidation of reduced substrates such as *ammonium/ammonia*, aliphatic amines, as well as urea (Fig. 9.1) led to fast accumulation of highly toxic (*nitric oxide*), mutagenic (*hydroxylamine or* "aminol") or explosive (*hydrazine*) intermediates that demanded detoxification by either reduction or oxidation reactions. Detox by further reduction of reduced intermediates would deprive the cell of precious reducing power without exploiting these intermediates catabolically. In contrast, detox by oxidation and channeling of the extracted electrons to a sufficient reservoir of intracellular electron acceptors would preserve catabolic opportunities. This was successfully accomplished by the modular coupling with QRP complexes facilitating high-throughput electron flow in and out of the quinone pool to respiratory acceptors, thereby generating enough proton-motive force needed to support reverse electron flow to fuel carbon and nitrogen assimilation. The fixation of carbon and nitrogen into biomass are the two costliest anabolic tasks cells have to master, and they can afford catabolism with narrow differences between their oxidation and terminal reduction potentials only when they exist in highly reduced environments and have access to methane (CH_4) and ammonium (NH_4^+). In our present predominantly oxic world, oxygen is available and well suited to serve as co-substrate and terminal acceptor at high-throughput energy- and electron-harvesting and electron-disposing ends, respectively, of the catabolic pipeline, which generates greater differences between catabolic oxidation and terminal reduction potentials. Broader implementation of this opportunity required the emergence of a great diversity of QRP complexes suited to match particular donor and acceptor environments, and this diversity could not have arisen at once during the Great Oxygenation Event (GOE) about 2.5 billion years ago. Therefore, the individual modular components of the pipeline must have evolved and been functional during anoxic times before the GOE, and some likely evolved separately in different genomic backgrounds. Subsequently, horizontal gene transfers then occurred at the "right time," the "right place," and the "right frequency" to fit cassettes poised at the proper redox potential into an expanded new module to provide the recipient cell with an advantage for succession in its present or emerging new environment.

Enzyme complexes generally operate bidirectionally, driven by the redox and energy gradients in which they operate. This is why individual cassettes could be "fitted" into different catabolic contexts as long as the intersections met with correct redox potentials, thereby creating diverse linear and branched modules with varying entering and exiting redox potentials that were accomplished, in part, by the emergence of a diverse complement perfectly suited for metabolic innovation. This modular design permitted metabolic collaboration between functional cassettes within cells, between different cells in a population and between populations of different cells, which provided for closed cycles as well as for segments of cycles. The extant nitrogen cycle more likely than not existed unclosed during the vast anoxic period before the GOE when copper was not largely bioavailable and the only known enzyme capable of reducing nitrous oxide (laughing gas) to dinitrogen, the copper enzyme complex nitrous oxide reductase, was not yet invented or operational. This missing connection between the inert reservoir of dinitrogen and the

branch of reductive N-oxide transformations must have increased the pool of nitrous oxide in the anoxic dinitrogen-rich atmosphere and contributed to global warming and ozone depletion thereby counteracting metabolic diversification and abundance of biomass. This is one reason why the evolved capability of cyanobacteria to extract electrons from water and generating increasing levels of free molecular oxygen as a byproduct was such a massive liberating event, today described as the exploding diversity of life following the GOE.

Before water was exploited as an external reductant from which electrons could be extracted by oxidation to generate free molecular oxygen (a.k.a., oxygenic phototrophy), water served as the dedicated intracellular source of oxygen that permitted the anoxic oxidation of nitrite to nitrate and the oxidation of hydroxylamine to nitrite, potentially via NO as an intermediate. Both reactions are known since decades to occur in oxic environments and molecular dioxygen was implicated as the oxidant. This all changed in the late 1990s and early 2000s with the discovery of peculiar bacteria affiliated with the planctomycetes that catabolized ammonium and produced dinitrogen in the complete absence of oxygen: the *an*aerobic *amm*onium-*ox*idizing (anammox) bacteria. In contrast to the then well-known aerobic ammonia-oxidizing bacteria (AOB), the anammox bacteria not only oxidized ammonium without oxygen as a co-substrate; they also did not terminate electron flow with oxygen as happens in aerobic AOB and ammonia-oxidizing Thaumarchaeota. Instead, the anammox process couples the extraction of electrons from ammonium with the reduction of N-oxides to oxygen-void nitrogen compounds by comproportionating NO and ammonium to form hydrazine (rocket fuel) as an energy-rich, albeit dangerous, intermediate. NO is obtained by reducing nitrite with electrons extracted from ammonium. In addition, in order to generate enough reducing power to drive carbon assimilation, anammox bacteria anaerobically oxidize nitrite to nitrate. The discovery of this anammox catabolism was proof for how central catabolic modules of the extant nitrogen cycle evolved and were functional in the absence of free dioxygen and that the intermediate products of their activities must have been available during evolutionary times before the GOE.

Before free molecular oxygen was available at sufficient concentrations, the *NO* radical was the only operative oxidant released during spontaneous interconversions of nitrogen species at different oxidation states. Nitrogen in its most reduced state, ammonium/ammonia [NH_4^+/NH_3], can oxidize successively, albeit very slowly, without catalysis into its N-alcohol, hydroxylamine ["aminol", H_2N-OH (NH_2OH)]; its N-aldehyde, hydrogen nitrosyl [nitroxyl; N(=O)-H, *HNO*]; its two N-acids, nitrous acid [hydrogen nitrite; N(=O)-OH, HNO_2] and hyponitrous acid (N(=N-OH)-OH, $H_2N_2O_2$); as well as its imine peroxide [hydroperoxy-nitrene; N = (O-O)-H, *HONO*]. Hyponitrous acid, which essentially is dimerized nitroxyl, can disintegrate into two molecules of HNO; however, spontaneous dehydration would yield water and nitrous oxide [$H_2N_2O_2 \rightarrow H_2O + N_2O$]. Of all these N-oxides, NO is by far the most reactive, capable of both accepting and donating electrons, and is particularly reactive with sulfur alcohols [Thiols; -SH]. Since the pre-cell stage of evolving metabolism, NO and Methyl-thiol [CH_3SH] have served as the primordial universal oxidant and reductant, respectively, and their interactions might have thus

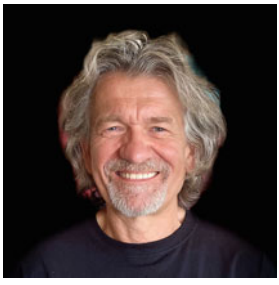
been the complementary (yin-yang) driving force facilitating the evolution of catalysis. It is thus no surprise to see that the complement of metalloenzymes involved in the controlled transformations of NO is by far the greatest of all enzymes that function in the extant N-cycle, facilitating one or two electron transfers in a single reaction (see Fig. 9.1a, b).

Interestingly, while the flow of nitrogen and electrons in the extant nitrogen cycle can be reconstructed by just examining microbes that utilize nitrogen in their oxidative and reductive branches of catabolic electron flow, this does not prove that all modules participating in the extant nitrogen cycle evolved in the ancestors of extant nitrogen-centric bacteria (ammonia- and nitrite-oxidizing bacteria, anammox bacteria) and archaea (ammonia-oxidizing Thaumarchaeota). In fact, it is more likely that some of the modules that utilize nitrogen oxides as electron acceptors in the reductive branch of catabolism evolved in microbes that harvest energy and reductant from reduced compounds that lack nitrogen, such as carbon-, sulfur- or iron-containing compounds. Some of the N compound-reducing modules in the reductive branch of catabolism are respiratory in that they contribute to the formation of proton-motive force (i.e., NrfAH and NrfABC, reducing nitrite; NarGH, reducing nitrate), while others do not contribute to the conservation of energy (i.e., nitrite reductases NirK and NirS; nitrate reductases NasAB and NapABGH). On the other end of the electron flow chain, it is unlikely that modules facilitating high-throughput oxidation of catabolic substrates such as ammonium/ammonia or methylamine were established before the modules providing detoxification of intermediates, and safe channeling of the extracted electrons to final acceptors, were functional. Indeed, the oxygen-dependent ammonia-oxidizing microbes utilize copper-containing membrane-bound monooxygenases (CuMMO), which were not functional as such in anoxic environments. In contrast, the anammox bacteria catabolize their substrates at considerably lower rates; the ammonium-oxidizing and hydrazine-forming module is soluble, sequestered in an internal compartment, the anammoxosome, employs iron as its catalytic transition metal, and it operates in the constraints of a fast product-consuming redox gradient (without this gradient, the module operates in the opposite direction, disproportionating hydrazine).

The nitrogen cycle with its many redox-active intermediates beautifully demonstrates how microorganisms evolved sophisticated metabolic inventory permitting electron transfers between both benign and highly harmful nitrogen intermediates. The modules are finely tuned to prevent accumulation of toxic intermediates near susceptible intracellular targets and are redundant and diverse enough to provide an opportunity to "call up" the module component to do the job under a range of conditions. The acquisition of inventory enabling cells to exploit catabolically the large diversity in oxidation states of nitrogen compounds including "mutagens, radicals, rocket fuel, and laughing gas" and "stringing metabolic modules" to support cellular metabolism provided a significant evolutionary avenue for organisms, successful in anoxic environments, to evolve and succeed (survive and prosper) in the expanding oxic surrounding of our blue planet. Dissection of the extant biogeochemical nitrogen cycle and inference in the evolutionary history of the major players in this cycle, organisms, and macromolecules, has, so far, provided beautiful

evidence that microorganisms and their activities are "the foundation stone of the biosphere."

The human invention of the dinitrogen-fixing Haber-Bosch process has, in less than 100 years, doubled the concentration of reactive nitrogen in the biosphere that before was entirely limited by the activity of diazotrophic microorganisms. While the reasoning behind the Haber-Bosch process was to inexpensively increase the food supply for humans, the unintended consequence has been a rapid acceleration of the many redox-active functions of nitrogen-cycling microorganisms, leading to steep increases in concentrations of harmful molecules like nitrate, NO, and nitrous oxide in many of Earth's ecosystems. Over 25% of the human population owes their lives to the Haber-Bosch process due to its success in increasing crop yields, yet climbing atmospheric levels of nitrous oxide, a greenhouse gas over 300 times more potent than carbon dioxide in holding heat, is leading to a warmer climate year upon year, thus putting the survival of humanity and multitudes of animal and plant species at great risk. This is a lesson to us that while microorganisms can adapt and evolve in response to any number of redox-active molecules that feed and regulate their catabolic modules, we are entirely dependent on microbial activities, their control over biogeochemical cycles, and the balance of reactive molecules from these cycles that evolved long before the first metazoans. Not only did the microbial nitrogen cycle pave the way for our existence, it is also the key that we must understand and work with for its continuation to guaranty "a safe operating space for humanity."



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Chapter 10
The Grand Microbial Variety Show

Aharon Oren

Abstract In the past decades, many novel microbial processes were discovered, and many old dogmas about the apparent limits to what microbes can do were upended. As a result, our understanding of the roles that microorganisms play in the global cycles of carbon, nitrogen, sulfur, and phosphorus has greatly increased. This essay explores a large number of processes that were recently discovered and processes for which important new insights were obtained. These include autotrophic nitrification; anaerobic oxidation of ammonia (the anammox reacti\on); diverse ways in which methane can be oxidized in anoxic environments; different ways in which chemo-autotrophic sulfur bacteria can cope with lack of oxygen; new insights in the anaerobic degradation of hydrocarbons; newly discovered possibilities for bacteriochlorophyll-driven anoxygenic photosynthesis; and retinal protein-driven photoheterotrophy. I also discuss the diversity in biochemical pathways used by different types of prokaryotes to achieve identical goals, as well as the question as to how some prokaryotes can make a living on chemical reactions that yield very low amounts of energy.

> Het werd een streven om steeds maar weer nieuwe, oogenschijnlijk ontoegankelijke, gebieden voor de microben te ontsluiten. De eene specialiteit onder de microben was nog niet ontdekt, of een andere, die nog verrassender kunststukken verrichtte, werd reeds aangekondigd: het microbiologisch schouwtoneel geleek één grootsch natuurwetenschappelijk variété!
>
> [It became a pursuit to continuously unlock new, seemingly inaccessible areas for the microbes. Barely had one specialist among the microbes been discovered when another one that performed even more surprising feats was already announced; the microbiological theater resembled one grand natural-science variety show.]—(Kluyver 1924; translation: Aharon Oren)

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_10

10.1 Introduction

The sentences quoted above were taken from a lecture entitled "Eenheid en verscheidenheid in de stofwisseling der microben" (Unity and diversity in the metabolism of the microbes), delivered by Albert Jan Kluyver at the general assembly of the Netherlands Chemical Society in April 1924. Kluyver is well-known for his concept of the unity in biochemistry (Kluyver and Donker 1926). Indeed, the general unifying principles that Kluyver identified, as expressed in his famous saying "From elephant to butyric acid bacterium—it is all the same!" still hold today. Many of the ideas exposed in his famous paper of 1926 can be found in the text of the lecture he gave 2 years earlier.

There can be no doubt that the greatest metabolic diversity is found in the prokaryotic world. Since the days of Sergei Winogradsky and Martinus Beijerinck who identified many novel ways in which bacteria can make a living, it is obvious that archaea and bacteria exploit many modes of energy generation that do not exist in the eukaryotic world. One could almost say that, as long as a chemical reaction based on compounds available in nature is sufficiently exergonic, some microbe can be found that will derive its energy from it. Chemical thermodynamics sets the limits. Kluyver was obviously fascinated by this "grand microbial variety show." His own studies on different processes of fermentation, anaerobic respiration, and methanogenesis, as well as many other discoveries of new types of metabolism identified by others in the first decades of the twentieth century, urged him to search for the unifying principles behind the metabolic diversity.

My personal fascination with the metabolic diversity displayed by the microorganisms made me decide to devote my career to the study of the prokaryotic world. It happened that my first microbiology teacher was Hans Veldkamp, who had spent part of this time as a student in Albert Jan Kluyver's laboratory (Konings and Kuenen 2003). When today I compare our knowledge of the metabolic diversity of the prokaryotes with what we knew when I started my studies in the early 1970s, I am amazed how many novel processes were discovered, especially in the last two decades. Many old dogmas about the apparent limits of what microbes can do were upended. Textbooks had to be updated and rewritten to include the often major contributions made by hitherto unknown processes to the global cycles of carbon, nitrogen, sulfur, and phosphorus. Still, the basic principle of the unity in biochemistry holds true. In this chapter, I present a personal selection of newly discovered ways used by prokaryotes to obtain energy and carbon for growth, all exciting new microbial "acts" that can be added to Kluyver's microbial "variety show."

10.2 Nitrification: Then and Now

After Sergei Winogradsky's early studies on the bacteria responsible for the nitrification process—the oxidation of ammonium ions to nitrate by chemolithotrophic prokaryotes—at the end of the nineteenth century (Winogradsky 1891, 1949), there was a general feeling that we know almost all there is to know about the basics of the process. Two groups of organisms jointly perform it: one type of organism oxidizes ammonium to nitrite using molecular oxygen as the electron acceptor, and a second type of organism then oxidizes the nitrite further to nitrate:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+; \Delta G_o' = -274.7\ \text{kJ}$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-; \Delta G_o' = -74.1\ \text{kJ}$$

The first reaction is performed by members of the *Betaproteobacteria* and the *Gammaproteobacteria* and the second one by members of the *Alphaproteobacteria* and the *Deltaproteobacteria*, as well as the deep branching *Nitrospirae* phylum. A chemolithoautotrophic nitrite oxidizer belonging to the phylum *Chloroflexi* (genus *Nitrolancea*) was characterized a few years ago (Sorokin et al. 2014).

The recent finding that chemoautotrophic members of the archaea play a major role in the oxidation of ammonium to nitrite came as a great surprise. Thaumarchaeota are now known to be the numerically dominant ammonia oxidizers in the ocean and in soils. The large number of marine Thaumarchaeota in the world's oceans, estimated at 10^{28} cells, suggests that they may play a major role in global biogeochemical cycles. The first isolate belonging to this group is chemolithoautotrophic *Nitrosopumilus maritimus*, obtained from the Seattle, WA, aquarium (Könneke et al. 2005; Qin et al. 2017); the first species whose name was validly published was *Nitrososphaera viennensis* isolated from garden soil, and the name of the class *Nitrososphaeria* is based on the name of this genus (Stieglmeier et al. 2014). Not all ammonia oxidizing Thaumarchaeota are necessarily chemolithoautotrophs; some not only transport organic carbon compounds, but they may even require organic carbon sources (Qin et al. 2014).

The Gibbs free energy yields of the two consecutive steps of the nitrification process are small. In addition to the low energy yield of the oxidation reactions (ammonium to nitrite, nitrite to nitrate), the nitrifying prokaryotes are faced with the high energy expense involved in the formation of reducing power for the autotrophic fixation by the Calvin-Benson-Bassham cycle. This is due to the high standard redox potential of the couples, 0.44 V for NO_2^-/NH_4^+, 0.43 V for NO_3^-/NO_2^-, as compared with −0.32 V for $NADP^+$/NADPH. Therefore, it would be advantageous to combine the two partial nitrification reactions in a single organism:

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2\ H^+; \Delta G_o' = -348.8\ \text{kJ}$$

Winogradsky himself had already recognized the problem very early in his career (Winogradsky 1891):

> Le fait que la production de nitrites devenait de plus en plus abondante à mesure des ferments, paraissait conduire à la conclusion que la formation d'acide nitreux serait leur seule function. Mais l'énigme, que comportait cette conclusion, empêchait de l'accepter, avant que l'on eût attaqué le problème de tous les côtés. Comment, en effect, s'expliquer qu'un organisme, possédant des moyens énergiques d'oxyations, termine son action par la production d'un corps chimique plus oxydable que celui du début. En admettant même qu'il ne soit capable de parfaire d'emblée l'oxydation complète de l'ammoniac, comment comprendre, qu'il paraisse totalement dépourvu d'action sur l'acide nitreux, alors que l'oxydation de ce corps pourrait lui lui fournir un surcroît d'énergie?
>
> [The fact that the production of nitrites became more and more abundant commensurate with the ferments, seemed to lead to the conclusion that the formation of nitrous acid would be their only function. But the enigma that was included in this conclusion prevents it to be accepted before one would have attacked the problem from all sides. How, in fact, could one explain that an organism that possesses the energetic machinery to perform oxidations should end its action with the production of a chemical compound that is easier to oxidize than the starting material? Even if one admits that it would not be able to finish from the outset the complete oxidation of ammonia, how can it be understood that it seems to be completely devoid of action on nitrous acid, while the oxidation of that substance would supply it with additional energy.]

This is one of the few cases in which Winogradsky discussed energetics-related considerations in his writings.

One of the possible solutions to the paradox of the division of labor is based on the kinetic theory of optimal design of metabolic pathways, which postulates the existence of an optimal length for a pathway that maximizes the rate of ATP production. Shortening long pathways could result in an increased growth rate. This is offset by a reduced growth yield if the shorter pathway has fewer ATP-generating steps. A complete nitrifier should gain more energy per mole of substrate but may grow at a slower rate than organisms carrying out the individual steps of the pathway. A complete nitrifier could have advantages over the classical two-step process when microbes grow slowly in clonal colonies (Costa et al. 2006; Santoro 2016). It is therefore not surprising that *com*plete *amm*onia *ox*idizers ("comammox" bacteria) were found in biofilms in an aquaculture treatment system, in a deep subsurface pipe, and in a bioactive filter at a drinking water treatment plant (van Kessel et al. 2015; Daims et al. 2015).

Complete ammonia oxidation to nitrate was found in a low-oxygen bioreactor fed with low concentrations of ammonium, nitrite, and nitrate. *Nitrospira* species developing in the reactor possessed genes for all the enzymes necessary for ammonia oxidation via nitrite to nitrate, including genes for ammonia monooxygenase (AMO) that are phylogenetically distinct from the known AMOs (van Kessel et al. 2015). A thermophilic comammox bacterium, "*Candidatus* Nitrospira inopinata," was obtained in co-culture with a bacterium affiliated with the *Hydrogenophilaceae*. Genes affiliated with the distinct ammonia monooxygenase and hydroxylamine dehydrogenase genes of *Nitrospira* are present globally in many environments.

Thus, *Nitrospira* performing complete oxidation of ammonium to nitrate may be a key component of nitrogen-cycling microbial communities (Daims et al. 2015).

10.3 The Anammox Reaction: From a Hypothetical Mechanism for Dinitrogen Fixation to a Major Process in the Global Nitrogen Cycle

The latest editions of textbooks of general and environmental microbiology mention the anammox reaction as part of the nitrogen cycle in nature. In the anammox (*an*aerobic *amm*onia *o*xidation) reaction, ammonium is oxidized in anoxic environments using nitrite as the electron acceptor, yielding gaseous nitrogen:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O; \Delta G_o' = -357.8\ \text{kJ}$$

The effect of the process is to some extent comparable to that of denitrification: biologically available bound nitrogen is converted to inert nitrogen gas.

The history of the discovery of the anammox process is interesting. Relatively few people may be aware of the fact that the reaction was already found in the microbiological literature 90 years ago. In those days the enzymatic mechanism of biological nitrogen fixation by nitrogenase was yet unknown. Different hypotheses were brought forward to explain the conversion of nitrogen gas to inorganic nitrogen compounds available to life. Thus, the anammox reaction is found in the opposite direction in the first edition of Marjory Stephenson's textbook "Bacterial metabolism" (Stephenson 1930; Oren 2015):

$$N_{2gas} + 2\,H_2O_{liq} = NH_4^+ + NO_2^-; -\Delta F = -85690\ \text{cals.}$$

In 1977 the same reaction was proposed so that ammonium and nitrite could together be used as energy source. In a fascinating paper entitled "Two kinds of lithotrophs missing in nature," a paper that one could call prophetical today, Engelbert Broda asked why the reaction:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O; \Delta G = -86\ \text{kCal}\ (= -360\ \text{kJ})$$

is not found anywhere in nature (Broda 1977). At that time it was generally assumed that ammonia-nitrogen can only be oxidized aerobically by ammonia monooxygenase, a reaction in which ammonia combines with one of the two atoms of O_2 to yield hydroxylamine, which is converted to nitrite. Broda passed away in 1983, so he did not live to see that the process he predicted indeed exists in nature.

The discovery of the anammox reaction was reported in 1995 based on the observation that ammonium was disappearing from an anaerobic denitrifying

fluidized bed reactor treating effluent from a methanogenic reactor. Nitrate and ammonium fed into the column were consumed with concomitant gas production (Mulder et al. 1995). It was soon discovered that nitrite rather than nitrate was the electron acceptor for the ammonium oxidizing bacteria that are affiliated with the *Planctomycetes* phylum (Strous and Jetten 2004). Now it is clear that the anammox process is a major link in the global nitrogen cycle, especially in the ocean where it causes major losses in available nitrogen in oxygen-depleted waters (Francis et al. 2007).

Among the varied company of new "actors" in the "grand microbial variety show," the anammox bacteria are maybe the most unusual. Hydrazine, a highly reactive compound used as rocket fuel, is an intermediate in the oxidation of ammonium to dinitrogen (Strous et al. 2006). The anammox bacteria possess an intracellular compartment named the anammoxosome, composed of a specialized membrane on which the redox processes occur with energy conservation in the form of a transmembrane proton gradient. Possibly to protect this membrane from reactive intermediates, this very dense membrane is built of linearly concatenated cyclobutane lipids. These lipids contain up to five linearly fused cyclobutane moieties with *cis* ring junctions. Such "ladderane" molecules were never yet encountered elsewhere in nature (Sinninghe Damsté et al. 2002).

10.4 Diverse Ways to Oxidize Methane in Anaerobic Environments

Just like the old dogma that ammonium cannot be oxidized in the absence of molecular oxygen, it long was believed that oxidation of methane in anaerobic environments is not possible. The rationale was very similar: the only known reaction in which methane could be oxidized was by methane monooxygenase, which catalyzes the oxidation of methane to methanol in a reaction in which the oxygen atom of methanol is derived from molecular oxygen, analogous to the formation of hydroxylamine from ammonia in the first step of nitrification.

Occurrence of anaerobic oxidation of methane was first postulated following the analysis of vertical profiles of methane abundance in anoxic marine systems. Methane consumption was correlated with a decrease in sulfate concentrations, suggesting that sulfate may act as the terminal electron acceptor. Based on methane profiles, radiotracer experiments, and stable carbon isotope data, it appears that a large fraction of the globally produced methane is oxidized to CO_2 in anaerobic marine sediments (Boetius et al. 2000). The process is often performed by dense aggregates of archaea (termed ANME—ANaerobic MEthane oxidizers) and sulfate-reducing bacteria, growing in clusters of which the anaerobic methane-oxidizing archaea are surrounded by sulfate reducers. Massive anaerobic oxidation of methane in the bottom sediments of the Black Sea results in the formation of up to 4-meter-high carbonate buildups produced by the activity of ANME archaea and sulfate

reducers of the *Desulfosarcina/Desulfococcus* group (*Deltaproteobacteria*) (Michaelis et al. 2002). The overall reaction is:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O; \Delta G = -40 \text{ kJ per mol methane},$$

calculated for a pressure of 8 MPa (780 m depth) (Boetius et al. 2000).

The ANME organisms have not yet been grown in pure culture. Therefore most information about their physiology was deduced from metagenomics data. The ANME organisms use a "reverse methanogenesis" pathway to produce cellular carbon and energy, and nearly all genes typically associated with methane production are present in archaeal methanotrophs (Hallam et al. 2004). The role of the sulfate-reducing bacteria associated with the ANME archaea is not fully clear, and the nature of the intermediate exchanged by the partners in the symbiosis was never unequivocally identified. Anaerobic oxidation of methane might not be an obligate syntrophic process but may be carried out by the ANME alone. Zero-valent sulfur is a key intermediate in marine methane oxidation, and it is formed by the methanotrophic archaea through a new pathway for dissimilatory sulfate reduction. The produced elemental sulfur (in the form of disulfide) is disproportionated by the associated *Deltaproteobacteria* (Joye 2012; Milucka et al. 2012).

Anaerobic oxidation of methane can also be coupled to nitrate reduction. An organism designated "*Candidatus* Methanoperedens nitroreducens" was enriched from a bioreactor fed with nitrate, ammonium, and methane. Methane activated by methyl-CoM reductase undergoes full oxidation to carbon dioxide via reverse methanogenesis. The genes for nitrate reduction were probably obtained by this ANME archaeon by lateral gene transfer from a bacterial donor. Electron transport in "*Candidatus* M. nitroreducens" probably involves cofactor F_{420} in the cytoplasm, quinones in the cytoplasmic membrane, and cytochrome *c* in the pseudoperiplasm. The nitrite produced from nitrate is further reduced to dinitrogen by anammox bacteria (Arshad et al. 2015; Haroon et al. 2013).

The range of electron acceptors that can be coupled with anaerobic methane oxidations is not restricted to sulfate and nitrate. Microorganisms found in the Eel River Basin in California can use oxidized manganese (birnessite) and iron (ferrihydrite) to oxidize methane (Beal et al. 2009). Iron-dependent anaerobic methane oxidation is performed by archaea related to "*Candidatus* Methanoperedens nitroreducens" (Cai et al. 2018). Some anaerobic methane oxidizers appear to be versatile organisms that can switch between different electron acceptors, depending on environmental conditions (Ettwig et al. 2016).

Undoubtedly, the most fascinating way to oxidize methane in an anaerobic environment is displayed by the organism described as *Methylomirabilis oxyfera*. Its strategy to obtain energy from methane in the absence of molecular oxygen is unique. Instead of searching for alternative pathways for the activation of methane, it uses the well-known oxygen-dependent conversion of methane to methanol mediated by methane monooxygenase. To do so, it has developed a way to generate itself

the oxygen required for the process. Its strategy is simple: if you cannot find oxygen when you need it, make your own!

The first indication for the existence of such an organism came from the study of a freshwater denitrifying microbial consortium (Raghoebarsing et al. 2006). The organism responsible for the methane oxidation uses nitrite as the electron acceptor according to:

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O;$$

$$\Delta G_o' = -929\ \text{kJ per mol}\ CH_4$$

Analysis of the "*Candidatus* Methylomirabilis oxyfera" genome sequence assembled from the metagenome, combined with isotopic labeling and biochemical studies, showed a unique way of coping with life in an anaerobic environment while using molecular oxygen in a key reaction in its metabolism. Nitrite is first reduced to nitric oxide, a reaction that is part of the conventional pathway of nitrate reduction in denitrification. However, instead of the subsequent reduction of NO to N_2O, the nitric oxide is dismutated to yield N_2 and O_2. The oxygen thus generated within the cell is then used for the activation of methane by methane monooxygenase. "*Candidatus* Methylomirabilis oxyfera" is thus a "cryptic aerobe." The reaction catalyzed by the nitric oxide dismutase is one of the very few biological reactions that generate molecular oxygen, the others being oxygenic photosynthesis, respiration of chlorate, and detoxification of reactive oxygen species (Ettwig et al. 2010; Oremland 2010).

10.5 *Thioploca*, *Beggiatoa*, and *Thiomargarita* Carrying Their Own Supply of Electron Acceptor for Anaerobic Respiration

An interesting way to enjoy life in the presence of molecular oxygen while using denitrification as the mode of anaerobic energy generation is by storing large amounts of nitrate inside the cells, to be used when oxygen is not available. This mode of adaptation to life in fluctuating conditions is found in a number of 'giant' prokaryotes, sulfur-oxidizing members of the *Gammaproteobacteria* that contain large intracellular vacuoles. The process was first discovered during the study of extensive mats of the autotrophic sulfur bacterium *Thioploca* found along the continental shelf off Southern Peru and North and Central Chile below the oxygen-minimum zone in the upwelling region, at water depths between 40 and 280 m. *Thioploca* is a multicellular filamentous organism that lives in bundles surrounded by a common sheath. *Thioploca* cells have a vacuole that occupies >80% of the cell volume. Here the cells accumulate nitrate at concentrations up to 0.5 M. The filaments are motile by gliding, and they transport the nitrate 5–15 cm

down into the sediment where they use it as electron acceptor for the oxidation of sulfide, growing autotrophically or mixotrophically (Fossing et al. 1995; Jørgensen and Gallardo 1999). Similar nitrate vacuoles were found in marine *Beggiatoa* filaments found at a Monterey Canyon cold seep and at the Guaymas Basin hydrothermal vents where sulfide concentrations are high and dissolved oxygen is low. Nitrate concentrations in the cells were estimated at 0.13–0.16 M, being 3000- to 4000-fold higher than ambient levels (McHatton et al. 1996). Another giant sulfur-oxidizing bacterium is *Thiomargarita namibiensis*. With cells measuring up to 0.75 mm, it is the prokaryote with the largest cells ever recorded. It was discovered in dense populations in Namibian shelf sediments underlying the oxygen minimum zone of the Benguela Current upwelling system. Like its close relative *Thioploca*, its cells possess a central vacuole in which nitrate is accumulated to a concentration of ~0.8 M (Schulz et al. 1999).

10.6 Cable Bacteria: Oxidizing Sulfide Using Remotely Located Oxygen

One of the most surprising findings in microbial ecology in the past decade is the discovery that oxidization of sulfide in anaerobic marine sediments can be coupled with reduction of oxygen in the overlaying aerobic layers, up to several centimeters higher. Filamentous "cable bacteria" can transport electrons over centimeter distances in sediments. The existence of electrical communication between the reduced and the oxidized parts of sediments was discovered when it was noticed that altering the oxygen concentration in the water overlying the sediment resulted in a rapid change in the sulfide concentration more than 12 mm below the oxic zone. This change could only be explained by transmission of electrons but not by diffusion of molecules (Nielsen et al. 2010). The electrical conductors are filamentous bacteria that transport electrons along their length. These cable bacteria are phylogenetically affiliated with the sulfate-reducing and sulfur-disproportionating *Desulfobulbaceae* (*Deltaproteobacteria*), and they probably oxidize sulfide by reversing the canonical sulfate reduction pathway. The cell envelope contains highly conductive fibers, and periplasmic cytochromes may be responsible for the electron flow (Kjeldsen et al. 2019; Meysman et al. 2019). Presence of the enzymes of the Wood-Ljungdahl pathway of CO_2 fixation (see Sect. 10.10) suggests that they may lead an autotrophic way of life (Kjeldsen et al. 2019). The existence of such living electrical cables shows that the action of some microorganisms is not limited to the micrometer scale but can have significant effects centimeters away (Nielsen et al. 2010; Pfeffer et al. 2012).

10.7 How to Degrade Diverse Hydrocarbons in the Absence of Molecular Oxygen?

In oxic environments, the activation of C–H bonds in hydrocarbons is mainly performed by O_2-dependent reactions. As for the oxidation of methane, the classical way of activating larger hydrocarbons is through the action of oxygenases that convert the hydrocarbons to the corresponding alcohols. However, there are many reports of anaerobic degradation of hydrocarbons. Thus, from oil wells and oil production fluids, moderately thermophilic sulfate-reducing bacteria could be enriched that utilize *n*-alkanes and alkylbenzenes under strictly anoxic conditions (Rueter et al. 1994). There are even observations that alkanes might be consumed in anoxic sediments below the zone of sulfate reduction where they are converted to methane under strictly anoxic conditions (Zengler et al. 1999).

Starting in the early 1990s, highly diverse ways were discovered in which bacteria, in most cases members of the *Betaproteobacteria* and the *Deltaproteobacteria*, degrade both aliphatic and aromatic hydrocarbons under anaerobic conditions (Rabus et al. 2016). Thus, a sulfate-reducing bacterium, designated strain Hxd3—"*Desulfococcus oleovorans*" (a name without standing in the nomenclature)—can grow anaerobically on hexadecane as sole carbon source (Aeckersberg et al. 1991). Alkanes are activated by carboxylation at the third carbon with subsequent elimination of the terminal and subterminal carbons, yielding a fatty acid that is one carbon shorter than the parent alkane (So et al. 2003).

Toluene can be anaerobically activated by the addition of fumarate to form benzylsuccinate that is further converted to benzoyl-CoA. This reaction is found in the denitrifying *Betaproteobacteria Thauera aromatica* and *Azoarcus* spp. (Biegert et al. 1996). Ethylbenzene can be anaerobically converted to phenylethanol by *Azoarcus* strain EbN1 (also known as "*Aromatoleum aromaticum*") by the action of ethylbenzene hydrolase (Kniemeyer and Heider 2001). Degradation of aromatic hydrocarbons such as benzene and polycyclic aromatic hydrocarbons in the absence of molecular oxygen is very slow. Bacterial cultures with doubling times of around 2 weeks were obtained that anaerobically degrade benzene, naphthalene, methylnaphthalene, and even phenanthrene, the largest polyaromatic hydrocarbon currently known to be degradable under anoxic conditions. Degradation of benzene and naphthalene is probably initiated by carboxylation to benzoate and 2-naphthoate, respectively (Merckenstock et al. 2016).

10.8 Newly Discovered Possibilities for Bacteriochlorophyll-Driven Anoxygenic Photosynthesis

Bacteriochlorophyll-based anoxygenic photosynthesis is found in many lineages of the domain Bacteria. Purple and green sulfur bacteria are photoautotrophs that use reduced sulfur compounds as the electron donors for autotrophic CO_2 fixation; others lead a photoheterotrophic life in which light is used as energy source, while carbon for cell growth is derived from organic substrates.

Studies of the distribution of phototrophic microorganisms in the water column of the Black Sea have shown that some green sulfur bacteria (class "*Chlorobia*") can live at extremely low light intensities. In water samples collected in 1988 when the 0.1% light level was estimated at 30–40 m, the interface between oxygen and sulfide was located at 80-100 m depth, and bacteriochlorophyll *e* and characteristic carotenoids derived from *Chlorobium* sp. were found in the water profile between 68 and 121 m depth (Repeta et al. 1989). Brown-colored sulfur bacteria identified as strains of *Chlorobium phaeobacteroides* were isolated from the chemocline at 80-m depth where light transmission was calculated to be 0.0005% of surface irradiance. The strains are obligate phototrophs and are adapted to growth at extremely low light intensities: the amount of light-harvesting pigments is increased, and they have a very low maintenance energy requirement (Overmann et al. 1992).

In his prophetic paper in which Broda predicted the existence of the anammox bacteria, Broda also asked why photosynthetic, anaerobic, ammonia-oxidizing bacteria do not appear to exist; such bacteria could theoretically oxidize ammonia to nitrogen gas, analogous to the oxidation of sulfide to elemental sulfur by purple and green sulfur bacteria (Broda 1977). Such ammonia-oxidizing anoxygenic phototrophs have still not been found, but nitrite was shown to serve as an electron donor by a strain of the purple sulfur bacteria genus *Thiocapsa* (*Gammaproteobacteria*) isolated from sewage sludge. Nitrite is oxidized to nitrate, and it is the highest-potential electron donor known so far to drive anoxygenic photosynthesis: $NO_3^-/NO_2^- = +0.43$ V, compare $S_o/HS^- = -0.27$ V and (for oxygenic photosynthesis) $O_2/H_2O = +0.82$ V (Griffin et al. 2007). The finding of nitrite-driven autotrophic growth in an isolate of *Rhodopseudomonas* (*Alphaproteobacteria*; a genus of anoxygenic photoorganotrophic bacteria), also obtained from a municipal sewage treatment plant, shows that the ability to use nitrite as an electron donor for photosynthesis is more widespread (Schott et al. 2010).

Another recently discovered reduced compound that can drive anoxygenic photosynthesis is arsenite. Isolates of arsenate-oxidizing *Ectothiorhodospira* (*Gammaproteobacteria*) were obtained from a hot spring and from sediment of the alkaline hypersaline Mono Lake, California, and from Big Soda Lake, Nevada. The As(III) oxidation may be a common phenomenon among soda lake purple photosynthetic bacteria (Hoeft McCann et al. 2017).

10.9 Retinal Protein-Driven Photoheterotrophy

Until the end of the twentieth century, it was generally assumed that photoheterotrophic growth based on light absorption by membrane-bound retinal pigments is restricted to *Halobacterium* and a few related genera of extremely halophilic members of the *Euryarchaeota*. The function of the light-driven proton pump bacteriorhodopsin in these archaea was already known in the 1970s. Therefore, the finding of similar membrane-bound retinal-containing proton pumps in marine members of the domain Bacteria came as a big surprise. Proteorhodopsin, the retinal protein of marine members of the *Proteobacteria*, was discovered in metagenomics studies of marine bacterioplankton. The functioning of this new rhodopsin pigment resembles that of the archaeal proton-pumping rhodopsins (Béjà et al. 2000). Genes encoding proteorhodopsin are distributed among bacterial taxa belonging to phylogenetically divergent lineages of *Alphaproteobacteria*, *Gammaproteobacteria* (de la Torre et al. 2003), *Flavobacteria* (Gómez-Consarnau et al. 2007), and others.

While it is clear that proton pumping by proteorhodopsins can lead to the formation of ATP driven by the transmembrane proton gradient, the importance of the pigment in the life of marine bacterioplankton is still far from clear. Proteorhodopsin-mediated photoautotrophic growth has never yet been demonstrated, and there are only very few cases in which light was shown to increase growth yield of marine bacteria growing heterotrophically. Growth yield of a retinal protein-containing strain of *Dokdonia* (*Flavobacteria*) was increased during light exposure (Gómez-Consarnau et al. 2007), but it is still not established as a general phenomenon. However, quantitative estimations of the amounts of retinal pigments in the marine environment show that microbial rhodopsins are major contributors to the capturing of solar energy in the Mediterranean Sea and the Atlantic Ocean. The highest rhodopsin concentrations were found above the deep chlorophyll *a* maxima, and proton-pumping proteorhodopsins may absorb as much light energy as chlorophyll *a*–based phototrophs, so that proteorhodopsins may be a major energy-transducing mechanism of harvesting sunlight in the surface ocean (Gómez-Consarnau et al. 2019).

10.10 Unity in Biochemistry: Diversity in Biochemical Pathways

The concept of unity in biochemistry (Kluyver and Donker 1926) implies that in all forms of life, the basic principles of the biochemical reactions are similar. In spite of this, the same metabolic goals such as aerobic degradation of simple sugars or autotrophic CO_2 fixation can often be achieved in different ways (Gottschalk 1985). It is not always clear why some microorganisms use a certain biochemical pathway, while others prefer a different sequence of reactions in spite of the fact that

it may yield less energy (for dissimilatory reactions) or may be energetically more costly (for assimilatory processes).

Sugars such as glucose can be degraded to pyruvate by the Embden-Meyerhof-Parnas glycolytic pathway via fructose-1,6-bisphosphate or by the Entner-Doudoroff pathway with 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate as intermediates. In the latter pathway, only one molecule of ATP is formed for each two pyruvate generated instead of two ATP in the classical glycolytic pathway. The Entner-Doudoroff pathway is also operative in barley and possibly in other higher plants (Chen et al. 2016). There are additional ways to degrade sugars, such as the oxidative pentose phosphate cycle in which three molecules of 6-phosphogluconate are decarboxylated to three ribulose-5-phosphate, which are then converted to one glyceraldehyde-3-phosphate and two fructose-6-phosphate.

For fermentative organisms, the difference between one and two ATP formed is highly significant. Still, besides the alcohol fermentation of yeasts (the Embden-Meyerhof-Parnas glycolytic pathway, yielding two ATP per glucose fermented), we have the alcohol fermentation by *Zymomonas* that yields the same end products with generation of 1 ATP only, as the organism uses the Entner-Doudoroff pathway (Swings and De Ley 1977). Another case in which the same fermentation products can be formed by using different metabolic pathways is the fermentation of lactate with the formation of propionate, acetate, and CO_2. In most propionate-forming bacteria, succinate formed by carboxylation of pyruvate is an intermediate; however, in *Anaerotignum propionicum* (basonym: *Clostridium propionicum*) and *Megasphaera elsdenii*, no such carboxylation reaction occurs, and the three-carbon compound acrylate is a key intermediate (Gottschalk 1985).

When the reactions of the reductive pentose phosphate cycle (the Calvin-Benson-Bassham cycle) were discovered in 1950, the cycle was proposed as the universal pathway for autotrophic carbon dioxide assimilation, the most important biosynthetic process in biology. However, a second pathway for autotrophic CO_2 fixation, the reductive citric acid cycle, had been discovered by 1966. The question must be asked why nature has devised many different ways to active the same goal (Berg 2011). Today we know no less than six different pathways used by different autotrophic prokaryotes for the assimilation of inorganic carbon into their biomass. These include:

The Calvin-Benson-Bassham cycle with ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoribulokinase as the key enzymes. To some extent, it is surprising that most autotrophic CO_2 fixation is mediated by RuBisCo: the enzyme has a low affinity for CO_2, its catalytic turnover rate is slow, and it has a wasteful oxygenase side reaction that generates phosphoglycolate, leading to photorespiration in oxygenic phototrophs. There appears to be a sharp upper temperature limit (~70 °C–75 °C) for the functioning of the cycle. This may be due to the heat instability of glyceraldehyde-3-phosphate and other intermediates of the cycle.

The reductive citric acid cycle, also known as *the Arnon-Buchanan cycle*. In this pathway, most reactions of the tricarboxylic acid cycle (the Krebs cycle) are reversed. Four carboxylation reactions are operative: carboxylation of acetyl-CoA to pyruvate, phosphoenolpyruvate to oxaloacetate, succinyl-CoA to 2-oxoglutarate,

and 2-oxoglutarate to isocitrate. This pathway was originally discovered in the green sulfur bacterium *Chlorobium* (*Chlorobi*), but it has since been found in anaerobic or microaerobic members of the *Aquificae* (*Aquifex*, *Hydrogenobacter*), *Proteobacteria* (especially *Deltaproteobacteria* and *Epsilonproteobacteria*), and the *Nitrospirae*. Some *Aquificae* that use this pathway can grow up to 95 °C.

The reductive acetyl-CoA pathway, also known as *the Wood-Ljungdahl pathway*. Here, one CO_2 is reduced to the level of a methyl group; a second CO_2 molecule is reduced to carbon monoxide bound to CO dehydrogenase, which also acts as an acetyl-CoA synthase, combining the two carbons to form acetyl-CoA. Acetyl-CoA is then carboxylated to pyruvate. As the CO dehydrogenase/acetyl-CoA synthase is highly oxygen sensitive, functioning of the pathway requires strict anoxic conditions. The Wood-Ljungdahl pathway is the preferred mode of autotrophic CO_2 fixation of acetogenic bacteria and methanogenic archaea, organisms living close to the thermodynamic limit. The pathway functions also in the anammox bacteria (*Planctomycetes*), in autotrophic sulfate-reducing bacteria such as *Desulfobacterium* (*Deltaproteobacteria*), and in autotrophic *Archaeoglobales* (*Euryarchaeota*). The pathway is compatible with life at the upper limits of temperature: it is used by a *Methanopyrus* strain that can multiply at 122 °C.

The 3-hydroxypropionate bi-cycle, also known as *the Fuchs-Holo bi-cycle*. Figure 5 in Berg (2011) gives a detailed description of this complex pathway. The CO_2 fixation steps are the carboxylation of acetyl-CoA to malonyl-CoA and of propionyl-CoA to (*S*)-methylmalonyl-CoA. The 3-hydroxypropionate bi-cycle operates in the green non-sulfur phototrophs of the *Chloroflexaceae* family (phylum *Chloroflexi*), which preferentially grow as photoheterotrophs. The key carboxylase activities of biotin-dependent acetyl-CoA/propionyl-CoA carboxylase are virtually irreversible and use bicarbonate as substrate. This may be advantageous during growth at high pH. However, the energy costs are high: seven ATP equivalents are needed for the synthesis of pyruvate and three additional ATPs for its conversion to triose phosphate.

The 3-hydroxypropionate/4-hydroxybutyrate and dicarboxylate/4-hydroxybutyrate cycles. Here acetyl-CoA and two inorganic carbons are converted to succinyl-CoA, the carboxylation reactions being the conversion of acetyl-CoA to pyruvate and phosphoenolpyruvate to oxaloacetate in the first case and conversion of acetyl-CoA to malonyl-CoA and of propionyl-CoA to (*S*)-methylmalonyl-CoA in the second case. The enzymes of the 3-hydroxypropionate/4-hydroxybutyrate cycle are oxygen-tolerant. The pathway is found in (micro)aerobic *Sulfolobales*, while the dicarboxylate/4-hydroxybutyrate cycle is found in mostly anaerobic members of the *Crenarchaeota* orders *Desulfurococcales* and *Thermoproteales*. Both cycles are energetically expensive, the anaerobic one being less costly. The thermotolerance of these pathways may be an important feature explaining why they were adopted by the hyperthermophilic crenarchaeotes (Berg 2011).

Another interesting case of multiple biochemical pathways used by different microorganisms to achieve the same goal is the incorporation of methane-derived carbon into the biomass of cells that grow on methane as single carbon source. None of the known methanotrophs uses RuBisCo and the enzymes of the Calvin cycle for

CO_2 fixation. In the methanotrophic prokaryotes, we know two different pathways for carbon fixation: the ribulose monophosphate pathway and the serine pathway. A third pathway is operative in methanotrophic yeasts the dihydroxyacetone pathway, also known as the xylulose monophosphate pathway, with as key reaction the formation of glyceraldehyde-3-phosphate + dihydroxyacetone from xylulose-5-phosphate and formaldehyde. The ribulose monophosphate pathway is based on the reaction of formaldehyde with ribulose-5-phosphate to yield 3-hexulose-6-phosphate that is then converted to fructose-6-phosphate. The formaldehyde is derived from the oxidation of methane via methanol. The 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase are the key enzymes. This pathway is found in methanotrophs with Type I membranes, which include members of the *Gammaproteobacteria* such as the genera *Methylococcus*, *Methylomonas*, and *Methylobacter* (Colby et al. 1979; Hanson and Hanson 1996). The ribulose monophosphate pathway is widespread in the prokaryotic world, not only in organisms that grow on methane, but it is also involved in formaldehyde fixation and detoxification, and in some archaea its enzymes catalyze the reverse reaction for the biosynthesis of pentose phosphate (Kato et al. 2006). Methanotrophs with Type II membranes such as *Methylosinus* and *Methylocystis* (*Betaproteobacteria*) employ the serine pathway that combines the assimilation of formaldehyde (formation of serine from glycine) and CO_2 (carboxylation of phosphoenolpyruvate to oxaloacetate) (Colby et al. 1979; Hanson and Hanson 1996).

10.11 How Much Free Energy Should a Reaction Minimally Yield to Support Microbial Growth?

The amount of Gibbs free energy needed to form one mole of ATP from ADP and inorganic phosphate is about −32 kJ under standard conditions and approximately −44 kJ when taking the true intracellular concentrations of the compounds into account (Thauer et al. 1977).

It is always surprising that many anaerobic prokaryotes thrive on dissimilatory reactions that yield even less free energy than needed for the synthesis of one ATP molecule. An example is the sulfur-reducing, acetate-oxidizing bacterium *Desulfuromonas acetoxidans*, which obtains its energy from the reaction:

$$2\ \text{Acetate}^- + 2\ \text{H}^+ + \text{S}_8 + 4\ \text{H}_2\text{O} \rightarrow 4\ \text{CO}_2 + 8\ \text{H}_2\text{S};$$
$$\Delta G'_\text{o} = -16.7\ \text{kJ per acetate oxidized}$$

Desulfuromonas forms robust growing syntrophic mixed cultures with phototrophic green sulfur bacteria that re-oxide the sulfide formed (Pfennig and Biebl 1976).

Syntrophic growth, i.e., the growth of two types of organisms together in which one partner consumes metabolic end products excreted by the other, is a well-known

process that even enables some bacteria to make a living from reactions that under standard conditions are endergonic. The process was discovered in a culture known as "*Methanobacillus omelianskii*." This culture produces acetate and methane from ethanol and CO_2:

$$2\ \text{Ethanol} + CO_2 \rightarrow 2\ \text{Acetate}^- + 2H^+ + CH_4; \Delta G_o' = -111.3\ \text{kJ}$$

The original culture was isolated by Vasily Omeliansky (1916) from feces of rabbits that first received 1% ethanol in their drinking water, later increased to 2%. We may assume that those rabbits quite enjoyed their life in Omeliansky's laboratory. Cultures performing the same reaction were later isolated from freshwater sediments and from sewage. In 1967, it was recognized that "*Methanobacillus omelianskii*" is a syntrophic co-culture of a bacterium (the "S-organism") that oxidizes ethanol and a methanogen that reduces CO_2 with hydrogen as the electron donor:

$$2\ \text{Ethanol} + 2\ H_2O \rightarrow 2\ \text{Acetate} + 2\ H^+ + 4\ H_2; \Delta G_o' = +19.4\ \text{kJ}$$

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O; \Delta G_o' = -130.7\ \text{kJ}$$

The sum of these two reactions is the "*Methanobacillus omelianskii*" reaction given above. By efficiently removing hydrogen so that it does not accumulate, the first reaction becomes sufficiently exergonic to enable ATP generation (Bryant et al. 1967).

The principle of syntrophic growth is simple: if you want to make a living from an endergonic reaction, team up with someone else and share energy resources. Such syntrophic cultures are often found in methanogenic environments where methane producing archaea keep the hydrogen pressure sufficiently low to allow the oxidation of NADH and $FADH_2$ by its partner:

$$\text{NADH} + H^+ \rightarrow \text{NAD}^+ + H_2; \Delta G_o' = +18.1\ \text{kJ}$$

$$FADH_2 \rightarrow \text{FAD} + H_2; \Delta G_o' = +37.4\ \text{kJ}$$

Examples of such interspecies hydrogen transfer processes are the anaerobic degradation of propionate (*Syntrophobacter wolinii*), butyrate (*Syntrophomonas wolfei*, *Syntrophospora bryantii*), and benzoate (*Syntrophus buswellii*), all endergonic reactions under standard condition (Stams 1994):

$$\text{Propionate}^- + 3\ H_2O \rightarrow \text{Acetate}^- + HCO_3^- + H^+ + 3\ H_2; \Delta G_o' = +76.1\ \text{kJ}$$

$$\text{Butyrate}^- + 2\,H_2O \rightarrow 2\,\text{Acetate}^- + H^+ + 2\,H_2; \Delta G_o' = +48.1\ \text{kJ}$$

$$\text{Benzoate}^- + 7\,H_2O \rightarrow 3\,\text{Acetate}^- + HCO_3^- + 3\,H^+ + 3\,H_2; \Delta G_o' = +58.9\ \text{kJ}$$

Methanogenic archaea that use the aceticlastic methanogenesis reaction (*Methanosarcina*, *Methanothrix*) obtain only little energy:

$$\text{Acetate} + H_2O \rightarrow CH_4 + HCO_3^-; \Delta G_o' = -31\ \text{kJ}$$

It is therefore surprising that two organisms cooperating in a syntrophic partnership can exploit even such a low-energy-yielding reaction. Analysis of a methanogenic enrichment culture grown at 60° C with a doubling time of 30–40 h showed the presence of two organisms rather than a single aceticlastic methanogen. Studies with ^{14}C-labelled substrates showed that the methyl group and the carboxyl group of acetate are both converted to CO_2 with formation of hydrogen gas that is subsequently used to reduce part of the CO_2 to methane by a *Methanobacterium* sp.:

$$\text{Acetate}^- + 4\,H_2O \rightarrow 2\,HCO_3^- + 4\,H_2 + H^+; \Delta G_o' = +104.6\ \text{kJ}$$

$$4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\,H_2O; \Delta G_o' = -135.6\ \text{kJ}$$

The first reaction can only proceed when the methanogenic partner keeps the hydrogen pressure below 10^{-4} atmosphere. It is a reversal of the reaction of homoacetogens that make a living from the formation of acetate from hydrogen and CO_2 (Zinder and Koch 1984). The organism responsible for the oxidation of acetate using the strongly endergonic reaction could be isolated in pure culture on a different substrate: ethylene glycol; it proved also capable of autotrophic growth as a homoacetogen (Lee and Zinder 1988).

Energy conservation in anaerobic prokaryotes that live on substrates that do not allow the synthesis of one mole of ATP per mole of substrate via substrate level phosphorylation is only possible by the generation of an electrochemical ion gradient across the cytoplasmic membrane followed by ATP synthesis via an ATP synthase. Based on the limitations for ion transport and ATP synthesis, it was often assumed that at least −20 kJ/mole of substrate are required for ATP synthesis. However, under certain conditions the minimum biological "energy quantum" that sustains life may even be lower (Müller and Hess 2017).

10.12 Can Indeed All Reactions with a Negative Gibbs Free Energy Change Be Exploited to Drive Microbial Growth?

The examples analyzed above create the impression that every possible exergonic reaction that uses compounds readily available in nature is used at least by some type of microorganism to make a living. This is indeed nearly always true.

Still, there are exceptions. The most notable is the simple equation:

$$0.5\ N_2 + 1.25\ O_2 + 0.5\ H_2O \rightarrow NO_3^- + H^+; \Delta G_o' = -32.6\ kJ$$

With the existing pressures of the gasses, the reaction is exergonic as long as the nitric acid concentration does not exceed 0.1 M (Broda 1975; Lewis and Randall 1923). Many of the above-discussed reactions with much lower free energy yields efficiently drive microbial life. The answer to the paradox must be sought in the highly stable nature of dinitrogen. A very high activation energy is needed to split the molecule, so that kinetic constraints rather than the free energy change of the overall reaction determine what is possible. That is also the reason why biological nitrogen fixation is so energetically expensive. If nature had invented an enzymatic system that can reduce the activation energy needed to split N_2 while still enabling conservation of the free energy change in the form of ATP and/or a transmembrane proton gradient, the world's ocean would probably have been a highly acidic solution of dilute nitric acid (Oren 2015). Thus, there still are limits to what the diverse prokaryotic world can do.

10.13 Final Comments

When some time ago I received the kind invitation from the editor of this volume to contribute a chapter for a book entitled "Microbes: The foundation stone of the biosphere," I did not immediately guess that he intended the stone in the center of the Dome of the Rock on the Temple Mount in Jerusalem, less than 4 kilometers away from my office. According to legend, that stone once was the only thing in existence. Interesting tales were told about this rock over the centuries. Mark Twain (Samuel Langhorne Clemens, 1835–1910), who visited the Holy Land in 1867 with a company of early tourists and pilgrims, commented in his travelogue: "The rock, large as it is, is suspended in the air. It does not touch anything at all. The guide said so. This is very wonderful" (Twain 1869).

Instead, my first thought was a reference to Psalm 118: 22:

אבן מאסו הבונים היתה לראש פנה

[The stone that the builders rejected has become the cornerstone] (translation: English Standard Version). Together with Isaiah 28: 16, on which it may be based,

this text is referred to several times in the New Testament. The lowly bacteria are truly the cornerstone of the world. Indeed, one cannot see them, but they are ubiquitous, and they even quantitatively dominate life on Earth. With estimated numbers of $4\text{-}6\times10^{30}$ cells, the total amount of prokaryotic carbon is 60%–100% of the estimated total carbon in plants. The Earth's prokaryotes contain about tenfold more nitrogen and phosphorus than do plants, and they represent the largest pool of these nutrients in living organisms (Whitman et al. 1998).

But the true "cornerstone" or "foundation stone" function of the prokaryotic world is based on its tremendous metabolic diversity. Nearly every process that is thermodynamically feasible is realized by at least some types of bacteria or archaea. As shown by the quotations at the opening of this chapter, this was fully realized by Albert Jan Kluyver in his famous essay on "Eenheid en verscheidenheid in de stofwisseling der microben" (Kluyver 1924), in which he referred to famous predecessors such as Sergei Winogradsky and Martinus Beijerinck, to whom we thank much of our basic understanding of the possibilities and limitations of microbial metabolism.

The examples presented in this chapter demonstrate that the true metabolic diversity exhibited by the prokaryotes is much beyond what was dreamt by Kluyver. Nature functions within the framework of his concepts of unity in biochemistry and metabolism of microorganisms (Kluyver 1924; Kluyver and Donker 1926). Many new processes were discovered in the past decades, some of these so important that textbook chapters on the biogeochemical cycles of carbon, nitrogen, sulfur, phosphorus, and other elements had to be rewritten. Old dogmas were upended, as exemplified by the discovery of anaerobic oxidation of ammonium ions (the "anammox" process) and of multiple ways to oxidize methane in anaerobic environments. These, and other recently obtained insights, make a renewed study of the functioning of the prokaryotic world truly fascinating.

In the same lecture presentation quoted in the opening sentences of this chapter, Albert Jan Kluyver commented:

> . . . een rechtgeaard microbioog mag nimmer de gelegenheid laten voorbijgaan om mede te werken aan het eerherstel der kleinste levende wezens.
>
> [. . . a genuine microbiologist should never miss an opportunity to contribute to the rehabilitation of the smallest living beings.]—(Kluyver 1924)

Today those smallest living beings no longer need a "rehabilitation."

Acknowledgments I thank Ronald S. Oremland (US Geological Survey, Menlo Park, CA, USA) and Rich Boden (University of Plymouth, UK) for their helpful comments.

Aharon Oren

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Part IV
Microbes Established and Sustain Life

Chapter 11
Microbes' Many Roles in Climate Change: Contribution, Consequence, Mitigation, and Model System

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Abstract Climate change is an imminent problem, one quite possibly catastrophic to the global ecosystem and human civilizations. Most understand that human activities are responsible for climate change, yet it is enhanced microbial processes by human activities that are truly causing it. The three major biogenic greenhouse gases (carbon dioxide, methane, and nitrous oxide) all have significant microbial sources, especially in agricultural practices. However, microbes are also at the forefront of climate change impacts due to their prevalence and numerous ecosystem functions that they are performing. Studies using simulation experimental settings like the Free-Air Carbon dioxide Enrichment (FACE) facility showed clear responses in microbial communities to increased carbon dioxide concentration and temperature, which are associated with disruptions in the soil ecosystem, increasing harmful algal blooms, and range shifting of vector-borne infectious diseases. It would then be useful to use mitigation strategies that target microbes in order to reduce greenhouse gas production, such as modifying agricultural practices to reduce emissions and increase carbon sequestration. Microbes can also be a good model system to study the consequences of climate change due to their rapid reproduction rates and large population sizes so that more realistic climate change simulation may be possible within a shorter time frame. All together, microbes are in a unique position for the issue of climate change as contributors, victims, mitigators, and a model system.

11.1 Introduction

Humans and all organisms are living in an era of climate change that scientists are now calling a climate crisis. Empirical evidence from various scientific disciplines almost unanimously points at anthropogenic activities as the cause of the current climate change. The autotrophic greenhouse gas carbon dioxide (CO_2)

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_11

concentrations have geologically oscillated between ~150 and 300 ppm for the last 800,000 years until the Industrial Revolution, which started to generate an unprecedented quantity of carbon dioxide into the atmosphere by burning fossil fuels and changing land cover and use. Since reaching 400 ppm on May 7, 2013, at Mauna Loa Observatory, Hawaii, whose data was used for the Keeling curve, carbon dioxide concentration has continued on an upward trend (415 ppm, February 2020) and is projected to be between 550 and 900 ppm by the end of this century. The global mean temperature has increased almost 1 °C since the Industrial Revolution, and many climate models predict over a 2 °C increase by the next century. The Intergovernmental Panel on Climate Change (IPCC) published the 5th Assessment Report on 2013–2014 putting 2 °C as a goal; however, with an updated understanding of the devastating effect on the global ecosystem and human civilization, a Special Report was issued with a new goal of 1.5 °C in 2017.

11.2 Contribution

The natural cycle of carbon is simply an exchange between autotrophy and heterotrophy, i.e., carbon dioxide in the atmosphere is fixed (reduced) into organic matter by autotrophs, and heterotrophs break down (oxidize) the fixed carbon back to carbon dioxide. These redox reactions support both the autotrophs and heterotrophs that perform them, since the organisms gain energy from these chemical reactions. Autotrophic microbes are divided into two general categories of photoautotrophs and chemoautotrophs. The photoautotrophs use energy from the sun's electromagnetic radiation and these organisms include algae, diatoms, Cyanobacteria, and anoxygenic phototrophic bacteria (e.g., purple sulfur bacteria and green sulfur bacteria). The chemoautotrophs derive their energy from chemical sources, and these organisms include nitrifying bacteria and iron-oxidizing bacteria. Heterotrophic microbes are the majority of aerobic and anaerobic organisms prevalent in the earth ecosystem.

While human activities are the ultimate reason for the imbalance of the carbon cycle—which increases carbon dioxide concentration in the atmosphere—it is microbes that facilitate the actual processes. Fossil fuels are stably stored organic matter from geological processes; thus the excessive removal of them from the ground and seafloor, and then burning them, is an obvious contribution to raising the atmospheric carbon dioxide concentration. Through the Green Revolution, humans have produced a tremendous quantity of crops from the agricultural fields, of which a significant portion was converted from forests or other natural ecosystems. The conversion reduces long-term carbon storage capacity of most natural ecosystems, as agricultural fields have shorter-term carbon storage in their crop biomass through conventional agricultural practices, such as tilling. Forests can store some of their carbon in cycles that last hundreds of years, contrasted with a few types of crops that may store carbon in cycles lasting less than 1 year.

Two more potent biogenic greenhouse gases—methane (CH_4) and nitrous oxide (N_2O)—are even more directly relevant to microbial activities, especially in agriculture. Methane is the second most important biogenic greenhouse gas because of its sheer quantity (10% of US greenhouse gas emission in 2017) and the fact that methane is around 25 times more potent than carbon dioxide in warming potential per equal mass. Methane has multiple man-made sources: land-use change, the livestock industry, and landfills. However, methane is produced from those environments actually by archaea populations belonging to *Euryarchaeota* phylum, which are abundant in anaerobic environments like wetlands, marine sediments, the rumens of ruminants, and termite guts. Rice is a major crop that grows in artificial wetlands called rice fields, and annual methane production from rice fields is estimated to be 20%–25% of total biogenic methane production. With increased meat consumption due to an increasing human population and their quality of life, methane production from ruminants is estimated to be about 20%.

Nitrous oxide is the least abundant (6% of US greenhouse gas emission in 2017) but most potent (~300 times to carbon dioxide) greenhouse gas and is mostly produced from two microbial processes: nitrification and denitrification. Nitrification is the process of oxidizing ammonia (NH_3) to nitrite (NO_2^-) to nitrate (NO_3^-), and denitrification is anaerobic respiration of organic matter using nitrate as an electron acceptor. In low oxygen conditions, ammonia-oxidizing bacteria and ammonia-oxidizing archaea use nitrite as an electron acceptor instead of oxygen—thus leading to the reduction to nitrous oxide ($NO_2^- \rightarrow N_2O$). Nitrous oxide is an intermediate in the denitrification process, and low oxygen and low organic matter seem to favor the nitrous oxide production pathway. As nitrogen is often naturally limited in terrestrial ecosystems, agricultural fields experience an excessive amount of anthropogenically associated nitrogen input as chemical fertilizer or organic forms (e.g., manure). The natural soil microbial processes from agricultural fields then introduce more nitrous oxide into the atmosphere. Increased nitrogen input also enhances overall soil microbial respiration through a stoichiometric response between carbon and nitrogen contents, thus increasing the release of soil carbon into carbon dioxide as well.

11.3 Consequences

Rapid environmental events, like those associated with the current climate change trends, force affected organisms to take one of these responses: migration, acclimation, adaptation, or extinction. Individual organisms and populations of organisms would make use of one or more of those responses based on their physiology and genetics. For example, vegetation is more prone to acclimation or adaptation over time, if not extinction, as migration usually is not a realistic nor effective response compared to the rapid movement available for insects or animals. Microbes are known for their hyperdiversity in taxonomic, phylogenetic, and functional dimensions, so they have an advantage in making use of any of these responses, which enables them to thrive in certain environmental events. Nevertheless, these response

strategies will result in loss of diversity, altered species interactions, shifted community structure, and often reduced ecosystem functions, all of which are observed in almost all studies concerning the biological consequences of climate change.

As we saw in the previous section, microbes contribute to climate change as contributors by producing an excessive amount of greenhouse gases with the help of human activities. At the same time, microbes are recipients at the forefront of the impacts of climate change due to their prevalence and tremendous contributions to our ecosystems' functions and services. There has been active research to better understand and predict the biological consequences of climate change in the future. Perhaps the most innovative experimental setting for such studies is variants of Free Atmospheric Carbon dioxide Exchange (FACE) for carbon dioxide manipulation. The FACE facilities implement pipes connected to carbon dioxide sources that surround vegetation of interest to maintain a local environment with desired carbon dioxide concentration. This makes it so that there can be a proper investigation into the responses of vegetation and associated microbes to elevated carbon dioxide concentrations that represent potential future time points. Our group studied soil microbial communities from a FACE facility called BioCON (Biodiversity, Carbon diOxide, and Nitrogen) in Cedar Creek Ecosystem Science Reserve in Minnesota. After 10 years of continuous carbon dioxide concentration at 560 ppm, we discovered that soil microbial communities responded very clearly: they showed increased biomass and shifted community structures. Moreover, the abundance of microbial functional genes responsible for nitrogen fixation (*nif*H gene) and labile carbon degradation (e.g., amylase, pullulanase, cellobiase, endoglucanase genes, etc.) was significantly increased in the plots with elevated carbon dioxide levels as compared to the plots with ambient carbon dioxide concentrations (control plots). This study suggested possible positive feedback responses in soil carbon and nitrogen cycles, with elevating the carbon dioxide levels resulting in more available carbon and a greater carbon-to-nitrogen ratio (C/N ratio).

Harmful algal blooms (HAB) represent another problem linked with climate change due to increased environmental temperature, surface stratification, atmospheric carbon dioxide concentration, reduced calcification from ocean acidification, altered hydrologic patterns, and more. Harmful algal blooms are usually local or regional problems. However, they are also global problems since they occur in many coastal regions and freshwater ecosystems around the world. There have been numerous studies on specific harmful algal blooming organisms including dinoflagellates (e.g., *Karenia* and *Cochlodinium*), diatoms (e.g., *Pseudo-nitzschia*), and cyanobacteria (e.g., *Microcystis* and *Cylindrospermopsis*) for their genetics, physiology, and ecology. Many studies have shown the shifting of local aquatic microbial communities by harmful algal bloom-causing organisms migrating and proliferating in response to changing local environmental conditions often related to climate change. Although most researchers agree that climate change and responsible anthropogenic activities (e.g., nutrient pollution) must be associated with the increasing frequency and intensifying harmful algal bloom events, specific causal mechanisms are still mostly elusive. This is understandable as the systems that need to be studied are very complex and dynamic, so appropriate research efforts require truly

multidisciplinary teams of atmospheric science, oceanography, molecular biology, ecology, and data science.

Emerging infectious diseases have been major news items in recent years including current COVID-19 (COronaVIrus Disease 2019), influenza in the current and many previous years, Zika fever in 2018, etc. Many of those diseases are vector-borne, and insect vectors are under the impacts of climate change and often respond through migration. For example, the British Chief Medical Officer in 2002 predicted that by 2050 the climate in England will be suitable for endemic malaria. Many tropical diseases are moving poleward in both the upper and lower latitudinal ranges. Examples include dengue fever, Zika fever, lymphatic filariasis (elephantiasis), African trypanosomiasis (Chagas disease), yellow fever, and other mosquito and tick-borne diseases. Mosquito species like *Aedes aegypti* and *Culex pipiens* and tick species like *Ixodes scapularis* are expanding their ranges, thus imposing new problems to the regions where those infectious diseases used to be of no threat. Very recently (November 2017), a new discovered invasive tick species, *Haemaphysalis longicornis*, has been observed in mainland USA, which is an example of the complexity of how vector migration can be influenced by multiple environmental and societal factors, which are again associated with the human activities causing climate change.

11.4 Mitigation

Responsible for producing all three biogenic greenhouse gases, microbes are major contributors to climate change. However, this also means that they can be a major mitigation strategy for greenhouse gas production. Emission reduction and sequestration enhancement would be two major greenhouse gas mitigation strategies. Land-use (re)conversion to forests or native vegetation would have multiple beneficial effects by reducing the emission of some greenhouse gasses and increasing soil carbon sequestration. Most of the agricultural practice improvements used for achieving sustainability focus on the modification of microbial processes. In principle, the emission of biogenic greenhouse gases can be reduced by using more efficient management practices for the flows of carbon and nitrogen. Efficient nutrient management is the most important aspect of emission reduction because nitrogen in fertilizer and manures is not always used most efficiently by crops and grazing plants. Instead, excess application of nitrogen increases soil microbial processes like respiration, nitrification, and denitrification liberating greenhouses gasses into the atmosphere and causes nutrient pollution contributing to consequent eutrophication in aquatic ecosystems. Thus, improved efficiency and optimization of nitrogen application to agricultural lands would reduce nitrous oxide and other greenhouse gas emissions. Some available practices for achieving these goals include using slow-release fertilizer forms, and nitrification inhibitors, and adjusting application rates based on more precise estimation of plant needs. Methane emission from rice fields can be reduced by several draining events during growth seasons.

There has been a new cultivar of rice developed with low root exudation rates, which would reduce methane emission as well. Cutting down meat consumption would also help reduce methane production from those ruminants. Improved agricultural practices that increase yields and generate higher inputs of residue carbon into the soil can lead to increased soil carbon storage. These practices include using improved crop varieties, extending crop rotation, and converting to reduced tillage or no-tillage. Reduced or no-tillage practice can also reduce carbon emission by reducing the use of heavy agricultural machinery.

11.5 A Model System in Research

As mentioned previously, the accurate prediction of climate change consequences is critical for consorted mitigation efforts. But accurate predictions are not easy to obtain even with innovative research facilities like FACE. Perhaps the most difficult aspect of climate simulation facilities is the realistic treatment of climate parameters, such as carbon dioxide concentration and temperature. For example, most FACE facility settings are based on elevated carbon dioxide concentrations being pumped into biological systems constantly for some designated period of time, but the real carbon dioxide concentration has been and will be increasing gradually with daily and seasonal fluctuations. However, it is very difficult to set up an experimental system with gradually changing climate parameters for the scales appropriate to most biological systems. Studies on microbial systems offer an exception because microbes have relatively short generation times and large population sizes. Several small-scale lab studies are available to compare abrupt versus gradually increasing schemes and all show in general rather distinctive responses. Our previous work with samples collected from a local lake showed a rather similar abundance but quite distinctive community structure patterns during a 90-day incubation for both carbon dioxide concentration and temperature. This pattern would have been due to the different responses of microbial communities to two treatment schemes. Abruptly increasing carbon dioxide concentration and temperature would exert more selective pressure, while gradually increasing those parameter values would provide more acclimation opportunities to the existing microbial communities. This was observed in an ordination plot (non-metric multidimensional scaling) with a significantly different degree of dispersion; samples from gradual increasing treatment were tightly clustered, while samples from abrupt treatment were scattered around the ordination space. Results available from several studies all indicated that under gradual treatments, less drastic effects on microbial responses were observed for such assessments as community structure, richness, and evolutionary adaptation. These suggest that the general trend of significant consequences of climate change observed from research at FACE-like facilities may be an overestimation. There have not been enough large-scale research data available as of yet, which is needed to provide a more conclusive understanding of how microbial responses to climate change may be different from the existing knowledge.

One very unique and important climate change research facility is found in the Grassland Soil and Water Research Laboratory (Temple, TX) of the US Department of Agriculture's Agricultural Research Service. The LYCOG (LYsimeter Carbon Oxide Gradient) facility accommodates a continuous carbon dioxide concentration from 250 to 500 ppm, simulating the pre-Industrial Revolution to the near future atmospheric carbon dioxide concentrations. Unlike discrete settings (e.g., ambient vs. elevated) provided by FACE-like facilities, findings from LYCOG facility showed less obvious responses to carbon dioxide concentration gradient in microbial diversity and community structure after 10 years of treatment. Again, these are results suggestive of research at FACE-like facilities possibly overestimating the effect of carbon dioxide concentration upon microbial responses to climate changes. These are two examples of how microbial systems can be the desired model system to study the impacts of climate change in more realistic experimental settings which may provide unexpected results thus augment our understanding.

11.6 Conclusion

Microbes are ubiquitous on Earth as they are found in every single ecosystem and have tremendous importance in all those ecosystems' health through the high abundance and versatile nature of the ecosystem functions that they provide. These characteristics of microbes put them in a unique position in the issue of climate change as contributors, victims, mitigators, and a model system for research. Multidisciplinary and multiphasic approaches and studies of every possible aspect of microbial activities associated with climate change are needed to properly address this climate crisis.

Sanghoon Kang

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Chapter 12
The Revolutionary Potential of the Hidden Half of Nature in Agriculture and Medicine

David R. Montgomery

Abstract Recent advances in microbial community ecology have reframed our view of the microbial world. In particular, advances in understanding the importance of horizontal gene transfer and symbiotic relationships with host organisms carry practical relevance for agriculture and medicine. In both areas, growing recognition of the potential to cultivate beneficial microbial communities presents opportunities to revolutionize conventional practices. Regenerative farming systems based on soil-health building principles can greatly reduce major impacts of agriculture's environmental footprint. And dietary practices that promote nutrient-dense foods and cultivate beneficial gut microbiomes can help address the distinctly modern chronic diseases that increasingly afflict humanity. Acknowledging the duality of the microbial world and adopting practices to promote development of beneficial communities and limit opportunities for pathogens could transform these two areas that remain central to human health.

12.1 Introduction

Recent advances in microbial community ecology have reframed our view of the microbial world in ways that carry practical relevance for agriculture and medicine. In both areas, growing recognition of the potential to cultivate beneficial microbial communities presents opportunities to revolutionize conventional practices. Acknowledging the duality of the microbial world and adopting practices to promote development of beneficial communities and limit opportunities for pathogens could transform these two areas that remain central to human health.

The way we see the world shapes how we act in it. And how we see microbes has changed both figuratively and literally over recent decades. It turns out that our century-long drive to annihilate microbial adversaries caused far greater collateral damage than Louis Pasteur and Robert Koch could have imagined when they first

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_12

vied to pin infectious diseases on particular microbes. In the century and a half since their day, growing understanding of the microbial world fundamentally altered how we see ourselves—simultaneously changing our views on the world of nature and the nature of our world (Montgomery and Biklé 2016).

The hidden half of nature that makes up its microbial domain lay unknown until a seventeenth-century Dutch draper indulged his passionate curiosity for making rudimentary microscopes. More than a century later, Louis Pasteur's nineteenth-century discovery that microbes fermented beer and wine opened the door to thinking that such small and inconsequential organisms could produce important, tangible results. And the subsequent rise of germ theory cemented in modern minds the popular perception of microbes as invisible foes, a view most recently reinforced by the disruptive global impact of COVID-19.

But our view of microbes broadened substantially in the decades before the recent pandemic as new technologies shed light on what particular ones did and how communities of them interacted. A revelatory advance came through scientific recognition and grounding of beneficial roles that microorganisms serve in relation to higher life forms—on the importance of microbiomes in the health of plants and people, for the well-being of our crops and ourselves. We've been learning how inherently cryptic microbial ecosystems are just as complex as those making up the natural world we can detect with our senses.

Too small to see and inhabiting realms often shielded from view, the microbial world turns out to run differently from how we imagined. We're only starting to discern and translate microbial dramas that have long played out beneath our feet and in the dankest depths of our bodies. Yet what we're learning has the potential to reshape key practices in agriculture and medicine (Montgomery and Biklé 2016). For it has become clear that maintaining the fertility and productivity of the soil, as well as the health of our own bodies, depends on the care and feeding of microbial allies, something that, in turn, depends on what we eat and how we grow it—on how we treat the land.

12.2 Key Advances

Much like the way discovery of plate tectonics transformed the earth sciences in the twentieth century, several recent advances stand out in transforming our understanding of the microbial world. Recognition of the major role horizontal gene transfer plays in microbial genetics revealed that microbes don't play the game of life in the way we're familiar with from our perch atop the macroscopic world. That microbes seem to swap genes the way we do stories means they play by different rules and helps make them incredibly adaptive. Consider too how over the past half century it generally took less than a decade or two for bacteria to start exhibiting resistance to new antibiotics.

We simply can't win an evolutionary arms race against organisms with a life span measured in minutes. So we need to think differently about them, and use microbial

ecology to out-compete and keep pests and pathogens off balance. Instead of breeding resistance into them and just delaying our own defeat, we can cultivate their competitors and stifle their opportunities to gain more than a toehold, in effect keeping bad actors at bay by preferentially seating their more beneficial brethren at the table. All in all, the tremendous adaptability of microbes means that we have to adjust our tactics to forge new strategies that favor those with interests common to ours.

A second major advance that influenced our view of the microbial world lay rooted in recognizing the evolutionary power of symbioses as a key dimension to inter-species competition—that life could be a team sport. For a century, Darwin's long shadow obscured the role of symbiotic relationships as biologists peered at life through the lens of individual competition and predator-prey relationships. But along the way, we discovered that much of the microbial world runs through highly evolved relationships that parallel the familiar dance of pollinators and flowers in a garden. And it turns out that symbiotic partnerships between plants and soil microbes are central to plant health, just as our gut microbiome is a key factor in determining human health. Recognition that microbial partnerships are fundamental to the health and well-being of our crops and ourselves motivates re-evaluating conventional practices in agriculture and medicine.

12.3 Agriculture

Selman Waksman and Robert Starkey were among the early researchers to identify the botanical importance of soil microbes. Their 1931 book *The Soil and the Microbe* recognized the key role soil life plays in decomposing once-living matter and keeping elements circulating from soil to plants and animals and back again (Waksman and Starkey 1931). After the realization that microbes use antibiotics to repel competitors in the soil, it took decades to crack the chemical codes passed between plants and soil life to reveal complex synergistic interactions central to plant health and defense.

In the meantime, growing adoption of now-conventional agricultural practices generally worked against the interests of microbial life that benefits the growth and health of crops. Indeed, new advances in soil microbial ecology go a long way toward explaining observations that animated early advocates for organic agriculture, like Sir Albert Howard (Howard 1940) and Lady Eve Balfour (Balfour 1943) who argued that mycorrhizal fungi played instrumental roles in crop health. But in their day, it remained unclear just how fungi and bacteria could partner with plants, and they could not identify mechanisms to support their views, which were consequently dismissed in mainstream circles.

Decades later, I was trained to think of roots as like straws that plants use to slurp up nutrients in soil water. But it turns out that roots are more of a two-way street, with material leaving as well as entering plants. Root exudates can account for more than a third of the carbon a plant captures through photosynthesis (Bais et al. 2006).

Plants don't do this to waste energy, they do it to recruit microbial partners in the zone around their roots—the rhizosphere. In this life-filled zone, root exudates nourish microbes that assist the botanical world with nutrient acquisition and signaling that aids plant defense. A 2016 review in *Advances in Agronomy* concluded that the plant microbiome was solidly established as crucial for maintaining plant health in both natural and agricultural systems through its influence on access to water and nutrients, as well as resistance to pests and disease (Reeve et al. 2016).

Of particular importance is that most terrestrial plants, including humanity's primary crops, develop symbiotic relationships with mycorrhizal fungi. Acting as root extensions, mycorrhizal fungi can take up immobile elements like zinc and phosphorous from the soil and deliver them to crops (Bolan 1991). The partnerships can be multidimensional and network communities of bacteria, fungi, and plants into mutually beneficial exchanges. For example, certain fungi and bacteria in the soil increase the availability of normally stable elements like iron (Nielands 1995) that mycorrhizal fungi then help plants take up (Antunes et al. 2012). The foundational nature of soil life in building soil health was highlighted by the recent discovery that root exudates are a primary source of soil carbon (Liang et al. 2017).

We've also learned that now-conventional practices undermine mutually beneficial relationships in nature's subterranean bazaar. Farming practices that reduce fungal colonization include frequent use of phosphorus and nitrogen fertilizers and frequent disturbance by tillage (Jansa et al. 2006). Field-scale and experimental studies show that increased use of inorganic fertilizers reduces the abundance and diversity of mycorrhizal fungi and selects for less mutualistic species (Johnson 1993; Egerton-Warburton and Allen 2000; Corkidi et al. 2002). Tillage disrupts soil food webs and fungal networks (Wardle 1995), reducing species richness in the soil and the overall diversity of soil fungi and bacteria (Anderson et al. 2017). Tillage also disrupts fungi that help stabilize and maintain the soil aggregates structuring the porosity that allows water to drain water down into the soil to where crops can take it up (Ritz and Young 2004; Jansa et al. 2006), instead of the water running off and stealing away fertile topsoil.

Conversely, farming practices that cultivate mycorrhizal partnerships can increase crop yields, enhance mineral micronutrient acquisition, and boost resistance to pests and pathogens. For example, a 2018 comparison of conventionally tilled and no-till fields showed that over a 12-year period no-till farming increased crop yields, microbial biomass, soil organic matter, and plant-available zinc in the soil while reducing runoff, thereby allowing more water to infiltrate into the soil (Nunes et al. 2018). Introducing a cover crop further increased each of these positive effects, as well as the amount of plant-available iron in the soil. A broader 2016 review of 54 field studies found that less intensive tillage combined with cover cropping greatly increased mycorrhizal colonization of crop roots (Bowles et al. 2016). Overall, tillage, fertilization, and crop diversity all influence fungal abundance and diversity (Verbruggen and Kiers 2010).

Adopting regenerative practices that cultivate beneficial soil life frames a new paradigm for agriculture rooted in building soil health—in using microbial ecology to support soil life. Practices based on the three central principles of conservation

agriculture—minimal disturbance (low or no-till), keeping living plants growing at all times (cover crops), and growing a diversity of crops—work synergistically to cultivate beneficial life (Montgomery 2017). In addition, microbial inoculants have been shown to be able to enhance the growth, health, and nutrient density of various crops (e.g., Lambert et al. 1979), although it can take finding the right microbes to partner with particular crops because different plants form symbiotic relationships with different fungi (Smith et al. 2011). A 2014 meta-analysis of the effects of mycorrhizal fungi on zinc uptake by crops that surveyed 104 articles reporting on 263 field trials (Lehmann et al. 2014) illustrates contemporary interest in potential agricultural applications.

Recognition of the role of soil organic matter in sustaining fertility also has been growing (Tiessen et al. 1994). Reviews of the effects of no-till farming on soil organic matter consistently report increases in the amount of organic matter in topsoil but mixed results for full soil profiles (Powlson et al. 2014; Haddaway et al. 2017). However, most such comparisons treat no-till as a stand-alone practice, while the efficacy of no-till for increasing soil organic matter appears to depend on integration with other practices, particularly in combination with both cover cropping and more complex crop rotations.

One long-term study in southern Brazil documented that 25 years of conventional tillage decreased soil organic matter to less than a fifth of the amount in native soils (Oliveira Ferreira et al. 2016). But soil organic matter levels recovered almost fully over two decades after switching to high-intensity, no-till farming using cover crops and a diverse rotation (Oliveira Ferreira et al. 2016). Similarly, regenerative farms in Ohio, South Dakota, Saskatchewan, and Ghana have combined practices based on minimal disturbance, cover crops, and diverse rotations to restore soil organic matter to levels comparable to native soil in those regions (Montgomery 2017).

But the individual practices don't work on their own nearly as well. For example, a recent UC Davis study showed how adding cover crops to regularly tilled fields does not necessarily increase soil organic matter (Tautges et al. 2019). The 19-year study compared the effects of nitrogen fertilizers, winter cover crops, and composted poultry manure in tilled corn-tomato and wheat-fallow crop rotations. Soil organic matter in conventionally managed fields did not increase and the addition of cover crops increased carbon in the topsoil, but produced offsetting losses deeper in the soil profile. Yet soil carbon levels increased overall by about two-thirds of a percent a year in the field that received both cover crops and composted manure. The authors attributed this striking difference to compost feeding life in the soil.

Working together the full system of conservation agriculture practices works to promote the care and feeding of beneficial soil life—and the economic viability of regenerative farms that rebuild soil health and thereby slash farmer expenses for diesel, fertilizer, and pesticides (Montgomery 2017; LaCanne and Lundgren 2018). Agricultural systems that employ all three principles are growing in adoption on farmland around the world. Global acreage under conservation agriculture rose from less than 3 million hectares in the early 1970s to about 180 million hectares in 2016, about 12% of global cropland (Kassam et al. 2019). This new system of farming holds the potential to regenerate soil health as a consequence of intensive farming

and thereby reverse the historical pattern of agricultural soil degradation that undermined past civilizations (Montgomery 2007).

12.4 Medicine

Soil life has also played a key role in modern medical advances. Soil bacteria have been our primary source of antibiotics so far (Abrahams 2002), including recent ones effective against antibiotic-resistant microbes (Pepper et al. 2009). Other drugs have come from the soil too. From 1983 to 1994, more than half of the new drugs for treating cancer were isolated from soil (Oliver and Gregory 2015). Beyond these direct medical connections between soil life and human health, there are striking parallels in the relationships of endemic microbiomes to their hosts in the rhizosphere and the human gut (Montgomery and Biklé 2016).

Much like our view of soil microbes, our understanding of our own microbial tenants has changed dramatically over recent decades. Expanding from the foundation of germ theory to include community ecology and symbiotic relationships, we came to understand our bodies as ecosystems in which the communities making up our microbiome play a far broader role in our health than the infectious pathogens we've mostly focused on controlling. While effective vaccines now protect against infamous scourges that have long plagued humanity—like polio, smallpox, and yellow fever—the past several decades have seen an explosion of revelations about the importance of the human microbiome in the constellation of modern chronic maladies that increasingly afflict public health. In particular, understanding how our diet and the metabolites our microbes make from it promote or quell inflammation brought new insights into preventing and treating the modern epidemic of chronic diseases (Montgomery and Biklé 2016). And while the human microbiome responds rapidly to differences in what we ingest, the health effects of diet are such that over half of all cancers have been considered attributable to diet (Rao and Agarwal 1999).

Many chronic diseases—like heart disease, type 2 diabetes, and breast, prostate, and colon cancer—are generally considered rooted in or influenced by chronic inflammation. Certain foods promote or quell inflammation through the nature of the metabolites that gut dwelling microbes transform them into. And certain phytochemicals can boost anti-inflammatory defenses and render malignant cells more vulnerable to immune system attack (D'Incalci et al. 2005). For example, medical researchers commonly attribute the cancer-suppressing effect of greater fruit and vegetable consumption to antioxidant and anti-inflammatory phytochemicals (Murthy et al. 2009). And the amount of dietary antioxidants and other phytochemicals that crops contain is connected to farming practices. For example, a 2005 review in the *European Journal of Nutrition* found that agronomic practices could double the content of individual phytochemicals in radishes and increase them in broccoli and cauliflower tenfold (Schreiner 2005). Connections run from soil health to human health through the ways we treat our soil and gut microbiomes.

In essence, there are two basic, complementary, approaches to cultivating beneficial life in the human gut: probiotics and prebiotics. Probiotics deliver particular live microbes through pills or foods that contain them (like yoghurt or fermented foods). Prebiotics are foods that feed particular varieties of microbes, like whole grains and vegetables, fiber-rich foods that nourish colonic fiber fermenters. Think of probiotics as introducing desirable microbes and prebiotics as feeding them to maintain their presence and abundance. Seen in this light, it makes little sense to consume probiotics if one does not follow up with a prebiotic diet to sustain those that do manage to land and take root in the colon.

While there is tremendous potential to explore using both approaches to building robust, self-sustaining microbial communities that support human health, there is no room to debate the utility of modern pharmaceutical drugs and antibiotics for treating acute illnesses—they work remarkably well. Yet there remains great potential to enhance human health through dietary shifts that support and supply a healthy gut microbiome with nutrient-dense, phytochemical laden fodder. Our new view of the microbial world is opening up new perspectives on what constitutes effective preventive medicine.

12.5 A New View of Nature

The common roles that endemic microbiomes play in the parallel worlds of the rhizosphere and gut suggest that they can be considered inside-out variations on one another (Montgomery and Biklé 2016). In the rhizosphere, plants outsource to their root microbiome key functions of digestion, nutrient acquisition, and chemical signaling, whereas in the human gut, our bodies rely on our internal inhabitants for similar functions. In agriculture and medicine, the still-emerging view of nature's hidden half as a rich universe of interacting microbial allies and foes shakes the foundation of conventional practices. To varying degrees, products and practices we've developed and relied upon for the past century can be replaced by or supplemented with new ones aimed at promoting beneficial life to benefit both soil and human health.

The striking reality remains that the health of both plants and people depend on the community ecology of microbial partners. Thinking ecologically about their care and feeding as central to our health reframes relationships we're still learning to navigate. Seeing the root zone and our gut are ecosystems influenced by diet, disturbance, and diversity invites insights and opportunities in agriculture and medicine based on the care and feeding of microbiomes. In particular, conservation agriculture and regenerative grazing practices can bring life back to degraded soils and enhance soil health (Montgomery 2017), and dietary choices better tailored to meet the needs of our colonic stowaways can serve up preventive medicine (Montgomery and Biklé 2016).

In short, recognition of the role of microbial ecology in the health of soils, plants, and people opens the door for revolutionary thinking across two broad areas of

human experience and interaction with nature. Regenerative farming systems based on soil-health building principles can greatly reduce major impacts of agriculture's environmental footprint. And dietary practices that promote nutrient-dense foods and cultivate beneficial gut microbiomes can help address the distinctly modern epidemic of chronic diseases that increasingly afflicts humanity. While it remains to be seen as to how our new view of the microbial world will reshape agriculture and medicine, the coming decades will see growing adoption of practices that complement and in some cases supplant conventional practices of today.

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Chapter 13
Microscale Carbon Cycling Between Bacteria and Algae Under the Sun

Xavier Mayali

Abstract Photosynthetic planktonic algae in the aquatic biosphere exert a profound influence on the carbon cycle and will in part determine our planet's response to climate change. The microbial world surrounding these organisms, also known as their microbiome, have direct cell-to-cell interactions with them and in turn affect small-scale cycling of elements one tiny volume of water at a time. The difficulty of studying the interaction of microorganisms at the single cell scale, as well as the lack of an approach to transfer this information to better understand large-scale biogeochemical processes, has hampered our ability to accurately predict the response of aquatic ecosystems to external factors such as pollution and climate change. In addition, these processes are not well-linked to those in other ecosystems (soil, groundwater, the atmosphere), which would be needed to help predict the global microbial biogeochemical response to our ever-changing climate. A coordinated scientific effort across agencies and beyond borders is now required to tackle this problem, as this is no longer an issue solely for curious academic researchers. Instead, as a national and international security issue, government agencies must increase their involvement in aquatic carbon cycling research to ultimately solve this issue of global importance. Our future depends on it.

13.1 History of Research on Aquatic Algae and Bacteria

Seventy percent of our earth is covered by water, most of it salty, such as oceans and seas. This has been the case for billions of years, and it is believed that life evolved in shallow seas. Most of early life was anaerobic: there was no oxygen, neither in the oceans nor in the atmosphere (Fenchel and Finlay 1994). This changed with the advent of oxygenic photosynthesis which first arose in cyanobacteria (formerly known as blue-green algae). These were (and still are) bacteria, and along with eukaryotic algae that evolved later, still dominate photosynthesis on today's earth

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_13

(along with trees and other plants on land). Photosynthesis, and for this essay I use that term in reference to oxygenic photosynthesis, is the proces by which organisms fix their own organic carbon from carbon dioxide using water and produce oxygen as a by-product. It is important to note that the appearance of oxygen-producing organisms on its own was not enough to oxygenate the atmosphere. In order for the atmosphere (and the ocean, in fact) to become oxygenated, there had to be a net burial of photosynthetically produced organic carbon. If there was no net burial, that organic carbon would all be remineralized to CO_2 by bacterial respiration, and it would be a zero-sum game: all the O_2 produced by photosynthesis would be respired back to CO_2, and the seas and atmosphere would remain devoid of O_2. Today, this process is still occurring and is called the biological carbon pump by oceanographers: microscopic algae in the surface waters fix CO_2 into organic carbon via photosynthesis, and before all this organic carbon is respired away by bacterial respiration, some of it gets buried in the deep ocean and we get a net production of O_2, both in the upper ocean, and in turn in the atmosphere. Unfortunately, we as humans have been reversing this process over the last couple of hundred years by drilling into the ocean floor, collecting old buried algae (we call this oil), and burning it to produce energy and sending CO_2 into the atmosphere. A majority of this CO_2 goes back into the ocean, but the biological carbon pump is not fast enough to catch up. This has led to, among other things, increased temperatures and ocean acidification.

Before the 1970s, bacteria in the oceans were believed to be insignificant to global biogeochemical cycles. It was thought that algae and cyanobacteria (collectively referred to as phytoplankton) fixed CO_2 and then this material was eaten by organisms in higher trophic levels or sank down to the bottom, but bacteria played little role in any of these processes. At that time, bacteria were believed to be simply decomposers of dead material, and their only interactions with phytoplankton was to provide remineralized nutrients for photosynthesis. This view radically changed, starting with the work of Lawrence Pomeroy in the 1970s who first measured that the highest metabolic activity in a volume of seawater seemed to be in the smallest-sized organisms (under 2 micrometers in diameter; Pomeroy 1974). Subsequent work discovered that bacteria in lakes and oceans were three orders of magnitude (1000X) more abundant than previously thought (Hobbie et al. 1977). In the early 1980s, the term “microbial loop” was coined in a seminal paper (Azam et al. 1983) that provided a framework to explain the role of bacteria in the biogeochemistry of the oceans, lakes, and seas: bacteria are constantly metabolizing organic carbon produced by phytoplankton and this feeds an entire, previously unknown ecosystem. Subsequent work in the 1990s discovered that aquatic viruses are major contributors to the biogeochemistry of the microbial loop (Fuhrman 1999).

Fast forward to today: aquatic microbiology is a dominant discipline, but microbial processes still lag behind in large-scale modeling efforts. Often, microbes are not even explicitly represented in such models, but in terms of biology, they are central to studies of aquatic carbon cycling. In particular, due to the fact that most aquatic microbes cannot be cultivated on their own, they are generally studied with genomic techniques, which enable the identification of their function through the

analysis of their gene sequences at the DNA, RNA, and protein levels. These types of studies have again revolutionized the study of aquatic microbiology, discovering new metabolisms in aquatic ecosystems and beyond. However, there remains a disconnect between the study of microbial metabolism in natural ecosystems, generally studied with molecular techniques, and empirical measurements of microbial activity. Specifically, attributing a specific biogeochemical flux to one or a set of microbes remains a challenge.

13.2 Algal-Bacteria Interactions and New Methodologies to Study Them

As discussed above, the primary interaction between aquatic algae and bacteria is the balance between photosynthesis and respiration, which controls whether a system is net autotrophic (O_2 producing) or heterotrophic (O_2 consuming). However, this is clearly an oversimplification, and we do not yet fully understand how the aquatic ecosystem works. For example, new metabolisms are constantly being discovered, including new ways for microbes to fix carbon (Figueroa et al. 2018). In the surface waters where light is present, it is also now becoming clear that heterotrophs are also able to use light energy (Gómez-Consarnau et al. 2019), and we do not fully understand how this affects the carbon cycle, including interactions with microalgae. Another underappreciated interaction is the impact of heterotrophic bacteria on the growth of microalgae through the production of growth-enhancing substances such as vitamins, hormones, and other yet-undiscovered compounds. It is relatively straightforward to test the impact of a bacterium on algal growth under controlled laboratory conditions, but determining how prevalent an interaction is in the environment, with unpredictable and ever-changing chemical, physical, and biological complexity, is much more difficult. Particularly daunting is the idea that microbial interactions generally occur at the cell-to-cell level. These interactions are often mediated, or at least strongly affected, by two mechanisms that exist at the very small scale: attachment between individual cells or a swimming behavior called chemotaxis (Smriga et al. 2016). It is these microscale interactions between individual cells within a tiny volume of water that drive the biogeochemistry of the world's aquatic ecosystems. To better study these interactions, we need to better integrate measurements in the context of the small scale and make them more practical, and we likely need to develop new tools with which we can better measure activities at the microscale.

One particularly useful advance of the past few decades which has greatly helped with the ability to link microbial identity with biogeochemical activity is collectively known as stable isotope probing (Radajewski et al. 2000). This approach requires the incubation of an aquatic sample with substrates highly labeled with normally rare heavy isotopes (most often ^{13}C, ^{15}N). The organisms (or specific biochemical components of the organisms, such as DNA, RNA, fatty acids, or proteins) that

incorporate those substrates can be detected, and even quantified, with various tools, providing a quantitative measurement. Combining this incubation approach with mass spectrometry can provide further detail, particularly if the instrument has single cell resolution, such as the NanoSIMS (Nanoscale secondary ion mass spectrometer). NanoSIMS stable isotope probing, also known as NanoSIP (Pett-Ridge and Weber 2012), enables the quantification of biogeochemical activity at the single cell level, which at least partially addresses two scientific needs: (1) linking activity and identity for microbial biogeochemistry and (2) studying microbes at the scale at which they behave (the single-cell scale). For more information, I refer the reader to a recent review on this topic (Mayali 2020). Although NanoSIP, unfortunately, cannot measure biochemical activities not linked to growth (such as catabolic processes, e.g., respiration), it still provides useful data on microbial biogeochemical activities coupled to substrate incorporation into biomass. Several other drawbacks include the fact that the instrument used for this type of spectrometry is very expensive, requires highly skilled technicians to operate it, and analytically is slow (i.e., not high throughput). Still, in my opinion, it remains a state-of-the-art method to measure microbial biogeochemical activity in situ at the single cell level and will continue to yield exciting discoveries over the next decades.

13.3 Algal-Bacteria Interactions in the Face of Climate Change

Most of the increased CO_2 from the atmosphere is absorbed by the oceans, which among other impacts causes ocean acidification (Doney et al. 2009). Phytoplankton photosynthesis supports aquatic carbon sequestration; thus these organisms directly impact and are impacted by increasing CO_2 concentrations. Climate change also has led to increasing ocean temperatures. A number of studies have investigated the impacts of temperature and CO_2 on carbon cycling in aquatic environments, but these studies are still too few and far-between and generally suffer from the caveats described above (lack of activity measurements, inability to link small and large scales). In particular, there is a lack of mechanistic studies that investigate how climate impacts small-scale biogeochemical interactions. For example, how is climate change going to impact the exchange of metabolites between microbial cells in the ocean? Do phytoplankton make more or less utilizable organic material when stressed by climate impacts? More research is needed in this area, even beyond studies related to climate change. More importantly, new approaches need to be developed that can bridge the small and large scales and that offer mechanistic and predictive abilities. Most likely, these methods will be computational in nature, since modeling can in some cases successfully investigate phenomena that are not possible to reproduce experimentally (Stubbendieck et al. 2016; Gould et al. 2018; Diener et al. 2020).

Over the last several decades, the fields of algal-bacterial interactions and aquatic microbial biology in general have been carried out primarily by academics for the sake of gaining fundamental scientific knowledge about our natural world. As such, this research has been sponsored by funding agencies that target basic science. In the United States, this is primarily the National Science Foundation, funded by the US government, as well as several philanthropic nonprofit organizations that have noticed this type of research was underfunded, with examples being the Gordon and Betty Moore Foundation and the Simons Foundation. Since microbes play such a central role in the carbon cycle which is undeniably affected by anthropogenically caused climate change, I would argue that we have left behind aquatic microbiology research as a purely academic exercise reserved for basic research. I suggest that climate change and our earth's response to it should now be considered in the realm of applied research and that government agencies should focus upon this area of research. Historically, national laboratories have taken the lead in addressing issues of national interests, such as the development of nuclear weapons, sequencing of the human genome, and atmospheric modeling for weather prediction. It would seem logical that the ocean's response to climate change would be something that is crucial to the US national security interests and that government laboratories should become involved with this work. Several decades ago, in fact, the US Department of Energy's (DOE) Office of Science was a major funder to oceanographic research. For example, the Food Chain Research Group at Scripps Institution of Oceanography in San Diego, California, where the "microbial loop" mentioned above was discovered (Azam et al. 1983), was DOE funded. Today, the DOE does not fund marine biogeochemical research unless it is directly relevant to applied energy production, such as algal biofuels. In addition, the National Oceanic and Atmospheric Administration (NOAA), whose mission is " to understand and predict changes in climate, weather, oceans, and coasts. . .," also does not fund microbial oceanography research, except when related to harmful algal blooms. As climate change continues to impact our planet over the next decades, the hope is that these US government agencies will find it in our national and international interest to work together and fund ocean microbial biogeochemistry research. Perhaps the issue is that the oceans do not belong to any nation (past the 12 nautical mile near the coast), and so no nation wants to take ownership of the problem. Global research issues need to be addressed by multinational efforts. The issue of ocean plastics exemplifies this unfortunate outlook leading to such "tragedy of the commons," in this case the Great Pacific Garbage patch that no single country can be blamed for. At the very least, we should be funding aquatic biogeochemical research at the same rate as terrestrial research, since they are each responsible for half of the O_2 production globally.

Acknowledgments Funding provided by the Department of Energy's Genomic Sciences Program Bioenergy Science Focus Area grant # SCW1039 under the auspices of the U.S. Department of Energy at Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

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Part V
The Basic Aspects of Microbial Symbioses

Chapter 14
Discovering the Symbiotic Nature of Microbial Life: Summarizing Milestone Publications from 1866 Through 1947

Christon J. Hurst

Abstract This essay summarizes twenty publications which I believe represented milestones along the path to understanding microbial symbiosis. Most of these are articles which I selected because they showed remarkable insight which was ahead of its time. Unfortunately, some people who believed they expressed insights came close but missed the mark. I follow the progress beginning with Mikhail Stepanovich Voronin's conclusion in 1866 that the nodular root growths on black alder (*Alnus glutinosa*), and the bulbous root outgrowths of lupine (*Lupinus*), are in some ways identical phenomena and in both cases the appearance is caused by a foreign organism. I include Simon Schwendener's then controversial conclusion published in 1868 that lichens are a composite which consists of a fungus growing together with an algae. Albert Bernhard Frank seems to merit credit with initially proposing the term 'symbiotism' in 1877. Harry Marshall Ward then presented in 1899 a landmark review "Symbiosis" in which he brought together understanding of the fungal symbiosis represented by mycorrhiza, plus the bacterial nitrogen fixation associated with leguminous nodules, and photosynthetic symbioses including lichens, *Azolla*, cycads, and protozoa. Included is Paul Ernst Christof Buchner's publication in 1921 of his landmark book on intracellular symbioses in animals. I then finish the essay with a summary of Howard H. M. Bowman's 1947 article on antibiosis, and his assumption that disease producing microorganisms were once non-pathogenic, but subsequently became adapted to a parasitic life in specific hosts.

14.1 Introduction

The identification and understanding of microbial symbioses began with determined efforts made during the last half of the nineteenth century. That important work was done by people who bravely were willing to face ridicule from other observers including harsh comments from their often disbelieving fellow scientists.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_14

Fortunately, the goal of understanding symbiotic associations was supported by perseverance in the direction of advancing scientific knowledge.

I have found two difficulties in preparing a review of old microbiology literature. The first is that, by my experience, very often old publications are cited as containing information which indeed was not present in those publications. The second is that a majority of these microbiology articles were published in German, which I barely can read. For those reasons, I began my review efforts for this essay by creating digital versions of the German language publications using optical character reading programs, then I used an internet translation program to create English language versions of those articles, and I worked from those translated texts.

Hopefully you will enjoy this summary of those old publications and find the process to have been insightful. I have included in chronological order publications by: Mikhail Stepanovich Voronin, Simon Schwendener, Albert Bernhard Frank, Heinrich Anton de Bary, Franz Friedrich Schindler, Jørgen Brunchorst, Karl Andreas Heinrich Brandt, Hermann Hellriegel and Hermann Wilfarth, Martinus Willem Beijerinck, Harry Marshall Ward, George T. Moore, Charles Bernard Lipman and Lawrence W. Fowler, Paul Ernst Christof Buchner, Edmund Newton Harvey, Lemuel Roscoe Cleveland, George E. Helz with Ira Lawrence Baldwin and Edwin Broun Fred, Frank Sherwood Taylor, and Howard H. M. Bowman.

14.2 "Über die Bei der Schwarzerle (Alnus Glutinosä) und der Gewöhnlichen Garten-Lupine (Lupinus Mutabilis) Auftretenden Wurzelanschwellungen" by Mikhail Voronin, 1866

Mikhail Stepanovich Voronin (Woronin 1866) concluded that the nodular root growths on black alder (*Alnus glutinosa*), and the bulbous root outgrowths of lupine (*Lupinus*), are in some ways identical phenomena and in both cases the appearance is caused by a foreign organism.

He thought that the nodular growths on black alder roots, which we now know are associated with endosymbiont actinobacteria of the genus *Frankia*, were reproductive organs of an endophytic fungal parasite which penetrates into the tree roots. He named that fungal parasite *Schinzia alni*.

Voronin mentioned the root nodules of lupine plants (genus *Lupinus*) to contain small rod shaped organisms that voluntarily were very motile in water, that those organisms would come to rest, would break apart, and then form new rod shaped organisms as their sprouts. He had no success in culturing those organisms. He said that the organisms in lupine nodules looked like either bacteria, vibrio or zooglea. His conclusion was that the organisms in lupine root nodules were a type of vibrio. We now recognize the microbes associated with lupines as being endosymbiotic alphaproteobacteria of several genera including *Rhizobium*.

14.3 "Untersuchungen über den Flechtenthallus II. Laub- und Gallertflechten" by Simon Schwendener, 1868

Simon Schwendener (1868) included as the addendum of this publication a conclusion that, based upon his detailed observations, at least some lichens are composed of algae and fungi. By my translation, his statement was "The decision as to whether and to what extent the assumption of a thallus formation due to the growth of parasitic fungi with algae is justified remains largely open to investigation. However, since the possibility of such a process and in some cases even the likelihood of it can no longer be disputed, the question arises whether or not all lichens may arise in the same way: whether the gonidia should be continuously [considered as a] typical algae and the colorless hyphae are to be regarded as mushroom hyphae [fungal hyphae], which [for the purpose of] the construction refer [to a required food for the Thallus]. As things stand at present, there are many things to be said for and against such a view, and the judgment of the individual will vary depending on the weight he attaches to the facts in question." He included among his named algae *Cystococcus humicola* and *Cystococcus Nägeli* both of which we now consider to be *Chlorococcum humicola*, along with three genera which we now consider to be cyanobacteria (phylum Cyanobacteria) and those were *Chroococcus*, *Gloeocapsa*, and *Nostoc*.

14.4 "Über die biologischen Verkältnisse des Thallus einiger Krustflechten" by Bernhard Frank, 1877

Albert Bernhard Frank is credited with initially proposing in this 1877 publication the term 'symbiotism' (Frank 1877). Frank had been studying lichens, which consist of a fungal thallus that we now understand typically to be colonized by either a cyanobacteria or an alga, and he said that together these two biological elements lead a double life. I included an image of one particular lichen, *Letharia vulpina* also known as the wolf lichen, as part of the photomontage image "Volvox reimagined" which I created and appears on the book cover for this series. *Letharia vulpina* is described as having a fruticose growth form, growing like a multiple-branched tuft or leafless mini-shrub.

Frank noted that there seemed to be a useful connection between what we now know as the lichen mycobiont, which typically is an ascomycete (member of the phylum Ascomycota) although some lichens do instead have a basidiomycete (member of the phylum Basidiomycota) as their mycobiont, and the lichens Gonidia. That part of a lichen not involved in reproduction is termed the thallus and composed of fungal hyphae. Gonidia is a term used to describe parts of the lichen which then still were considered to be 'brood cells' or reproductive organs of the lichen. The sexual reproduction structures of lichens were termed apothecia and contained the gonidia. Frank thought that it was wrong to consider the Gonidia as being analogous

to their related algae. We now know that the gonidia indeed are the algal or cyanobacterial member of the lichen partnership. In the lichen partnership, the photosynthesizing organism is phototrophic and termed to be a photobiont, with algal photobionts being termed phycobionts, and cyanobacterial photobionts are called cyanobionts. The phototrophic member of the lichen association supplies metabolic energy through photosynthesis, the fungus does in turn offer a supportive matrix and also may protect the photobiont from radiation and dehydration.

Frank wrote about symbiosis in this publication, using the German equivalent word 'Symbiotismus', as being a cohabitation between species and suggested that in his opinion there were stepwise levels of symbiotic association from the loosest to the deepest possible necessary connection. The lowest stage would in his opinion be called Pseudoparasitism 'Pseudoparasitismus' and represented by epiphytes. The next higher level would be parasitism 'Parasitismus' in which one member only takes and does not give, and even destroys. Frank believed that the term symbiosis 'Symbiotismus' would represent the highest level of association in which both beings connectedly exist in a way that provides one-of-a-kind services to each other, such that the two individuals seem to lose the concept of being separate organisms and that relationship would be similar to a commensalism 'Commensalismus'. Frank suggested that the term 'Homobium' could be used to describe the combination of those two symbiotic organisms.

14.5 "Ueber Symbiose" by Anton de Bary, 1878

Heinrich Anton de Bary was a surgeon, botanist, microbiologist, and mycologist. He is considered both a founding father of plant pathology and the founder of modern mycology. We most remember Anton de Bary for a lecture "Ueber Symbiose" [About Symbiosis] which he presented in 1878 to a meeting of the German Association of Naturalists and Physicians, at a convention which that group began on September 11th in Cassel, Germany. The name of the city eventually was changed to Kassel. The text of his lecture was published both as a journal article (de Bary 1878) and then later as a book (de Bary 1879). I have relied upon the text of the lecture as published in 1878 for the purpose of preparing this essay.

de Bary mentioned during his lecture what he considered to be a few examples of parasitism, mutualism and commensalism.

14.5.1 Parasitism

de Bary defined one phenomenon of symbiosis as being complete parasitism, in which an animal or a plant undergoes its entire life process while residing either on or within another organism, during which the living body of the host serves as nutrient material for the parasite. de Bary stated that the opposite end of the same

spectrum was represented by parasites which can spend segments of their lives independently. He mentioned that part time obligation as applying to some of the entomopathogenic fungi which cause the disease muscardine. The fungal genera which cause muscardine are *Aspergillus*, *Beauveria*, *Cordyceps*, *Hirsutella*, *Metarhizium*, *Penicillium*, and *Sorosporella*. de Bary indicated that parasitic relationships were antagonistic and represented a struggle between host and parasite, with a diversity of possible outcomes depending upon the individual case. One of the examples that he included was the disease trichinosis, which is caused by the metazoan genus *Trichinella*, whose symptomatology for its host can vary from very limited to lethality.

14.5.2 Cooperative Relationships, Mutualism and Commensalism

de Bary defined these as following the appearance of parasitism, but being quite different.

The examples of cooperative relationships which de Bary gave included when, based upon my translation "Many smaller animals are settled on larger ones and live from the waste of the latter: from the desquamated epidermis parts, feathers, hair, and the like." de Bary referred to these situations, again based upon my translation, as "van Beneden's mutualists; they stand in relation to mutual support for the hosts who [they] inhabit; by taking their food from the garbage they" take care of the toilet "of the same." We now consider these relationships to represent mutualism.

de Bary also mentioned, again by my translation, that "Other small animals settle on or close to larger ones in order to live their lives from the crumbs that fall from the large table: from the excess of the food material that the large one brings in for its own use." We have come to consider such an interaction as being classically commensal, with that word derived from medieval Latin and defined as sharing a table together (the prefix com-, meaning "together," and the adjective mensalis, meaning "of a table."), characterized by a symbiotic relationship in which one species is benefited while the other is unaffected.

My interpretation of de Bary's intention becomes difficult at this point because, by my translation, he mentions "These are van Beneden's commensuals, blackheads." I presume de Bary intended that to be a mention of the parasitologist Pierre-Joseph van Beneden, and reference to the disease which we know as histomoniasis that is caused by the pleomorphic protozoan *Histomonas meleagridis*. *Histomonas meleagridis* lives in the cecal lumen and liver parenchyma of birds, where it causes extensive necrosis and one of the symptoms is cyanosis or darkening of the animals head. That darkening of the birds head has resulted in histomoniasis being given the name 'blackhead disease'. *Histomonas meleagridis* naturally resides in eggs of the nematode *Heterakis gallinarum*, which is itself a cecal parasite of birds, and thus birds acquire *Histomonas meleagridis* during inadvertent ingestion of *Heterakis*

gallinarum eggs contained in either feces or soil. *Histomonas meleagridis* also can exist as larvae in earthworms, which presents another feeding related route of acquisition by birds. Pierre-Joseph van Beneden often is credited with having introduced the terms mutualism and commensalism, although I found first listing of these terms in a publication by Bernhard Frank (1877).

de Bary mentioned epiphytic plants such as orchids, and their host plants, when considering mutualistic and commensal relationships. de Bary suggested that the epiphytes could be considered commensuals of the host plants even though the epiphytes are chlorophyll-containing plants and therefore should be able to satisfy their own energetic needs. By my interpretation, his words were: "At the most, one could still call them commensals of the latter; but this designation applies to all non-parasitic plants that live in the same place [i.e., on either a tree or some other physically supportive host plant]".

de Bary then mentioned the symbiotic association between *Azolla* and a cyanobacterium, *Trichormus azollae* (previously named *Anabaena azollae*).

Although the cyanobacterium can exist on it's own, that cyanobacteria typically is found within cavities inside the leaves of *Azolla*. In this classically symbiotic relationship, the cyanobacterium receives carbon compounds from the *Azolla* plant in exchange for supplying fixed nitrogen to the *Azolla*. We humans traditionally have used that particular relationship in agriculture by having the growth of *Azolla* in the water of rice paddies provide a source of fixed nitrogen for the rice plants.

de Bary also mentioned the symbiotic association between *Nostoc*, in reference to a genus of cyanobacteria which we now know by the name *Anabaena cycadae*, and its host plants of the order Cycadales. *Anabaena cycadae* invades the cycad plant structures termed coralloid roots, whereafter the *Anabaena cycadae* then resides and fixes nitrogen within the middle cortex of those coralloid roots.

de Bary additionally mentioned lichens, which are a symbiotic association between a fungus and either an algae or cyanobacterium "For most lichens, however, things are very different [for] ... the algae [which] can exist independently. Not only can they be artificially isolated and they can continue to vigorously vegetate and reproduce on their own, but the algae forms can also be found spontaneously in many cases growing on their own without being part of a lichen body." and "The lichen fungus behaves the other way round. As has already been said, he is incapable of independent training without the algae and quickly dies if he does not find it, because he depends on her carbonic acid [I did not quite understand that, because it is the fungal component rather than the algae which produces the carbonic acid] assimilation to obtain the building materials for his growth. But it [the fungal component of the lichen] does not simply settle on or in the algae, but grows around it, absorbs it into its own body and then, with most lichens, in turn increases its mass so enormously that it is by far the main component of the common [whereas] the algae form a small fraction, or much less. After the two codependents have divided, the mushroom [fungal] owner, host, is the algae guest. However, the landlord is dependent on the guest to live—which also happens otherwise. Accordingly, the guest is treated with the utmost care: not only [should] his growth not be impaired, but, as we will see later, it is often prominently promoted in comparison with the

solitary state, and kept in step with that of the accommodation provider. Finally, the [fungus], in turn, not only ensures the attachment of the body to the substrate by penetrating it, often deeply into hard rock; but it also leads the necessary parts of the axis to the common household."

de Bary termed all three of these associations, with *Azolla*, cycads, and the structure of lichens, as being mutualists and, by my translation "... the guest and the accommodation provider they serve are mutually beneficial".

de Bary mentioned two additional symbiotic relationships. One of these was an association considered to be a classical example of mutualism, which is the birds which sit on large terrestrial mammals and eat Hematophagous arthropods. He considered that relationship between symbionts as being similar to *Utricularia nelumbifolia*, which is a large carnivorous perennial tropical epiphytic bladderwort that grows in water-filled rosettes of some bromeliad species. As per my translation, regarding the insect consuming birds and the *Utricularia* "The indignation must fade when a further look shows that this is not an unlawful act or imposition, but a special case of an appearance that recurs everywhere in the living nature".

14.6 "Ueber das Zusammenleben von Thieren und Algen" by Karl Brandt, 1881

Karl Andreas Heinrich Brandt (1881) published in many places the text of a lecture which he had presented "Ueber das Zusammenleben von Thieren und Algen". An English translation of that lecture title would be ["About the coexistence of animals and algae"]. Brandt's name also is sometimes listed as Andreas Heinrich Karl Brandt. Brandt examined many groups of animals that contained chlorophyll. Specifically, he studied: amoeba, *Coleps*, the protists that once were grouped as heliozoa, *Hydra*, Monothalamids, *Paramecium*, a planarian which I presume to be *Convolutriloba retrogemma* commonly called the red planaria, *Spongilla*, *Stentor*, *Stylonychia*, and *Vorticella*. Brandt determined that the supposed chlorophyll bodies of these animals are morphologically independent, unicellular beings which he named as being zoochlorella and zooxanthellae. Brandt determined that the photosynthetic microbes could be squeezed from these animals, after which the photosynthetic microbes were able to live independently and some of the microbes subsequently could be taken into animals. He also determined that the photosynthetic microbes were providing nutrients to the animal. In his conclusions, Brandt said that instead of the photosynthetic microbes being parasites, from a physiological point of view the animals were the parasites.

Brandt also published a shortened English version of that presentation in The Popular Science Monthly (Brandt 1882). Thomas Krueger has published his own translation of Brandt's 1881 publication and Krueger included additional information about Brandt along with some of Brandt's illustrations (Krueger 2017).

14.7 "Zur Kenntniss der Wurzelknöllchen der Papilionaceen" by Franz Schindler, 1884

Franz Friedrich Schindler (1884) noted that legumes formed root nodules when there was a lack of available nitrogen in the soil, and that the nodules were both more numerous and larger in nitrogen-poor soil as compared to nodulation in soil that contained adequate nitrogen. By my translation, I would quote Schindler as having stated "... it seems so certain that they [the nodules] cannot be regarded as "pathological excesses"; they rather belong to the normal life of the plant, and for this reason alone the organisms observed therein cannot be identified with parasites in the ordinary sense of the word. The closest assumption is that one is dealing with a phenomenon of symbiosis" "Vienna, December 30, 1883."

14.8 "Ueber die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze" by Bernhard Frank, 1885

Albert Bernhard Frank (1885) first described in this publication the role of an ectosymbiosis between plant roots and fungal hyphae, and he is credited with initially presenting in this 1885 publication the term mycorrhiza. Frank had been commissioned by the King of Prussia (Wilhelm I) to develop practical methods for truffle cultivation. Wilhelm I also became the first German Emperor. Although the truffle project was not successful, eventually that project led Frank to this elucidation of the nature and development of mycorrhizae.

Frank described this observed plant and fungal interaction as being a relationship in which the entire root system of a tree was enclosed by fungal mycelium, with there being a core to the structure that represents the actual tree root and a bark that is organically intergrown with the fungal hyphae.

Frank indicated that there was no evidence of fungal growth on the radicle of the seedlings, and that both the taproot and initial side roots likewise were fungus-free. Fungal growth gradually could be seen to appear on those first and subsequent side roots. Fungal hyphae initially attached themselves to the root epidermis at individual points, and the fungus then developed branches that crawled on the root and connecting the fungal coat with the root. His description included knowledge that this fungal coating completely envelops the root as if the fungus were a mantle (also termed a cloak), the fungus also covered the vegetative point of the root and the fungus grows with the root tip behaving in every respect like a peripheral tissue that is organically connected to the root. Frank also indicated that the formation of root hair seemed to have been made impossible by the firmly attached fungal covering. But, to a certain extent, root hairs were replaced by formation of the fungal mantle. The external cells of the fungal mantle extended outward and spread between the surrounding soil particles. Frank believed that the organic connection between the

root and the fungal mycelium formed a morphologically independent organ, with their being an interdependence of the growth of both parts which revealed a close relationship of physiological functions existing between the plant and fungus. By my translation, I would quote Frank as stating "The whole body is therefore neither tree root nor fungus alone, but similar to the lichen thallus, a union of two different beings into a single morphological organ, which can perhaps be aptly referred to as a mushroom root, a 'mycorhiza'".

Frank's opinion was that, in so far as the mycelium are concerned, the fungus must undoubtedly be regarded as a parasite to the living root because of the manner in which the fungus attaches and penetrates into the growing root. He believed that the underlying nutritional needs of the fungus, as it applied to all parasitic fungi, mainly would relate to assimilating carbon-containing nutrients which the tree prepares through its chlorophyll-containing organs. On the other hand, the fungus would take its own mineral nutrients from the soil.

Frank mentioned that this type of relationship previously was only known from the gonidia of lichens and some lower algae (cyanobacteria) enclosed in higher plants. He did note that although there was a difference in character between the symbiosis of lichens versus his discovered fungal-plant root interaction, the biological character of his observed mycorhiza was almost exactly parallel in biological character to the lichens. That is to say, by my translation " ... concerning both the needs and services which this plant-fungal cooperative provides for the nourishment of both parts, the root fungus is the lichens hyphae and the tree is analogous to the lichens gonidia."

14.9 "Ueber die biologische Bedeutung der Wurzelknöllchen bei den Papilionaceen" by Franz Schindler, 1885

Franz Friedrich Schindler (1885) identified that the root nodules of legumes are sites where new formation of protein occurs. He also concluded that the produced protein was formed within the nodules for the purpose of that protein later being consumed in other parts of the plant. He believed that the more leafy a clover plant was in general, the more and larger were the root nodules produced by that plant. Schindler stated that while a potato tuber is a place of storage, the root nodules of legume plants are with respect to proteins both a place of production and storage. We have since come to learn that plant tubers actually do produce starch, and that plant tubers thus are not just simply sites of carbohydrate storage.

Schindler modified the statements within his earlier publication (Schindler 1884) by saying that root nodules were also occasionally observed when legume plants grew in nitrogen-rich solutions.

Schindler indicated a belief that, together with his colleagues, they had proven the root nodules are actually normal organs of leguminous plants. He also believed that

there was a connection between the formation of nodules, nutritional activity of the plant, and development of the plants fruits.

Schindler noted that when the above ground parts of bean plants were covered to the point of completely darkening the foilage, which would cause assimilatory work of the green leaves to cease, then formation of root nodules by those covered plants was remarkably limited as compared against the nodulation activity of similar plant specimens that had remained exposed to sunlight. Although, if the foliage of the darkened plants was again uncovered, then eventually those plants which had experienced darkness would catch up with their unshaded plants in terms of number and size of root nodules. Schindler believed that his observations from the shading experiments indicated growth of the nodules to have been tied to an influx of building material from the sunlight-assimilating leaves.

Schindler believed that the emergence of root nodules was not a pathological appearance, and that it was important to share this conclusion with scientists who believed that the nodules are caused by a parasitic infection with the nodule-bearing plants then presumed ill because of the nodules.

Schindler mentioned that a great difficulty in assessing their own results was the need to understand their observed symbiosis to be of a different nature than Frank's "Mycorhiza". We since have come to learn that there are endomycorrhiza which serve a similar purpose as do the symbiotic bacteria associated with the root nodules of legumes.

Schindler stated, as per my translation, that "I have not yet spoken about one point, namely about the bacterial-like organisms which, since Woronin's [Voronin] discovery, have become so regular [regularly noted] and general [generally found] in the nodules that they must be regarded as an integral component of the latter." By reading further in this essay, you will learn that Jørgen Brunchorst had a very opposite and empahtically negative opinion regarding the idea of bacterial organisms being beneficially associated with legume root nodules.

14.10 "Ueber die Knöllchen an den Leguminosenwurzeln" by Jørgen Brunchorst, 1885

Jørgen Brunchorst (1885) presented his observations regarding the root nodules of leguminous plants. He began the text of that article by giving a summary of previous studies regarding root nodules, mentioning that while many researchers had considered the contents of root nodules to represent a type of bile, some other people had believed the nodules to represent a fungal association. Brunchorst thought that the nodules might be organs for storing and organizing proteins.

By his assessment, Brunchorst noted that the root nodules contained structures which he said looked as though 'bacterial like bodies', and we credit Brunchorst with giving us the name 'bacteroid' as a descriptive term for those structures. Brunchorst believed, however, that the contents of the bacteroids was not bacteria. He had

noticed that the young nodules initially began looking as though they contained rod-shaped bacteria. And, although early in their development it was the bacteroid structures which contained those things which looked like small bacteria, eventually the bacteroids developed into something which contained no resemblance to bacteria.

Brunchorst believed that rather than actual bacteria being involved in the bacteroids, the bacteroids were natural formations and normal organs that play a role in the plant's substance balance. By my translation, I would quote Brunchorst as stating "If one assumes such activity of the bacteroids, it cannot be astonishing that they approach the real organisms [bacteria], in appearance and chemical behavior in such a way that they have so far been confused with them, while their development and their dissolution [of the bacteroids] at the end of the vegetation [plants life cycle], as set out above, shows that they are structures of the plasma of the legumes themselves whose similar physiological function leads indeed [to a] most morphological resemblance to bacteria. After all, it is a very interesting case that the plasma of higher plant cells can take on such a microorganism-like shape."

Brunchorst similarly thought there was no reason to regard the bacteroids as being either fungal spores or fungal organs. By my translation, I would quote Brunchorst as stating "On the other hand, it is undoubted that, as Frank [presumably Albert Bernhard Frank] says, they [the bacteroids] are actually surrounded by a membrane" and that "This alone would make it unlikely that you would have mushroom galls in front of you in the nodules". Brunchorst further believed that his findings, again by my translation "... force us to assume that we have it in the nodules to be normal organs of the legumes, the bacteroids have to do with equally normal organs of the cell plasma. It must always be noted what is beyond all doubt that the nodules are only there for the bacteroids, only because of them [the bacteroids, do the nodules] have a right to exist."

Thusly, although Brunchorst used the word 'symbiose' in reference to the root nodules, and Brunchorst is credited for establishing a hypothesis that a symbiosis takes place through and in the nodules, he believed it was a symbiosis between the legume and a fungus and that this symbiosis had nothing to do with the bacteroids. Brunchorst believed that the nodules were organs which gave legumes an ability to utilize any nitrogen-containing organic matter in the soil. Brunchorst developed his opinion after studying *Lupinus luteus*, *Trifolium pratense*, *Vicia sativa*, and a few other legume species. The plant group previously known as Leguminosae we now consider to be Fabaceae. Microbiologists have since kept the term bacteroid. We also have come to know that root nodule cells contain symbiosomes which enclose the bacteria.

14.11 "Untersuchungen über die Stickstoffnahrung der Gramineen und Leguminosen" by Hermann Hellriegel and Hermann Wilfarth, 1888

Hermann Hellriegel and Hermann Wilfarth (1888) set for themselves the goal of developing a new hypothesis about the uptake of nitrogen by papilionaceae, which we presently know as the subfamily Papilionoideae within the family Fabaceae. It had been known that the growth of crop plants generally was directly related to the amount of nitrogen added to the soil. But, that also was known to be a correlation which did not hold true for the papilionaceae. This particular publication covered research data from 1882 through 1887.

Hellriegel and Wilfarth had determined in 1882 and 1883 that papilionaceae could grow well in nitrogen free soil, and similarly could grow in nitrogen-free liquid nutrient solutions. Prior to these studies by Hellriegel and Wilfarth, the root nodules on legume plants had long been regarded as pathogenic formations similar to galls. Gradually, though, the notion became widespread among the scientific community that these root nodules were normal structures belonging to the plant. Some observers had considered the nodules to be used for assimilation of nitrogen, while other observers believed the nodules were used for storage of nitrogen compounds.

Hellriegel and Wilfarth included in their publication as background information some mention of findings by other scientists. In particular, a discovery by Berthelot (whom I might guess to have been Pierre Eugène Marcellin Berthelot) that nitrogen becomes bound in non-sterilized soil, and the bound nitrogen then exists in a form that cannot be washed out by water. Berthelot also had shown that a sterilized soil does not similarly bind nitrogen. Berthelot determined this accumulation of free atmospheric nitrogen to represent assimilation by microorganisms that were present in the soil, and that the assimilated nitrogen was deposited in the form of protein substances. Berthelot presumed this nitrogen fixation was due to the activity of bacteria which existed in the soil, albeit proof that this process of nitrogen binding was due to the activity of bacteria had not yet been obtained.

Hellriegel and Wilfarth also understood that part of the nitrogen present in soil existed as complex organic compounds such as plant residues and humus substances which are converted into ammonia, and they attributed that knowledge to Boussingault (whom I might guess to have been Jean-Baptiste Boussingault). Jean-Baptiste Boussingault additionally has been credited as identifying the basic idea of the biological nitrogen cycle by demonstrating that plants do not absorb nitrogen as an element from the air, but rather plants obtain nitrogen from soil in the form of nitrates.

Hellriegel and Wilfarth cited Albert Bernhard Frank as having concluded from his own work that the process of binding nitrogen into soil compounds is favored by the presence of living plants. Hellriegel and Wilfarth mentioned that while another opinion existed, which was for the necessary nitrogen simply to be absorbed from air by moist soil, that opinion clearly could not explain why absorption of the necessary

nitrogen was so much easier with the growth of clover-like plants and legumes as contrasted with the growth of cereal crops.

Hellriegel and Wilfarth included the knowledge that if you grow gramineae in a field for sequential years without the supply of nitrogenous fertilizer, then yield of the graminea will decrease every year. The same reduction in yield happens if cultivation of legumes is repeated for a long time. But, by adding nitrates (in addition to the necessary mineral fertilizer), the decline in harvests can be reduced in the case of the gramineae although not in the case of legumes. That indicated the lack of productivity observed by subsequent plantings of legumes was not due to a shortage of nitrogen availability. It also had been known from previous studies by other scientists that growing either lupines or red clover (*Trifolium pratense*) as crop resultingly would increase the level of soil nitrogen.

Hellriegel and Wilfarth found that if legume plants were otherwise supplied with nitrogen, then nodule formation and growth of the plants were not necessarily interdependent. Their plants were able to grow normally, produce flowers and produce fruit without starting a single root nodule. Thus, they concluded that legume plants were able to beneficially absorb and process the nitrates available to them in soil even absent root nodules. The legumes did, however, have a supplemental second source from which they could cover their nitrogen requirements if the amount of nitrogen compounds already available in the soil was not sufficient. This second source provides elemental nitrogen from the atmosphere. While the legumes do not per se have the ability to assimilate free nitrogen from the air, the participation of living microorganisms in the soil is absolutely necessary in order that this second source can provide for needs of the plant. That second source was linked to both the availability of soil microbes and to root nodule formation. Root nodule formation was always accompanied by a nitrogen gain for the plant, which did not derive from the original nitrogen content of the soil at the start of the tests. Their statements included certainty that the given observations did not support assumption for the root nodules to serve the plant merely as reserve containers for storing nitrogen-containing nutrients through the time of plant fruiting. Instead, it was much easier to combine the concept of nitrogen storage with the view that the nodules are assimilation organs of the plant. Resultingly, the papilionaceae were, following harvesting, able to leave in the soil a significant amount more nitrogen than had been present in the soil when the papilionaceae had begun to grow.

Hellriegel and Wilfarth concluded from their studies that the root nodules of legumes seemed to be plant organs. They attributed nitrogen accumulation by the legumes to involve a symbiosis between the legume and microorganisms which promoted development of the legumes by promoting nitrogen uptake. Hellriegel and Wilfarth presumed, although admittedly without supporting fact, that some types of fungi could convert free nitrogen of the air into different compounds and that these different forms of bound nitrogen were unequally assimilable for the different types of higher plants. Hellriegel and Wilfarth believed that in order to make free nitrogen available to the legumes for nutritional purposes, it is not enough just to have any microorganisms in the soil, but it is necessary that certain species of the latter enter into a symbiotic relationship with the former. Their final presumption was that the

involved root nodule microorganisms were fungi. Hellriegel and Wilfarth found value in observation that the nitrogen gain in the soil was almost exclusively in the form of organic compounds. They also believed that subsequently studying either some bacteria or fungal hyphae contained within those root nodules would lead to clarification as to how microbial behavior participated in the process of nitrogen uptake by the legumes.

The understanding by Hellriegel and Wilfarth regarding symbiosis was of a conceptual relationship in which two creatures exert a mutually beneficial influence on their life's activities. They presumed that the legumes have an ability to enter into a symbiotic relationship with certain types of fungi as represented by the root nodules. Hellriegel and Wilfarth assumed that their observed ability belongs to the legumes, but not to either the gramineae or other agricultural crops. They perceived nothing in their studies to have indicated that the cereals were able to draw a remarkable quantity of their required nitrogen from a source other than the soil. We since have learned that the Poaceae and indeed most vascular plants do utilize symbiotic microbial nitrogen fixation in the form of arbuscular mycorrhizae, as will be discussed later in this essay. We also have since learned that the legumes form endomycorrhiza of the vesicular-arbuscular type.

14.11.1 A Summary of the Experiments Presented in This Publication by Hellriegel and Wilfarth

Hellriegel and Wilfarth found that when legumes were grown in non-sterilized, nitrogen-free soil, the appearance of numerous well-formed nodules was generally observed on the legume roots. The appearance of root nodules also went hand in hand with vigorous growth of the plants and vigorous availability of nitrogen.

Hellriegel and Wilfarth then set up initial hypotheses which included that legumes could directly assimilate atmospheric free nitrogen, while the barley and oats made no use of that convenient opportunity. Another hypothesis considered was that the legumes, unlike their other test plant species, are able to take their nitrogen from the deeper layers of the subsoil. Experimentally, they eliminated that latter hypothesis by growing legume plants in the absence of subsoil.

Hellriegel and Wilfarth used chemically washed, thermally dry sterilized, quartz sand to culture barley, oats, and peas. They also grew summer turnip, white mustard, red clover, buckwheat, yellow lupine (*Lupinus luteus*, European yellow lupine), serradella (*Ornithopus sativus*), vetch and horse bean. The dry sand sometimes was amended with calcium carbonate, and the sand was moistened with a nutrient solution containing potassium monophosphate, potassium chloride, magnesium sulphate, plus sometimes sodium chloride and calcium nitrate. The plants then were supplied with distilled water. When the gramineae and legumes were grown under identical conditions, but without addition of nitrates, legumes growing in the washed sand often were able to develop normally while the Gramineae remained

nonproductive. Some of their test plants also received an aqueous soil infusion as a source of microbial inoculum. Heating the soil infusion to 70 °C, or boiling the infusion, before administering it to the plants was used as a means of destroying microbes naturally present in the infusion.

In their experiments, the growth of barley and oats (both family Poaceae, then known as Gramineae) was found to be strictly dependent upon the amount of nitrates added to the soil. However, they observed that growth of peas failed to show a similarly dependent relationship to presence of nitrates in the soil. Chemical analysis revealed that the crop products obtained from their pea plants contained considerably more nitrogen than was found in the given nitrates, seeds and soil together. Hellriegel and Wilfarth concluded that, in addition to any nitrogen present in the soil made available to their pea plants, the peas managed to find another source from which they were able to acquire nitrogen in abundance.

14.11.2 Hellriegel and Wilfarth's Final Conclusions as They Specifically Were Stated, with Computer Assisted Translation

1. The legumes behave fundamentally different from the Gramineae with regard to the absorption of their nitrogenation.
2. With their nitrogen requirements, the Gramineae are solely dependent on the assimilable nitrogen compounds present in the soil and their development is always in a direct relationship to the available nitrogen stocks of the soil.
3. In addition to soil nitrogen, the legumes also have a second source from which they can cover their nitrogen requirements in the most extensive way or, as far as the first source is not enough, they can supplement them.
4. This second source provides the free, elemental nitrogen in the atmosphere.
5. The legumes do not per se have the ability to assimilate the free nitrogen in the air, but the participation of living microorganisms in the soil is absolutely necessary for this.
6. In order to make free nitrogen available to the legumes for nutritional purposes, it is not enough just to have any lower organisms in the soil, but it is necessary that certain species of the latter enter into a symbiotic relationship with the former.
7. The root nodules of legumes are not to be regarded as mere reserve stores for protein substances, but are related to the assimilation of free nitrogen.

14.12 "Die Bacterien der Papilionaceen-Knöllchen" by Martinus Beijerinck (1888)

Martinus Willem Beijerinck (1888) began his publication with a brief review of the literature on root nodules of legumes (family Fabaceae). Beijerinck then described the results from his efforts at isolating a bacterial species that was associated with legume root nodules. Beijerinck used gelatin plates on which he was able to grow the bacteria from those nodules, after first grinding the nodules on cleaned glass plates. Instructions easily still can be found for preparing gelatin plates to culture microorganisms (Merten 2020). Beijerinck assigned the name *Bacillus Radicicola* to his newly found bacteria. Beijerinck capitalized both the genus and species names which he assigned to his discovered bacteria.

The concepts presented by Beijerinck included a belief that the bacteroids found inside of root nodules were shaped protein bodies which the plant cultivates from *Bacillus Radicicola*, and *Bacillus Radicicola* was a species of metamorphic bacteria which migrates into the roots from the outside. He concluded that these bacteria must penetrate into the plant cell lumen by an existence of pores in the cell wall, that this process represents an infection of living cells of the root pericambium, and the produced nodules then serve for the purpose of local protein accumulation. Beijerinck believed the bacteroids are stored within the cytoplasm where the bacteroids are better served with a lower than ordinary oxygen tension and also that juices of the papilionaceae cells were their favorite food for the bacteroids.

Beijerinck had found that germinating Papilionaceae (leguminous plants whose flowers have butterfly-shaped corollas) seeds placed between the *Bacillus Radicicola* colonies on gelatin plates greatly promote growth of the bacterial colonies. He believed that tissues of the Papilionaceae roots must be exerting a strong attraction on the *Bacillus Radicicola*, and he regarded these roots as bacterial traps. Beijerinck specifically mentioned the well-known fact that there are either none or very few root nodules on legume plants that have been grown in humus-rich soil. Beijerinck suggested that the papilionacea roots probably release substances into the soil that attract *Bacillus Radicicola*, and if there is no nodule formation as in the case mentioned, then this secretion of attractive substances may have been either abnormal or completely absent.

Beijerinck defined the interactions of *Bacillus radicicola* with plants belonging to numerous genera of the family Fabaceae, those genera were *Caragana*, *Cytisus*, *Ervum* (which now seems to be synonymous with *Vicia*), *Lotus* (that is not a reference to the water plant *Nelumbo nucifera* which commonly is called Lotus), *Lupinus*, *Ornithopus*, *Phaseolus*, *Pisum*, *Robinia*, *Trifolium*, and *Vicia*. He found that the bacteria cultivated from different Papilionaceae species are very similar, but not always completely identical. Although the external appearance of root nodules differed depending upon the species of legume being examined, Beijerinck assessed the fact that the inner structure reveals the same level of microbial training was not to be surprising for, and I translate his words directly, " ... educations that are obviously as old as the legume group itself".

Beijerinck believed that the metamorphosed *Bacillus Radicicola* bacteria present in the bacteroids hence had lost their ability to develop aside from serving their function as shaped protein bodies. Those metamorphic bacteria seemed linked to the normal, culturable form of *Bacillus Radicicola* which he commonly found in soil.

Beijerinck mentioned four characteristics about the root nodules that he studied:

First—the nodules seemed to go through two phases, development and exhaustion;

Second—during development, the bacteria that have entered the cells are more or less completely enclosed by the protoplasm, gradually lose their vegetative power, and finally change into bacteroids which are unable to grow. Those bacteria which were present in the nodules but not included in the cell cytoplasm, on the other hand, remained capable of growth (he used the terms cytoplasm and protoplasm interchangeably although we now, 110 years later, sometimes make a distinction between those two words);

Third, exhaustion can take place in two ways: it can be based either on a normal drainage process by the plant, which seemed to be exhaustion of the nodule, or on bacterial overgrowth. During normal emptying of the nodule, the bacteroids seemed to leave behind either some peculiar strongly light-refractive bacteroid-like remains, or some microsome-shaped bodies which like the bacteroids themselves are unable to grow. In bacterial exhaustion, on the other hand, there was an overgrowing by countless easily cultivated individuals of *Bacillus Radicicola* still present within the nodules in addition to the plant cells containing vesicular bacteroids that were unable to grow or develop;

Fourth—development of the nodules can stop at all stages, they eventually either come to a state of rest or fail due to exhaustion. Beijerinck noticed that other bacteria existed in healthy and also decaying root nodules. Another one of the organisms which he identified was *Bacillus fluorescens putidus* which now is named *Pseudomonas putida*.

Beyerinck said that, although the nodules cannot be understood in the same sense as are normal plant organs such as roots, stems, and leaves, existence of the nodules shows such an analogy that it appeared certain the nodules represented a nutritional function for the benefit of the plant. He included the surmise that, regardless of their numerous associated disorders and some noted exceptions, emptying of the protein supply from the bacteroids must be regarded as representing a normal life cycle of the nodules for at least herbacaous plants.

Beyerinck proposed that the question should be, is it permissible to conclude from the foregoing findings that the nodules are completely useless for the bacteria? He believed that such a view is not correct and that, at least in certain cases, life in the nodules primarily benefits the bacteria. Beyerinck observed that the nodule plasma clearly produces proteins but Beyerinck could not get his isolated and aerobically growing bacteria to produce nitrogenous compounds. It was only later understood that the enzyme complex responsible for nitrogen reduction, termed nitrogenase, is inactivated by oxygen. Therefore, the process of nitrogen fixation requires conditions that are either anoxic or nearly anoxic and his presumption of their being lower oxygen tension within the nodules clearly was correct. In symbiotic associations, the

nitrogen fixing microbes are sequestered in differentiated cells which limit exposure of the nitrogenase to oxygen.

We now know Beijerinck's discovered bacteria to be members of the genus Rhizobium, class Alphaproteobacteria. The rhizobia are soil bacteria that form an endosymbiotic nitrogen-fixing association with the roots of legumes, and additionally with some other plants. Beijerinck mentioned in his publication *Melampyrum pratense* and *Rhinanthus major*, which are members of the family Orobanchaceae and do not seem to form root nodules. He did not mention the plant genus *Parasponia*, which belongs to the family Cannabaceae and does develop *Rhizobium* root nodules.

Beijerinck proposed, as per my translation, "Why, we continue to ask, did the Papilionaceae use the root bacteria to create protein stores? Don't they show in their seeds that they can achieve this purpose even without bacteria?" and, "For the time being we take the protein supply of the bacteroids as a given, but we will come back to its origin at the end and only then can we fully indicate the probable meaning of the nodules."

14.13 "Symbiosis" by Marshall Ward, 1899

Harry Marshall Ward (1899b) wanted to distinguish differences between parasitism and symbiosis. He suggested that symbiosis could be considered in a broad sense, and should not be used to describe interactions between species that only represented either temporary associations or transient encounters. In his words "When we come to inquire as to the processes which lead to enhancement of the functional activity of one organism by another living symbiotically with it ..." And, he talked about symbiosis as including associations in which neither species could survive alone.

Ward included in his presentation the importance of nitrogen fixation and nitrogen cycling. Among his examples were the fungal symbiosis of mycorrhiza and the roots of plants, plus the bacterial nitrogen fixation associated with leguminous nodules that was achieved by "Hellriegel and Willfarth's cultures" which we know to be effected by *Rhizobium*. Ward included the nitrogen cycling which occurs when *Bacillus ramosus*, which we now call *Erysipelatoclostridium ramosum*, is added together with *Nitrosomonas* and *Nitrobacter*.

Wards review included several photosynthetic symbioses. Among his examples were the formation of lichens, which are composite organisms that exist as a mutualistic relationship between either an algae or a cyanobacteria living among the filaments of fungi. Ward also mentioned microbial symbioses in the plant world including among those the photosynthetic symbionts which were then termed algae but we now know as cyanobacteria, which live intracellularly within the stems of *Gunnera*, in the roots of Cycads, and either within or on the thallus of *Anthoceros* (hornworts), *Blasia* (liverwort), *Azolla* (mosquito ferns), and *Lemna* (duck weed). The cyanobacteria in those partnerships provide fixed nitrogen to the plant, while the plant provides fixed carbon for the cyanobacteria.

Ward also included some examples for photosynthetic microbial symbioses which then were known in animals. Those were the mutualistic association between sponges and what we now term cyanobacteria, and the mutualistic association between *Hydra* and its endosymbiont algae *Chlorella*. He included among those animals the "Green Infusoria", for which I presume he intended relationships such as the endosymbiotic cyanobacteria that exist in amoeba of the genus *Paulinella*. We now consider amoeba to be protists rather than animals.

Ward indicated that another example of derived advantage might be when an aerobic microorganism prevents the access of oxygen to an anaerobic microorganism. In this regard, he mentioned the fixation of atmospheric nitrogen accomplished by *Clostridium pasteurianum* as facilitated by other organisms that can scavage oxygen to create adequately anaerobic conditions for the *Clostridium*. The scavanging of oxygen is important because the nitrogenase which fixes nitrogen is rapidly degraded by oxygen.

Ward talked about 'metabiosis' as representing instances when one organism prepares a more suitable environment for another. We now consider metabiosis to be a form of commensalism. Wards examples of metabiosis included when one species modifies a substance, after which a different organism subsequently can utilize that modified substance, with this representing the provision of definite food materials by one for the other. His suggestion was that these metabiotic associations might be a 'half-way house' to symbiosis, with an example being when one organism can hydrolyze starch via diastase although the subsequent build-up of sugar eventually might become enzymatically inhibitory, after which another organism would ferment the sugar to alcohol and thereby remove the inhibition. We now use the term diastase in reference to any α-, β-, or γ-amylase, which are hydrolases capable of breaking down carbohydrate. The specific example which Ward gave was for sake production, when *Aspergillus* acts upon the starch in rice, which prepares the way for yeast to then ferment the sugar into alcohol.

Ward also suggested that there might be mutualistic cooperations in which either species could 'carry on' alone in a given situation although they did better when acting together. We now define 'mutualism' as representing relationships when each organism derives a net benefit. 'Commensal' is a relationship in which one species gains benefit while the other is not necessarily harmed. Ward suggested the term 'Antibiosis' to represent antagonism.

Ward was a prolific writer, and in that same Annals of Botany volume he included the third part in his series of articles about the microorganisms found in the Thames River (Ward 1899a) plus information on methods for culturing algae (Ward 1899c).

14.14 "Soil Inoculation for Legumes; Reports upon the Successful Use of Artificial Cultures by Practical Farmers" by George Moore, 1905

George T. Moore (1905) studied the culturing and then agricultural application of root nodulation bacteria for assisting the productivity of legume crops. Historically, soil transfer had been proven beneficial to provide the appropriate microbes for achieving effective root nodulation in legumes. However, soil transfer also can result in the inadvertent movement of pathogens that cause such plant diseases as wilts and blights.

Moore noted that some strains of rhizobial bacteria produced nodules but did not benefit nitrogen assimilation by leguminous crops. Interestingly, some other bacterial strains seemed to fix nitrogen without the formation of root nodules.

Experimentally, Moore tested nodule-forming bacteria isolated from the common pea (*Pisum sativum*), grew that isolate for 2 weeks in nitrogen-free media, and then used that culture for experimentally inoculating seeds of: Crimson clover (*Trifolium incarnatum*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), berseem clover (*Trifolium alexandrinum*), alsike clover (*Trifolium hybridum*), sweet clover (*Melilotus albus*), cowpea (*Vigna unguiculata*), alfalfa (*Medicago sativa*), broad bean also called fava bean (*Vicia faba*), common bean (*Phaseolus vulgaris*), fenugreek (*Trigonella foenum-graecum*), hairy vetch (*Vicia villosa*), scarlet vetch (*Astragalus coccineus*), yellow vetch (*Vicia lutea*), blue lupine (*Lupinus angustifolius*), and white lupine (*Lupinus albus*).

In every case, with exception of the lupines, Moore's cultured bacteria successfully resulted in the production of root nodules.

This agricultural bulletin also provided a summary of field reports from different farmers who used a dried product containing Moore's culture of microorganisms to facilitate root nodulation and thus to promote growth and productivity of: alfalfa, beans (wax beans, green beans, refugee beans, and green podded bush beans all of which are different varieties of *Phaseolus vulgaris*), berseem clover, cowpea, field and garden peas (*Pisum sativum*), hairy vetch, peanut (*Arachis hypogaea*), red clover, soybean (*Glycine max*), sweet pea (presumably *Lathyrus odoratus*, but only the common name was given and that could have been reference to a variety of *Pisum sativum*), and velvet bean (*Mucuna pruriens*).

14.15 "Isolation of *Bacillus Radicicola* from Soil" by Charles Bernard Lipman and Lawrence Fowler, 1915

Charles Bernard Lipman and Lawrence W. Fowler (1915) reported in this publication their discovery that *Bacillus Radicicola* was a natural soil organism. Lipman and Fowler found the microbe could be isolated from soil, grown in laboratory medium, and then a suspension of the cultured bacteria successfully used to inoculate sterile

soil that contained surface-sterilized *Vicia* seeds. The bacterial culture medium which they used was soil extract agar, which is prepared by adding agar and maltose to an aqueous extract of soil.

14.16 "Tier und Pflanze in Intrazellularer Symbiose" by Paul Buchner, 1921

Paul Ernst Christof Buchner published in 1921 the first edition of his masterful reference book on intracellular symbioses in animals. Buchner presented an understanding that on both sides of the partnership, the symbiont and its host, there must have been acquisition of properties without which the symbiotic relationship cannot occur.

Buchner includes a history of the discovery and understanding that many animals which curiously seemed to produce chlorophyll did instead contain photosynthetic microbial symbionts. Specifically, Buchner mentions photosynthetic endosymbioses of protozoa, Anthozoa, Cnidaria, Porifera and Turbellaria, with zoochlorella (*Chlorella*) and zooxanthellae (dinoflagellates).

Buchner mentioned an interesting endosymbiosis by the gastropod group opistobranchia, which is the acquisition of chloroplasts from zoochlorella by the sea slug *Elysia chlorotica*. We now understand that to be an occurrence by which chloroplasts from ingested algae *Vaucheria litorea* are incorporated into the host cells through phagocytosis, after which the chloroplasts become a part of the animals own cellular content. It would seem that the endosymbionts can be maintained if the slug possesses appropriate nuclear DNA sequences that possibly have been acquired by horizontal gene transfer, but otherwise continual feeding on algae is necessary for sustaining presence of the chloroplasts. This method of acquiring chloroplasts is termed kleptoplasy, which derives from ancient Greek and means the chloroplasts have been stolen.

Buchner did mention an occurrence of endosymbiotic zooxanthellae in echinoderms, but I could not find subsequent information about that occurrence. Buchner additionally mentions that unfavorable situations can result in the loss of photosynthetic symbionts, with the symbionts either being attacked and degraded or ejected from the host. That ejection most notably causes the process which we term to be coral bleaching.

Buchner was very much a philosophical luminary, including understand that the bioluminescence of ctenophores was an intrinsic characteristic which occurred within photocytes. The luminescence produced within photocytes is a reaction of luciferin and luciferase. Photocytes are specialized cells found in a range of multicellular animals including annelids, arthropods including insects, ctenophora, cnidaria, and fish. Although some fungi are bioluminescent, producing the effect called foxfire, those fungi do not have specialized luminescent cells. A contemporaneous summary of knowledge regarding bioluminescence, and the fact of their

being intrinsic bioluminescence that was not due to symbiotic organisms, was published in 1920 by Edmund Newton Harvey (1920).

Buchner mentions the symbiont of *Molgula*, which I presume is a reference to *Nephromyces*. *Nephromyces* is a genus of apicomplexan that are symbionts of the ascidian genus *Molgula* (sea grapes) and that seems to be a beneficial symbiosis rather than a parasitic one. This particular relationship would thus seem to be an exception among apicomplexans, which usually are parasitic upon their animal hosts. *Nephromyces* lives in the renal sac of its host, wherein there are high concentrations of the nitrogenous waste product urate. The *Nephromyces* cells contain urate oxidase, and thus the *Nephromyces* may be using waste product from its host animal as a nitrogen source.

Buchner also mentions endosymbioses of insects including omnivores, wood eating insects, and insects that consume sap. Those symbionts often assist the host by providing important metabolic products such as amino acids which the host otherwise could not obtain from its limited dietary range. The pseudovitellus of aphids is mentioned, and that is a mycetome that also exists in other insects such as cicadas which similarly subsist by ingesting plant phloem sap.

The title of Paul Buchners book would suggest that it mentions intracellular symbionts both of plants and animals, but by my reading this book covers animal hosts. It is possible that some of the species which we now consider to be either animals or microbes were, at the time this book was written, considered to have been plants. Paul Buchner's book went into several editions by slightly different titles.

14.17 "The Production of Light by the Fishes *Photoblepharon* and *Anomalops*" by Newton Harvey, 1922

Edmund Newton Harvey was working at Princeton University when he published this article in which stated that symbiotic bioluminescent bacteria were housed in the light organs of two marine fish species, *Anomalops katoptron* and *Photoblepharon palpebratus*, and the light emitted by those organs was produced by their symbiotic bacteria.

These two fish species are termed "Flashlight fish", and the bioluminescent bacteria are housed in organs located below the fish eyes. These organs constantly produce light but the fish can choose to either reveal or conceal that light. *Anomalops* fish rotate their luminous organ upward to expose the light and downward to conceal the light. *Photoblepharon* fish have a tissue covering which can be opened to reveal the light and then closed to conceal the light, effectively blinking. Other scientists had come close to understanding the microbial nature of that light production, but it was Harvey who in 1922 clearly stated it as being fact.

We have since come to learn that the luminous organs of many other fish and also squid similarly house bioluminescent symbiotic microorganisms.

Two years before that publication, in 1920 Harvey had published a very important monograph titled "The Nature of Animal Light" (Harvey 1920). In his 1920 publication, Harvey presented some history of our knowledge regarding bioluminescent microorganisms. Those include bioluminescence associated with bacterial decay of fish which I have not seen, with fungal decay of wood which is called foxfire and fascinated me the first time that I saw it when I was a Boy Scout on a camping trip, and with dinoflagellates in the ocean which my brother saw light up the ocean waves that washed over the deck of his navy ship at night. These microbial phenomenon had been observed and examined as a curiosity for millennia. Bioluminescence certainly is not limited to microbes, and indeed many macroscopic organisms produce their own bioluminescence. Our common fascination with bioluminescent animals includes chasing fireflies.

The modern scientific studies which led to our understanding of bioluminescence seem to have begun with the work of Raphaël Horace Dubois in the late 1800s. It was presumed for decades that this illumination was either a fluorescence or a phosphorescence. Eventually, we came to understand bioluminescence as being the product of an enzymatic oxidation by luciferase acting upon a luciferin. Harvey had studied the light organs of invertebrates and the chemistry of their luminosity including his making detailed examinations of luciferin and luciferase reactivity. Harveys observations (Harvey 1920) extended the existing knowledge that luciferins and luciferases from closely related species will react together and produce light, but mixing together luciferins and luciferases from species that taxonomically are more distant will not produce light.

I presume that the bioluminescent marine bacteria *Vibrio harveyi* was named in honor of E. Newton Harvey. That bacterial species lives commensally in the gut of some marine animals and is a pathogen of many marine animals.

Other examples of symbiotic bioluminescent microbes include the bacterial species *Aliivibrio fischeri*, which is a mutualistic symbiont of various marine animals including the Hawaiian bobtail squid *Euprymna scolopes*. Presumably that microbes luminescence is of benefit to the squid (Visick et al. 2000). The bacteria *Photorhabdus luminescens* is an intestinal symbiont of entomopathogenic nematodes belonging to the family Heterorhabditidae. *Photorhabdus luminescens* is transmitted by regurgitation into insects that have been attacked by the nematode. The bacteria then kills the insects by producing toxin, and secretes enzymes that decompose the insect (Ciche and Ensign 2003). The decomposed insect serves as a nutritional source for both the bacteria and its host nematode. The ecological role of the bioluminescence produced by *Photorhabdus luminescens* is not yet understood. Insects that are infected with *Photorhabdus luminescens* do glow.

14.18 "Symbiosis Between Termites and Their Intestinal Protozoa" by Lemuel Cleveland, 1923

Lemuel Roscoe Cleveland was working at Johns Hopkins University in Baltimore, Maryland, studying the relationship between termites and their intestinal protozoa. He examined the intestinal contents of termites in a museum collection and determined that whenever wood was present so were protozoa, and whenever protozoa were present so was wood. Thus, there was a clear positive correlation between a wood-feeding habit and the presence of protozoa. Cleveland discovered through experimentation that the relationship between wood-feeding termites and their intestinal protozoa was temperature labile. Incubation of termites for 24 h at 36 °C resulted in death of the harbored intestinal protozoa. The incubated termites survived this thermal treatment, and Cleveland described those surviving termites as being defaunated. The defaunated termites subsequently were able to ingest their normal diet of wood but unable to derive sustenance from ingesting that wood, and the termites instead seemed to die from starvation. Feeding either fungus-digested cellulose or humus to the defaunated termites allowed the termites to survive. Permitting defaunated termites to have coresidence with unincubated termites resulted in the defaunated termites becoming reinoculated with protozoa, a process which Cleveland described as being a reinfection of the termites. Following coresidence, the defaunated termites with their population of reacquired intestinal protozoa were able to survive as normal on a diet of either wood or cellulose. Starving termites by denying them wood resulted in a change of the intestinal protozoa population, those protozoa which ingested wood were eliminated from the intestine but the non-wood-ingesting protozoa remained abundant. The starved termites subsequently could ingest wood but lacked ability to digest that wood. Cleveland surmized that the wood-ingesting protozoa possibly split the cellulose into cellobiose, the cellobiose into glucose, and then created glycogen from the glucose. He presumed that the termites were obtaining sustenance from the products generated by those protozoa.

14.19 "Strain Variations and Host Specificity of the Root-Nodule Bacteria of the Pea Group" by George Helz, Ira Lawrence Baldwin, and Edwin Broun Fred, 1927

George E. Helz, Ira Lawrence Baldwin, and Edwin Broun Fred (1927), working at the Department of Agricultural Bacteriology, University of Wisconsin, tested seventeen different strains of bacteria which induce formation of root nodules in legume crops. Their goal was to increase the harvest yields of garden pea (*Pisum sativum*) although their selection of studied plants was wider in scope than peas alone, with inclusion of broad bean (*Vicia faba*), hairy vetch (*Vicia villosa*), lentil (*Lens esculenta*), and sweet pea (*Lathyrus odoratus*).

The results published by Helz, Baldwin, and Fred confirmed earlier discoveries that some rhizobial strains show host dependent differences in their effectiveness for supporting plant growth. Specifically, a culture originally isolated from broad bean (*Vicia faba*) produced better results on *V. faba* than did a culture originally isolated from peas. The organism isolated from peas, on the other hand, gave much better results on both peas and sweet peas than did the *V. faba* culture.

More importantly, Helz et al. (1927) found one bacterial strain which produced nodules on all five of the host species studied, but that ability for inducing nodulation somehow did not benefit the plants nitrogen economy. Ninety five years later we learned the reason for that curious discovery when Matthew B. Crook and colleagues published their revelation that a naturally existing plasmid results in rhizobial strains which enhance competitiveness for nodule occupancy, but impair nitrogen fixation, resulting in reduced benefit to the host plant (Crook et al. 2012).

14.20 "The Conquest of Bacteria from 606–693, 2nd edn" by Sherwood Taylor, 1940

Frank Sherwood Taylor had a Doctorate in History and the Method of Science, and he wrote interesting books on many different aspects of science. I found a copy of his book on bacteria (Taylor 1940) in a used bookstore when I was a graduate student. Taylor presented an informed summary about the history of research into bacterial disease, the body's immunologic responses, chemical disinfectants, and also antimicrobial chemotherapy. Taylor's inspiration for that book may have been his brothers death from tuberculosis. This book holds particular interest for me because it describes using antibiosis for controlling infectious disease of humans. The techniques mentioned were chemotherapy using laboratory-developed synthetic compounds and also naturally antibiotic compounds

Arsphenamine. originally named Salvarsan, initially was designated "606" because it was the sixth in the sixth group of compounds synthesized for testing. It was discovered in 1909 by Paul Ehrlich, Alfred Bertheim, and Sachachiro Hata, marketed originally in 1910 by Hoechst AG, and was the first truly effective cure for infections caused by *Treponema pallidum*. William H. Benton, who was a dear friend of mine and for a while was my laboratory technician, had been a United States Air Force Hospital Corpsman. Bill once told me that it was always possible to identify by x-ray if someone had received buttock injections of Salvarsan for treatment of syphilis, because the injections left a pattern of metallic dots that looked like shotgun pellets.

This book also describes Kl730, which later became known as Prontosil, as discovered by Bayer chemists Josef Klarer, Fritz Mietzsch, and Gerhard Domagk. The compound Kl730 gave rise to the sulphonamide class of antibiotics. At the time when that book was written by Taylor, the sulphonamides which still serve us well were relatively new, as was Sulfanilamide which consists of an aniline derivatized with a sulfonamide group. Sulfanilamide acts by inhibiting the formation of folic

acid. Sulfanilamide's present usage includes treatment for yeast and bacterial infections.

M&B 693 is sulphapyridine and was discovered in 1937 by Lionel Whitby at the British firm May & Baker. It is Sulfanilamide linked to a pyridyl group and was able to reduce the *Neisseria meningitidis* death rate by nearly eighty percent, plus it was effective in treating infections caused by *Neisseria gonorrhoeae*. The most famous patient treated with M&B 693 was Winston Churchill, for whom the antimicrobial compound cured a bacterial pneumonia.

Taylor lamented that the amount of money spend on weapons of war and defenses against them was so much greater that the amount of money spent on combating infectious diseases, and I quite firmly agree with that.

14.21 "Antibiosis" by Howard Bowman, 1947

Howard H. M. Bowman (1947) said that the term symbiosis defines a partnership between two organisms, with a subset of symbiotic partnerships represented by the term antibiosis. Antibiosis is an association of two species in which one is harming the other, with an example being the production of antimicrobial substances. Bowman included in this article a historical review of the discovery and developments of penicillin (Fleming 1929) and streptomycin (Schatz et al. 1944).

Bowman explained that millions of microorganisms live in the soil and most of these microbial organisms are supported by the wastes and debris of the bodies of higher plants and animals that live on the earths surface. Some of the minute forms of life that live in the soil are also dangerous enemies of the higher organisms. Resultingly, "It is now recognized that all the higher plants and animals suffer from the ravages of microbes such as bacteria, fungi and protozoa; and the more highly evolved the plant or animal, the more numerous are its microbial parasites. Many of these disease-producing agents are closely related to harmless forms which lead independent lives in soil or water."

This led Bowman to present the assumption that disease producing microorganisms were once non-pathogenic, but subsequently became adapted to a parasitic life in specific hosts. The pathogenic microorganisms produce toxic substances which injure the host until the host either succeeds in building up a resistance to the pathogen or the host finally is killed. There also would be situations in which the host survives the attack without killing its parasite, and the host then remains a carrier of the pathogen. These disease-producing microorganisms naturally spread from host to host through routes that include water, dust or excreta of the host.

Bowman surmised that microbial populations naturally present in soil will be antagonistic and attack the pathogens. "It has been found that even the common *Rhizobium*, a bacillus that forms nodules on the roots of leguminous plants, cannot thrive outside the nodules of the roots. [Selman] Waksman found that the normal soil microbes will quickly kill it when it is placed alone in soil. It survives only when protected in the security of its nodules on the plants." Bowman also mentioned that the organism which causes Texas Cattle Fever, specifically a parasitic alveolate now

named *Babesia bigemina* which is carried by a tick, is not able to live in climates which are cold enough to kill the ticks. He stated that presumably the parasite cannot survive in pasture soil unless the parasite is protected within the body of the tick.

Bowman mentioned the discovery of penicillin by Alexander Fleming, which occurred in 1929. Fleming had noticed that *Staphylococcus* growing on a culture plate were being lysed by a contaminating Penicillium mold. Fleming modestly suggested that perhaps the antibacterial substance in a broth culture of the mold might be used clinically to help infections caused by those bacteria which he found susceptible to it. We now understand that in the struggle for survival which is represented by antibiosis, microorganisms produce many antimicrobial compounds and also evolve defenses against those antimicrobial compounds which are produced by other organisms.

Acknowledgement I wish to thank Kerry Oliver for reviewing this essay and also for his helpful suggestion that I include Paul Buchner's book.

Christon J. Hurst "Have Passport–Will Travel"

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Chapter 15
Microscopic World and the Phenomenon of Symbiosis in the Natural Environment

Vladimír Klaban

Abstract This chapter presents a perspective of the interactions that exist between microorganisms. Those interactions cover a broad spectrum, ranging in nature from collaborations to competitions, and they are a key aspect of the biosphere's foundation. Included in this chapter are brief mentions and some history of the following concepts: mutual relationships of microorganisms including synergism, syntrophy, and commensalism; reciprocal and unilateral antibiosis; amensalism alias allelopathy; bacteriocins and zymocins; plus microbial competitions including parasitism and predation.

15.1 Mutual Relationships of Microorganisms

Let us imagine that on a beautiful day under sunny skies we are sitting with friends in the backyard. We do not realize that all around us there exists an invisible life of microorganisms in various environments. Microorganisms colonize the leaves and stalks of plants, the upper layers of soil that we see and as well the roots of plants. They are present on the surface of animals, on human's skin and as well in their gastrointestinal tract. Likewise, microorganisms are also present in high altitude environments, in regions with perpetual snow, they live in the lakes, seas, and oceans, even on the very bottom of those water bodies. They are also present in the atmosphere. The so-called hyperthermophilic microorganisms can be found in hot springs. Microorganisms thus immensely broaden the Earth's region called biosphere. Microorganisms in many of these environments are very active. They decompose all kinds of compounds and substrates not just for their own nutrition, but also for the beneficial consumption of other organisms. In natural environments, microorganisms form all kinds of mutual interrelationships. And right here we arrive at a very used and useful term of symbiosis.

From the etymological point of view, the word symbiosis means "life together," in our case the mutual coexistence of microorganisms, derived from the Greek words

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_15

"syn" meaning "together," and "bios" meaning "life." Often though the word symbiosis is understood as a mutually beneficial relationship for two organisms. In the more general sense, it means any relationship between organisms irregardless of whether positive or negative. The interrelationships may be any of various different characteristics and oftentimes it is difficult to exactly differentiate between the various types of relationship categories. Quite often these relationships between microorganisms are more complicated than those in the animal world or even in human society. In the following text, we will deal with some important relation types, especially with synergism, commensalism, antibiosis, amensalism, competition, parasitism, and predation. These can exist in various combinations. It should be stressed that there are even more sorts of mutual relationships.

15.1.1 Synergism and Syntrophy

The term synergism is derived from the Greek word "syn," meaning "together," and "ergon" meaning "work," literally "co-operation." It denotes a situation where certain strains of microorganisms grow better together than on their own. Synergism and mutualism are similar in that both populations derive advantage from their mutual, localized relationship. However, unlike mutualism, synergism represents an association in which each of the two participants is capable of living independently of the other in their natural habitat. Therefore, the synergic relationship is open and in that sense, one of the involved populations can be relatively easily replaced by another one. In some situations, however, it is hard to determine if the relationship is rather of an essential nature and therefore could be considered as mutualism. Slater (1978) presented a synergic degradation of cyclohexane (which is one of the most stable of cycloalkanes) by organisms of the genera *Nocardia* and *Pseudomonas*. In that pairing, *Nocardia* supplies *Pseudomonas* with the degradation products of cyclohexane, while *Pseudomonas* provides *Nocardia* with biotin.

Syntrophism (syntrophy), from Greek "syn" = together and "trofe" = nutrition, is one of the forms of synergism and sometimes also called "cross feeding." It is a special form or case of symbiotic cooperation between two metabolically different kinds of bacteria, which are mutually dependent on each other with their common aim being to degrade a certain substrate. An example would be when one of two kinds of bacteria makes a certain vitamin, amino acid, or a nutritional factor, which is essential or beneficial for the life and multiplication of the other microorganisms and vice versa. Thus, two kinds of microorganisms help each other by the synthesis of essential growth factors. A classical case of syntrophism was described in 1940 by Ernest Frederick Gale (1940). It is a relationship between *Enterococcus faecalis* and *Escherichia coli*. Neither of the microbes is able to transfer the amino acid arginine into the biogenic amin putrescine. *Enterococcus faecalis* can transform arginine into the amino acid ornithine, which can then be used by *E. coli* to form putrescine. Although *E. coli* alone is able to utilize arginine and to make agmatine by its

decarboxylation, it cannot produce putrescine without the help of the other bacterial population.

Another well-known example of syntrophy was published in 1939 by Horace Albert Barker. Barker isolated an organism which he named *Methanobacillus omelianskii*. It appeared that microorganisms alone could use ethanol and carbon dioxide to produce acetate plus methane. Thermodynamically, the reaction is feasible. However, later it turned out that these reactions are in fact carried out by two kinds of organisms. *Acetobacterium woodii* forms hydrogen and acetate from ethanol. The other microbe is *Methanobacterium bryantii*, which uses hydrogen and carbon dioxide (but no ethanol) to make methane. These two microbes were originally isolated and stored together. That also suggests a close (somatic, physical) relationship between the two partners. Another example is the syntrophic oxidation of butyrate. The overall reaction of butyrate to methane and carbon dioxide is this:

$$2\,CH_3CH_2CH_2COOH + 2\,H + 2\,H_2O = \;\; 5\,CH_4 \;\; + 3\,CO_2.$$

Under standard conditions, the above reaction produces free energy, 177 kJ/2 mols butyrate. This reaction is catalyzed in microbial culture by three different, but mutually cooperating kinds of bacteria. First, one kind of bacterium transforms butyrate into acetate and hydrogen. Then another bacterial species produces methane from hydrogen and carbon dioxide. In the end, the acetate is transformed into methane and carbon dioxide by a third bacterial species. Another syntrophic oxidation of acetate, producing methane plus carbon dioxide, already had been reported in 1936 (Barker 1936).

15.1.2 Commensalism

The word commensal is derived from Latin "com" = simultaneously and "mensa" = table, also meaning meal. The word, introduced by Edouard Van Beneden, could be also interpreted as "sharing the table." Commensalism denotes interaction, or coexistence, of two or more organisms. One of them is the commensal, i.e., the one which benefits from this relationship. The host, which oftentimes is a macroorganism, is not in any way harmed or negatively influenced. In nature, there exist different forms of commensalism, ranging from loose relationships to more lasting and stronger ones. In the case of stronger relationships, the commensal is located in an immediate vicinity of the host, perhaps even on its body surface or in body cavities. Typical human commensals are some species of coliform bacteria in the gastrointestinal tract. Similar situations exist in animals. The microflora of the mouth or the skin can serve as another example. Based on the type of commensalism, participant microorganisms can be divided into ectocommensals and endocommensals.

Ectocommensals live on plant surfaces, but mostly on animals and other organisms. There exist also microscopic commensals that live on the surface of microorganisms. The host organisms provide nutrition to the ectocommensals in the form of

their metabolism's products. In most cases, the mutual relationship is rather weak. Endocommensals typically are microscopic organisms that mostly colonize gastrointestinal systems of terrestrial animals and humans, but there certainly also are endocommensals of aquatic animals and some endocommensals colonize plants. The so-called endocommensal protozoa can feed on bacteria in the intestinal tract. Endocommensal organisms are dependent entirely on the inner environment of the host and they are unable, with small exceptions, to live independently in the open environment, where they would quickly perish.

Some interesting and remarkable examples of commensalism include the fact that commensalism can also develop such that an unaffected population of microbes, during its growth, adjusts its surrounding environment in such a way that another population benefits from it because the modified environment is more suitable for requirements of the second population. For example, when a population of facultatively anaerobic microorganisms uses oxygen, it lowers the surrounding oxygen content and thus creates an environment more suitable for an obligatory anaerobe. Thus, in this case the obligatory anaerobe benefits by the metabolic activity of the facultative anaerobe. The production of growth factors creates another platform for many commensal relations between microbial populations. A certain microbial populations may synthetize and secrete growth factors (for instance, amino acids and vitamins) that can be utilized by other microbial populations. For example, *Empedobacter brevis* secretes an amino acid, cysteine, which *Legionella pneumophilla* in the water environment uses for its multiplication.

Another type of commensalism among populations is the conversion of organic molecules by one population into a substrate suitable for another population. For example, certain fungi produce extracellular enzymes that can convert complex polymeric compounds, such as cellulose, into smaller substances and even into glucose. These simpler compounds can then be used by other microorganisms for their nutrition when those other microorganisms do not possess their own enzymes for degradation of the complex organic molecules.

Transformation of insoluble compounds into soluble ones, and the subsequent conversion of soluble substances into gaseous compounds, forms a basis for certain types of commensal relationships in which gaseous substances can then be beneficial for other microbial populations. For instance, methane produced by bacterial populations in sediments can be useful to methane-oxidizing bacteria in a water environment. Under certain conditions bacteria of the genus *Desulfovibrio* can supply compounds to *Methanobacterium* which then is capable of using those compounds to reduce carbon dioxide to methane.

Also, an activity of one microbial population can release a compound without its chemical transformation for use by another population. As an example, it is possible to denote an acid production by one microbial population, which can release compounds bound to soil particles or otherwise not accessible to the other population. Such desorption processes are most likely frequent in the soil where many substances are bound either to mineral particles or to a humic substance. *Entylia* uses a form of commensalism where the commensal is found directly in the host's body cavity without causing any harmful or negative effect to the host. For instance,

coliform bacteria live in the digestive tract, specifically in the intestinal system of people and animals.

15.2 Antibiosis: Reciprocal Versus Unilateral

The term "antibiosis" was first used in 1889 by the French mycologist Jean Paul Vuillemin, then director of the Museum of Natural Sciences of the Medical faculty, University of Nancy (France). His definition of the term was "one being destroys another one in order to preserve its own." Vuillemin defined antibiosis in a wider biological context which at first did not even include microorganisms, just plants and animals. In his assessment of antibiosis, H. Marshal Ward (1899) defined antibiosis as a phenomenon whereby in an association of two organisms one of them damages the other. Later, in 1928, Georges Papacostas and Jean Gaté (1928) defined antibiosis in the following manner: "...when we consider an association of two microbes, it always involves an inhibition, either a reciprocal one, or a unilateral ...." Howard *H. M. Bowman valuably presented a review of the literature on antibiosis in 1947* (Bowman 1947*).*

15.3 Amensalism Alias Allelopathy

The term allelopathy, a synonym to amensalism, dates back to Hans Molische (1937). A typical example of amensalism is the biosynthesis of antibiotics which represents a pronounced antagonistic influence upon microbial species. In 1970, William L. Brown with coworkers proposed the term allomons for allelopathic substances (Brown et al. 1970).

Amensalism represents a relationship between organisms of a microbial community in which a specific microbe, the so called inhibitor, produces or releases a specific substance (or substances) which suppresses either the growth or some metabolic activity of another organism. For example, organotrophic (chemoorganotrophic) organisms are sensitive to NH_3. Similarly, microbial alkalinization of the environment may negatively affect some microbial species.

In nature, an accumulation of hydrogen sulfide (H_2S) occurs exclusively in anaerobic environments. Proteins as well as amino acids represent the source of H_2S. Also, in nature, desulfurization bacteria reduce sulfates to hydrogen sulfide. In general, these processes take place in the muddy bottoms of ponds and water reservoirs, but they also occur during the process of biological decontamination of wastewaters or in the digestive tract of animals. Sensitive microorganisms can be negatively affected by the presence of H_2S and that can change the species composition of microorganisms inside the digestive system of animals.

Other microorganisms can form hydrogen peroxide (H_2O_2), when even low H_2O_2 concentrations may be bacteriostatic. Yet other bacteria may synthetize both organic

and inorganic acids, thus strongly affecting the pH of the environment. *Thiobacillus*, for instance, produces significant amounts of sulfuric acid that lowers greatly the pH of the environment, which in turn causes changes in the composition of the surrounding microbial community. Another example can be the production of lactic acid by the lactic acid bacteria. That acid production also causes a significant reduction in the number of microbial species originally present in a given environment.

Another well-known example of amensalism is the production of ethanol, which negatively affects many species of microorganisms. For example, production of ethanol by certain species of yeasts inhibits the growth of many sensitive bacteria. Bacteriocins, zymocins, mycotoxins as well as antibiotics all belong to the realm of amensalism.

15.4 Bacteriocins

The term bacteriocin was introduced in 1953 by Jacob and coauthors (Jacob et al. 1953) as denomination for a group of proteins with antibiotic characteristics. These are the products of certain strains of bacteria and they have lethal effects on other strains within the same species. The effect of bacteriocins is also dependent on the presence of specific receptors on the surface of sensitive cells. The receptors are structures onto which bacteriocins bind and they are located in the bacterial cell wall. Bacteriocin-producing strains are called bacteriocinogenic strains.

The synthesis of bacteriocins is determined genetically by certain genes. The best known and the earliest described bacteriocins are the so-called colicins, which are produced by some strains of *Escherichia coli*. They affect sensitive bacteria by various mechanisms. For instance, colicin E2 decomposes the DNA of the attacked cell, while colicin 3 splits ribosomal RNA. Other colicins bind to the surface receptors of bacteria and block the flow of nutrients into the cell. Yet another colicin inhibits bacterial growth by blocking the permeation of ions through the cytoplasmatic membrane. It is assumed that bacteriocin production gives bacteria a definitive advantage in their living environment because they thus can eliminate other microorganisms with which they would otherwise have to compete for nutrition. In a similar fashion, yeasts produce antimicrobial substances akin to bacteriocins, called zymocins. Bacteriocins can also be used for the biological identification of bacteria of the same species. The names of bacteriocins are derived from the names of those bacteria which produce them, for example, species of the genus *Proteus* form proticins, *Pseudomonas aeruginosa* produces pyocins and so on.

15.4.1 Biosynthesis of Bacteriocins and Their Lethal Effect

In 1877 Louis Pasteur, along with Jules Francois Joubert (Pasteur and Joubert 1877) systematically studied antagonistic interaction between bacteria. They discovered that common bacteria such as *Escherichia coli* can inhibit the growth of *Bacillus anthracis* (anthrax bacteria) when both of them are simultaneously inoculated into urine, used as a culture medium.

Bacteriocins are generally stated as being either proteins or peptides, which exhibit bactericidal effect on other closely related strains of bacteria. It appears, however, that some bacteriocins are composed of a combination of various proteins or are proteins with either lipid or saccharide components. Bacteriocins produced by Gram-positive bacteria are most likely synthetized at first as prepeptides. Later a leader peptide is separated from the rest of the prepeptide. This leader peptide then exhibits biological activity. According to Anthony P. Pugsley (1984a, b) colicins exhibit two main types of lethal effects. Some of them form channels in the cytoplasmatic membrane, while others inhibit nuclease activity following their entry into sensitive cells. However, in view of the small molecular mass of bacteriocins of Gram-positive bacteria it is assumed that they mostly affect the cellular membranes.

Every kind of colicin is adsorbed onto a receptor molecule of the outer membrane. This phase of attack represents the first part of an interaction with sensitive cells. It appears, though, that many bacteriocins of Gram-positive bacteria possess a relatively small adsorption specificity. It is because the cellular wall of Gram-positive bacteria allows passage of relatively large molecules that bacteriocin attack against Gram-positive bacteria does not really require the need for a presence of bacteriocin receptors as opposed to the outer membrane of Gram-negative cells. Colicin production is always determined by plasmids. As stated earlier, the best-known bacteriocins are colicins. They are produced by certain strains of *Escherichia coli* and related species of the family Enterobacteriaceae. Immediately after contact with colicin, the growth and multiplication of sensitive cells stop. Depending on the general conditions and on the colicin type, in about 20 min the attacked rod-shaped bacteria start to increase their volume and along with their loss of light refraction they assume an ovoid or a spherical shape.

15.4.2 Characteristic Properties of Colicins

Colicins represent a group of antibiotic substances, which are characterized by several common properties:

(a) All of them are either proteins or peptides.
(b) The structure of colicin molecules is coded by genes localized in special plasmids, the so-called Col plasmids.
(c) Colicins are formed during growth of so-called colicinogenic cultures.

(d) Theoretically, each molecule of any colicin is capable of killing one sensitive bacterial cell.
(e) The spectrum of the lethal effect of colicins is limited. Colicin-synthetizing bacteria are immune against the effect, although these bacteria do possess colicin binding receptors, they are not affected by it.

There exist three molecular mechanisms that make bacteria insensitive to a given colicin: resistance, tolerance, and immunity. Each bacterium that does not have a colicin receptor on its surface is resistant to the colicin. Colicin-tolerant describes a bacterium that is not sensitive to it, although it has functional receptors in its outer membrane and can specifically bind the colicin. Tolerance is caused by a loss mutation of the sensitive strain, which blocks some of the stages that follow after the colicin binds to the receptor.

Immunity constitutes the most specific mechanism of non-sensitivity. It emerges when a colicinogenic bacterium produces—along with colicin—a specific low molecular protein of acidic nature, the so called immune substance. The immune substance is capable of binding to the C-terminal region of the respective colicin and forms with it an inactive complex. Some strains of *Shigella sonnei* also have the ability to synthetize colicin.

15.4.3 Yeasts and Zymocins

Some yeasts of certain species or genera can form toxic products, which kill sensitive yeasts of the same species or genus. From Greek, these have been named zymocins meaning zyme = yeast, and from Latin caedere = to kill. Zymocins are also called either killer factors (toxins) or killer proteins. Another proposed term is mycocin as a certain analogy to bacteriocins. The ability of certain yeasts to produce zymocins is referred to as a killer phenomon. Nevertheless, other yeast cells of its own strain are immune to those zymocins. These substances were first detected by M. Makower and E. A. Bevan in 1963 (Bevan and Makower 1963) in *Saccharomyces cerevisiae*. From the point of view of zymocin production *Saccharomyces cerevisiae* is the most intensively studied species. Four different killer toxins have been described in this microorganism, designated as K1, K2, K3, and K28 and coded by double-stranded RNA (ds RNA) of the viruses ScV-L (so called L-virus *S. cerevisiae*), and ScV-M. The killer protein can link to beta-1,6 glucan in the cellular wall of a sensitive yeast cell. No energy is needed for this reaction. The killer protein then penetrates to the plasma membrane. It is assumed that eight molecules of killer protein mutually join together and form a circular octamer that causes a pore in the membrane. This pore impairs the function of the yeast's membrane so much so that various ions and molecules, including those of ATP, can leak out. This leakage actually causes the cell's death. However, it was also discovered that killer strains can be "cured"—to deprive them of their ability to kill sensitive cells. That successful curing can be

achieved either by exposing the cells to cycloheximid or by exposure to the temperature of 40 °C.

It is of interest that zymocins were found only in the wine strains of *S. cerevisiae*. However, their production also has been detected in other genera of yeasts, for example, *Cryptococcus*, *Debaryomyces*, *Kloeckera*, *Kluyveromyces,* and *Pichia*. Zymocins are determined genetically and are formed by yeasts containing in their cells a special virus, the RNA of which codes for their synthesis. It is assumed that the zymocin-forming yeasts hold a certain ecological advantage over other yeasts. Zymocines, or rather the current killer strains, can sometimes influence negatively even classical biotechnological processes. Such instances have been described in beer brewing where the presence of killer strains led to a significant slowdown of the fermenting process and at the same time, there were reports of negative sensory qualities of the produced beer. Experiments designed to examine if the killer strains could prevent contamination by foreign or undesirable yeasts also have been conducted. However, quite a few disadvantages have resulted in this approach not being followed in practical applications.

15.5 Microbial Competition

Competition is a type of interactive association between two microorganisms. There are situations in which both species require the same nutrient or even the very same living space for their existence. Since the nutrient exists in the environment only in a certain quantity, the two microorganisms grow only at suboptimal speed, as they have to share the limited resource. This type of competition is called exploitative.

Then there is interferential competition, which is direct. In many cases, it is provoked by a greater aggressivness of behavior of some organisms, thus preventing the other species from obtaining the necessary amount of a nutrient. It is also possible that one type of organism displaces the other one from their common space. This is a case of an exclusive competition. Furthermore, taken from a different point of view, there can be interspecific competitions occurring between populations of different species, as well as an intraspecific competition between individuals of the same species, within a given study population.

Competition exists among all groups of organisms and it represents an important biological regulatory mechanism in nature. It eliminates the less viable individuals. At the same time it reduces the density of individuals in a given population. It can contribute to the process of speciation, the formation of new and distinct species in the course of evolution.

15.6 Parasitism

Parasitology is a science of human and animal parasites. In a wider sense, some authors consider parasitology to be a part of microbiology. It deals with protozoa, worms, and insects parasitizing on humans or on animals. Furthermore, it describes the life cycles of parasitic organisms, diseases caused by them, and also proposes the pertinent treatment. There are two branches of parasitology, human and veterinary.

15.6.1 Definition of a Parasitic Organism

Parasite is a word derivation from the Greek word parasitos. In parasitism very often a tiny parasite attacks a much larger organism, the host. The parasite lives at the expense of its host, but in general, it does not kill it. On the other hand, predators first kill their victim and only then devour it. Depending on the host, the parasites are classified either as zooparasites or as phytoparasites. Intracellular parasites live in somatic cells or in phagocytes, often causing chronical infections (for instance, bacillar dysentery, brucellosis, and tuberculosis). On the other hand, extracellular parasites are quickly destroyed by phagocytosis and they harm the host only as long as they are outside of phagocytes.

However, there does not exist any clear cut distinction between parasitism and predation, because there are many intermediary forms. The same is true for commensalism and parasitism, where a commensal microorganism can convert to a parasitic relationship.

15.6.2 Ectoparasite and Endoparasite

There are plant parasites as well as animal parasites, and microbial parasites. An equal term for an ectoparasite is an exoparasite. Generally, it represents every organism that parasitizes on the surface of a host's body. On the other hand, an endoparasite lives inside the body of the host. Parasites can be classified as either facultative or obligate. As the name suggests, a facultative parasite can live even beyond its perhaps customary existence with a host organism. However, under certain conditions, it will enter into a parasitic relationship with its specific host. Obligate parasites are absolutely unable to live and multiply without nutrition received from the host organism. Obligate parasitism exists among plants and animals. Also, viruses belong to the category of obligate parasites. Each parasite has a certain spectrum of hosts. That means a parasite always seeks a host, which suits it based upon the parasite's nutritional requirements. Some parasites are highly specialized and therefore they can have just one type of a host. These cases exist, especially with viruses, including phages and also with fungal parasites.

15.6.3 Mycoparasitic Fungi

These attack other fungi. They were first described as early as around the year 1800 by mycologists who were interested in the diseases of plants. Mycoparasitic plants can act as natural enemies of the phytopathogenic fungi that are causative agents of plant diseases. For this reason research of some prospective fungi for the protection of agricultural crops and fruit trees has been going on since the early twentieth century and is still active now.

Some mycoparasitic fungi are biotrophic meaning that they grow on live mycelium of other fungi, and most of those biotrophs belong to the class Zygomycetes. Other mycoparasites are known for necrotrophy, meaning they attack and destroy their hosts. Some mycoparasitic fungi include mitosporic genera *Gliocladium*, *Pythium*, and *Trichoderma* as well as genera from the former class Zygomycetes, specifically the genera *Dicranophora*, *Spinellus*, and *Syzygites*.

Special attention is reserved for the species *Pythium oligandrum*, which differs diametrically from the other species of the genera *Pythium* by its mycoparasitism. It attacks fungi and enzymatically decomposes the mycelia and also some reproductive organs of the attacked fungi, and then uses the products of those enzymatic degradative processes for its own nutrition. From another point of view, the pathogenicity of *P. oligandum* is brought about partly by its direct antagonistic action (= its mycoparasitism) and partly by its formation of antimicrobial compounds. This fact was confirmed by Nicole Benhamou et al. (1999) when *Phytophthora megasperma* was inactivated at distance by *P. oligandrum* without any direct contact with this pathogen. Later, in 2001 Benhamou with her coworker Chantal Garand (Benhamou and Garand 2001) proved that a protein called oligandrin, produced by *P. oligandrum*, causes a systemic resistance of tomatoes against infection by a fungus of the genus *Fusarium*.

However, *P. oligandrum* must possess a certain ability to distinguish between its own cells and the cells of a phytopathogenic fungus. Otherwise, it could destroy its own organism by its cellulolytic enzymes. This question of how this recognition occurs has not been solved yet. Thus, *P. oligandrum* acts as a biological fungicide (biofungicide). Commercial preparations created on the basis of this fungus are already used not only in agriculture for plant protection, but also in medicine for the treatment of various mycotic ailments.

15.7 Predation

Let us imagine a relationship between a predator and its prey. One organism, the predator, violently destroys and then consumes another organism, the prey. This is a common natural event not only in the animal kingdom, but also in amongst microscopic organisms. On the microscopic level, the predators of bacteria are, for example, myxobacteria, protozoa, and some fungi. For instance, a flagellate of the genus *Ochromonas* of the family Chromulinaceae consumes not only whole bacteria, but also yeast and algae. Bacteria also constitute a frequent source of nutrition for

Infusoria, which is a collective term that describes minute freshwater aquatic pond creatures such as ciliates, euglenoids, protozoa, unicellular algae, and small invertebrates.

Theoretical models of predator–prey relationships were suggested in 1925 by the American biophysicist Alfred J. Lotka (1925), with an example being his publication "Elements of Physical Biology" (Lotka 1925). An Italian mathematical biologist Vito Volterra (1926) also dealt with a similar subject. Those efforts led to a finding that the interaction between predator and prey has a regular (cyclical) fluctuation.

An interesting microbial relationship has been described between the ciliate *Paramecium bursaria* and a yeast *Schizosaccharomyces pombe*. This biocenosis was studied in laboratory conditions and a periodical oscillation of representatives of both species was noted. An increase in the number of predator cells in that mixed species population typically leads to a fast reduction in the cell number of the prey population, i.e., the yeast. That consequence greatly lowers the nutrition availabile for the *Paramecium,* stops development of the *Paramecium* population and the number of *Paramecium* organisms then drops significantly. That reduction in predator population can possibly lead to an increase of the number of yeast cells and thus the whole situation would cyclically repeat.

In other microbial populations, a certain balance between the predator and the prey populations develops. The numbers of both species then do not periodically change and instead stay at relatively constant levels. The situation in the rumen of ruminants is quoted as an example of such balance. The lysis of intact bacteria by the action of myxobacteria having the ability to secrete extracellular enzymes is sometimes also presented as a predation, as previously mentioned.

15.7.1 Bdellovibrio bacteriovorus *as a Predator Bacterium*

From Greek bdella = leach and from Latin vibrare = tremble. This predator bacterium was described in detail by Heinz Stolp and Mortimer P. Starr in 1963 (Stolp and Starr 1963). It is a tiny, curved, rod-shaped, strictly aerobic bacterium, about 0.3–04 × 0.8–1.2 μm in size. It obtains energy by oxidation of amino acids and acetate via the citric acid cycle. It features a biphasic life cycle. The cycle is characterized by an alternating nongrowing phase, with a free-living predator phase in which the organism reproduces. Although *Bdellovibrio bacteriovorus* has only one flagellum, it is very mobile in the water environment. In one second it can cover a distance equal to 100 times its length, while *E. coli*, for instance, at the same time travels over a distance of only ten times its length. Parasitic strains of *Bdellovibrio bacteriovorus* have a unique ability to parasitize some bacteria and they attack only Gram-negative bacteria.

Bdellovibrio bacteriovorus penetrate into the periplasmatic space of their target, between the cellular wall and the cytoplasmatic membrane. Within this space, they use the host cell as a substrate for *Bdellovibrio* development and multiplication. Gram-positive bacteria lack the periplasmatic space and thus Gram-positive bacteria cannot be infected by *Bdellovibrio*. Penetration of the cellular wall of the host

bacteria by *Bdellovibrio bacteriovorus* occurs by enzymatic action of this predatory bacterium, whereby *Bdellovibrio* enzymes open up a hole in the wall. Through these holes *Bdellovibrio* enters the host and settles down in the periplasmic space. Following initiation of the infection, the host bacterium turns into an osmotically stable spherical bdelloplast. *Bdellovibrio* elongates up to 20 times its original length within that bdelloplast and this very long organism then forms cells that are later released into the environment thanks to a subsequent lysis of the infected host cell.

The *Bdellovibrio* developmental process, starting from penetration, takes about 4 h and requires an actively metabolizing host cell. The burst size (the number of progeny per one host cell) depends on the size of the host cell. It varies in amongst microbes from about four *Bdellovibrio* cells with *Escherichia coli* up to about 20 *Bdellovibrio* cells in case of the much larger bacterium *Aquaspirillum serpens.* In amongst microbes there is quite often a rather vague resolution between parasitism and predation. For example, the interaction between *Bdellovibrio* and a sensitive Gram-negative cell is considered by some investigators as parasitism and by others is considered as a predation.

It is worth noting that *Bdellovibrio bacteriovorus* also attacks *Microcystis aeruginosa* and some other cyanobacteria (blue-green bacteria). Such infection of cyanobacteria could be of ecological importance. *Bdellovibrio* are relatively common in nature and can be isolated from contaminated or wastewaters by methods similar to those employed for examination of phages, with samples of water or soil suspensions first filtered through a membrane filter, which holds back larger bacteria and lets through *Bdellovibrio*. The filtrate is then inoculated onto the surface of an agar substrate containing a host strain of bacteria. Following incubation, the *Bdellovibrio* contained in the inoculum form plaques (negative colonies) on the otherwise coherent growth, termed a lawn, of the host bacteria and *Bdellovibrio* can be also isolated from these plaques. The plaques are round transparent spots, in which the host bacteria have been killed by the predatory *Bdellovibrio*.

Bdellovibrio are capable of quite intensively attacking their host in laboratory experiments; however, this parasitic relationship is quite weaker in the natural environment. This parasitism of bacterium by *Bdellovibrio* is partially inhibited by the presence of clay mineral particles, a fact established in 1978 by Margaret Roper and Kevin Marshall (Roper and Marshall 1978). The clay particles possibly form a sort of coating layer around the *E. coli* cells, preventing direct access of the predator *Bdellovibrio* to the host cells. Also, it has been established that even the *Bdellovibrio* can be attacked by specific bacteriophages (Hashimoto et al. 1970).

15.7.2 Myxococcus xanthus

Myxococcus xanthus belongs to Myxococcaceae family, alias Gymnomycota. These microorganisms are known for their slippery mobility. They produce a whole range of antibiotics and lytic compounds. Live host cells that they attack are killed by antibiotic substances secreted by the *Myxococcus* and then the host cells lyse by the action of a group of extracellular enzymes (Keane and Berleman 2016) mostly

composed of proteases, lipases, and nucleases. It is easy to cultivate these bacteriolytical myxobacteria on the surface of mineral agar substrates. It was established that *Mycococcus xanthus* uses genetically determined chemotaxis to attack the prey and that the predation is stimulated by a close contact with the prey's cells. The myxobacteria also predate on cyanobacteria, as described by C. Burnham et al. (1981), he experimented with a cyanobacterium of the genus *Phormidium* and *Myxococcus xanthus*.

In general, myxobacteria are quite effective antagonists for phytoplankton, because they limit phytoplankton multiplication. Predation has been studied in detail for higher animals but studied relatively rarely in prokaryotes. It is assumed that predation could also have led to the origin of eukaryotic cells. In addition, there also exist cellulolytic myxobacteria that decompose plant polysaccharide cellulose. However, they are very hard to cultivate.

15.7.3 Protozoan Predation

Protozoa prey on bacteria and this activity plays a very important role in the regulation of bacterial populations in water environments, specifically in a quantitative limitation of bacterial numbers. Agneta Andersson and her coworkers (Andersson et al. 1986), and Patricia Menon along with her coworkers (Menon et al. 2003), estimated that protozoa (inclusive of flagellates and ciliata) can kill up to 90% of autochthonous and allochthonous bacteria in fresh water and seawater ecosystems. The predation speed depends on the temperature and on the very nature of the predation population. The speed of predation grows proportionally with the temperature between 12 and 22 °C. Often, Gram-positive bacteria are predated upon less than are the Gram-negative ones.

Amoebas belong to the broad group of organisms known as *Rhizopoda* and, more recently, to the group Tubulinea. They are one-cell eukaryotes that consume microbial prey by phagocytosis and at the same time they play an important role in microbial nutritional chains. Amoebas are natural grazers of cyanobacteria and for their growth they require presence of heterotrophic bacteria as the source of cobalamin, i.e., vitamin B_{12}. The best-known species include *Amoeba proteus* and *Amoeba leningradensis*.

15.7.4 Carnivorous Fungi

There are predatory fungi that can catch microscopic organisms, for instance, nematodes. The prey microorganism is caught and killed in loops of the mycelia. The fungus then sucks nutrients out of the prey. Some of the species and genera of carnivorous fungi include *Zoophagus insidians*, *Zoophagus pectosporus*, *Stylopage*, *Zoopage*, and also *Harpella* as well as *Arthrobotrys*. They can be found in the soil, in decomposing plant material, and in water. Their prey can be nematoda or *Rhizopoda*,

especially amoeba. The predatory fungi can live parasitically either on their host's surface or inside the host's body.

However, predation is not the only means of nutrition used by carnivorous fungi. If suitable prey is unavailable, these fungi can feed by regular organotrophic nourishment. Much of the research on carnivorous fungi was conducted by C. L. Duddington in Great Britain, with an example being Duddington and Wyborn (1972), and by Charles Drechsler in the USA, who discovered and studied many predatory fungi during the period between 1935 and 1959, with examples of his work being Drechsler (1941) and Drechsler (1934). However, the genus *Zoophagus* was described as early as 1911 by Hermann Sommerstorff (Sommerstorff 1911). Predation in nature is in some respects a positive phenomenon, as it liquidates weak and sick animals that would otherwise perish just the same.

A special type of predation is cannibalism, when animals of the same species devour each other. Another term used to describe this is intraspecies predation. It was established that, for instance, the Gram-positive sporulating bacterium *Bacillus subtilis* does in certain times kill cells of its own species and feeds upon them (González-Pastor 2011).

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Chapter 16
Symbiosis in a Rapidly Changing World

K. M. Oliver and C. H. V. Higashi

Abstract While the intensification and globalization of agriculture and commerce have provided humanity with numerous benefits, they have simultaneously escalated systemic risk to ecological systems. Global climate change, acting in negative synergy with other anthropogenic stresses, is expected to rapidly increase ongoing losses of biodiversity and profoundly redistribute enduring species. This chapter provides an overview of microbial symbiosis in a rapidly changing world. The ability of many multicellular organisms to relocate, acclimate, or adapt to these changes will depend on their microbial symbionts. But for other groups, specialized symbionts may expose acute vulnerabilities that limit climate resilience unless compensating mechanisms are engaged. We review a number of globally relevant symbioses to examine responses to elevated temperature, drought, ocean acidification, and other climate-driven challenges. While several widespread interactions, including mycorrhizal fungi associated with plants, show the clear potential to mitigate specific threats, others, such as photosymbiotic corals and the obligate nutritional symbioses of arthropods are likely to be among the first mass casualties of climate change.

16.1 Introduction: A Critical Contradiction

The Dickens classic, *A Tale of Two Cities*, begins with the famous line "it was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness. . ." highlighting the contradictions of the time surrounding the French Revolution in Paris and London where societal advances occurred in a milieu of revolutionary fervor and state reprisals. While such inherent contradictions have likely been recognizable in most societies at any given age, today this sentiment is playing out on a global scale, where human abundance and their collective actions are running headlong toward the cliff of environmental catastrophe.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_16

On the black side of the ledger, there has arguably never been a better time, as a matter of probability, to be alive as a human. By many metrics, including infant mortality, life expectancy, poverty reduction, dissemination of knowledge, and declines in violence, the human condition has improved substantially over past centuries (Pinker 2018). These gains have been facilitated by the astonishing pace and success of scientific discovery and associated applications including the printing press, engines, vaccines, antibiotics, pasteurization, batteries, electricity, transistors, computers, refrigeration, steel, light bulbs, communications, combine harvester, nitrogen fixation, birth control, molecular biology, and sequencing. Along with other evidence-based solutions, such as sanitation, the green revolution, and disease eradication programs, these efforts have likely saved billions of lives and improved the lives of countless more. Of course, these observations about the human condition are meant as broad strokes, and improvements are clearly not distributed evenly within or among populations, with many critical issues requiring novel and more equitable solutions.

On the red side of the ledger, anthropogenic global climate change (GCC) is set to create unprecedented challenges for humanity and potentially escalate ongoing losses of biodiversity to levels last observed during the biotic crisis 66 million years ago. As many as one million animal and plant species are currently at risk of extinction, with rates of loss expected to accelerate going forward (IPBES 2019). Biodiversity declines, however, are difficult to gauge, in part, because only about 14% of the estimated 9 million extant eukaryote species are known to science. While invertebrate animals represent roughly 80% of described species (and 95% of estimated species), the conservation status of less than 1% is known (Collen et al. 2012). For groups that are better known, including terrestrial vertebrates, population declines and range contractions have been substantial (Ceballos et al. 2020; Ceballos et al. 2017). For example, nearly three billion birds have vanished since 1970 in the United States and Canada (Rosenberg et al. 2019). Drivers of diversity declines include habitat loss and fragmentation, pollution from pesticides and fertilizers, overexploitation, and exotic species introductions; processes largely associated with the intensification and globalization of agriculture. There is a tendency to think that the consequences of GCC will increase gradually over time, but extreme events have already caused the abrupt degradation of some ecosystems (Smale et al. 2019). Forecasting future biodiversity losses is even more challenging, owing to uncertainties in how to model species interactions, evolution, dispersal, and other key biological mechanisms (Urban et al. 2016). Nonetheless, climate-mediated biodiversity declines have been predicted to occur in punctuated waves of die-offs as ecological assemblages could collapse before 2030 in the oceans and begin by 2050 in terrestrial systems (Trisos et al. 2020).

Of course, the expansion of the human footprint and biodiversity declines are not unrelated. Some of the forces, including market-based globalization, argued to foster economic growth and reduce poverty in developing nations, also escalate systemic risk to ecological systems. For instance, global commerce has been built on the back of the combustion engine, together with the ability to extract large reservoirs of carbon-based energy. This carbon was deposited over millions of years as buried

organisms decomposed anaerobically. But in the geological instant from the Industrial Revolution to present, enormous quantities of carbon have been released into the atmosphere threatening to create a hothouse planet. The elevation of near-term economic incentives far above the value of ecosystem services, which humans rely on, and climate considerations, in which humans occupy a surprisingly narrow niche (Xu et al. 2020), has created our age's most critical "contradiction." Essential services that nature provides include air production, sustaining air quality, freshwater availability and distribution, wood fuel, wild food production, sinks for carbon emissions, nutrient cycling and soil conditioning, pollination, coastal storm surge protection, biological pest control, plant-based medicines, and the physical and psychological benefits of natural spaces.

Climate models have been remarkably accurate since the early 1970s (Hausfather et al. 2020). Hence, the deployment of science and reason has not only accurately described the threat for 50 years, but provided a variety of strategies likely to mitigate risks (Riahi et al. 2017). Yet responses by the major carbon emitting nations have been grossly inadequate, hampered by a suite of countervailing forces. First, the tools of reason and science are hard to bring into play when they conflict with the interests of power structures. But proposed climate solutions directly threaten the lucrative economic models of the energy sector and its many interconnected industries, which often exert outsized influence over government policies (Gilens and Page 2014). Second, science does not deal in certainties, opening the door for "merchants of doubt" to sow confusion and craft messaging that impedes evidence-based solutions (Oreskes and Conway 2010). Third, GCC is a "tragedy of the commons" problem (Hardin 1968). Individual countries are motivated to act self-interestedly with respect to the shared atmosphere, and may avoid acting for the common good unless all major polluters follow suit in a verifiable manner with sanctions for moochers. Fourth, the large expenditures of monetary and political capital required at present to mitigate damage will not show observable dividends for decades (Samset et al. 2020). One effect is that major factors influencing carbon emissions remain heavily biased to their continued use. For example, despite decades of convincing evidence for GCC, fossil fuels remain grossly underpriced. A 2019 International Monetary Fund (IMF) working paper estimated global fossil fuels subsidies at US$5.2 *trillion* in 2017, which represents 6.5% of global gross domestic product (Coady et al. 2019).

16.1.1 Out of the Frying Pan into the Fire

The 2015 Paris Agreement represents the latest global effort to address greenhouse gas emissions. While it is promising that most of the 196 member countries have ratified the accord, few have hit climate targets and the lack of enforcement mechanisms, suggests that at least for the near future, global mitigation efforts will remain patchy at best. The most aspirational goal of limiting warming to 1.5 °C above pre-industrial levels (Relative Concentration Pathway (RCP) 1.9) is essentially

fantastical; net greenhouse emissions would need to be cut by 50% *this* decade and 100% by around 2050. This would require a transformation of the global energy system at a nearly unimaginable pace and scale. While a 1.5 °C increase itself looks dire, losing, for example, 70–90% of coral reefs, effects get significantly worse across all metrics with increases of 2.0 °C and beyond (IPCC 2014, 2018). The present "base case scenario" (RCP 2.6), where temperatures are potentially kept below 2.0 °C, also requires large near-term cuts and net-zero by 2075. The "business as usual" (RCP8.5) scenario leads to a 5 °C increase by 2100, but continued market-based transitions away from more polluting fuels are predicted to result in "only" a 4 °C increase without other major efforts. The most likely scenario, involving modest mitigation, leaves the planet warming to somewhere between 2.5 and 3.0 °C by 2100 (IEA 2019). However, these estimates do not include amplifying feedback mechanisms, including the increasing probability of forest fires, or breaching irreversible "tipping points" such as the thawing of permafrost (Lenton et al. 2019). The Arctic is warming twice as fast as the global mean increasing odds of the latter (NOAA 2020). Even if CO_2 emissions were abruptly and completely halted, the global mean temperature will increase for decades, sea levels will rise for centuries, and the planet would not return to pre-industrial temperatures for thousands of years (Zickfeld et al. 2013; Mauritsen and Pincus 2017).

Whatever the final amount of warming, effects will be dramatic, punctuated, and unevenly distributed across the planet. Many human populations face dire near-term consequences, including those inhabiting low-lying islands and coastal regions exposed to flooding, those dependent on vanishing meltwater, or those where the combination of heat and humidity will soon make outdoor work, then existence untenable. New models suggest that in the next 50 years, under the best-case scenario (RCP 2.6), 1.2 billion people will no longer live within the "human comfort niche" where humans thrived for the past 6000 years (Xu et al. 2020). Along with increasing probabilities of extreme weather events (Meehl and Tebaldi 2004) and threats to food supplies (Ukkola et al. 2020; Dahlke et al. 2020; Costanza et al. 2014), these changes are certain to result in massive population displacements.

Yet, it is not just humans who will be on the move, GCC will impose a "universal redistribution of life on Earth" (Urban 2015; Pecl et al. 2017). The extent of relocation will depend on the timing and extent of human responses to GCC and continued rates and distributions of habitat loss along with other anthropogenic stresses. Of course, some organisms have flourished under human expansion. These include *Coffea arabica*, which has vastly increased in range and abundance since human discovery, although GCC is beginning to reverse this trend (Imbach et al. 2017). Also included are many disturbance-adapted organisms, and species exploiting the global commercial infrastructure as a dispersal mechanism. Organisms without such traits, especially those with small endemic ranges, or that flourish only under a narrow range of environmental conditions, have limited options, and are expected to fare less well. For example, some coral reef systems, where major losses are expected no matter what actions are taken, show high levels of endemism in supported communities (Roberts et al. 2002). In terrestrial systems, many plant and arthropod species are being extinguished before being known to science. Not

only is deforestation concentrated in the tropics, where diversity is centered, but tropical ectotherms have narrow thermal tolerances compared to temperate species, rendering them especially vulnerable to warming (Hansen et al. 2013; Deutsch et al. 2008). Tropical plants do not have narrower thermal tolerances, but sit nearer to their thermal limits, making them vulnerable through a different mechanism (Sentinella et al. 2020). In between, are the many species with larger ranges or environmental tolerances, as well as those in relatively protected sites, including those legally protected, unsuitable for development, or in regions, like northeastern North America, which have shifted from net deforestation to reforestation (Rudel et al. 2020). These species will have opportunities to relocate, acclimate, or adapt to the human-reconfigured planet. Along with altering the timing of activity and use of microhabitats, organisms will relocate to higher elevations in terrestrial systems and deeper depths in marine systems, as well as range shifts and contractions toward the poles; some will also respond to GCC with combinations of phenotypic plasticity and adaptive genetic changes (Sgro et al. 2016; Scheffers et al. 2016; Pecl et al. 2017; Parmesan 2006; Hastings et al. 2020).

16.2 Symbiotic Saviors or Achilles Heels?

One possible route to widespread and rapid acclimation and evolutionary change in the face of climate disruption is through symbiosis, defined as the intimate and prolonged living together of dissimilar organisms (Oliver and Russell 2016). Symbionts often confer novel properties on their hosts, including tolerance to a wide range of biotic and abiotic stressors, which may increase odds of survival in a more variable world. On the flip side, the net effects of climate change on organisms are driven by species interactions, often surprisingly complex ones, rather than the reactions of individual species. Hence specialized interactions, including many symbioses, may be particularly vulnerable to climate perturbations (Wernegreen 2012; Renoz et al. 2019; Corbin et al. 2017; Blois et al. 2013).

One challenge in forecasting the roles of microbial symbionts in climate adaptation is that microbes are rarely considered in discussions of climate change, even though temperature is a key determinant of microbial community diversity (Thompson et al. 2017; Cavicchioli et al. 2019). Microbes perform major roles in biogeochemical cycles, including carbon, nitrogen, and oxygen. They have modulated past climates, and in turn, have repeatedly adapted to changing climates. Moreover, bacteria and archaea are exceptionally abundant (estimated at 1.2×10^{30}), occurring, mostly in biofilms, across diverse marine and terrestrial habitats (Flemming and Wuertz 2019). They are also the only known life forms in the vast subsurface of the planet, as well as some aboveground extreme environments, including those that are very hot, cold, or salty, and ones that are highly acidic or basic. Some of these microbes may have exaptations that facilitate eukaryote persistence in the forthcoming world.

Historically, microbes represent the dominant organisms on the planet; life began approximately 4 billion years (Ga) ago and was entirely microbial for the first 2 Ga (Knoll and Nowak 2017). The earliest microbes were likely chemoautotrophs deriving energy from the oxidation of inorganic compounds, and the first ecosystems were anaerobic, with patchy distributions restricted to sources of geochemical energy (Judson 2017). Sunlight was abundant and the evolution of oxygenic photosynthesis 2.7 Ga, preceded by anoxygenic photosynthesis a billion years before, was certainly among the most important evolutionary innovations. Photosynthesis greatly improved productivity and allowed ecosystems to exist independent of geochemical sources. Although it took time, oxygenic photosynthesis led to the oxygenation of the atmosphere (2.3 Ga) and later the oceans. This Great Oxidation Event created the protective ozone layer, and created a plethora of new niches ranging from anoxic to oxygen rich (Judson 2017). Even more impactful, the availability of oxygen as an energy source opened the door to the rise of large, multicellular organisms; a transition enabled by the endosymbiotic acquisition of an alpha-proteobacterium that became mitochondria in eukaryotes (Lane and Martin 2010). These large-bodied organisms themselves became niches for microorganisms, which in turn, provided novel capabilities to their hosts, enhancing existing functions or creating novel ones. For instance, oxygenic photosynthesis likely evolved just once in the ancestors of cyanobacteria, but was shared broadly among eukaryotic lineages via symbiosis, cumulatively representing the bulk of primary productivity across the planet (Venn et al. 2008). In another example, fungi and plants co-invaded the land, with each partner using the other for successful terrestrial establishment, with coevolutionary interactions driving diversity and major evolutionary events in each group (Lutzoni et al. 2018). Increased energy and mobility further created opportunities for predation and parasitism, leading to arms races around the evolution of defenses and counter-defenses that likely drove the diversification of multicellular eukaryotes into “endless forms most beautiful” (Judson 2017; Darwin 2009).

Today, most multicellular eukaryotes interact intimately with microorganisms. For example, tissues exposed to the environment, including animal guts, are typically colonized by microbes, although at highly variable degrees of specificity and specialization (Engel and Moran 2013; Douglas 2014). While gut microbes may not be critical for every species, they often increase the digestive efficiency of hosts by breaking down recalcitrant polymers or toxins, facilitating nutrient and energy uptake, and modulating immune function, often by providing colonization resistance against ingested pathogens (McFall-Ngai et al. 2013; Ley et al. 2008a, b; Hammer et al. 2017; Gould et al. 2018). Climate-induced changes in the abundance, composition, or function of microbiomes have the potential to impact these common phenotypes and hence host fitness. There will be opportunities for microbe-mediated acclimation to GCC, but also the conversion of beneficial or commensal microbes into pathogens (Alberdi et al. 2016). Studies are limited, but heat-stressed individuals have been associated with decreased digestive and immune performance, although it is difficult to show causation because of feedback between host and microbiome (Sepulveda and Moeller 2020).

Across diverse taxa, including those of human hunter–gatherers, gut microbial communities have been shown to fluctuate with seasonality and latitude or altitude either directly through changes in environmental conditions (temperature, water availability) or indirectly due to changes in physiological state (e.g., hibernation) or food supply that occur in association with these changes (Smits et al. 2017; Sepulveda and Moeller 2020). But since gut microbial community composition varies among animal taxa and trophic level, effects of climate-associated variables are also likely to vary among host taxonomy and niche. For example, members of the bacterial phylum Firmicutes often dominate in vertebrate guts and elevated temperatures often reduce, by unknown mechanisms, their abundance across a range of endo- and ectothermic taxa (Sepulveda and Moeller 2020; Fontaine et al. 2018; Bestion et al. 2017). In contrast, the guts of invertebrate animals are typically enriched with members of the Proteobacteria, but these tend to positively correlate with temperature (Sepulveda and Moeller 2020; Moghadam et al. 2018). Thus, while gut associates are certain to be impacted by a warming world, the observed variability renders it difficult to make general predictions about these associations.

Highly specialized symbioses, on the other hand, often share characteristics across taxa that allow for general predictions regarding responses to GCC. Many specialized symbioses are widely distributed across the planet with symbionts performing key roles in the exploitation of particular niches. In marine systems, many animals have symbioses with photosynthetic bacteria or eukaryotes (informally algae) while those that live beyond the reach of sunlight may partner with chemosynthetic bacteria (Venn et al. 2008; Dubilier et al. 2008). In terrestrial systems, most plants are associated with mycorrhizal fungi or nitrogen-fixing bacteria important in nutrient acquisition (Smith and Read 2008), while animals specialized on nitrogen-poor diets have N-fixing, N-recycling, and N-provisioning symbionts (Hansen et al. 2020), those restricted to vertebrate blood have B-vitamin provisioning symbionts (Vogel and Coon 2020), and those consuming difficult to digest plant polymers harbor microbes that help degrade these substances (Wertz and Béchade 2020). There are also symbionts with varying degrees of specialization that confer conditional benefits, including defense against natural enemies, often by the production of toxins (Oliver and Perlman 2020; Florez et al. 2015).

Key climate challenges that specialized symbiotic organisms encounter include increasing temperatures, water variability, extreme weather events, elevated CO_2 levels, ocean acidification, and hypoxia. These are often exacerbated by other anthropogenic stresses, especially habitat loss and pollution. These factors may impact the abundance and distribution of specific symbioses, by, for example, converting mutualistic associations into parasitic ones. Symbiotic organisms may respond to climate change by reorganizing relationships: expelling symbionts, switching symbiotic partners, or acquiring completely novel symbionts. Host and symbiont genotypes may also acclimate or adapt to changing conditions, but the shorter generation times of the latter suggest they may be first responders. Any emerging symbiotic solutions have the potential to be shared widely among macroscopic organisms via horizontal transfer, but since stresses will come in many forms,

multiple symbiont solutions would be required. Also, eukaryotic organisms are already relocating in response to GCC, but for the majority of organisms with environmentally acquired microbes, less is known about the movement of their symbionts. Presumably, organisms that associate with less specific microbes may experience fewer constraints on migration, while mismatches in dispersal ability between plants and more specific symbionts may curtail dispersal ability.

16.3 The Early Results Are in, and Symbiotic Animals Are Not Faring Well

16.3.1 Widespread Losses of Corals

Photosynthetic symbionts are widely distributed across the planet and eukaryote taxa, including animals and fungi, as well as plants and microbial eukaryotes where plastids derive from ancient symbioses (Venn et al. 2008; Palmer 2003; Falkowski et al. 2004). In shallow tropical oceans, where sunlight is abundant, benthic animals have become abundant through photosymbiosis. However, warming oceans pose a general threat to photosymbiotic animals, as many, including corals, sponges, and anemones, expel their photosynthetic symbionts during thermal stress. This phenomenon, called bleaching, is mediated by oxidative stress in one or both partners and often results in the loss of host color (Oakley and Davy 2018).

Now unfortunate icons of the threats posed by GCC, corals are a symbiosis between anthozoan animals (Phylum Cnidaria) and photosynthetic dinoflagellates in the Symbiodiniaceae, such as *Symbiodinium*. Coral reefs, engineered by scleractinian stony corals, are hotspots for production and biodiversity despite covering just 0.1% of the ocean surface (Roberts et al. 2002). Their associated ecosystem services (e.g., coastal protection, food products, and tourism) are valued in the trillions of USD, and sustain roughly 10% of the world's human population (Costanza et al. 2014). However, stress from a variety of factors, including warming and pollution, can convert microbial mutualists into parasites (Vega Thurber et al. 2014; Morris et al. 2019; Hughes et al. 2017a; Fitt et al. 2001; Baker et al. 2018). Since most of the animal's energy is obtained from the photosymbionts, extended periods of loss result in partial to complete coral mortality. Effects of bleaching radiate outward, for example, homogenizing coral-supported communities (Richardson et al. 2018).

In addition to gradual increases in surface temperature, heat waves are causing ever more widespread, severe, and frequent mass bleaching events, limiting corals' capacity to recover (Hughes et al. 2018). Three pan-tropical mass bleaching events occurred between 1997 and 2016 with just 1 °C warming (Hughes et al. 2017b). And the Great Barrier Reef suffered its most widespread bleaching event in 2020; the third event in just 5 years. The latter is especially troubling given that it occurred in the absence of a normally facilitating El Niño event (Stone 2020). Intensifying

storms are also major drivers of coral loss in some reef systems, including the Great Barrier Reef (Lam et al. 2018). More recently, ocean hypoxia, due to warming and nutrient pollution, has emerged as possibly the most imminent threat to corals (Hughes et al. 2020). In the end-Permian biotic crisis, which saw losses of up to 96% of marine species, temperature-dependent hypoxia was likely the major cause of most extinctions (Penn et al. 2018). Ocean acidification also reduces the rate of skeleton formation of stony corals and other organisms that produce calcium carbonate skeletons or shells (Hoegh-Guldberg et al. 2007).

Corals have exhibited limited resilience to these anthropogenic stressors, which act in negative synergy, with responses derived from the animals as well as the symbionts (Carballo-Bolanos et al. 2020; Apprill 2020). Besides temporary switches to heterotrophy (Grottoli et al. 2006), corals can exhibit acclimatization. For example, thermal preconditioning, even without shifts in symbionts, can reduce susceptibility to bleaching (Bellantuono et al. 2012). Can corals adapt? While heritable variation in thermal tolerance in corals has been documented (Dixon et al. 2015), 4–8 year sexual generation times imply a limited capability for rapid adaptation. Instead, resilience is more likely to emerge from partnering symbionts. The environmentally acquired Symbiodiniaceae taxa associating with corals vary considerably in thermal tolerance and hence provide opportunities for symbiont switching. After bleaching events, more thermally tolerant symbionts, including *Durusdinium* species (clade D in the A-I scheme), can replace less tolerant species. This can lead to shifts in the relative abundance of specific community members or complete symbiont replacement (Boulotte et al. 2016). Symbiont switching, however, may offer only a temporary reprieve depending on the amount of warming, or not be an option for some species. For instance, corals living in the Persian Gulf, among the warmest regions, appear inflexible to changing partners, possibly because they already associate with the most tolerant partners (Howells et al. 2020). Moreover, some coral-symbiont associations appear more coevolved, which may make partner swapping a less viable strategy (Tamar 2006; Stat et al. 2009). Since symbionts have much faster replication times than their hosts they are more likely to adapt to rapidly changing conditions. Genetic variations in nutrient and thermal tolerance are known for photosymbionts, and rapid adaptation in the laboratory has been documented (Chakravarti et al. 2017; Bayliss et al. 2019), but it remains unclear whether symbiont adaptation will play key resilience roles in natural systems.

About a quarter of corals are "depth generalists" suggesting some diversity may be maintained in "deep reef refugia" (Bongaerts et al. 2010). Recently, 13% of shallow-reef (<30 m) hard coral species associated with the Great Barrier Reef were unexpectedly found at depths below 45 m (Muir et al. 2018). Similarly, thermal-resistant corals from warm bodies of water may also persist and even be used in ecological restoration efforts (Morikawa and Palumbi 2019). In summary, corals and the diverse biotic and human communities they support are in deep trouble. While symbiont replacement, adaptation or relocation may provide a temporary or partial reprieve, expected atmospheric warming in the coming decades is predicted to result in the loss of more than 99% of the world's reefs (IPCC 2018). Climate-mediated bleaching will similarly challenge other photosymbiotic animals, including already

threatened species, such as the iconic giant clams currently listed as "vulnerable" due to the overharvesting of their decorative shells.

16.3.2 Obligate Symbionts as "Achilles Heels" for Many Terrestrial Arthropods?

Invertebrate animals, especially arthropods, dominate terrestrial ecosystems with respect to diversity, abundance, and ecosystem services. These animals are often infected with specialized symbionts that play key roles in nutrient acquisition and host defense (Oliver and Martinez 2014). Early results indicate that GCC may harm these specialized symbioses in particular, where the loss of function, or of the symbiont itself, may curtail the distribution of the animal host or its resilience to a changing climate. For instance, some insects have highly specialized gut symbionts, such as the southern green stink bug, *Nezara viridula*, which harbors specific bacterial symbionts in midgut crypts required for insect development (Tada et al. 2011). Warming treatments of just 2.5 °C reduced symbiont abundance and host fitness to levels similar to those observed for aposymbiotic bugs; while warming of 5 °C completely eliminated development to adulthood (Kikuchi et al. 2016). Related insects have also been shown to lose their specific crypt-associated gut symbionts with 5 °C increases in temperature (Prado et al. 2010) suggesting this phenomenon may be common for this type of symbiosis.

Most obligate nutritional symbioses, which occur in tens of thousands of terrestrial arthropod species, are even more specialized. For example, most plant sap-feeding, and many blood-feeding insects, harbor maternally transmitted intracellular symbionts that provision amino acids (sap) or B vitamins (blood) that occur in insufficient quantities in their respective diets (Vogel and Coon 2020; Moran et al. 2008; Duron et al. 2018). These associations are typically mutually obligate, with symbionts restricted to host cells (bacteriocytes) and organs (bacteriomes) and displaying sophisticated metabolic integration and regulation (Wilson 2020; Buchner 1965; Baumann 2005). Ancient infection with these symbionts provided numerous arthropod groups with the metabolic machinery to exploit previously unusable niches, within which they subsequently diversified. However, a lifestyle restricted to specific host tissues combined with transmission bottlenecks, which limits effective population sizes, comes with long-term consequences. These include elevated mutation rates and the accumulation of deleterious mutations; processes that result in gene inactivation, loss, and eventually extreme genome reduction (Wernegreen 2017; Moran and Bennett 2014; Bennett and Moran 2015). For instance, one general pattern is that the large majority of host-restricted organisms, obligate symbionts included, show degenerated aminoacyl-tRNA synthetase domains, which can lead to inaccurate translation resulting in a global decline in protein quality (Melnikov et al. 2018). Not only have obligate symbionts lost

specific pathways that might provide climate resilience, but those retained show reduced function, especially under stressful conditions.

Take aphids, a group of ca. 5000 phloem-feeding insects, which have a 100+ million-year association with the bacterium *Buchnera aphidicola* and show genomes at varying degrees of degradation (Chong et al. 2019). Many *Buchnera* proteins show reduced thermal stability compared to free-living relatives and the symbiont must compensate for this via the constitutive expression of heat-shock chaperones (DnaK/GroEL) that prevent protein misfolding (van Ham et al. 2003; Fares et al. 2002). These findings suggest that *Buchnera* limits the thermal tolerance of their hosts, which has been hypothesized to at least partially explain why aphids are largely restricted to the temperate northern hemisphere (Perkovsky and Wegierek 2016). Indeed, a recent study finds that aphid species show variation in responses to heat stress and a key determinant of aphid fitness is the heat sensitivity of *Buchnera* (Zhang et al. 2019). Even within aphid species, a single bp change in the promoter of the *Buchnera* small heat shock protein IbpA can result in dramatic changes in aphid and *Buchnera* fitness at varying temperatures (Zhang et al. 2019; Dunbar et al. 2007). Heat also negatively impacts nutritional symbionts in ants (Fan and Wernegreen 2013), psyllids (Hussain et al. 2017), roaches (Sacchi et al. 1993), weevils (Heddi et al. 1999), and whiteflies (Shan et al. 2017).

Most studies documenting the effects of temperature on obligate symbioses have used brief exposures to high temperatures (i.e., heat shocks). While studies investigating how long-term exposure to warmer mean temperatures will impact obligate nutritional symbionts are needed, heat shock experiments are also informative as climate models predict more frequent, intense, and longer maximum temperatures (Meehl and Tebaldi 2004). Depending on the availability of microclimates and behavioral responses, tolerance to "new" extremes and temperature variability will likely be key determinants of the future ranges and viability of arthropods with obligate symbioses (Woods et al. 2015; Vasseur et al. 2014; Sunday et al. 2014).

Hence, unless compensating mechanisms are employed, obligate partners are likely to serve as an "Achilles heel," turning hundred-million-year-old mutualists into parasites. In contrast to marine organisms, switching to more thermally stable obligate symbionts is less likely to be a viable general strategy in terrestrial arthropods, in part due to transovarial, rather than environmental, transmission. And, in general, obligate symbiont switching is rare (Bennett and Moran 2015). Intriguingly though, the few aphid groups that have lost *Buchnera* and switched to different symbionts (bacteria or fungi) reside in tropical and subtropical regions suggesting switches may have been temperature related (Fukatsu et al. 1994; Chong and Moran 2018; Buchner 1965). For obligate symbionts in terrestrial systems that are environmentally transmitted, symbiont swapping may be a viable tactic. For instance, herbivorous leafcutter ants inhabit low-to-mid-elevation tropics owing to dependence on a cold-sensitive *Attamyces* fungus, which they have cultivated for millions of years as a food source. However, their northernmost limit is expanded by behaviorally modifying fungal garden depth in conjunction with using cold-tolerant fungal strains (Mueller et al. 2011).

16.3.3 Facultative Symbionts May Mitigate Damage to Obligate, Nutritional Symbionts

Insects with bacteriocyte-associated obligate symbionts are frequently also infected with facultative heritable symbionts, which while not required for host reproduction often provide conditional benefits (Oliver et al. 2010; Moran et al. 2008; Buchner 1965). In pea aphids, the facultative symbiont *Serratia symbiotica* protects against thermal stress, possibly by lysing and releasing protective metabolites that stabilize *Buchnera* (Russell and Moran 2006; Burke et al. 2010). Specific strains of other pea aphid facultative symbionts, including *Regiella* and *Fukatsuia* have also been reported to confer thermal tolerance by supporting *Buchnera* (Heyworth et al. 2020). That thermal tolerance is also conferred by heritable *Rickettsia* symbionts in whiteflies (Brumin et al. 2011) suggests this phenotype may be widespread in sap-feeding insects. However, more work is needed in understanding the mechanisms underlying thermal tolerance and its importance in natural populations. In other cases, facultative symbionts are known to supplement the failing functions of degraded obligate symbionts, often via the provisioning of B vitamins and, in the process, becoming incipient co-obligate symbionts (Russell et al. 2017; Monnin et al. 2020; Meseguer et al. 2017). These newly dependent symbionts potentially provide additional functions, including tolerance to climate-associated stresses. Genome fragments of functional significance may also move among symbionts or from symbionts to the host genome (Manzano-Marín et al. 2020; Husnik and McCutcheon 2018). If these retain function after transfer, then these too potentially provide resilience.

16.3.4 Protection Services by Facultative Symbionts May Fail in a Warmer World

In addition to interacting with obligate symbionts, facultative heritable symbionts are also associated with mediating diverse ecological interactions. For example, numerous facultative symbionts, inhabiting diverse insect systems, provide protection against pathogens, parasites, and as noted above, thermal stress (Oliver and Moran 2009). The genomes of facultative symbionts are typical of intermediate size between obligate symbionts and free-living relatives and retain capabilities for multiple functions, variable tissue tropism, occasional horizontal transmission, mediating competition with other microbes, and other features associated with navigating a host-restricted lifestyle (Moran et al. 2008). Unlike obligate symbionts, infections are not typically fixed within a population, and specific infections can be dynamic over space and time. Variable infection frequencies create the potential for identifying correlations between symbiont prevalence and climate variables, such as temperature (Morag et al. 2012; Doremus et al. 2018; Charlesworth et al. 2019). Temperature has also repeatedly been found to impact within-host abundance of

facultative symbionts, which may affect their function or transmission rates (Corbin et al. 2017).

Early results show that temperature is a key determinant in the success of symbiont-mediated defenses. For example, a common aphid symbiont, *Hamiltonella defensa*, confers resistance against hymenopteran parasitoids (Vorburger 2014; Oliver and Higashi 2019). The likely mechanistic basis for these anti-parasitoid defenses are eukaryotic toxins encoded on a bacteriophage called APSE, with variable levels of protection depending on the strain (Oliver et al. 2009; Degnan and Moran 2008; Brandt et al. 2017). However, symbiont protection across a range of *H. defensa*/APSE strains has been shown to fail under modest increases (+5–7 °C) in temperature (Doremus et al. 2018).

Most studies investigating the effects of temperature on facultative symbioses have used either heat shocks or increases that are ≥5 °C above controls and held at constant temperatures or under diurnal regimens. However, a recent study examined the effects of just a 2.5 °C increase, consistent with near-term IPCC predictions, on the anti-parasitoid defensive symbiosis in pea aphids (Higashi et al. 2020). This study also used treatments that mimicked daily fluctuations at the site of aphid collection by cycling between average daily lows and highs, and varied the timing of warming because models and empirical findings indicate that nighttime temperatures are increasing faster than daytime temperatures (Davy et al. 2017). Ectothermic animals may respond differently to daytime and nighttime warming as the former moves organisms nearer to their thermal limits and results in greater daily temperature variation, while night warming minimizes these, and may even relieve constraints of cooler nighttime temperatures (Speights et al. 2017). However, the aphid study found that regardless of the timing of warming (uniform vs. day vs. night) an increase of just 2.5 °C was enough to substantially decrease symbiont-based protection (Higashi et al. 2020). Surprisingly, the daily maxima for the no-warming control and nighttime-warming treatments were identical, indicating that higher temperature *per se* was not the cause of symbiont failure. Also, while outcomes of host–parasite interactions are often mediated by temperature (Thomas and Blanford 2003), here only symbiotic organisms were harmed by warming. If this pattern holds for other protective *Hamiltonella*, then thousands of aphid species may be more vulnerable to common enemies as the planet warms.

One caveat is that insect symbionts, including aphid defensive symbionts, are primarily studied in laboratory settings. In the field, many factors, both selective and nonselective, can influence symbiont dynamics (Oliver et al. 2014). While *Hamiltonella* has been shown to confer defensive benefits in the field (Smith et al. 2015; Rothacher et al. 2016; Ives et al. 2020), protective benefits do not appear to be the only force underlying their prevalence across populations (Smith et al. 2015). A recent longitudinal field study found that warmer temperatures, not parasitoids, were the best predictor of *Hamiltonella* infections throughout a sampled season (Smith et al. 2020). Some *Hamiltonella* strains have been shown to confer tolerance to heat stress, which may be the basis for symbiont persistence in this study (Russell and Moran 2006; Doremus et al. 2018). While apparently less common than symbiont-based defenses, some pea aphid clones are endogenously resistant to parasitoids, yet

temperature does not affect this mode of resistance (Martinez et al. 2014; Doremus et al. 2018). With warming, there may be selection for aphid clones, especially those with endogenous anti-parasitoid protection, that carry thermally protective strains.

Another common protective symbiont in insects is *Spiroplasma*, which protects *Drosophila* flies against parasitic nematodes and parasitoid wasps via the production of variable RIP (ribosome-inactivating protein) toxins (Xie et al. 2010; Jaenike et al. 2010; Hamilton et al. 2016; Ballinger and Perlman 2017). A recent study found that *Spiroplasma* protection against parasitoids performs better at cooler temperatures (18 °C) compared to 25 °C (Corbin et al. 2020). Temperatures above 25 °C, however, were not examined in this study, and especially cool temperatures were not examined in the aforementioned aphid studies; hence optimal symbiont performance may occur within a system-specific range of temperatures. In protective symbioses that use toxins to harm parasites (Oliver and Perlman 2020), production of defensive compounds could be modulated by temperature directly, or indirectly through the suppression of bacterial abundance. Alternatively, toxins are often heat-labile, and hence may only function properly within a range of temperatures.

The heritable symbiont *Wolbachia* represents the most common and widespread symbiont on the planet, infecting an estimated 52% of arthropods (Weinert et al. 2015). While long recognized as the "master manipulator" of host reproduction as a means of infecting new hosts (Werren et al. 2008), some strains also confer protection against pathogens, especially viruses (Martinez et al. 2017; Hoffmann et al. 2015; Hedges et al. 2008). The basis for pathogen protection is unclear but *Wolbachia* infection may modulate innate immune function, or compete with pathogens for limiting nutrients (Yin et al. 2020). Given that the innate immune function of hosts can vary with temperature (Murdock et al. 2012), temperature variation may impact *Wolbachia*-mediated pathogen resistance via changes in hosts or symbionts. A recent study found that across a range of doses, *Wolbachia* protection against an RNA virus (*Drosophila* C virus) was stronger at 18 °C compared to 25 °C (Chrostek et al. 2020). In this study, higher temperatures increased viral loads, but not *Wolbachia* titers; so higher viral replication, rather than more effective symbiont protection may partially explain the observed results. Interestingly, preinfection assays showed the inverse; flies reared at 18 °C were not protected by *Wolbachia*, while those developing at 25 °C received protection (Chrostek et al. 2020). In the latter assay, viral loads were not affected by treatment, but *Wolbachia* titers were 33% lower when reared at cooler temperatures.

The combination of anti-pathogen activity and reproductive manipulation by *Wolbachia* has emerged as a promising approach to control arthropod-vectored diseases, such as dengue (O'Neill et al. 2019; Nazni et al. 2019). One reproductive manipulation, called cytoplasmic incompatibility (CI), occurs when *Wolbachia*-infected males mate with *uninfected* females and the resulting offspring are not viable. This increases the fitness of infected females relative to uninfected females, leading to the spread of the symbiont, and any accompanying anti-pathogen traits, into the target insect population. These approaches typically involve artificially introducing anti-pathogen strains from *Drosophila* into vector species, such as mosquitos. Hence it is important to distinguish between natural and engineered

associations when generalizing. While engineered vectors typically show reductions of pathogen infection or transmission, variation in phenotypes, infection costs, and transmission rates have been observed, which can arise from any of the interacting participants (Ross et al. 2017; Ross et al. 2019b). But since higher temperatures and heat shocks generally suppress both the strength of manipulative phenotypes and the transmission efficiency (Corbin et al. 2017), warming may limit the ability to drive pathogen-suppressing symbionts into vector populations, especially in tropical regions where these diseases cause the most harm. Studies investigating the effects of temperature on pathogen blocking strains are limited, but one study has shown that the loss or retention of the driving mechanism (CI) under field conditions depended on symbiont strain (Ross et al. 2019a). Hence, identifying microbial strains that are robust to warmer temperatures will be important to the successful deployment of symbiont-mediated disease suppression as heat waves and ever-increasing mean temperatures may work to erode their efficacy.

In natural systems, the effects of warm temperatures on anti-pathogen traits and manipulative phenotypes may generally limit the spread of *Wolbachia* into warmer regions and at warmer times of the year. *Wolbachia* infection frequencies are highly variable in natural populations, but generally appear to be more common in cooler, temperate regions (Woodhams et al. 2020; Sazama et al. 2019; Charlesworth et al. 2019). Another widespread reproductive manipulator, *Cardinium*, in contrast, shows positive correlations with surface temperature, including in Culicoides biting midges that are important disease vectors (Morag et al. 2012; Charlesworth et al. 2019). However, specific strains appear to perform better under cooler conditions. In the parasitoid, *Encarsia suzannae*, warmer temperatures reduced *Cardinium* densities and the strength of CI, while cooler temperatures strengthened CI even though symbiont densities were also reduced (Doremus et al. 2019).

In total, these studies show that temperature has large effects on the phenotypes of facultative symbionts. Given the importance of these animals as medical vectors and agricultural pests, it is critical to understand how GCC will impact these symbioses. Early results indicate that thermal stress is likely to generally reduce the strength of symbiont-conferred phenotypes, which may impact host fitness, abundance, and distributions across diverse systems, with effects that reverberate through food webs. On the hopeful side, facultative symbiont genomes encode diverse bioactive factors, and climate variables may select for resilience-conferring phenotypes. The presence of these factors on mobile elements, including bacteriophages, indicates their potential to be shared within and among symbiont lineages (Touchon et al. 2017; Lynn-Bell et al. 2019). The further ability of the symbionts to move horizontally among host lineages creates opportunities for the widespread transfer of climate-mitigating traits across this hyper-diverse group (Oliver et al. 2010).

16.4 The Responses of Photosymbiotic Organisms to Climate Change Will Be a Key Determinant of the Future Diversity and Distribution of Life

In addition to their presence in numerous animals, photosymbioses also occur in seaweed and phytoplankton, the latter including diatoms and dinoflagellates, as well as nonsymbiotic cyanobacteria. As noted above, photosymbiotic corals are bleaching in response to anthropogenic stresses with negative effects on the communities they support. But production by corals pales relative to that of phytoplankton, which globally, represent roughly 45% of net primary production, despite representing less than 1% of biomass (Field et al. 1998).

Plastid-bearing phytoplankton occupy shallow waters (<200 m) and have high turnover rates across a massive surface area. As such, they exhibit the potential for rapid responses to climate variation on a global scale, which is expected to impact energy transfer throughout food webs (Winder and Sommer 2012). And as major sinks for carbon, changes in their abundance and species composition affect biogeochemical cycling that further impacts climate (Sabine et al. 2004). Hence, understanding the fate of phytoplankton in our warming and acidifying oceans is critical.

Phytoplankton abundance and distribution are determined largely by ocean upwelling and circulation with growth rates depending on sea surface temperature, nutrients, light availability, and species interactions. While predictions are complicated, surveys using satellite-based ocean color sensors show consistent, climate-tracking patterns, including shifts in the magnitude, timing, and length of seasonal blooms (Winder and Sommer 2012; Racault et al. 2012; Friedland et al. 2018; Behrenfeld et al. 2006). While some studies report a decline in global phytoplankton since the 1950s (Boyce et al. 2010), this is not a consensus view (McQuatters-Gollop et al. 2011). Warming enhances ocean stratification, which in turn reduces the supply of limiting nutrients, resulting in regional biomass reductions. In tropical waters, for example, warming produces delayed winter blooms that occur over shorter durations and with reduced biomass (Gittings et al. 2018). Marine heat waves, and associated stratification, can decrease local production by phytoplankton and result in widespread losses of productive habitats, such as kelp forests (Smale et al. 2019). Another factor that may decrease phytoplankton abundance in warm waters is that consumers (e.g., viruses and grazers) often increase in biomass relative to producers and hence can exert top-down control on the phytoplankton population (O'Connor et al. 2009).

In addition to warming, the oceans have absorbed about 31% of anthropogenic CO_2, causing ocean acidification; pH is down 0.1 unit since the Industrial Revolution, and predicted to drop 0.3–0.4 more units by 2100 (Hurd et al. 2018; Gattuso et al. 2015). Rising CO_2 levels in the oceans intuitively lead to increases in primary production via phytoplankton. However, this capacity is variable within and among taxonomic groups, and overall, increases in photosynthesis appear slight at best, constrained by limiting nutrients and other stresses (Mackey et al. 2015). Furthermore, some phytoplankton, including diatoms, are negatively impacted by

acidification. Diatoms have unique cell walls made from silica and are responsible for 25–45% of ocean primary productivity and export roughly 40% of particulate carbon to the deep ocean for storage (Boyd et al. 2019). Ongoing acidification, however, hinders their ability to form strong shells making them smaller and less effective at carbon sequestration (Petrou et al. 2019).

Looking forward, increased rates of melting of sea ice means more sunlight penetrating ocean waters in the Arctic. This will lengthen the growing season of phytoplankton at high latitudes, and lead to higher phytoplankton biomass, which in turn will further warm surface waters by pigment-induced changes in radiant heating, triggering additional positive feedbacks (Park et al. 2015). In response to increasing temperatures and changes in circulation, entire communities of common cool-adapted phytoplankton are projected to move poleward before 2100, which may have large effects on food webs (Barton et al. 2016). Assemblages of foraminifera (zooplankton), which live in darker, cooler waters, have already moved poleward relative to pre-industrial distributions (Jonkers et al. 2019). In addition to relocation, some species have been shown to adapt to modest increases in temperature and acidification, but often with costs, such as smaller, carbon-depleted cells (Schlüter et al. 2014; Irwin et al. 2015). Acidification may also alter competition among phytoplankton species, as well as antagonistic interactions. For example, acidification may select for nuisance species, such as toxic microalga over those providing ecosystem services, or impact the composition of viruses attacking phytoplankton, which are key factors controlling blooms (Riebesell et al. 2018; Highfield et al. 2017). Continued inaction leading to sustained warming may result in changes in wind patterns, water temperatures, ice cover, and circulation that would transfer nutrients from surface waters into the deep ocean, starving current ecosystems (Moore et al. 2018).

In terrestrial systems plant cells contain chloroplasts, photosynthesizing organelles of endosymbiotic origin that facilitated the dominance of this group with respect to biomass and primary production (Bar-On et al. 2018). Vegetation and soils are major carbon sinks, absorbing roughly 25% of anthropogenic CO_2. Plant communities also perform key roles in oxygen, nitrogen, and water cycles, and strongly impact the diversity and distribution of land animals. The thermal tolerances of plants are governed by biogeographic and evolutionary histories as well as local environments and species interactions, and are generally predicted to become increasingly threatened by GCC (Lancaster and Humphreys 2020).

Ecological research networks, synthesizing long-term vegetation data, show strong effects of GCC on primary productivity, carbon flux, and advancing spring phenological events (Franklin et al. 2016). Shorter scale analyses show rapid shifts in plant distributions, mostly to higher latitudes and elevations (Kelly and Goulden 2008; Corlett and Westcott 2013). Changes in land use, and associated effects on succession dynamics, have also reduced the resilience of forest communities (Franklin et al. 2016). While growth is increasing in forests that are temperature limited, widespread mortality is also increasing due to "hot droughts" where warmer temperatures extract water from plants even without net changes in precipitation; conditions that also contribute to wildfires and insect-mediated disease outbreaks

(Overpeck 2013). As with phytoplankton, increased CO_2 can contribute to enhanced growth in plants, called CO_2 fertilization, which if widespread would exert mitigating effects on GCC. However, forests appear limited in their ability to use the excess CO_2. Carbon intake depends on nutrient and water availability, and changes in these factors are predicted to slow carbon sequestration going forward (Jiang et al. 2020; Green et al. 2019).

In addition to the domesticated chloroplasts, virtually all plant species interact with microbes that retain at least some independence. Many of these emerge from the soil microbiota, which itself is highly diverse and plays key roles in climate feedback via the cycling of soil organic carbon and other nutrients; although uncertainty remains regarding whether soils will ultimately be a sink or a source of greenhouse gasses in the future (Jansson and Hofmockel 2020). Warming can change the composition of soil microbiota, favoring specific bacterial species or fungi, which in turn can influence system function (DeAngelis et al. 2015; Classen et al. 2015). The rhizosphere, the soil zone surrounding plant roots, contains a number of plant beneficial or conditionally beneficial microbes that play important roles in mitigating drought, nutrient, and thermal stress and may enhance plant fitness in climate-affected soils (Mendes et al. 2013; Jansson and Hofmockel 2020).

The most widespread plant symbioses involve their mostly obligate associations with mycorrhizal fungi (MF). About 90% of plants receive MF-mediated benefits such as water, nutrients, or protection in exchange for organic carbon derived from plant photosynthesis. Four types of MF are recognized (Tedersoo et al. 2020; Steidinger et al. 2019; Brundrett and Tedersoo 2018): (1) Arbuscular endomycorrhiza; AMF are found in roughly 78% of plant species, are common in warm tropical forests, and produce structures called arbuscules that form in root cortex cells and facilitate nutrient, especially phosphorous, transfer. (2) Ectomycorrhiza; EcMF occur in only 2% of (mostly) woody plant species yet occupy the roots of nearly 60% of tree stems, especially those in nitrogen-poor boreal and temperate forests. EcMF are characterized by a Hartig net of in-growing hyphae that penetrate the root epidermis and cortex mobilizing nitrogen and other nutrients. (3) Orchid MF are found in the largely epiphytic Orchidaceae (ca. 10% of plants) and are characterized by coiled structures called pelotons that colonize root cells. (4) Ericoid MF occur in acidic soils associating with 1.4% of plants in the Ericaceae (heaths) and characterized by hyphal coils in root epidermal cells. Vegetation with MF store about 350 gigatons of carbon globally, mostly by AMF and EcMF, compared to 29 GT in non-MF plants (Soudzilovskaia et al. 2019).

Belowground, extensive networks of MF connect conspecific and heterospecific plants facilitating nutrient transfer among plants (Tedersoo et al. 2020). Remarkably, up to 40% of the carbon in the fine roots of any given individual plant can be obtained from the photosynthetic products of its neighbors (Klein et al. 2016). Intra- and interspecific plant communication, such as warning signals and kin recognition, also occurs through MF networks (Tedersoo et al. 2020). Together, nutrient exchange and communication influence plant–plant interactions and community dynamics, and by increasing the number of trading partners, the costs of nutrients can be lowered while increasing the stability of the association (Wyatt et al. 2014;

Argüello et al. 2016). Cross talk between plants through MF networks can also assist recovery and succession of forests following disturbance events (Song et al. 2015).

The composition of mycorrhizal types is increasingly recognized as an important factor in biogeochemical cycling and ecosystem function. For example, MF are major determinants of soil carbon stocks, which store more carbon than the atmosphere and vegetation combined. Ecosystems composed largely of plants with EcMF store more carbon than those with AMF, by (1) producing a larger biomass of recalcitrant mycelia and, (2) imposing nitrogen-limitation on free-living soil microbes that slows saprotrophic decomposition (Averill et al. 2014). Globally, agriculture and other land use changes have reduced EcMF-associated vegetation, reducing carbon sequestration in the soil (Soudzilovskaia et al. 2019). Climate change may further drive declines in EcMF abundance (Parrent et al. 2006). For example, coast live oaks, *Quercus agrifolia*, form tripartite symbioses with AMF and EcMF, but drought differentially impacts fungal colonization abilities, such that water-limited plants may become increasingly reliant on AMF (Querejeta et al. 2009). Anthropogenic changes, including GCC, nitrogen pollution, and fire suppression have induced continent-wide shifts toward AMF in US forests (Jo et al. 2019; Averill et al. 2018). Even with climate mitigating strategies, declines in EcMF are predicted in North American pines over the next 50 years with negative implications for biogeochemical cycles (Steidinger et al. 2020).

Although studies are typically of limited duration and biased to the northern hemisphere, MF show potential to improve plant resilience to warming, elevated CO_2 levels, and rainfall variability (Mohan et al. 2014; Compant et al. 2010; Bennett and Classen 2020) by several mechanisms. First, elevated levels of atmospheric CO_2 can increase hyphal colonization and rates of nutrient exchange. As noted above, however, this does not necessarily correspond with increased production as plants are more often nitrogen, rather than carbon limited. Here the type of MF is important. In plant species with AMF, nitrogen limitation indeed inhibits CO_2 fertilization, whereas biomass increases regardless of nitrogen availability in plants associating with EcMF (Terrer et al. 2016). Similarly, temperature often positively correlates with MF abundance and activity, and may affect the structure of the hyphal networks, affecting storage versus growth dynamics (Mohan et al. 2014; Hawkes et al. 2008). Third, as soils dry, nutrient uptake is often reduced. Experimental studies show that MF alleviate water stress under both drought and variable water conditions (Bowles et al. 2018; Augé 2001). Furthermore, AMF may improve stomatal conductance (rate of CO_2 entering, H_2O vapor exiting) and photosynthetic rates, increase antioxidant activities, or regulate channel proteins involved in water transport that reduce water stress (Quiroga et al. 2017; Li et al. 2019; Augé et al. 2015). While the effects of drought on MF abundance and activity are mixed, overall MF plants are more productive compared to non-MF plants during drought (Mohan et al. 2014). Increased usage of fertilizer and fossil fuels have also increased reactive nitrogen deposition in soils. This impacts ecosystem functioning, leads to nitrate leaching and subsequent water pollution, as well as the release of N_2O; a potent greenhouse gas that also damages the ozone layer (Vitousek et al. 1997; Montzka

et al. 2011). It is important to note that AMF can also reduce N_2O gas emissions and nutrient leaching from soil (Bender et al. 2014; Bender et al. 2015).

Nitrogen-fixing bacteria, associated with roughly 7% of plant species, represent another widespread group of rhizosphere symbionts (Steidinger et al. 2019). Instead of extracting nutrients from the soil, nitrogen fixers convert abundant, but unusable N_2 gas in the atmosphere into forms that plants can assimilate. These are common in legumes (Fabaceae) and other dicotyledonous angiosperms occurring in nitrogen-poor soils. Nitrogen-fixing symbionts appear limited by temperature and soil pH to warm, arid regions (e.g., tropical savannas and xeric shrublands) and with continued warming are expected to increase in abundance in regions such as the arid southwest of North America (Liao et al. 2017). These expansions could partially offset the massive losses of evergreen trees also predicted for this region (McDowell et al. 2016). Unfortunately, an expansion of nitrogen-fixing symbioses has the potential to severely worsen climate change if soil N_2O emissions outweigh carbon sequestration, but uncertainty surrounds model estimates (Kou-Giesbrecht and Menge 2019).

In addition to rhizosphere-associated symbionts, plants are routinely infected with aboveground endophytes, usually fungi or bacteria, that spend at least part of their life cycle inhabiting plant tissues (Hardoim et al. 2015). Often with blurred boundaries, endophyte lifestyles vary from opportunistic to obligate, with variable routes of transmission. Many interactions are commensal, but others are beneficial or conditionally beneficial, often improving plant fitness by conferring tolerance against biotic or abiotic stress. A meta-analysis across 42 host plants and 94 endophyte strains found that endophytes increased host plant biomass during nitrogen deficiency, drought, and high salinity (Rho et al. 2018). Fungal endophytes, in particular, are known for roles in mediating abiotic and biotic stress and hence may play a prominent role in mitigating climate stress (Rodriguez et al. 2009; Kivlin et al. 2013). The clavicipitaceous endophytes (Class 1) are associated with about 25% of grass species and confer protection against herbivores and drought stress (Rudgers et al. 2009; Clay and Schardl 2002). Those that promote drought tolerance can expand host ranges into drier habitats (Afkhami et al. 2014). The more diverse and taxonomically widespread non-clavicipitaceous endophytes (Classes 2–4) are harder to generalize, though infection phenotypes include drought and thermal tolerance (Rodriguez et al. 2008; Redman et al. 2002; Arnold et al. 2003).

16.5 Conclusions

The aggregate actions of humans have created prominent evolutionary forces that will, to varying degrees, impact ecosystems across the planet. The ability of multicellular eukaryotes to relocate, acclimate, or adapt to these changes will often depend on their microbial symbionts. In some cases, symbionts will provide a much-needed assist, but for many highly specialized associations, they will serve as an "Achilles heel" exposing acute vulnerabilities.

Changes in the abundances, identities, and distributions of plants and phytoplankton will shift sites and quantities of primary production, and affect energy transfer and species interactions across impacted ecosystems. For plants, these shifts will be hindered or helped by their symbionts depending on the interaction. For instance, by conferring tolerance to abiotic stresses, endophytes and MF may expand their hosts' ranges into warmer and more arid regions. And while nitrogen-fixing bacteria and EcMF tend to invade new territories alongside their woody hosts, those with AMF tend to form novel associations in introduced ranges (Nunez and Dickie 2014). Those plants relocating with symbionts in tow are likely to disrupt native associations (Rodriguez-Echeverria 2010), which could have systemwide effects. Also, microbe-mediated plant–soil feedbacks are among the most important determinants of soil carbon and nitrogen, yet also the least understood (Classen et al. 2015). Hence understanding plant microbiota is critical for predicting plant responses to climate change, and associated climate feedback, but also for efforts aimed at conserving plants (Carthey et al. 2020).

Failing symbioses often occupy niches that only symbiotic organisms can occupy, so replacement will occur only if other symbiotic organisms are able to move in, or over time, if new symbioses form. In terrestrial systems, the widespread loss of sap-feeding insects would re-open this challenging niche for the first time in tens of millions of years. However, since the majority of sap feeders are specialized to particular plant groups, even those fortunate species carrying thermally stable obligate symbionts would be unlikely to switch to vacated niches. This is because the same genome degradation that hampers thermal tolerance also results in inflexible nutrient provisioning (Hansen and Moran 2014). The minority of sap-feeding arthropods that are both food–plant generalists and harbor thermally tolerant obligate symbionts may be able to broadly take advantage of this opportunity. However, this homogenized cohort of replacements would further diminish associated communities as many natural enemies are also highly specialized.

In marine systems, the loss of hard corals creates opportunities for other photosymbiotic organisms, such as soft corals and sponges, which show greater tolerance to warm temperatures and acidification since most of the likely ecological replacements are non-calcifying (Wee et al. 2019; Norstrom et al. 2009; Bell et al. 2013). Unfortunately, these potential replacements too often show limited resilience to repeated bleaching events, and are predicted to reduce the diversity of supported communities (Slattery et al. 2019; Cruz et al. 2015). Hence, these would likely be short-term replacements that provide inferior ecosystem services.

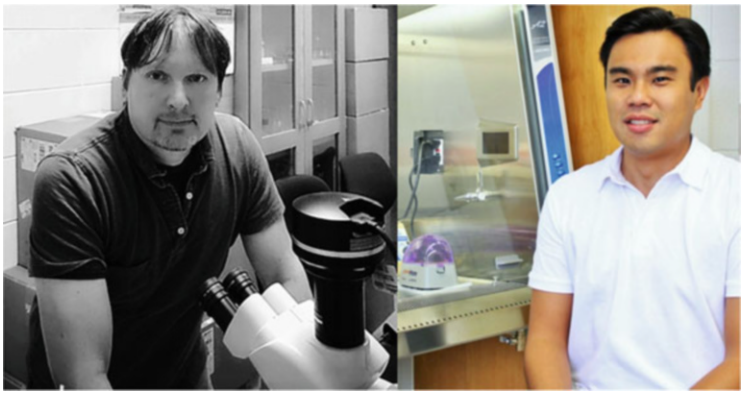
Climate challenged microbial symbioses will also disrupt macro-symbioses. Another group of Symbiodiniaceae-harboring anthozoans, the sea anemones engage in macro-symbioses with a variety of fish and invertebrates, most notably clownfish, providing protective services in exchange for nutritive excrement. As with other photosymbiotic animals, anemones bleach when stressed resulting in smaller animals and reduced abundance. In turn, this decreases the fecundity and growth rates of clownfish (Apprill 2020). Clownfish show preferences for unbleached anemones, and may be able to behaviorally adjust, assuming healthy hosts remain available (Scott and Dixson 2016). In terrestrial systems, the decline of sap-feeding insects

would similarly impact the many ant species that engage in protection schemes in exchange for sugary honeydew.

A few symbiotic organisms may be better prepared for a changing climate. For a variety of marine habitats where light is limited, primary production occurs via the chemosynthetic subseafloor microbial biosphere and aboveground chemosynthetic symbiotic animals, which create usable energy from the oxidation of inorganic compounds (McNichol et al. 2018; Dubilier et al. 2008). Some of these systems, like hydrothermal vents exhibit highly variable environmental conditions, so community members likely have plasticity to respond to changing conditions (Robidart et al. 2011). Vents occupied by symbiotic giant tube worms, for example, show large temporal variations in nutrients and temperature (Girguis and Childress 2006). Vents are also subject to a range of natural disturbances, ranging from the chronic (e.g., clogging of conduits) to the catastrophic (e.g., volcanic eruptions), with the largest anthropogenic impacts arising from deep-sea extraction, which, in contrast to warming and acidification, could be rapidly ceased (Van Dover 2014). However, effects of GCC on near-surface photosymbioses and resulting hypoxia, combined with changes in ocean circulation patterns, as seen with past climate shifts, may combine to disrupt the delivery of oxic water from the surface to the deep sea, and also become limiting even for these isolated systems (Vrijenhoek 2013; Childress and Girguis 2011).

In summary, climate change is set to reorganize life on Earth. Whether this is a temporary retreat to higher ground or a full-on planetary reset, will depend on the timing, extent, and success of human-led mitigation efforts. It is certainly not heartening to observe the ongoing losses of corals and other photosymbiotic animals in the oceans, and to recognize that losses of obligate nutritional symbioses and other specialized interactions possibly will soon follow suit in terrestrial systems. While some microbes, including thermally tolerant photosymbioses, arthropod facultative symbionts, as well as the endophytes and MF of plants are likely to provide some resilience, it is not clear how effective these will be with 3 °C of warming and in the context of all of the other changes that will be occurring simultaneously, including more frequent heat waves, droughts, and forest fires. However, it is instructive to keep in mind that microbes have survived every biotic crisis and there is every reason to expect they will persist until the sun runs out of hydrogen and becomes an earth-destroying red giant. Thus, one safe bet is that whatever shape macroscopic life takes on the other side of climate disruption, microbes will continue to be an important force in their ecology and evolution.

Acknowledgments We thank Chris Hurst for the opportunity to contribute to this volume. This work was funded by NSF award 1754302 to KMO.



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Chapter 17
Diversity–Function Relationships and the Underlying Ecological Mechanisms in Host-Associated Microbial Communities

Catalina Cuellar-Gempeler

Abstract Microbes have colonized, exploited, and inhabited animal and plant tissues since these macroorganisms first appeared on Earth. The resulting host–microbe interactions have far reaching consequences for animal and plant ecology and evolution. However, how ecological mechanisms maintain microbial functions beneficial for the host is still an open question. While some hosts keep diverse communities, others limit membership to very few, functionally efficient, taxa. Here, I outline the broad range of diversity–function relationships in host-associated microbial communities and propose two approaches based on ecological theory to better understand how and when to expect diversity to result in function. Specifically, I combine models from the Biodiversity-Ecosystem Function (BEF) literature and assembly processes with evidence from host-associated microbial communities. If we expand our perspective of diversity–function relationships, we will improve our ability to predict the effect of environmental change on microbiomes and to manage microbes for the benefit of human health and agriculture. Importantly, we may get closer to answering whether microbes or their hosts are in control of the biosphere.

17.1 Introduction

Since the origin of life, microorganisms have altered the environment by regulating the availability of oxygen, nitrogen, and other resources (Gruber and Galloway 2008; Van Der Heijden et al. 2008; Falkowski et al. 2008), influencing the evolutionary trajectories of most metazoan groups (Yeoman et al. 2011; Fitzpatrick et al. 2018). In fact, Earth had been ecologically dominated by bacteria for over 3 billion years before the first animals and plants (Knoll 2003). Moreover, bacteria have colonized, exploited, and inhabited animal and plant tissues since the inception of Kingdoms Animalia and Plantae (Dethlefsen et al. 2007; Krings et al. 2007;

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_17

Zilber-Rosenberg and Rosenberg 2008; Brinker et al. 2019). Resulting host–microbe interactions may be the key to understand major animal and plant evolutionary events (McFall-Ngai et al. 2013; Foster et al. 2017). For example, the radiation of ruminants (McFall-Ngai et al. 2013), specialization of insects' diets (Janson et al. 2008; Hongoh 2010) or the invasion of land by plants (Martin et al. 2017), were likely triggered by microbes giving hosts access to otherwise inaccessible nutrients. In these and other niche diversification events, microbial communities altered host evolutionary trajectories by persistently performing complex functions. Even though it seems key to understanding the evolution of life on Earth (Foster et al. 2017; Zeng et al. 2017; Henry et al. 2019; van Vliet and Doebeli 2019), we still struggle to identify the ecological mechanisms that maintain function in host-associated microbial communities.

With the advent of better and cheaper molecular techniques, we have moved beyond regarding microbes as a "black box," and started considering complex interactions between microbial species and their environment in closer detail (Blaser 2014; Widder et al. 2016; Julliand and Grimm 2016). In the last 20 years, studies taking advantage of new sequencing technologies have revealed how microbial diversity and composition change in time and space (Gonzalez et al. 2012; Lauber et al. 2013; Shade et al. 2013). In fact, the microbial communities associated with hosts vary dramatically in composition and function across time and space (Turnbaugh and Gordon 2009; Costello et al. 2009; Louca et al. 2016a). Part of this variation responds to changes in the host's development, health status, and the surrounding environment (Kraal et al. 2014; Oh et al. 2016; Dunphy et al. 2019), yet a large proportion remains unexplained (Huttenhower and The Human Microbiome Project Consortium 2012; Louca et al. 2016a, Cao et al. 2017). This lack of explanatory power highlights important gaps in our understanding of microbial community dynamics and their consequences for host–microbe interactions.

An important aspect of this gap is the role diversity plays in shaping microbial function. In the literature, microbial diversity is often equated to function (Reese and Dunn 2018). Traditionally, gut microbial communities with high diversity are assumed to be more efficient contributors to digestion and harder to invade by pathogens, just as more diverse forests and coral reefs are considered to be more productive and unlikely to recruit invasive species (Balser et al. 2006; Allison and Martiny 2008). Within their guts and other body parts, most hosts maintain diverse communities that are assumed to correlate with high functional stability, functional diversity and, in general, benefits to host fitness (Hongoh 2010; Philippot et al. 2013; McFall-Ngai et al. 2013). However, diverse microbial communities have also been linked to a higher likelihood of cheater strains, opportunistic pathogens, and unstable dynamics (Coyte et al. 2015; Foster et al. 2017; Coyte and Rakoff-Nahoum 2019). Some hosts filter the incoming microbes to such extent that community membership is restricted to one or few species. An extreme example is the Bobtail Squid's selection of a single species, a highly functional symbiont within its light organ (Nyholm and McFall-Ngai 2004). We need to consider this mismatch between diversity and function if we aim to understand when hosts should maintain diverse

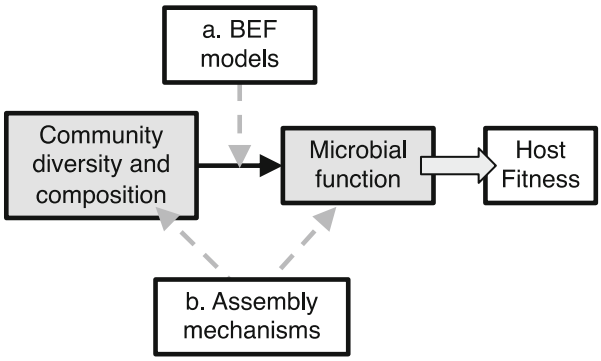

Fig. 17.1 Proposed conceptual framework of factors influencing microbial diversity and function in the context of host associations. (a) Biodiversity-Ecosystem Function (BEF) models propose mechanisms that can explain how diverse communities can yield functions that influence host fitness. (b) Because these functions depend on which species are present, assembly mechanisms affect how many and which species obtain community membership within a host, with consequences for their functional attributes

communities and when they should invest in stringent filtering and other systems to optimize function.

Here, I outline the broad range of diversity–function relationships in host-associated microbial communities and propose two approaches based on ecological theory to better understand how and when to expect diversity to result in function (Fig. 17.1). Ecology has considered the effect of biodiversity on ecosystem function (Biodiversity-Ecosystem function relationship, or BEF relationships) for decades, finding evidence of positive relationships between plant diversity and ecosystem functions like productivity, biomass accumulation, and stability (Hooper et al. 2005; Cardinale et al. 2012). I review the mechanisms proposed by the BEF literature to explain the contribution of diversity to function in host-associated microbial communities (Fig. 17.1a). Because many host-associated microbial communities seem to deviate from this positive BEF relationship, I expand on this view by considering negative selection BEF relationships and differentiating between broad and specific functions. Then, I discuss how assembly processes influence diversity, including host-imposed filters, microbe–microbe interactions, and colonization from the surrounding environment (Fig. 17.1b). I ask when can assembly result in a decoupling of the diversity–function relationship and when does it favor positive BEF relationships. If we expand our perspective of diversity–function relationships, we may be able to better understand how ecological interactions between microbes and their hosts have shaped the ecology and evolution of the biosphere.

17.2 What Are the Functions That Microbes Provide to Benefit Their Hosts?

Microbial communities provide many different functions to their hosts. In this section, I outline the relationship between the diversity of microorganisms and their functional output. In an effort to organize this information, I classify microbial functions into four major categories based on the contribution they make to the host fitness. Microorganisms can (1) supply scarce nutrients, (2) prevent disease, (3) facilitate morphogenesis and development, and (4) extend the host's phenotype. There are other ways to classify microbial function in the context of host associations (see, for example, McFall-Ngai et al. 2013, Christian et al. 2015), and this is not meant to be a review of the extensive literature. Instead, by examining some select studies, I aim to show the widespread range of species-rich and species-poor microbial communities that provide hosts with function.

17.2.1 Nutrient Provision

Nutrient provision has been documented for a long time and includes the acquisition of environmental nutrients by plant roots and the transformation of ingested nutrients in the animal gut (Hacquard et al. 2015). Procuring more and typically inaccessible nutrients can increase individual host's fitness by increasing reproductive outputs, population persistence (by increasing growth or reducing mortality), and community diversity for plants and animals (by reducing competition).

Overall, the rhizosphere and root endophytic microbial community, including bacteria and fungi, are species rich and contribute to plant growth, productivity, and carbon sequestration through nutrient acquisition (Philippot et al. 2013; Bulgarelli et al. 2013). Rhizosphere diversity and function are particularly well-studied in agricultural crops, where it is clear that diverse microbial communities contain the functional capabilities to transform nutrients, breakdown compounds toxic to the plants, and respond to environmental fluctuations (Xu et al. 2018; Yurgel et al. 2019).

Two important plant–microbe systems central to rhizosphere studies are the rhizobia–legume (Sprent et al. 1987) and mycorrhizae associations (Martin et al. 2017). In the rhizobia–legume association, nitrogen-fixing bacteria synthesize ammonia from atmospheric nitrogen which the plant obtains in exchange for carbohydrates and mineral nutrients (Kiers et al. 2003). Although plants can be infected with multiple strains of rhizobia, their interaction seems to be mostly antagonistic, resulting in lower plant productivity (Barrett et al. 2015). On the contrary, diversity in mycorrhizal infections seems to result in functional complementarity. In exchange for carbohydrates from the plant host, each species of fungi extends the reach of plant root systems in different ways, collectively improving access to nitrogen and phosphorus for the plant (Ferlian et al. 2018). Both of these host–microbe

associations depend on tight regulation via signaling secretion systems (Schmitz and Harrison 2014; Nelson and Sadowsky 2015), but only *Rhizobium*–legume associations are known for strict sanctions to cheater microbes (Kiers et al. 2003; Sachs et al. 2010; Oono et al. 2011).

Microbial communities in the animal gut break down complex polymers into digestible molecules, and provide essential vitamins and amino acids otherwise unavailable from the host's diet (Koh and Bäckhed 2020). In humans, the gut microbiome assists in the breakdown of dietary products, like complex polysaccharides, and production of essential nutrients, such as short-chain fatty acids, vitamins (B and D), and essential amino acids (Ley et al. 2008b; Qin and MetaHIT Consortium 2010; Kamada et al. 2013). More generally for mammals, the highest gut diversity is attributed to herbivores, particularly ruminants and fermenters, where these functions are essential to the breakdown of a plant diet, the host's sole source of nutrients (Ley et al. 2008a; Godon et al. 2016). These microbial communities are generally very diverse, ranging from hundreds to thousands of species (Reese and Dunn 2018), indicating that microbes may complement each other in their metabolic pathways leading to more effectively break down of food and more diverse production of metabolites (Henson and Phalak 2017; Coyte and Rakoff-Nahoum 2019).

Nonetheless, some animals with defined diets hold species-poor communities in their gut, potentially limiting membership to those that provide effective transformation of specific molecules. For example, bees host five to nine core bacterial species in their guts (Engel et al. 2012; Raymann and Moran 2018), while aphids consuming sap have been reported to have 8 core species (Smith et al. 2015) and up to 21 facultative bacterial symbionts (Gauthier et al. 2015). An extreme example is the lack of resident microbiota in caterpillars, suggesting that the host benefits from producing the necessary enzymes instead of hosting potentially dangerous microbes (Hammer et al. 2017).

17.2.2 Disease Prevention

In the context of human health, microbes were regarded solely as pathogens for a very long time, and understanding their role in preventing disease is a relatively newer perspective (Casadevall and Pirofski 2015). It is clear now that microbial ability to prevent and combat disease has direct applications in human health, such as combating *Clostridium dificile* infections with fecal transplants (Eiseman et al. 1958; Bojanova and Bordenstein 2016; Ooijevaar et al. 2019; Jin Song et al. 2019). Applications extend into other areas like agriculture, where inoculations of protective microbes in crops have the potential to increase yield and block phytopathogens while reducing the use of toxic compounds (Pérez-García et al. 2011; Busby et al. 2017). Whether in animals or plants, these defensive properties rely on interactions between protective microbes and invasive pathogens (García-Bayona and Comstock 2018). Those interactions include competition for resources, competitive interference, modification of abiotic conditions, and priming of the host immune system.

While some of these depend on resident microbial diversity, others depend on specific taxa.

A diverse microbial community is more likely to occupy all local niches, hindering the recruitment of pathogens just because there is no ecological space left to occupy. For example, diverse endophyte communities mediate resistance against pine rust (Ganley et al. 2008), leaf necrosis in cacao trees (Arnold et al. 2003), and smut in corn (Lee et al. 2009) likely by outcompeting fungal pathogens on plant tissue and priming the host immune system (Alabouvette et al. 2009; Rodriguez Estrada et al. 2011; Hartley et al. 2015). The specific mechanisms can be competition for space, as in endophytes, or for resources, like coliforms in the human gut starving enterohaemorrhagic *E. coli* strains by monopolizing organic acids, amino acids, and other nutrients (Momose et al. 2008a, b; Fabich et al. 2008; Leatham et al. 2009). A useful approach to test whether multiple species are required to defend a host, is to compare vulnerability to pathogen infection in hosts inoculated with one or more symbionts. For example, the resistance of hydra to fungal pathogens is only achieved when inoculating axenic hydra with a mixture of bacteria from its natural microbiome, but not with individual bacterial taxa (Fraune et al. 2015).

In other cases, individual taxa that are strong competitors against a pathogen are sufficient to protect the host (Van der Waaij et al. 1971). These effects have been found to be strongest with taxa related to the pathogens, likely because they compete more strongly for the same resources. For example, *E.coli* produces a bacteriocin specific to enterohaemorrhagic *E coli* (Schamberger and Diez-Gonzalez 2002; Hammami et al. 2013). In the case of defense against *Clostridium difficile* infections, the pathogen was found to be inhibited when a bacterium from the same genus, *Clostridium scindens* depleted bile acid resources in the gut (Buffie et al. 2015).

Resident microbiota can also protect the host by altering the surrounding environment in a way that disproportionately disadvantages incoming pathogens. For example, the presence of *Lactobacillus crispatus* in the female genital tract has been linked to protective effects against HIV infections, potentially because of reduced inflammatory responses (Gosmann et al. 2017). *Lactobacillus* sp. and other normal microbiota in the vagina maintain a low pH that constrains the invasion of urinary tract pathogens (Turovskiy et al. 2011; Hickey et al. 2012). Gut microbiota can also alter their surrounding pH by producing short-chain fatty acids to inhibit intestinal pathogens (Cherrington et al. 1991; Shin et al. 2002). In many cases, the beneficial microbes that establish conditions like low pH must arrive earlier than potential pathogens for the host to be protected (known as “priority effects” in ecology, Toju et al. 2018).

Microbes can also stimulate the development of the host’s immune system (Rakoff-Nahoum et al. 2004; Mazmanian et al. 2005). This is of particular interest in the vertebrate intestine, where commensal bacteria promote epithelial barrier function, macrophage recruitment, and cytokine precursors (Kamada et al. 2013). For example, germ-free mice and mice deficient in Nod2 and TLR signaling adaptors are unable to produce antimicrobial peptides (Kobayashi et al. 2005; Vaishnava et al. 2008). These mice also have reduced intestinal motility and are unable to control bacterial growth within their mucosa. A healthy microbiome–epithelium relationship

may thus allow hosts to control the identity of their microbial partners, through secretion of immune effectors and specific bioactive molecules (Douglas 2010; Hooper et al. 2012; Sommer and Bäckhed 2013).

17.2.3 Morphogenesis and Development

In addition to stimulating the immune system, microbial communities can also influence the timing and outcomes of transitions in the host's life. In plants, microbial communities can influence their host's phenology by altering flowering, fruiting, and germination timing and magnitude (Panke-Buisse et al. 2015). Importantly, microbes can mediate the effect of environmental stress on plant phenology (Yang et al. 2009; Hubbard et al. 2012). In a multigenerational study of drought responses in *Brassica rapa*, flower and fruit production were highest when soil microbes colonizing their roots were preadapted to drought (Lau and Lennon 2012). Hopefully, these associations can contribute to plant fitness in the face of climate change.

In animals, these transitions include larval settlement and morphogenesis in rocky shores, a key step in the life history of diverse intertidal invertebrate taxa. Although it is clear that larvae respond to surface-bound cues and that receptors are evolutionarily old (Hadfield 2010), the specific mechanisms behind these settling behaviors remain unknown. Bacteria may be providing larvae with direct benefits such as increased attachment due to adhesive properties of bacterial exopolysaccharidse (EPS). Alternatively, bacteria may be releasing signals that represent beneficial environmental conditions, or reflect conspecific density (Hadfield 2010). Larval settlement depends on diverse microbial communities for some invertebrate taxa, while for others, it responds to the presence of specific bacterial taxa. For example, the marine tubeworm *Hydroides elegans* can settle on biofilms composed exclusively of *Pseudomonas lutoviolaceae* (Huang et al. 2012) and scleractininan coral prefers to settle in shallow waters, where older biofilms (>8 weeks) are dense and diverse, with high abundances of *Gammaproteobacteria* (Webster et al. 2004). In contrast, barnacle settlement is less clear with some studies reporting larval settlement is induced by bacterial biofilms, while in others it is constrained by bacterial biofilms (Wieczorek et al. 1995; Hadfield 2010). Regardless of the diversity or the specific mechanism, it is clear that microbial communities can play important roles in animal development with dramatic consequences for the adult, its reproductive output, and longevity (McFall-Ngai et al. 2013; Gould et al. 2018; Douglas 2019).

17.2.4 Extended Phenotype

Next generation sequencing techniques renewed interest in the study of the relationships between genotypes and phenotypes of interacting organisms at fine levels,

known as the extended phenotype (Dawkins 1982; Hunter 2018). Central to this new attention is microbial communities associated with hosts, where the extended phenotype can be broadly defined to include many of the microbial functions described above, namely, access to new sources of nutrients, defenses against disease, and complexities of life histories (Hunter 2018). I will use a narrower definition here to categorize functions that extend the phenotype in other, and more radical directions. I include here the Bobtail Squid-*Vibrio fisheri* association, the coral–symbodinium symbiosis and the increased tolerance to abiotic stressors that endophytes provide for host plants, yet many others could have been featured here.

The benefits of light production are key to the survival of the bobtail squid in its natural habitat. *Vibrio fisheri*'s bioluminescence provides the bobtail squid (*Euprymna scolopes*) with the ability to use counterillumination to avoid predators (Nyholm and McFall-Ngai 2004). This association is unique in its horizontal but tightly selective transfer, due to its chemical (mucus, peptide secretion) and physical (active cilia creating countercurrents) constraints that favor *V. fischeri* over all other bacterial colonists (Nawroth et al. 2017). The squid obtains the symbiont from the seawater, and the initial contact triggers the development of the light organ, where the bacteria are housed (Koropatnick et al. 2004; McFall-Ngai 2015). Therefore, in this system, both partners shape each other's biology in a wonderful display of animal–microbe dialog (Schwartzman and Ruby 2016).

Coral associations with zooxanthellae are key to a multitude of marine fish and invertebrates that find resources and refuge among the coral reef. The coral–zooxanthellae association is based on the host providing shelter and nutrients like ammonium, while the zooxanthellae are primary producers and provide a carbon source in areas of the ocean that are typically nutrient poor. The resulting ecosystem is an oasis and refuge for marine life and one of the most beautiful sights on planet Earth. In contrast to the tight *V. fisherii*–Bobtail Squid association, different species of corals associate with many different species of zooxanthellae in the genus *Symbodinium* (Toller et al. 2001). Patterns of association indicate zooxanthellae tolerance to heat and depth stress, as well as coral taxonomic preferences (Kennedy et al. 2016).

Plant growth-promoting bacteria are those that stimulate growth and facilitate strong stress responses. Microorganisms associated with roots have been shown to increase drought resistance and tolerance to temperature extremes (Stone et al. 2018), facilitating the range expansion of otherwise susceptible species (Geisen et al. 2017; Ramirez et al. 2019) and the survival of endangered species in changing habitats (West et al. 2019; David et al. 2019). Some of the proposed mechanisms behind these functions include biofilm EPS secretion that resists the diffusion of water oxygen and nutrients and secretion of heat shock proteins (McLellan et al. 2007; Sandhya et al. 2009; Grover et al. 2011; Kumar Meena et al. 2015; Ortiz et al. 2015). Phyllosphere bacteria can also confer drought and heat resistance (Costerton et al. 1994; Auerbach et al. 2000) with the additional benefits of UV (Jansen et al. 1998; Jacobs and Sundin 2001) and frost protection (Lindow et al. 1982; Wisniewski et al. 1997; Attard et al. 2012). Physiological studies on symbionts suggest that most of these properties are provided by few species (Lindow et al. 1982; Auerbach et al.

2000; Attard et al. 2012), and that to protect itself from multiple stressors, a plant would need to maintain a diverse community (Arnold et al. 2003; Bae et al. 2009). However, some of these taxa are not strong competitors and would be eliminated under normal conditions. For example, ice-nucleating bacteria that reduce the freezing point of water (Duman and Olsen 1993) are outcompeted under nonfreezing conditions and excluded from phylosphere communities (Lindow et al. 1996; Stockwell and Stack 2007). Maintaining microbial protection against multiple stressors may require host intervention including complex hormonal secretion patterns and stomatal closures (Stone et al. 2018).

17.3 How Does Diversity Result in Function?

It is increasingly clear that host-associated microbial communities range from hundreds to single species, suggesting that sufficient function to benefit a host's fitness can result in different points of a diversity gradient. Ecologists have been addressing the issue of how diversity results in function for a long time, laying a rich body of literature. Therefore, I draw from this ecological literature to interpret the broad range of diversity–function relationships in host-associated microbial communities.

17.3.1 Does Diversity Lead to Function? The Biodiversity–Ecosystem Function Relationship

One way to understand this wide range of functional communities is to use the shape of the biodiversity–function relationship to infer the ecological mechanisms that result in function. The biodiversity and ecosystem function (BEF) has been central to the study of ecology for several decades (Hooper et al. 2005; Cardinale et al. 2012), revealing several possible ways in which species contribute to function, including positive, asymptotic, negative, and idiosyncratic relationships (Scherer-Lorenzen 2005). In this section, I review these mechanisms and the role they play in host–microbe interactions.

Many host-associated communities seem to have positive diversity–function relationships (Fig. 17.2a). For example, the human infant's gut microbial community increases in diversity and function during the first 3 years of life (Odamaki et al. 2016; Hill et al. 2017). Mechanistically, the accumulation of microbial species may correspond to the addition of complementary functions, resource use efficiency, and increased access to nutrients for the host (Ottman et al. 2012). These trends are equivalent to the complementarity model in plant and animal BEF relationships, where species with different functional traits contribute to an overarching function (Loreau and Hector 2001; Hooper et al. 2005; Fox 2005; Balvanera et al. 2006;

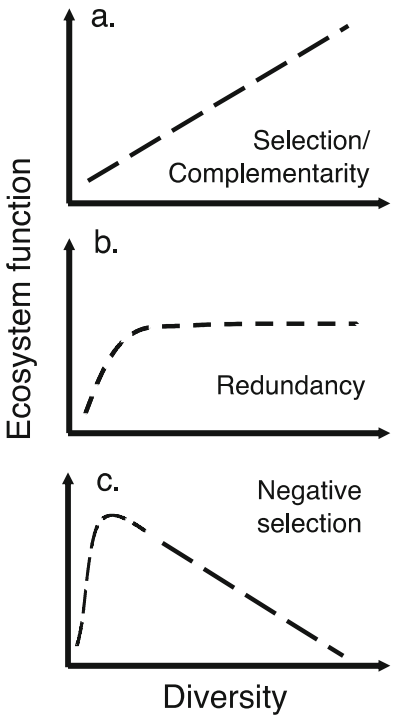

Fig. 17.2 Expected effect of diversity on function under the complementarity or selection (**a**), the redundancy (**b**), or the negative selection (**c**) models. Positive relationships can result from selection or complementarity, and enhanced by diverse colonist pools (**a**). Asymptotic BEF relationships result from colonist pools diverse in species but not in functions (functionally redundant), and local coexistence of functionally redundant species (**b**). Negative linear relationships result from negative selection (**c**)

Cardinale et al. 2012). Evidence from a study that followed a single infant for 2.5 years supports this model, showing a gradual increase in phylogenetic diversity was linked with progressive enrichment in pathways involved in carbohydrate metabolism, lactate utilization, breakdown of glycans from breast milk, and use of plant-derived polysaccharides (Koenig et al. 2011). On the other end of human development, species are lost from gut communities as we reach old age, resulting in reduced production of anti-inflammatory secondary metabolites, limited carbon utilization pathways, and increased rates of invasion by opportunistic facultative anaerobic pathogens (Biagi et al. 2010).

Other hosts maintain a taxonomically diverse and variable microbial community with stable functional profiles throughout their lifespan. This relationship corresponds to the redundancy model, where there is an upper limit to the positive effect of diversity on function, such that there is a saturating curve (Cardinale et al. 2011). Because coexisting species contribute similar or equal functions, loss of diversity does not always result in loss of function (Allison and Martiny 2008; Guillemot et al. 2011). For example, Louca et al. (2016a) found that bromeliad aquatic microbial communities exhibited high variability in taxonomic composition but similar functional attributes (coefficient of variation of 2–3 for OTU and 0.2–0.6 for gene abundance). Of all the taxa available to colonize the bromeliad's fluid, they found remarkable redundancy in functional processes associated with the breakdown of dead matter. This type of redundant relationship has been repeatedly found in

host-associated microbial communities, such as algae surfaces (Burke et al. 2011), salamander skin (Barnes et al. 2020), bovine rumen (Weimer 2015), and the adult human intestine (Rakoff-Nahoum et al. 2014). The prevalence of this redundancy model is unsurprising, since theory predicts that redundancy will evolve in all ecosystems through a combination of competition, and stochastic processes (Scheffer and van Nes 2006, but see Barabás et al. 2013, Vergnon et al. 2013), and it has been proposed as a paradigm in microbial ecology where functional diversity and variability are constrained by energetic and stoichiometric factors (such as the availability of electron acceptors for respiration, Raes et al. 2011, Nelson et al. 2016, Louca et al. 2016b).

In contrast to these positive diversity–function relationships, some hosts maintain species-poor microbial communities. For example, insects with specific diets, such as bees and aphids, are often found in association with low diversity microbial communities (Cariveau et al. 2014; Smith et al. 2015; Gauthier et al. 2015). Because increased diversity or changes in composition potentially reduce function and thus impact host's fitness, these hosts have little variation in their microbial communities. Therefore, we will probably not find these hosts harboring a diverse community, and test its effect on fitness. Nonetheless, we can follow a simple thought experiment to explore what is going on here: imagine a scenario where the functional and efficient species is a poor competitor, and its functional rates and abundance decrease if local resources are occupied by other, less functional species. Then, it would be beneficial for the host to strictly control membership to functional species, constraining recruitment of other taxa.

This scenario corresponds to the negative selection model where increased diversity results in diminishing ecosystem function rates (Fig. 17.2c, Jiang et al. 2008). Negative selection has been registered in environmental bacteria, where certain functions decrease along biodiversity gradients. For example, single species often provide important rate-limiting functions such as chitin and cellulose degradation that drive processes like litter decomposition and nutrient cycling in lakes (Peter et al. 2011). Functions under negative selection have more specific metabolic pathways, narrow phylogenetic constraints, or energetically expensive reactions (Delgado-Baquerizo et al. 2016). A potential example in host–microbe interactions is nitrogen fixation by rhizobia in association with *Acacia* sp. plants. Barrett and collaborators (Barrett et al. 2015) used a manipulative experiment to show that increased rhizobia diversity resulted in lower nitrogen provision due to increase competition. Nitrogen fixation is extremely energetically intensive and few taxa maintain the genes for the necessary nitrogenase enzyme (see Chap. 8 in this volume).

Studies that manipulate host-associated microbial diversity provide direct insight into how diversity influences microbiome function. Most of these experiments include the inoculation of sterile hosts with microbial isolates, while controlling for diversity (number of species) and composition (species identities). A common finding is that, while diversity correlates with function, species composition also has an important effect, suggesting that many functions are driven by specific species or specific networks of species. For example, Faith et al. (2014) found that germ-free

mice benefitted from diverse bacterial communities inoculated in their guts, by registering higher regulatory T cells, modulation of adiposity, and higher cecal metabolite concentrations. However, the effect of diversity was strongly modulated by the presence of a few taxa like *Collinsella aerofaciens, Subdoligranulum variable*, and several *Bacteroides* strains. Instead of supporting the complementarity model we described above, these findings support the selection model (Grime 1998) where, as diversity increases, the chances of adding species that are particularly efficient at performing a function increases too (Fig 17.2a).

In other systems, a single keystone species maintain diversity and supports the entire function. For example, Niu and collaborators (Niu et al. 2017) inoculated maize roots with communities assembled from 7 strains of bacteria and found that *Enterobacter cloacae* is a keystone species that maintains microbial diversity and protects the plant against the pathogenic fungi *Fusarium verticilloides*. In the absence of *E. cloacae*, the community is dominated by *Curtobacterium pusillum* and the resulting low diversity facilitates growth of *F. verticilloides.* Here, while the function of pathogen defense correlates with diversity, a specific species maintains community function and diversity corresponding to the keystone species BEF model (Scherer-Lorenzen 2005).

The truth behind diversity–function relationships lies somewhere between observational studies and manipulative experiments. Observational studies provide a glimpse into natural host–microbe interactions and manipulative experiments generate extended gradients in diversity and composition to directly test their role in function. However, both approaches have limitations. On one hand, observational studies are unable to distinguish between effects from competing drivers of diversity and function such as diet, physiological, or morphological changes over time (Faith et al. 2014). On the other hand, manipulative experiments have been criticized for being artificial, and not representing natural host–microbe, or microbe–microbe relationships (Scherer-Lorenzen 2005). Only by combining observational studies of natural gradients in diversity and controlled manipulative experiments can we fully understand how microbial communities assemble and provide function within hosts.

17.3.2 Broad and Specific Functions

Traditionally, the study of biodiversity–function relationships in ecology focuses on broad functions like primary productivity, or biomass accumulation (Hooper and Dukes 2004; Cardinale et al. 2012; Leibold et al. 2017). The majority of evidence supports positive biodiversity–function relationships (Balvanera et al. 2006; Tilman et al. 2014). Many of these studies focus on plant communities with a variety of life histories, where adding species increases resource use efficiency, leading to higher biomass and productivity. While primary productivity and biomass accumulation may be important in the context of host-associated microbial communities, these describe only a few of the functions that have effects upon host fitness.

Common to animal gut environments, the degradation of plant and animal matter is a different type of broad function where distinct metabolic pathways are required to break down large polymers into small molecules that can be then consumed and reused as secondary metabolites (Henson and Phalak 2017; Coyte and Rakoff-Nahoum 2019). Microorganisms at each point in a degradation line contribute to the overall degradation rate. If each of these contributions constitutes a specific function, then degradation can be considered as a broad function that encompasses all necessary metabolic pathways and results in the provision of nutrients to the host. This differentiation between the broad function of degradation and specific functions may be key to drawing generalities in diversity–function relationships.

The whole must be viewed as the combination of its individual parts. Here, I define a broad function as the outcome of multiple specific functions, each utilizing its own more narrowly delineated specific metabolic pathways. Many of these specific functions are subject to more narrow phylogenetic constraints or energetically expensive reactions than broader functions (Delgado-Baquerizo et al. 2016). Depending on how specific functions contribute to the whole, broad functions can be represented either as parallel contributions from independent specific functions (Fig. 17.3a) or occurring in series, where each contribution depends on the previous one in a sequence (Fig. 17.3b). Broad functions in parallel include biomass accumulation, or competitive exclusion of pathogen invaders (Casadevall and Pirofski 2015; García-Bayona and Comstock 2018). Broad functions in series may include degradation pathways where each microbe uses the products of the previous reaction to fully break down complex polymers in an animal's gut (Henson and Phalak 2017, Coyte and Rakoff-Nahoum 2019).

Each specific function has its own relationship with diversity (gray squares and dotted arrows in Fig. 17.3): some will have positive relationships, while some will be asymptotic or negative (Fig. 17.2). However, how the shape of a specific diversity–function relationship contributes to the broad function is currently unknown. Their input likely depends on whether specific functions are independent or dependent upon one another. For specific functions contributing in parallel (Fig. 17.3a), the broader function will reflect the biodiversity–function relationship of the majority of its specific functions. For specific functions contributing in series, however (Fig. 17.3b), I would expect that even one specific function under negative selection could dramatically impact the diversity–function relationship at the level of the broad function. Unfortunately, none of these models of functional contributions have been tested empirically yet.

Similar to the role of scale in defining patterns of diversity and function (Levin 1992; Bond and Chase 2002), distinguishing between broad and specific functions may play a major role in defining diversity–function relationships. At the smallest scale, perhaps a specific metabolic transformation can be linked to an enzyme, an enzyme to a gene, and a gene to one or more genomes. For metabolic processes, functional rates will likely depend on how many microbial species have a functional gene of interest, and how active those genes are. While this is fitting for degradative functions, it is more difficult to assign a specific function for other activities, such as microbes that protect their hosts from disease. In that latter case,

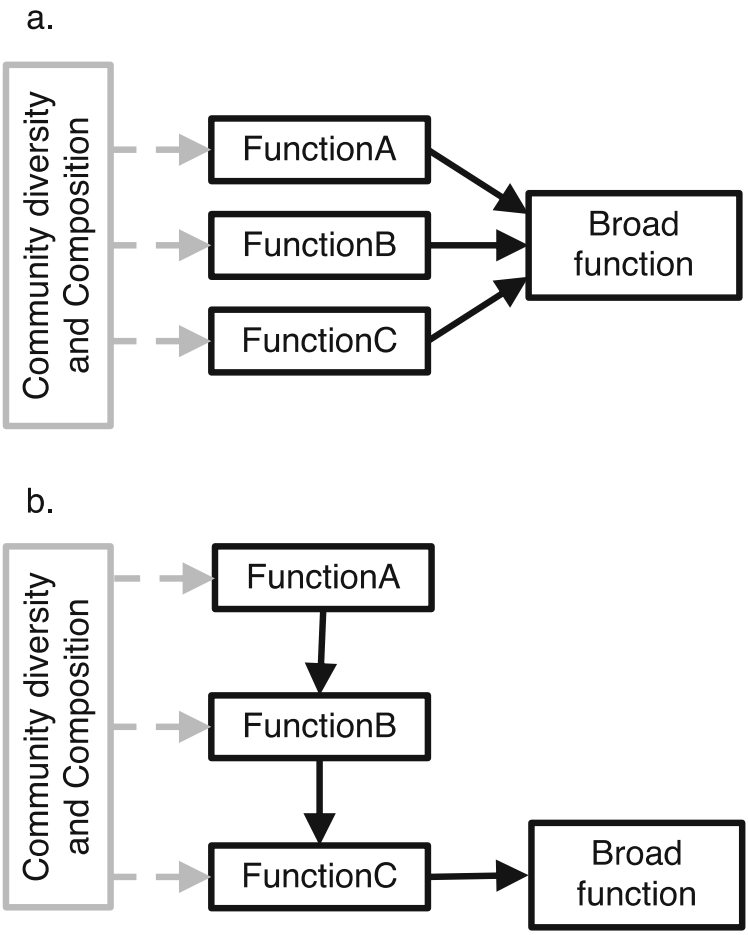

Fig. 17.3 Conceptual model for specific functions contributing to broad functions. (a) Specific functions can contribute in parallel, by independently increasing broad functional rates. (b) Specific functions can contribute in series, where one function's products are the next function's reagents, until the compound has been fully transformed

specific functions depend on the symbiont's niche space as well as the complexities of microbe–microbe and host–microbe interactions (Hurst 2016; Foster et al. 2017; Coyte and Rakoff-Nahoum 2019).

A comfortable intermediate scale for broad functions could focus on the functional categories described in Sect. 17.2. The broad function can then be measured in such outcomes as nutrient availability, disease symptoms or survival, developmental timing or metamorphosis success, and extended phenotype products. Intermediate scale functions can then be easily linked to specific functions and the largest scale broad function, host fitness.

Host fitness should be the broadest scale of microbial function in the context of host-associated microbial communities. Microbes can increase host fitness by providing nutrients, defense, regulating development, or extending the host's phenotype, but they can also decrease fitness when cheaters or pathogens invade communities. As I describe below, the host can have some control over microbial assembly with enough filtering to avoid cheaters and pathogens but permissive enough that beneficial species can be recruited and thrive. Importantly, there are at least two limits to the fitness effects of microbial communities on hosts. First, there are limits to the fitness effects of microbial communities on hosts equivalent to life history trade-offs (Zera and Harshman 2001). Gould et al. (2018) found that fruit fly microbiome diversity accelerated development and reproduction. However, these flies that reproduced more also died sooner, suggesting the effects of microbiomes on host fitness share the same life history constraints traditionally considered in biology (Zera and Harshman 2001).

A second trade-off occurs when the same microbial community can result in contrasting fitness outcomes, demonstrating that each functional category does not happen in isolation. In endophyte–plant associations, for example, there seems to be a trade-off between pathogen defense and tolerance to environmental stress. Diverse communities of endophytes can protect the plant by out crowding fungal pathogens (Bae et al. 2009; Alabouvette et al. 2009; Rodriguez Estrada et al. 2011), yet more endophyte species can also result in water loss, compromising survival in drought scenarios (Arnold and Engelbrecht 2007). Similarly, *Wolbachia* is an intracellular endosymbiont that associates with insects, providing essential metabolites, but interferes in the host's reproduction by biasing sex ratios (Brownlie et al. 2009; Correa and Ballard 2016). Perhaps here we can consider the dynamic nature of host-associated microbial communities, and the possibility that different assembly processes can result in functional outcomes that better fit the host's condition and its surrounding environment. Whether hosts or microbes control these dynamics may vary, depending on the specific assembly mechanisms underlying each microbial community.

17.3.3 Assembly Contributions to Diversity and Function

When an individual host is born, its tissues must be colonized by microbes incoming from the surrounding environment and conspecifics. Thereafter, microbial communities are the result of the interplay between the host tissue selecting for microbial species, microbe–microbe interactions, and continued colonization of the surrounding environment or neighboring hosts (Fig. 17.3, Adair and Douglas 2017, Miller et al. 2018). A useful approach to explain how these processes influence diversity and function is to study mechanisms of community assembly (Shafquat et al. 2014; Adair and Douglas 2017; Leibold et al. 2017). Although studies are beginning to consider the relative contribution of different assembly processes on host-associated communities (Costello et al. 2012; Miller et al. 2018; Reese and Dunn 2018),

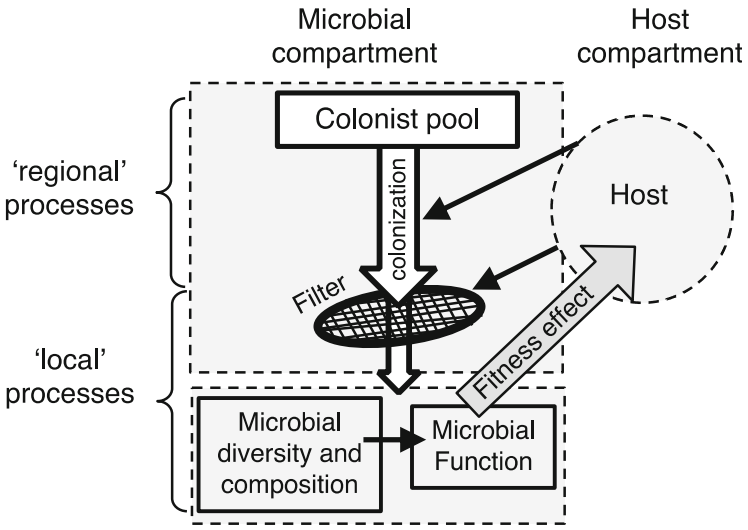

Fig. 17.4 Assembly processes that influence host-associated microbial communities. Regional processes include the diversity and composition of the colonist pool, as well as colonization rates. Local processes include habitat filtering imposed by the host and microbe–microbe interactions. The resulting local community diversity and composition defines its function, with consequences for host fitness. The host could control microbial assembly at the colonization and filtering steps (see Miller et al. 2018). Black arrows represent this host feedback

predicting changes in microbial function remains an unexplored frontier (Schlaeppi and Bulgarelli 2014).

Patterns and processes described in Sects. 17.3.1 and 17.3.2 only consider local-scale processes, including the effects of host tissue characteristics and microbe–microbe interactions that control diversity and community composition. Importantly, dispersal and colonization are traditionally disregarded, even if they can influence diversity and function. In fact, there are several scenarios where dispersal processes can decouple diversity from function. In this section, I will first cover how host-associated habitats filter microorganisms (Fig. 17.4, Filter), then discuss evidence of microbe–microbe interactions within hosts (Fig. 17.4, arrow between "filter" and "microbial diversity and composition"), and, lastly, how dispersal can decouple local diversity–function relationships (Fig. 17.4, "Regional processes").

When microorganisms come into contact with the host, recruitment to local communities depends on the tissue's physicochemical conditions, and host feedback, equivalent to habitat filters in the ecological literature (Fig. 17.4 "Filter," Shafquat et al. 2014, Kraft et al. 2015, Adair and Douglas 2017). These mechanisms of control can allow the host to avoid invading pathogens or communities with lower functional rates. Some host filters include general barriers to microbial recruitment such as low pH in the gut (Huttenhower and The Human Microbiome Project Consortium 2012; Beasley et al. 2015), high salinity in the skin (Chen and Tsao 2013), or root exudates in the rhizosphere (reviewed in Munoz-Ucros et al. 2020).

Other host filters can be very specific constraints including constitutive secretion of antimicrobial compounds and adaptive immunity. Constitutive chemicals are common in the marine environment, such as antimicrobial peptides in the arminin family, produced by Hydra species and resulting in low bacterial diversity and abundance (Franzenburg et al. 2013) or halogenated furanones, produced by red algae that interfere with cell signaling in bacteria (Longford et al. 2019). Physical filters have received less attention, but can be important in maintaining a core microbiome. For example, the proventriculus is a microporous (0.2 μm) valve that acts as a bacterially restrictive filter controlling movement between the crop and the midgut of some insects, including the Sonoran desert turtle ant (*Cephalotes rowheri*) (Lanan et al. 2016). Another example of physical filters can be found in the Bobtail Squid (*Euprymna scolopes*) using ciliary action to control water flow on the surface of the luminous organs creating an extreme type of biological filter that can exclude all but one bacterial species, *V. fisheri* (Nyholm and McFall-Ngai 2004). Other hosts are able to distinguish between resident microbiota and foreign invaders. Adaptive immunity in humans includes a dynamic molecular dialog with resident microbiota that seems to allow for discriminatory responses to pathogens and foreign microbes (Lee and Mazmanian 2010). Most host filters reduce diversity by constraining which species are recruited to their tissues, while ultimately increasing microbial function in terms of host fitness benefits.

In fact, failure of host control via habitat filters can result in disease and reduced fitness. For example, human skin is a dry, salty environment, full of sebaceous glands, and characterized by variations in temperature that favor typical skin bacteria like *Staphylococcus* spp. (Chen and Tsao 2013; Oh et al. 2016). Atopic dermatitis occurs when the host fails to produce the epithelial barrier protein filaggrin, inducing changes in the skin microbiome that include colonization and proliferation by *S. aureus* and other pathogens with an associated immune response (Chen and Tsao 2013). Similarly, disease in the corals *Porites astreoides* (Meyer et al. 2014), and *Montastrea lanceolata* (Sunagawa et al. 2009) are associated with increased bacterial diversity. In contrast, other types of disease or dysbiosis are associated with reduced diversity which can be explained by the proliferation of dominant taxa that outcompete beneficial microbes. For example, the gut parasite *Crithidia* reduces richness and abundance of core taxa in the bumblebee (Cariveau et al. 2014).

Other host habitats are permissive to microbial colonization without resulting in disease. Even when receiving colonists from other hosts and the surrounding environment, these communities' diversity and function can be under the control of microbe–microbe interactions that limit invasion by pathogens and cheaters. Some habitats open to colonization are strongly dependent on early arrival of colonists that monopolize resources and space, a mechanism known as priority effects. Flower nectar microbes, known for their influence in pollination, are a model system for priority effects, where bacterial early arrival constrains yeast recruitment and yeast early arrival lowers the pH and limits bacterial recruitment (Vannette and Fukami 2017; Toju et al. 2018). Priority effects may be common in other systems, such as seaweed surface bacteria, where the order of arrival follows a lottery model (taxa arrive randomly at new sites and early recruits monopolize

resources and exclude others) resulting in high variability across algae tufts (Campbell et al. 2015).

In contrast to these competition-based priority effect models, cooperation should benefit different species within the microbial community and promote coexistence, but it has been difficult to prove in the wild. Using in vitro experiments, *Bacteroidetes thetaiotaomicron* and *B. ovatus* isolated from the human gut were shown to secrete extracellular enzymes to process polysaccharides like inulin, to the benefit of other bacterial species and with reciprocal fitness effects that seem to balance the cost of enzyme production (Rakoff-Nahoum et al. 2016). However, the question of whether competitive or cooperative microbe–microbe interactions characterize host-associated microbial communities has been contentious (Coyte and Rakoff-Nahoum 2019), limiting our understanding of their role in microbial function within hosts. Some authors have suggested that microbe–microbe networks and complementary effects, rather than individual species, maybe building blocks of microbial function within hosts, calling for further study into species interactions networks (Rolig et al. 2015; Gould et al. 2018).

In many cases, communities remain open to colonization throughout the life of the host. Instead of communities dominated by initial colonizers, these hosts' microbial communities reflect the diversity and potentially changing composition of the pool of species available for colonization (Fig. 17.3, "Colonist pool"). For example, neighboring corals harbor more similar microbial communities than do coral heads that are far away from each other, indicating that neighbors exchange more colonists and microbial dispersal is limited in space (Dunphy et al. 2019). Similarly, nectar microbes are more similar in nearby flowers visited by the same hummingbirds, suggesting dispersal is limited and driven by hummingbird vectors (Belisle et al. 2012, 2014). In social hosts, this dispersal limitation results in increased similarity in microbial communities within cohabitating humans (Song et al. 2013) and primates living in groups (Perofsky et al. 2017). The pool of environmental bacteria from the surrounding habitat is an important source of microbial colonists for many hosts, including tadpoles (Louca et al. 2016a; Correa et al. 2020), fruit flies (Blum et al. 2013), and crabs (Cuellar-Gempeler and Leibold 2018, 2019). Even with increasing evidence of the role of dispersal in shaping host-associated microbial communities, we know little of its functional consequences.

One way to address this issue is to recognize that the influence of immigrants on function should depend on the identity, traits, and diversity of colonists (Spasojevic et al. 2018). If the pool of microbial colonists contains diverse species that are functionally redundant, then hosts can benefit from permissive filters where many different species can survive (Fig. 17.3 "Colonist pool"). However, the colonist pool can also add species that contribute to diversity but not to function (Spasojevic et al. 2018). The addition of ineffective colonists could result in negative diversity function because incoming colonists increase diversity but decrease function by utilizing resources. This is one way in which dispersal and colonization can cause the decoupling of diversity and function. Hosts that do not invest in strong filters could counteract this issue with behavioral mechanisms that result in symbiont choice. For example, fungus-growing attine ants choose the fungus that is most

related to their original strain, suggesting there is symbiont fidelity driven by ant behavior (Mueller et al. 2004). Therefore, permissible habitats within hosts can be feasible in three colonist pool scenarios: when the microbes have little influence on the host's fitness (Louca et al. 2016a), when the variability in microbial composition is inconsequential for function (such as redundant communities) or when variability is beneficial for its diversity in functions (upon dietary changes, for example, Davenport et al. 2014). This reasoning indicates that, to further our understanding with respect to the role of dispersal on microbial function in the context of hosts, we should consider the functional characteristics of the colonist pool as well as the assembly processes that control local diversity and function.

17.4 Conclusions

Understanding how different species come together and deliver a functional outcome that impacts host fitness is central to our understanding of host-associated microbial communities. Given the broad range of microbial communities that can contribute to host function, I believe this is an opportunity to challenge the way we look at diversity–function relationships. We should consider the mechanisms through which species contribute to a specific function and how the specific functions contribute to broader functions. This type of conceptual framework could lead to a more predictive study of host-associated microbial function. Moreover, we should also consider the ecological mechanisms that result in microbial diversity and function, namely, host–microbe and microbe–microbe interactions, as well as microbial dispersal and colonization. A clearer understanding of host-associated microbial diversity and function will improve our ability to predict the effect of environmental change on microbiomes (for example, in coral reefs, Ainsworth et al. 2010) and to manage microbes for the benefit of human health (Kamada et al. 2013) and agriculture (Grover et al. 2011; Munoz-Ucros et al. 2020).

Ultimately, we should be able to understand when a host needs to select for low diversity microbial communities (Fig. 17.3, black arrows represent host feedback). Based on the proposed framework, we could say that hosts should invest (evolutionarily or ecologically speaking) in filtering mechanisms when either: (1) only relatively few species are functionally efficient but these are poor competitors (function corresponds to the negative selection BEF model), or (2) the colonist pool contains ineffective species or dangerous potential pathogens. Similarly, hosts should have fewer filtering constraints and allow for permissive and diverse communities when either: (1) functions are complementary, (2) broad functions have specific functions in parallel, or (3) the colonist pool is redundant and functionally efficient. Interestingly, it is possible that filtering to prevent disease results in decreased function in other areas of the microbiome, reflecting trade-offs and revealing the limits of microbial benefits to fitness. Eventually, a broader picture can emerge, where ecological processes that shape microbial communities also result in functions that can benefit the host's fitness. A constant dialog between microbes

and their hosts can shape evolutionary and ecological processes of host filtering as microbial communities in the surrounding environment change over time and space. Whether it is the microbes or hosts that can respond faster to this feedback should dictate which is the driver of the biosphere.

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Chapter 18
Darwinian Medicine: We Evolved to Require Continuing Contact with the Microbiota of the Natural Environment. Evolution Turns the Inevitable into a Necessity

Graham A. W. Rook

Abstract The immune system requires data inputs, especially in early life. A lack of these inputs contributes to increases in immunoregulatory disorders such as those in which the immune system attacks inappropriate targets (harmless allergens, self-components, or gut contents) or fails to switch off unnecessary background inflammation (resulting in chronically raised biomarkers of inflammation, and cardiovascular, metabolic, and psychiatric disorders). We can use an evolutionary approach to identify inputs that the immune systems of evolving humans inevitably received, because these are the inputs on which we may be in a state of "evolved dependence". The maternal microbiota is clearly one major source of data for the developing immune system, and we now understand that caesarean deliveries, lack of breastfeeding, unvaried diets, and misuse of antibiotics are distorting mother-to-child transmission of the microbiota. However, the main focus of this essay is the crucial role of inputs from the natural environment, which have received much less attention despite the fact that epidemiology reveals strong health benefits of contact with nature. These inputs from nature, received via the airways, gut, and skin, provide crucial signals that set up immunoregulatory circuits. The natural environment also provides other inputs including epitopes to guide retention and expansion of useful lymphocyte clones, information about the microbiota of the environment into which the individual is born, signals that drive background activation of the innate immune system, spores of gut-adapted strains that can replace those lost because of antibiotics or poor diet, bacteriophages that modulate the microbiota, and DNA via horizontal gene transfer that increases metabolic flexibility of the gut microbiota. By analysing these microbial inputs and their mechanisms of action, while also identifying the lifestyle changes that are disrupting them, we can expect to discover novel prophylactic and therapeutic strategies.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_18

18.1 Introduction: Evolution and Exposure to Microbes

Hints that human health might depend on certain types of contact with the natural environment can be traced back to the nineteenth century when it was noted that hay fever was uncommon amongst farmers (Blackley 1873). This observation had been forgotten more than a century later when it was observed that children with multiple older siblings had a reduced risk of developing hay fever (Strachan 1989). It is now widely accepted that lifestyle changes that have reduced our interactions with microorganisms have contributed to faulty regulation of the immune system, and several overlapping hypotheses (for example, hygiene hypothesis, old friends hypothesis, and biodiversity hypothesis) have been developed to explain these observations (Strachan 1989; Rook et al. 2004; von Hertzen et al. 2011). The defects in immunoregulation have contributed to startling increases in two types of illness (Rook et al. 2013). First, there are disorders where the immune system is attacking targets that it should not attack, such as our own tissues (autoimmune disease), gut contents (inflammatory bowel disease), or harmless allergens (allergic disorders). Secondly, there are disorders associated with a failure to terminate redundant inflammatory responses. This leads to persistently raised biomarkers of inflammation, and predisposes to metabolic, cardiovascular, and psychiatric disorders.

But which organisms are involved? At first, the medical profession hypothesised that the older siblings were providing contact with the common infections of childhood, and that these might be necessary for maturation of the immune system (Strachan 1989). However, when we consider this proposition in relation to human evolutionary history, it is clearly wrong. The common infections of childhood are crowd infections that require large populations to become endemic, and rapidly disappear from small ones such as hunter–gatherer groups, or small island populations (Black 1966). Measles, for example probably did not affect human populations until sometime between 300 BC and the end of the Roman empire so we cannot be in a state of evolved dependence on measles. But as will be discussed below, the unfortunately named hygiene hypothesis makes this mistake and also confuses the public and the media by exaggerating the minor role of home hygiene (Bloomfield et al. 2016). The old friends hypothesis, which forms the background to this essay, suggests that to understand the precise nature of our dependence on contact with microorganisms, and the identity of the microorganisms that matter, we need to think about vertebrate and human evolution (Rook et al. 2004, 2017; Rook 2013), and to take seriously the observations of Blackley (1873).

18.1.1 Evolution of the Microbiota

The first cellular life forms evolved on earth approximately 3.8 billion years ago. Eukaryotic life followed about 1.5 billion years ago when an organism resembling an alpha-proteobacterium started to live inside another organism (an endosymbiotic

event), and gave rise to the mitochondrion (Imachi et al. 2020). Then between 540 and 520 million years ago there was the extraordinary Cambrian evolutionary explosion that resulted in the appearance of most of the existing animal phyla. The earliest vertebrates appeared 20–30 million years after the Cambrian explosion. Most experts think the crucial endosymbiotic event that created the mitochondrion occurred only once, so ultimately humans, like all eukaryotic life forms, evolved from a blend of 2 or more microbes. Recent biotechnology has revealed that about 65% of human genes originated in Bacteria, Archaea, and eukaryotic microbes (Domazet-Loso and Tautz 2008). This is strikingly true of the genes enabling synthesis of the neurotransmitters that are crucial to the brains of which we are so proud (Iyer et al. 2004). So we evolved from Bacteria, Archaea, and eukaryotic microbes, and we took most of our genes from them. But we also carry a vast community of them within our bodies.

Our guts contain symbiotic organisms (the microbiota) that are at least as numerous as the human cells in our bodies, and 30% or more of the small molecules in our peripheral blood, many of which have profound effects on our physiology, are products of the metabolism of these microbes (Wikoff et al. 2009). How did this situation evolve? Early in evolution, the organisms that inevitably found their way into the gut were separated from the host by a chitin barrier (Nakashima et al. 2018), a structure that persists in arthropods and annelids. In chordate invertebrates, such as tunicates, the chitin mesh is embedded in a mucin gel, and the gut bacteria are still rigorously separated from the gut epithelium. In the most primitive vertebrates (the ray-finned fish) a more substantial mucus layer is secreted by intestinal goblet cells, and this mucus covers the epithelium. However, the mucus layer is still separated from the lumen by a chitin membrane. Finally, in mammals the chitin layer is lost entirely and complex mucus layers interact with, and nourish organisms, many of which adhere to the mucus and modulate the function of the underlying cells (Nakashima et al. 2018). It is interesting that this parallels the situation in plants where organisms are attracted and nourished by molecules secreted from the roots, and then take part in symbiotic two-way signalling and exchange of nutrients.

18.1.2 Evolution of the Adaptive Immune System

This complex mucin barrier, devoid of the chitin mesh, allowed a much more intimate exchange of signals and metabolites between host and microbiota, and a much more complex community of organisms, with far greater numbers and diversity. Vertebrates co-evolved with this microbiota, which took on roles in the development and function of essentially all organs, including the brain. For example, studies of mice have revealed that germ-free animals delivered into a sterile environment by Caesarean section have abnormal brains and abnormal reactions to stress. These abnormalities seemingly can only be corrected by re-installing a normal microbiota in the early weeks of life (Sudo et al. 2004; Diaz Heijtz et al. 2011). Managing, tolerating, and "farming" this physiologically essential microbiota, while

simultaneously excluding pathogens, was the role of the immune system. Most evolutionary biologists now believe that the adaptive immune system evolved in parallel with this complex microbiota precisely because the innate immune system could not do the job (Pancer and Cooper 2006; discussed in Rook et al. 2017). The innate immune system relies on inherited germ line-encoded pattern recognition receptors (PRR), but rapid bacterial evolution can give rise to pathogens with structures not recognised by existing PRR. The innate immune system can try to catch up by duplicating the gene for a PRR and selecting a modification of its structure that is able to recognise the new pathogen, but clearly this process is too slow, quite apart from the fact that the genome would eventually become cluttered with massive numbers of duplicated PRR genes. The development of the adaptive immune system in vertebrates provided a way to create a very large repertoire of different receptors with a minimal increase in genetic complexity. This is achieved by somatic hypermutation involving the genes encoding the receptors of B and T lymphocytes. These random mutations create large numbers of distinct T and B lymphocyte clones bearing a huge diversity of receptors, but this creates several potential problems. For example, random mutation could result in vast numbers of useless lymphocytes that recognise nothing and so waste metabolic resources and space. Worse still, there might be lymphocytes that recognise the host's tissues and so mediate autoimmunity. However, the diversified receptors generated by mutation are expressed clonally. Each lymphocyte clone expresses only one receptor, so that if a receptor turns out to be useless or autoreactive, the relevant cell line can be eliminated. The autoreactive cells are mostly eliminated in the thymus, where self-antigens are expressed. However, in order to decide which other lymphocyte clones to keep for managing and tolerating the microbiota, while eliminating pathogens, the adaptive immune system requires data from the microbiota that is picked up from mother and family, and data from the environment. The subtlety of this arrangement is that each new individual develops an immune repertoire that is matched to the microbial world into which he or she is born.

Therefore, just like the brain, the adaptive immune system is a learning system, and like the brain, it must receive appropriate data inputs, and these must be received early in life, and then maintained and updated throughout life. These inputs come from the microbiota of mother and family, and also from the natural environment. Deprivation or corruption of these inputs, for example by depletion or distortion of the microbiota, is now known to have widespread physiological consequences. Thus, if we can determine which microbial data inputs are essential to our health, and which lifestyle changes are disrupting these inputs, we can expect to identify prophylactic and therapeutic strategies.

18.1.3 Evolution Turns the Inevitable into a Necessity

Which microbial data inputs are essential? The subtitle for this essay is "Evolution turns the inevitable into a necessity". For example, crucial functions can be

outsourced to "inevitably present" microorganisms. The classic example is a laboratory experiment where a culture of amoebae became infected with a bacterium. At first, both species in this mixed culture were handicapped but after 5 years the two organisms became mutually dependent and could no longer survive alone (Jeon 1972). Each of them had lost some crucial genes because the encoded functions could be outsourced to the other organism, which was *inevitably* present. However, evolved dependence can clearly arise in two different ways. First, a species might evolve in the presence of something (our need for oxygen for example), and so incorporate that something into its physiology from the start. Alternatively, as in the experiment with the amoeba described above, something might appear later and cause the pre-existing germ line-encoded function to become redundant. For example, most mammals can synthesise vitamin C, but in humans and some other species the gene encoding an essential enzyme is corrupted (Nishikimi and Yagi 1991). We lost an enzyme required for making vitamin C because the diet of evolving humans "inevitably" contained adequate supplies of it. Unfortunately, for sailors on long sea voyages before vitamin C was recognised, the presence of vitamin C in the diet turned out not to be inevitable after all and scurvy was common.

Both mechanisms apply to the human need for collaboration with, and exposure to microorganisms. Genetic analyses indicate that about 65% of our genes originated with the Bacteria, Archaea, and unicellular eukaryotes (Domazet-Loso and Tautz 2008), and we evolved in a world in which Bacteria, Archaea, and eukaryotic microbes are dominant life forms (Bar-On et al. 2018). Thus, it is well-established that we evolved from some microorganisms, and that we have incorporated others into our physiology as symbionts, especially in the gut. But as will be described below, we have also evolved dependence on continued exposure to microbes that were, at least in the past, inevitably present in the environment.

So evolution turns the inevitable into a necessity, but it is equally important to take note of the reverse concept: evolution tends not to *turn the non-inevitable* into a necessity because obviously this can lead to gene–environment mismatch. Sometimes human development results in lifestyle changes that evolution could not "predict" (such as the long sea voyages without sources of vitamin C), but sometimes we scientists assume inevitability that was not there. For example, the belief that we are in a state of evolved dependence on infection with helminths seems to be an error of this type. Helminths need to keep the host alive, so they downregulate inflammation in order to avoid fatal immunopathology. So, it was argued that humans accumulated mutations to partly offset the immunoregulatory strategies of helminths, with the consequence that without helminths our immune systems are too pro-inflammatory (Bilbo et al. 2011). But different helminth species live in blood, tissues, bladder or gut, and each species downregulates inflammatory responses via a different mechanism. Moreover, the loads of helminths differ wildly between individuals, even when they live in similar geographical locations. So there is no constant "inevitable" factor that could drive germ line-encoded dependence on helminths (discussed in Rook et al. 2017). Rather than becoming written into germline mutations, intermittent or temporary environmental or infectious stresses are coped with via epigenetic adaptations that can fade over several generations, or

be renewed as required. Therefore, it is not a surprise that allowing multiple sclerosis (MS) patients to become infected with helminths they would have encountered in childhood can stop progression of the disease (Correale and Farez 2011), whereas trials of helminth therapy for MS in locations where helminths have not been endemic for several generations are failing (Fleming et al. 2019; Charabati et al. 2020). Evolution turns the occasional into an option (via epigenetics), not into a germ line-encoded necessity.

So we are neither in a state of evolved dependence on the common infections of childhood, nor on helminth infections. Similarly, it is not possible that we are in a state of evolved dependence on the microbiota of the modern home. Until relatively recently our homes were derived from the natural environment, and built with natural timber, thatch, and mud- or dung-based plaster. In sharp contrast, the modern home is constructed from concrete, plastic, and biocide-treated timber and plasterboard (Sahlberg et al. 2013). The quite different microbiota of such a home cannot be part of our evolutionary heritage so it is not surprising that detailed studies do not find that modifications of the microbiota of modern homes by domestic cleaning practices are linked to immunoregulatory disorders (Weber et al. 2015). As will be discussed in detail later in Sect. 18.3, the relevant aspect of the microbiome of the home is the extent to which it resembles that of the natural environment. . . . or of the shelters and homes of our ancestors (Kirjavainen et al. 2019). Confusion about the role of domestic cleaning has been exacerbated by the fact that the cleaning agents themselves damage the lungs of children, but that is a separate issue unrelated to the microbiome (Parks et al. 2020). These errors in our thinking have delayed progress. Clearly, we need to ask ourselves which microbes really were inevitably present during an evolutionarily significant part of our past. Then amongst these organisms, we may find some on which we are in a state of evolved dependence. Finally, we will need to explain the mechanism of this dependence so that we can provide the relevant organism or mimic its function with novel medicines.

18.2 Microbiota from Humans and the Natural Environment

We receive the necessary microbial inputs from other humans (especially our mothers), and from the natural environment (Fig. 18.1). The fact that the gut microbiota is a crucial part of our physiology, and that its composition is a major determinant of health is now well-known and has been subject to numerous recent reviews (Lynch and Pedersen 2016; Davenport et al. 2017). Moreover, we now understand many of the lifestyle changes that are limiting or corrupting the transmission of the microbiota from family to child. These factors include caesarean deliveries, lack of breastfeeding, inappropriate hygiene that limits mother–child transmission, poor diet, and antibiotics during pregnancy and infancy. This last point is particularly worrying. The relative risk of obesity is linearly related to the

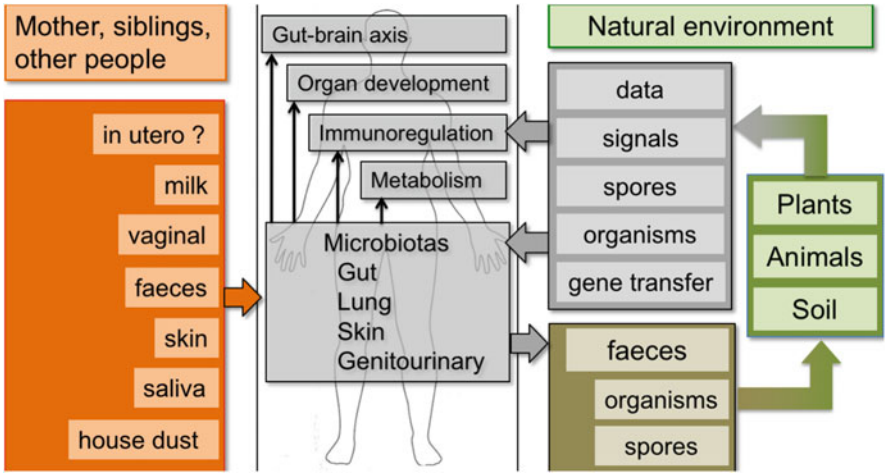


Fig. 18.1 The microbial world of mankind. The various microbiotas have co-evolved with us as partners that play essential roles in all aspects of our physiology. Thus, we are in a state of evolved dependence on their presence. These organisms come from mother and family, but also from the natural environment. In addition to the organisms themselves, they supply data and signals that are needed for setting up the immune system, and in particular, for fine-tuning the mechanisms that regulate the immune system

number of exposures to antibiotics in infancy (Shao et al. 2017). The same is true for psychiatric (Köhler-Forsberg et al. 2019), and allergic disorders (Strzępa et al. 2018), and there is perhaps an association between antibiotic use and some autoimmune diseases (Strzępa et al. 2018; Dydensborg Sander et al. 2019; Kemppainen et al. 2017). These findings are particularly worrying because a recent study of 8 low- and middle-income countries revealed an average of 11 antibiotic prescriptions by the age of 2 years (Fink et al. 2019). This will hasten the switch to a Western pattern of metabolic, psychiatric, and inflammatory disorders.

However, this essay concentrates on the role of exposure to the microbiota of the natural environment, and mentions the human microbiotas only when exposure to the natural environment is a major determinant of the constitution, integrity, and function of the human microbiota. For example, the natural environment can supply organisms to replace those eliminated by antibiotics. Many epidemiological studies suggest that exposure to the green environment is beneficial for health, and evidence is particularly strong for the same three types of health problems that are affected by antibiotic use, as summarised below: metabolic, psychiatric, and inflammatory disorders.

18.2.1 Metabolic Syndrome and Obesity

Metabolic syndrome, obesity, and cardiovascular disease constitute one of the major groups of health problem that plague modern humans. A longitudinal study based on

four clinical examinations over a 15-year period of 6076 individuals, aged 45–69 years at baseline, who participated in the Whitehall II study revealed a 13% lower risk of metabolic syndrome in people living within 500 m of green space (de Keijzer et al. 2019). Such longitudinal data reinforce the classical study of approximately 40×10^6 UK citizens which suggested that living in proximity to green space reduced the risk of cardiovascular disease and prolonged overall survival (Mitchell and Popham 2008).

18.2.2 Psychiatric Disorders

Psychiatric disorders constitute another major group. A study of approximately 1 million Danish citizens found that living close to high levels of green space during childhood reduced the risk of most mental illnesses later in life. In contrast, for those most deprived of green space during childhood, the risk of mental illness was up to 55% higher (Engemann et al. 2019), and similar results for depression and anxiety were reported in a large study in the Netherlands (Maas et al. 2009). Clearly, there are many possible explanations for this relationship, but evidence suggesting a role for the microbiota of the natural environment is described later.

18.2.3 Allergies

In the late nineteenth century, it was noted that farmers were less likely to develop hay fever than were city dwellers (Blackley 1873). Since then hundreds of epidemiological studies have confirmed that exposure to the farming environment in early life diminishes the risk of allergic disorders (Ege et al. 2011; Stein et al. 2016), while other studies have shown that merely living in proximity to green spaces lowers the risk of allergic sensitisation (Hanski et al. 2012). Importantly, some of these papers include immunological findings that strongly indicate cause and effect, rather than merely chance association (Hanski et al. 2012; Stein et al. 2016). These mechanistic points will be discussed later.

18.3 What Organisms from the Natural Environment Do We Meet?

In the past, we encountered organisms from the natural environment in our homes. The homes of our evolving ancestors were caves or shelters that were part of the natural environment, or constructed with timber, leaves, and mud taken from that environment. Even more recently, mediaeval homes were built with natural timber,

and walls were plastered with mixtures of mud, animal hair, and dung, while roofs were often thatched. The microbiota of such homes was similar to that of the natural environment with which we co-evolved, and harmless to humans. In contrast, the modern home, built with plastic, concrete, and biocide-treated timber and plasterboard can become toxic (sick building syndrome) when damp or deteriorating, because an unusual microbiota appears that has nothing to do with the microbiota of our evolutionary past. The secondary metabolism of such organisms can be toxic to humans (Andersson et al. 1998; Sahlberg et al. 2010). However, we can benefit from measures that transport the microbiota of the natural environment into our modern homes. One way of achieving this is to keep cats or dogs (Fujimura et al. 2010). A large Danish study found a dose–response relationship between the number of household cats and dogs during the first year of life and reduced manifestations of asthma, allergic rhinoconjunctivitis, or eczema) (Hesselmar et al. 2018). Similarly, the more the microbiota of the home resembles that of the farm environment, the lower the risk of asthma in children (Kirjavainen et al. 2019). But direct contact with the natural environment may be a better way to enhance intake of organisms from air, soil, and water via the airways, gut, and skin, as discussed below.

18.3.1 Air

When total numbers of organisms in air were counted (i.e. not only the cultivable ones) levels of 10^2/litre or more were regularly encountered over a grassy field on clear sunny days, and estimates approaching 10^3/litre have been reported above shrubs and some grasslands (reviewed in Burrows et al. 2009). The air in facilities housing agricultural animals can contain still higher numbers, reaching 10^4–10^5 archaea and bacteria in every litre (Nehme et al. 2009). Since the average adult breathes about 11,000 l of air each day, the input over a 24 h period can be anything from 10^6 to 10^9 organisms, and even 10^{10} if working hard and breathing heavily in some environments, but the issue is complicated by the fact that deposition in the airways depends on particle size. Large particles tend to impact on surfaces in the upper airways, such as the nasal turbinates while small particles may enter the more distal airways and interact with host tissues by diffusion. The microbial diversity of air is comparable to that of seawater, soil, and the human gut, but in a recent study only 9–17% of the airborne microbial sequences were identifiable (Gusareva et al. 2019). Thus, we really do not know what organisms are encountered via the airways, though tropical air contains a relatively higher proportion of fungi with only traces of phage and archaea (Gusareva et al. 2019). Clearly, soil organisms can enter the air via dust in dry conditions, but we now know that raindrops impacting soil cause tiny explosions of soil organisms to enter the air, and organisms are always present in the air we breathe (Joung et al. 2017). They will also settle on the food that we eat.

18.3.2 Soil

Food also supplies us with organisms from the soil, especially in farmers' markets where food has been subjected to little, if any, washing. But it turns out that soil consumption (geophagy) is an evolved behaviour, and extremely ancient in an evolutionary sense. It is likely that all vertebrates do it, especially in early life, and the green iguana has been much studied in this context (Troyer 1984). More relevant to humans is the fact that many primate species eat soil, including the closest relatives of humans, gorillas, orangutans, and chimpanzees (Krishnamani and Mahaney 2000; Sing and Sing 2010). Similarly, there is some archaeological evidence that early hominids included soil in their diets (Clark 2001). So what about modern humans? In the 1990s, it was noted that in western Kenya ~70% of 207 schoolchildren aged 5–18 years consumed soil on a daily basis (median ingestion of 28 g/day, range 8–108 g) (Geissler et al. 1997). This behaviour was more prevalent in girls and continued into adolescence. It has been noted in numerous other cultures, on all continents (Sing and Sing 2010; Geissler et al. 1997). Geophagy is also common in pregnancy, not only in undeveloped rural cultures, but also as a manifestation of the psychological disorder "pica" in Westernised ones. Babies of all cultures consume astonishing quantities of soil if adults do not intervene (Ngure et al. 2013). Interestingly soil was also an integral part of food preparation in many cultures, possibly because added soil adsorbed toxic glycoalkaloids present in foods based on acorns or early varieties of potatoes (Sing and Sing 2010). However, this does not explain the consumption of soil as an independent dietary component. Some authors suggest it was a desperate act driven by starvation but this is clearly not usually the case. Others suggest that it is an evolved behaviour that increases intake of minerals. But since evolution turns the inevitable into a necessity it is reasonable to suggest that whatever the initial motivation, intake of soil was an important source of inevitable microbial intake.

18.3.3 Skin, Wounds, and Abrasions

In addition to intake of microorganisms via the airways and via the oral route, we can assume that early humans encountered environmental microorganisms in cuts and abrasions much more often than does modern urban man, and would not have had the facilities for effective wound cleaning. Therefore, it is interesting that when tested, probiotic *Lactobacilli* were shown to be active via a parenteral route (subcutaneous) in models of arthritis and colitis (Sheil et al. 2004). Similarly, subcutaneous immunization with a saprophytic environmental soil mycobacterium blocked stress-induced colitis and behavioural changes, and partially inhibited stress-induced changes to the microbiota. These effects were dependent upon induction of Treg (Reber et al. 2016). Parenteral administration is not pursued by pharmaceutical

companies because of the regulatory hurdles to be overcome. It is much easier to take a product to market as a food supplement.

18.3.3.1 Non-specific Beneficial Effects of Vaccines

We can also regard the overwhelming evidence for the non-specific beneficial effects of vaccines as further evidence for the usefulness of parenteral exposure to micro-organisms. Many vaccines increase overall survival for reasons that are independent of the target infection. For example, BCG vaccine saves lives via mechanisms that in some ways have nothing to do with tuberculosis (Aaby and Benn 2020). Mechanisms are discussed later.

18.3.3.2 The Microbiota of Sweat

We should also consider the natural skin microbiota that our ancestors would have carried. These would have included ammonia oxidising bacteria (AOB) that are exquisitely sensitive to alkylbenzene sulfonate detergents, and so are rare on modern skin. But AOB convert the ammonia in sweat into nitrite and nitric oxide, which are readily absorbed, and relevant to many physiological pathways (Whitlock and Feelisch 2009). Interestingly butyrate, which is an important component of body odour, is a product of lipases from Corynebacteria, Staphylococci, and Micrococci acting on lipids secreted by sebaceous glands on the skin (Stilling et al. 2016). Was this also a significant role of our ancestors' skin microbiota?

18.4 Mechanisms of Benefit from a Green Environment

It is therefore inevitable that we will encounter the microbiota of the natural environment, and as outlined earlier there are epidemiological indications that exposure to this microbiota correlates with a reduced incidence of a wide variety of medical conditions. But why is this so, and are there data that go beyond epidemiological associations and that offer clear mechanisms to consolidate the hypothesis? I discuss below the many mechanisms that we can now identify.

18.4.1 Data for the Developing Adaptive Immune System

As already explained when discussing the evolution of the adaptive immune system, it is a learning system that requires inputs of data to enable selection and expansion of relevant lymphocyte clones that recognise organisms present in the world into which the individual is born (Fig. 18.2). Thus, each individual develops and expands

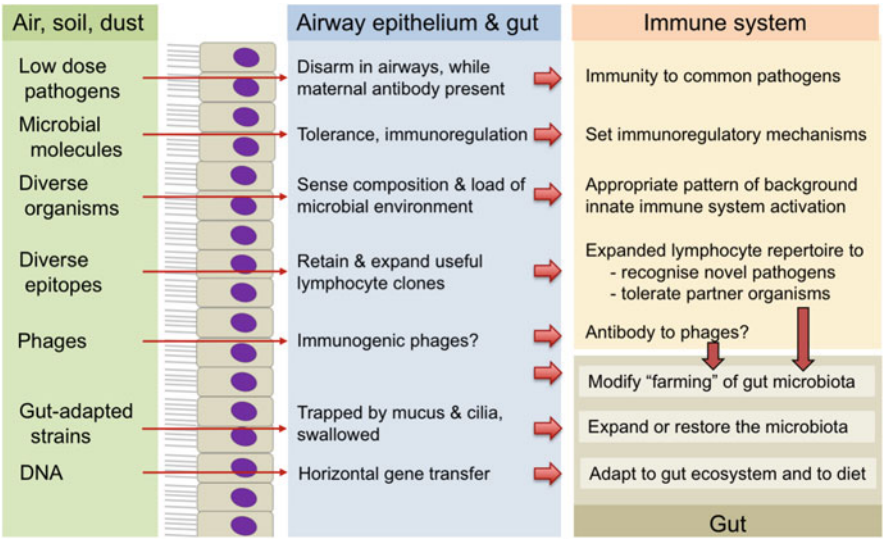


Fig. 18.2 Data supplied by the natural environment. The developing immune system requires inputs of data in order to select and expand relevant lymphocyte clones from those generated by random mutation. This process creates a repertoire of lymphocyte clones relevant to the world into which the individual is born. The data also set appropriate activation of the innate immune system (sometimes known as "trained immunity"), and expand the repertoire of tolerated organisms. Further data are supplied as DNA by horizontal gene transfer

a custom-made repertoire, and eliminates the lymphocytes that recognise nothing. The diversity of this repertoire needs to be large. All biological entities are built from variants of the same building blocks, so the more diverse the lymphocyte repertoire, the more likely it is to include memory cells that by chance recognise a novel virus, such as HIV (Su et al. 2013). Such T cells are not found in the blood of newborns, and seem to be induced by cross-reactivity of the T cell receptor with environmental antigens. They can be induced by vaccinations, and this probably represents one of the mechanisms that cause vaccines, particularly live polio, measles, and BCG, to benefit health more broadly than can be accounted for by protection from the targeted infection (Aaby and Benn 2020).

18.4.2 Modified Regulation of the Microbiota

This extended repertoire of lymphocytes is also needed for controlling and tolerating the gut microbiota (Fig. 18.2). Many workers have studied the role of the innate immune system in the "farming" of the microbiota. But as outlined above, evolutionary biologists suggest that the adaptive immune system evolved precisely in order to assist the innate immune system with this task (discussed in Rook et al. 2017). Thus, if dendritic cells (DC) lack the Class II major histocompatibility

complex (MHC) genes so that they cannot activate T cells, there is rapid and severe gut inflammation (unless the animals are germ free) that can be mitigated by antibiotic treatment (Loschko et al. 2016). Similarly, selective IgA-deficiency, although often superficially asymptomatic, is in fact accompanied by increased incidences of infectious and inflammatory disorders. The microbiota of these individuals has decreased overall diversity as well as altered relative abundances of specific microbial taxa (Catanzaro et al. 2019). Animal models have shown that the role of IgA is subtle, and different for different bacterial species. *Bacteroides fragilis* needs to be coated with IgA if it is to colonise the right niche (Donaldson et al. 2018). Similarly, IgA bound to *Lactobacillus rhamnosus* enhanced the ability of those bacteria to potentiate differentiation of Treg cells via TLR regulatory proteins, RALDH2 and secretion of IL-10 and TGF-β (Mikulic et al. 2017) (The Toll-like receptors, TLRs, are proteins that have a key role in the innate immune system and they recognise structurally conserved molecules derived from microbes that usually are expressed on sentinel cells such as macrophages and dendritic cells). On the other hand colonisation by beneficial *Clostridia* was antagonised by inappropriate IgA targeting (Petersen et al. 2019). It is interesting that chronic inflammation associated with metabolic syndrome or inflammatory bowel disease is accompanied by increased faecal levels of flagellin (Tran et al. 2019). Flagellin is the main component of bacterial flagella and induces inflammatory gene expression via TLR5 and the NLRC4 inflammasome, and also provides motility that helps bacteria to penetrate the intestinal mucus layer. Intraperitoneal injections of flagellin have been shown to induce intestinal anti-flagellin IgA, and to ameliorate diet-induced obesity and protect against the colitis that normally appears in IL-10-deficient animals (Tran et al. 2019). Thus, the adaptive immune system coordinates with the innate immune system and is indeed involved in "farming" the microbiota, and we should suspect that any input to the immune system that modifies the response to any microbial component will inevitably modulate the microbiota too.

18.4.3 Low Dose Pathogen Exposure

It is inevitable that amongst the organisms that are breathed in there will be low doses of potential pathogens (Fig. 18.2). This provides an opportunity for the immune system to develop immunity to these organisms, particularly in early life when the infant might still be protected by maternal antibody. The airways kill or disarm the respired organisms which are then taken in by the lymphoid tissue of Waldeyer's ring, or exposed to acid in the stomach before being sampled by the dendritic cells in small bowel (Schulz and Pabst 2013). The array of mechanisms used by the airways to disarm the inhaled pathogens is impressive. Bacterial cell wall LPS, acting via TLR4 induces release of nasal mucosa-derived exosomes containing inducible nitric oxide synthase. These exosomes transfer the enzyme to neighbouring epithelial cells which then increases release of nitric oxide (Nocera et al. 2019). Bacterial attachment also leads to release of cathelicidin (also known as

LL-37), which is taken up by infected cells. Cathelicidin is one of well over 100 human antimicrobial peptides (AMP) that also include defensins, β-defensins, lysozyme, lactoferrin, secretory leukocyte proteinase inhibitor, elafin, and RNase 7 (Hiemstra et al. 2016). Entry of bacteria and cathelicidin into the cell triggers activation of the NLRP3 inflammasome and a cascade of events including the activation of caspase 1, death of some infected cells, and release of the pro-inflammatory cytokines IL-1β and IL-18. These events enhance inflammation and recruit neutrophils. Under the influence of cathelicidin and other AMP, the neutrophils form networks of extracellular fibres consisting mainly of DNA to which some AMPs such as neutrophil elastase and cathepsin G adhere. These AMP-armed Neutrophil Extracellular Traps (NET) contribute to inactivation of microorganisms (Hiemstra et al. 2016).

18.4.4 Determine the Nature of the Microbial Environment

Data from the natural environment also inform each new individual's immune system about the balance and load of different types of organism in that individual's environment (Fig. 18.2). To take an extreme example, in tropical rainforest air, fungal spores account for up to ~45% of particulate matter, whereas this is not so in modern urban environments (Elbert et al. 2007).

In general, Gram-negative bacteria are recognised by TLR4 that detects their endotoxin (LPS), and Gram-positive organisms by NOD2, which recognises their cell wall peptidoglycans, and by TLR2 which recognises many components including lipopeptides. However, TLR2 forms heterodimers with other TLR (notably TLR1 and TLR6), and also recognises a vast range of molecules from bacteria, viruses and protozoa, and perhaps even some forms of LPS. Bacteria are also detected by human TLR8 and TLR7 which recognise single-stranded RNA. Expression of TLR7 is intracellular within airway epithelia and airway smooth muscle, leading to secretion of pro-inflammatory cytokines and type-I IFNs (Dong et al. 2016). The air can also contain Archaea. These organisms do not seem to trigger NOD2, TLR2, or TLR4, but their RNA, like that of bacteria, causes TLR8-dependent inflammasome activation (Vierbuchen et al. 2017).

The fungal cell wall is quite different from that of bacteria or archaea, and contains chitin (β-(1–4)-poly-*N*-acetyl-D-glucosamine; Ascomycota and Basidiomycota) and chitosan (similar but partially de-acetylated; Zygomycota). These are bound by numerous PRR, such as Fibrinogen C domain containing 1 (FIBCD1), RegIIIc, Toll-like receptor (TLR) 2, dectin-1, and the mannose receptor. Fungi also contain glucans that cross-link chitin or chitosan polymers that can be recognised by dectin-1 (Camilli et al. 2018). We tend to forget the fungi because they are not very numerous in the gut microbiota, but they are large organisms and so constitute a significant fraction of the microbial bulk. It is likely that exposure to greater loads of airborne fungi will therefore modulate the "farming" of the

microbiota by the immune system, quite apart from leading to a different pattern of activation of innate immune mechanisms in the airways.

This huge topic will not be reviewed further here, except to note that in addition to the microbial molecules mentioned above, various PRR detect peptidoglycan monomers, teichoic acids, porins, mycolic acid, mannose-rich glycans, flagellin, and many others. Thus, the PRR expressed in the airways play a crucial role in setting up appropriate background immune activation, matched to the load and identity of the microbes detected (Netea et al. 2016). But perhaps even more important is the role of airway PRR in setting up immunoregulation, discussed in the next section.

18.4.5 Signals to Set Up Immunoregulation

Studies of protection from allergic disorders by exposure in childhood to the farming environment have begun to highlight the importance of early exposure to microbe-derived molecules containing muramic acid, a component of many bacterial cell walls, or LPS derived from Gram-negative bacteria (Fig. 18.3). For example, levels of muramic acid in the home, or levels of LPS in the child's mattress during the first year of life correlated with a lower prevalence of school-age asthma, and mattress

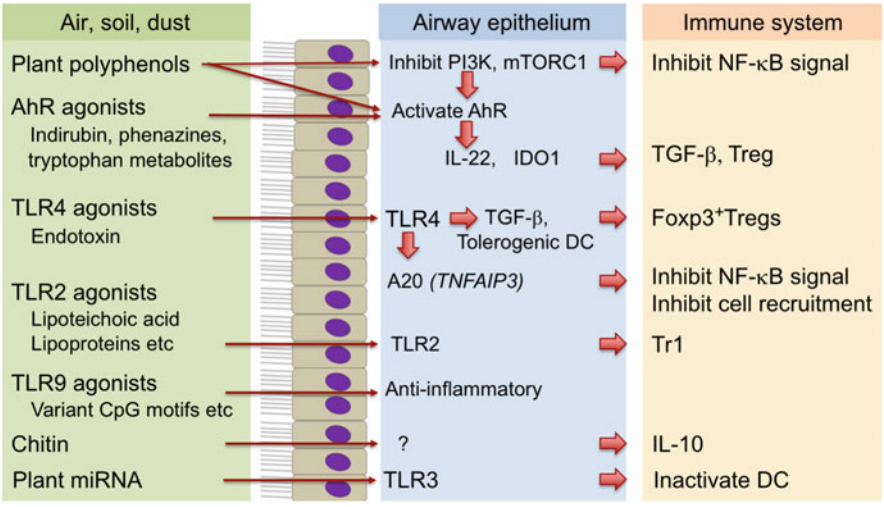


Fig. 18.3 Signals from the natural environment that drive immunoregulation. Signals such as that provided by endotoxin (LPS) initially drive inflammation, but if repeated in small doses they drive "endotoxin tolerance", which ultimately results in raised activity of anti-inflammatory pathways and mediators, and increased regulatory lymphocytes (Tr1, Treg), and mediators (IL-10). Most, perhaps all, pathogen-associated molecular patterns (PAMPS) do this, and so balance the activation of the innate immune system known as "trained immunity". Similarly, molecules of plant origin that often accompany microorganisms in biogenic aerosols and pollen grains can also provide anti-inflammatory signals

LPS was also inversely associated with atopic sensitization (Weber et al. 2015). Interestingly, the more the microbiome of a Finnish home resembles that of the farm environment rich in species of animal origin, the lower the risk of asthma in the children (Kirjavainen et al. 2019). Other studies had also shown that contact with cows and pigs protects against allergic disorders (Riedler et al. 2001; Sozanska et al. 2014). Moreover, the peripheral blood cells of children from homes with farm-like microbiomes released lower levels of inflammatory cytokines in response to bacterial cell wall components in vitro (Kirjavainen et al. 2019).

We now understand that signals provided in early life by conserved microbial components such as LPS are necessary for two distinct reasons. First, they set up the appropriate background level of innate immune system activation, sometimes referred to as "trained immunity", via a series of epigenetic mechanisms elegantly reviewed elsewhere (Netea et al. 2016). Secondly, and crucially, they set up anti-inflammatory and immunoregulatory pathways, and it is these mechanisms rather than trained immunity that are the focus of this review.

18.4.5.1 Endotoxin Tolerance and Immunoregulation

The first glimpse of these mechanisms was interpreted as "endotoxin tolerance". It was observed that animals would survive a lethal dose of endotoxin if they had previously been exposed to one or more lower doses. More recently we understand that this is not really what endotoxin tolerance is about (Fig. 18.3). In an animal model LPS was shown to induce Treg via tolerogenic dendritic cells and TGF-β (Jia et al. 2018). Endotoxin in dust protects against allergy through induction of A20 in lung epithelial cells (Schuijs et al. 2015). A20, the product of the *TNFAIP3* gene, is a potent inhibitor of the NF-κB signalling pathway. The relevance of this to immunoregulation in humans is demonstrated by the observation that mutations in the *TNFAIP3* gene that lead to A20 deficiency cause an early-onset auto-inflammatory disease (Zhou et al. 2016).

LPS also sets up immunoregulatory circuits via the gut. Autoimmune and allergic disorders are much more prevalent in Finland and Estonia than in genetically similar populations in Russia. It has emerged that the LPS of the dominant Gram-negative bacteria in the guts of Finnish and Estonian children fails to trigger TLR4 and so fails to set up endotoxin tolerance and the accompanying immunoregulatory mechanisms, while the Russian children have organisms that provide a potent TLR4 agonist (Vatanen et al. 2016).

18.4.5.2 Tolerance to TLR2 Agonists and Immunoregulation

Endotoxin is not the only microbial component to which tolerance can be induced, and that can help to set up immunoregulatory circuits, including Treg (Fig. 18.3). It is also possible to induce tolerance to TLR2 agonists such as lipoproteins from Gram-negative and Gram-positive bacteria, lipoteichoic acid from Gram-positive

bacteria, lipoarabinomannan from mycobacteria, and zymosan from yeast. Interestingly, lipoteichoic acid from a human-derived strain of *Lactobacillus paracasei*, acting via TLR2, was shown to correct age-related gut leakiness and inflammation, and increase mucin production and increase the abundance of a mucin-degrading bacterium *Akkermansia muciniphila*, an organism associated with protection from obesity and diabetes (Wang et al. 2019). Similarly, a TLR2 agonist administered to mice induced both TLR2 tolerance and attenuation of the autoimmune disorder Experimental Autoimmune Encephalomyelitis (EAE). This tolerance was accompanied by reduced Th17 cells and an increase in splenic type 1 regulatory T cells (Anstadt et al. 2016). Moreover, the same authors find that patients suffering from Multiple Sclerosis have abnormally low circulating levels of a bacterium-derived TLR2 agonist, when compared to healthy donors (Anstadt et al. 2016).

18.4.5.3 Immunoregulation via TLR9

There is evidence that signals via TLR9 might sometimes exert anti-inflammatory effects. This intracellular TLR detects unmethylated CpG motifs. These are relatively common in microbial genomes, and they drive an inflammatory response. However, several workers have identified variants of the CpG motif and other microbial DNA sequences, that have lost their pro-inflammatory effects, or become anti-inflammatory (Krieg et al. 1998; Hiramatsu et al. 2014). A recent study found that the genomes of a large range of *Lactobacillus* species are rich in these immunosuppressive motifs, when compared to several pathogens (Mazhary et al. 2020). Lactobacilli are ubiquitous, associated with food and people, but also notably with flowers, animals, insects, and soil containing fermentable matter such as grass (silage). We know that at least part of the probiotic effects of lactobacilli require the presence of TLR9, which is well expressed in the gut and airways (Rachmilewitz et al. 2004). This raises the possibility that organisms such as lactobacilli breathed in from the natural environment can exert immunoregulatory effects in this way.

18.4.5.4 Immunoregulation via Other Pattern Recognition Receptors

There is some evidence that tolerance can also be driven via other PRR, perhaps because the cytokine IL-1β is able to do it (Alves-Rosa et al. 2002), and many microbial components induce its release. Under some experimental conditions exposure to chitin, present as described above in fungal cell walls, is associated with release of anti-inflammatory IL-10, or with suppression of anaphylaxis and reduced allergen-specific IgE (Elieh Ali Komi et al. 2018). The latter effect might be attributable to a broad anti-inflammatory mechanism, but it could be due to induction of a Th1 bias, with reduced Th2-mediated allergic pathways. For example, signals from TLR7 can reduce airway inflammation, by promoting a Th1-bias that reverses airway hyperresponsiveness and reduces airway remodelling (Dong et al. 2016).

18.4.5.5 Immunoregulation via the Aryl Hydrocarbon Receptor

The airways contain a number of cellular sensor systems that can monitor the content of biogenic aerosols in inhaled air (Fig. 18.3). For example, many microbial pigments such as phenazines and naphthoquinones are Aryl Hydrocarbon Receptor (AhR) agonists and have been shown to regulate inflammation and antibacterial responses (Moura-Alves et al. 2014). However, even microbes with little direct AhR agonist activity can trigger the AhR via their metabolites. Tryptophan can be metabolised to produce AhR ligands that drive production of IL-22 by activated cells that are abundant at mucosal surfaces such as DC, T cells, and innate lymphoid cells (ILC). The protein IL-22 regulates reactions to microbial pathogens, especially in respiratory and gut epithelial cells, and plays a major role in resistance to colonization by fungi and by some bacterial species (Zelante et al. 2014). But IL-22 also activates host indoleamine-2,3-dioxygenase 1, which generates further tryptophan-derived AhR agonists. These drive production of TGF-β and Treg and so contribute to setting up immunoregulation (Bessede et al. 2014; Quintana et al. 2008).

18.4.5.6 Contribution of Non-microbial Biogenic Particles

The cellular sensor systems in the airways that monitor the content of biogenic aerosols will also detect molecules present in plant and pollen fragments. For example, the PI3K/Akt/mTORC1 signalling system plays a role in inflammatory pathways via NF-κB. Natural products from algae and higher plants (in addition to products of bacteria and fungi) can inhibit the activities of these protein kinases, and the overall effect is thought to be anti-inflammatory (Moore 2015).

Plant polyphenols can also exert anti-inflammatory effects via the Aryl hydrocarbon receptor (AhR). Thus, quercetin, resveratrol, and curcumin interfere with metabolic degradation of the endogenous AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ). This increases FICZ levels and indirectly activates the AhR (Mohammadi-Bardbori et al. 2012).

There is some evidence that miRNA from plants can trigger anti-inflammatory effects via TLR3 (Cavalieri et al. 2016). These miRNA target dendritic cells and reduce the ability of those cells to respond to inflammatory mediators and to drive T cell responses. A preparation of plant miRNA injected intravenously was able to attenuate Experimental Autoimmune Encephalomyelitis in a mouse model (Cavalieri et al. 2016).

18.4.6 *Spore-Forming Organisms That Set Up Immunoregulation*

The previous sections make the point that microbial components such as LPS provide signals via TLR and other PRR in the airways and gut that set up immunoregulatory pathways. This process does not necessarily imply colonization, and can be mediated by fragments of dead microorganisms in inhaled dust. However some strains, including several spore-forming organisms, need to colonise in order to drive immunoregulation. Spores can persist in the environment for hundreds, perhaps thousands, of years (Nicholson 2002). Therefore, wherever humans (or indeed other vertebrates with similar guts and diets) have lived, the environment is seeded with gut-adapted strains (Fig. 18.4). This is important because a recent study revealed that the spore-forming strains within the human microbiota are more diverse than non-spore-forming bacteria and show a higher species turnover or a greater shift in relative abundance over the course of a year (Browne et al. 2016). This implies that when a gut organism becomes extinct as a result of dietary inadequacy or antibiotic misuse (Sonnenburg et al. 2016), it can be "reinstalled" via spores from the natural environment, and this could rescue immunoregulation. For example, many spore-forming *Clostridia* are able to drive expansion of Treg populations both in vivo and in vitro (Narushima et al. 2014; Cekanaviciute et al. 2018). The likely relevance of this to humans is indicated by the observation that spore-forming organisms from the guts of normal donors will drive proliferation of

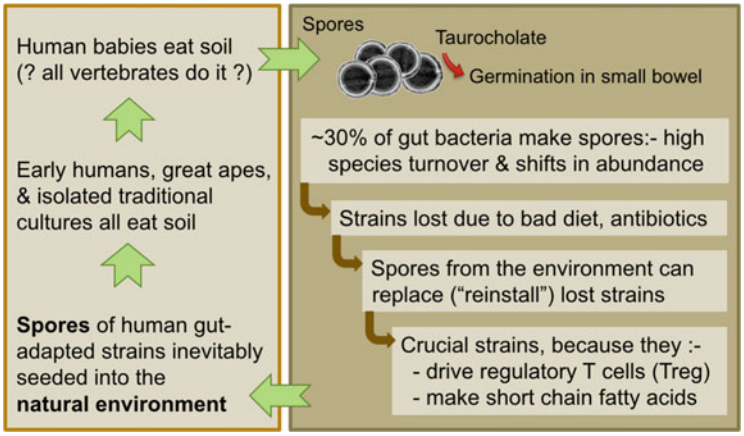


Fig. 18.4 The spore cycle. About 50–60% of bacterial genera in the gut produce spores that can survive in the environment for decades, centuries, or longer. Thus, wherever humans (or other vertebrates with similar guts) have lived, the environment is seeded with gut-adapted strains. The spore-forming gut microbiota show more frequent changes in abundance, or even disappearance (probably due to bad diet or antibiotics), than the non-spore-forming organisms. But they can be reinstalled by contact with the natural environment. It is noteworthy that young vertebrates, great apes, human babies, and even adults in traditional human societies, all eat soil. The spore-forming organisms perform several essential metabolic and immunoregulatory functions

Treg in an in vitro system, whereas spore forming organisms from MS patients failed to do so, and even opposed this property of organisms from normal donors (Cekanaviciute et al. 2018).

18.4.7 Spore-Forming Organisms as Probiotics

Other spore-forming organisms from the environment might also be important despite not being prominent components of the human microbiota, and despite mediating imperfectly understood functions, not all of which involve immunoregulation. Spores in soil have tended to be studied by environmental microbiologists and ecologists, and the soil has been regarded as the natural habitat of the spore-forming organisms such as *Bacillus* spp., despite awareness of the fact that many of them can germinate and replicate in the intestinal tracts of insects and other animals (Nicholson 2002). Recently, it has been reported that spores of *Bacillus subtilis* can germinate in the small bowels of mice and rabbits (Casula and Cutting 2002; Tam et al. 2006). Moreover, after germination they replicated in the small bowel, and then re-sporulated as they entered the colon. The same was observed in humans. *Bacillus subtilis* strains were obtained from biopsies of human ileum and from faecal samples (Hong et al. 2009a). There is therefore the possibility that *B. subtilis* and other environmental spore-forming species should be regarded as gut commensals rather than soil microorganisms (Hong et al. 2009b). *B. subtilis* is an important stimulus for development of the gut-associated lymphoid tissue (GALT) in rabbits and sporulation of live bacilli within the GALT was considered critical to this process (Rhee et al. 2004). We know little about the role of *Bacillus* spp. in humans, but bacillus spores have been used as probiotics for many years (Casula and Cutting 2002; de Castro et al. 2019). A recent study in Thailand noted that *Staphylococcus aureus* was never found in the faecal or nasal microbiota of individuals whose faecal microbiota included *Bacillus* species (Piewngam et al. 2018), and a preliminary study in the Philippines suggested that treatment for a week with about 2×10^9 spores of *Bacillus clausii* strains accelerated the termination of acute childhood diarrhoea (de Castro et al. 2019).

18.4.8 Strains That Adapt to Diet

Whether derived from the air or from soil, a subset of the organisms from the natural environment can colonise the host, and supplement the microbiota (Fig. 18.2). Indeed it has proved possible to reconstitute the gut microbiota of germ-free mice using organisms from soil, though when caged with animals bearing a normal mouse microbiota the mouse-gut-adapted strains rapidly replaced the soil-derived strains (Seedorf et al. 2014). Many bacterial species such as *Lactobacilli* are found both in soil and in the human gut but are these the same strains? The guess is that the answer

is sometimes yes, sometimes no. Analysis at the strain level is difficult, but different strains of the same species can have quite different properties. Nevertheless, there is increasing evidence that exposure to soil organisms is relevant.

The composition of the gut microbiome is strongly influenced by diet. For example, rural African villagers have strains of *Xylanibacter*, and *Treponema* that are entirely absent from the guts of European children, and indicate an ability to use indigestible carbohydrates such as xylane, xylose, and carboxymethylcellulose that are present in a diet rich in roots and tubers (De Filippo et al. 2010). From where did the African children acquire these organisms? An experiment with caterpillars provides a clue. The microbiome of caterpillars does not resemble that of the leaves on which they feed. The caterpillars are not endeavouring to become leaves. Rather, their microbiome resembles that of the soil beneath the plant, where organisms that can digest the leaves are found (Hannula et al. 2019). It is probable that the African communities picked up organisms that digest the foods they eat from the soil in which the food was grown.

18.4.8.1 Fermented Foods

Moreover, in the absence of refrigerators fermentation by environmental organisms was inevitable, and in scavenged meat or fallen fruit the process would have begun before the food was gathered. This would increase exposure to the organisms from the soil capable of metabolizing the food in question. Learning to control these fermentation processes involving bacteria (e.g. lactic fermentation), yeast (alcoholic beverages), or moulds (oncom, tempeh) so as to develop palatable non-toxic storage options was a crucial cultural advance. Analyses of residues in pottery reveal that fermented beverages containing alcohol were in use at least 9000 years ago (McGovern et al. 2004) and probably a great deal earlier (McGovern 2009). These techniques, together with lactic fermentation of vegetables (Sauerkraut, Kimchi etc.) and olives (Benitez-Cabello et al. 2016) or fermentation of meat, add nutritional supplements such as vitamins and lactoferrin (Swain et al. 2014; Leroy et al. 2013; Selhub et al. 2014; Breidt et al. 2013). Moreover, fermented foods provide microbiological diversity as well as bioactive peptides and phytochemicals such as flavonoids that modulate the intestinal microbiota (Lu et al. 2013). Microbiota from foods can transiently colonise the gut (David et al. 2014), and since there are relatively few organisms in the ileum, ingested organisms can form a significant percentage of the organisms present at this site, or even transiently outnumber the resident ones (Derrien et al. 2015). This is important because the ileum is where mucosal dendritic cells sample the gut contents and "inform" the immune system about the antigenic repertoire of gut contents (Schulz and Pabst 2013).

These points raise interesting issues for modern humans. Even when we do eat fermented foods, these are mostly fermented in controlled factory conditions, so we take in few fermenting organisms from the natural environment. How many European citizens have encountered the soil in which bananas, dates, or pineapples were grown? Are we failing to pick up organisms that we require for the inactivation

of pharmacologically active components of exotic foods, as well as reducing the diversity of microbial intake, and the supply of data to the immune system?

18.4.9 Horizontal Gene Transfer

In addition to microbial molecular signals, and colonising organisms, the natural environment can provide microbial genes (Hehemann et al. 2010; Smillie et al. 2011). For example, enzymes acquired by horizontal gene transfer (HGT) from marine seaweed-associated bacteria enable the gut microbiota of Japanese individuals to metabolise seaweed carbohydrates (Hehemann et al. 2010). The frequency of genes in the human microbiome that appear to have been acquired by HGT is remarkably high (Liu et al. 2012; Yaffe and Relman 2019), and transfer can occur between species that diverged in evolution millions of years ago. The natural environment thus constitutes a resource of genetic diversity for the microbiota and facilitates adaptation to a changing diet (Forsberg et al. 2012; Smillie et al. 2011). The process of HGT must also help organisms from the natural environment to adapt quickly to the gut (Fig. 18.2), so such environment-derived gut strains might rapidly appear to differ from the environmental precursor (Sousa et al. 2017).

18.4.10 A Microbiota of high Biodiversity. . .Why Is It Beneficial?

Contact with the natural environment is likely to increase the biodiversity of the microbiota, and there is a strong correlation between this biodiversity and health. Most illnesses are accompanied by reduced biodiversity of the microbiota, and a progressive reduction in biodiversity heralds decline in the elderly (Claesson et al. 2012). Why is this so? Diversity is certainly important for providing data to the immune system as described above. But it is also possible that it is not only the diversity per se that is important, but also the increased likelihood of the presence of essential species driving, for example, immunoregulation (Narushima et al. 2014; Cekanaviciute et al. 2018) or production of short-chain fatty acids (Dalile et al. 2019), or other necessary functions not yet discovered. Compared to Italian children, African children from villages in Burkina Faso had higher levels of SCFA (short-chain fatty acids), and their microbiota showed more species richness and biodiversity (De Filippo et al. 2010). Perhaps such richness and diversity reduce the probability of loss of essential functions of the symbiotic microbial community during periods of stress, dietary change, or antibiotic use? Ecologists have observed that species diversity buffers against excessive change-induced damage to environmental ecosystems, because the presence of many species increases the probability

that these include some that can adapt quickly to the new conditions (de Mazancourt et al. 2008). Perhaps the same is true of the gut microbiota?

Another possibility is that biodiversity inhibits potentially dangerous biofilm formation. Biofilms are communities of one or more species that adhere to each other and to surfaces. The "decision" to switch from planktonic to biofilm growth is regulated by environmental factors and quorum sensing. The normal healthy symbiotic gut microbiota forms physiological biofilms associated with the mucus layers of the large bowel. But the crucial point is that the physiology of an organism changes when it switches to biofilm mode, and with that switch some organisms become pathogenic as well as resistant to antimicrobials and resistant to the immune system. For example, most pathology caused by *Candida albicans* follows the switch from yeast to hyphal forms that occurs when it makes biofilm (Tsui et al. 2016). In patients with IBD (inflammatory bowel disease), gut microbiota can bypass the mucus barrier and abnormal biofilm is found adherent to the epithelial surface. Organisms from such biofilm can translocate across human intestinal epithelial cell monolayers in vitro, whereas bacteria from the microbiota of healthy donors do not (Buret et al. 2019). It is possible that high gut microbiota biodiversity affects the quorum sensing signals, and stops potential pathogens from switching to biofilm.

18.4.11 Modulation of the Gut–Brain Axis

Being deprived of contact with green space during childhood increases the risk of mental illness in later life (Maas et al. 2009; Engemann et al. 2019). Is this an effect of exposure to environmental microbiota, or is it due to other factors such as relaxation, sun, and exercise? There is abundant experimental evidence that the gut microbiota has potent effects on brain function (Mayer et al. 2014; Rook et al. 2018). For example, exposing pregnant rats to a nonabsorbable antibiotic leads to behavioural abnormalities in the offspring (Degroote et al. 2016), and even the adult mouse brain can be modified by antibiotics. Administering a broad spectrum antibiotic mixture to adult mice reduced hippocampal neurogenesis, and memory retention (Möhle et al. 2016). These defects could be treated by reconstituting a normal microbiota, particularly when supplemented with a probiotic mixture (VSL#3). Similarly, germ-free animals, although of uncertain relevance to humans, have abnormal stress responses that can be corrected by early restoration of the microbiota, but cannot be corrected by normalization of the microbiota in adulthood (Sudo et al. 2004; Diaz Heijtz et al. 2011). Interestingly, transfer of microbiota from depressed humans to germ-free or antibiotic-treated rodents induces depression-like behavior in the recipient animals (Kelly et al. 2016; Zheng et al. 2016). So these experiments establish the potential relevance of the microbiota, which is inevitably influenced by environmental inputs to the microbiota itself, and to the immune system that controls it. Many microbial components that modulate brain function have been identified (Caspani and Swann 2019).

Is there more direct evidence that organisms from the natural environment are involved in control of the gut–brain axis? A recent study used a mouse model in which a standardised stressor leads to colitis, behavioural changes (anxiety and fear) and an altered microbiota. Pre-immunising these animals by the subcutaneous route with a heat-killed soil-derived environmental saprophyte (*Mycobacterium vaccae*) partially stabilised the microbiota, and eliminated the colitis and behavioural effects (Reber et al. 2016). This protection was dependent on regulatory T cells (Treg) (Reber et al. 2016), implying that contact with such soil organisms via cuts and abrasions could affect mood. Another study exposed mice to very low doses of dust from a soil with high microbial biodiversity, and saw changes in the gut microbiota, and reduced anxiety, which they attributed to colonisation with a soil-derived anaerobic spore-forming butyrate producer (Liddicoat et al. 2020).

Human epidemiological support comes from the observation that adults who were brought up in an urban environment without pets had a greater inflammatory response to a standard laboratory stressor (TSST) than did adults who had been brought up on farms in the presence of animals (Bobel et al. 2018). Finally, there are suggestive data from administration of probiotics to human subjects. While not strictly environmental organisms, fermented foods are essentially metabolised by organisms of environmental origin, such as our refrigerator-deprived ancestors would have consumed. The subjects' brains were monitored by functional magnetic resonance imaging during exposure to an emotional stimulus, before and after 4 weeks consuming either a probiotic mixture, or a control material. The subjects who had consumed the probiotic mixture showed significant changes in the activity of brain regions that process emotion and sensation (Tillisch et al. 2013). This and other experiments have inspired numerous clinical trials of probiotics as treatments for psychiatric diseases. A recent meta-analysis of eligible studies ($n = 34$) found that the beneficial effects in depression are clearly significant (Liu et al. 2019).

18.4.12 Bacteriophages

Bacteriophages provide another possible mechanism that would enable the microbial environment to modulate the microbiota and therefore immunoregulation. Phages are the most numerous biological entities in the gut and approximately 90% of the human gut virome consists of virulent bacteriophages predicted to target major taxonomic groups of gut bacteria (Shkoporov et al. 2019). Some phages (lytic) lyse the bacteria or archaea that they infect, while others (temperate phages) can either trigger lysis, or alternatively, integrate into the host DNA or persist within the host as a plasmid. Either way the phage exerts profound effects on the function and survival of the host organisms. Integrated prophages can express genes that increase the fitness of the bacteria and protect them from infection by lytic phages. They may also supply bacteria with genes that are involved in the metabolism of toxins and polysaccharides, or in antibiotic resistance. Some phages cause changes in the

O-antigen component of the LPS of Gram-negative bacteria (Van Belleghem et al. 2018).

The critical role of phages in modulating the composition of the microbiota is suggested by several observations. First, there is evidence that phages bind to intestinal mucus via Ig-like domains, and contribute a layer of phage-mediated immunity. Moreover, the rate of replication of different organisms in the gut is enormously varied (Korem et al. 2015), and it seems likely that phages are at least partly responsible. Differing replication rates can potentially lead to misinterpretations of the relative importance of bacterial species. An organism that is present in low numbers because rapid proliferation is counteracted by rapid phage-mediated lysis might be providing more signals to the physiology of the human host than an organism present in greater numbers, with a low replication rate and low turnover.

More evidence of the crucial role of phages is provided by the observation that *Clostridioides difficile* infection can be treated using sterile faecal filtrates (Ott et al. 2017). This study did not identify the components of the sterile filtrate responsible for the cure, but phages must be strong candidates. Alcoholic liver disease provides another example. This condition is partly mediated by a cytolysin released by certain strains of *Enterococcus faecalis*. In a mouse model bacteriophages that specifically targeted cytolytic *E. faecalis* abolished alcohol-induced liver disease (Duan et al. 2019). Finally, compared to matched healthy controls, children who developed autoantibodies characteristic of T1D (type 1 diabetes), or who developed clinical disease, had less diverse bacteriophages in their guts and less species richness (Zhao et al. 2017).

So could the natural environment influence our gut bacteriophages, and if so, how? We know that there are about 10^9 phages/g of soil (Batinovic et al. 2019), and they are also found in drinking water where their presence is used as a test for water purity and for contamination with human-derived waste. Phages are found on all body surfaces including the skin, airways, urinary tract, and gut, and they can penetrate epithelial surfaces via rapid transcytosis and enter eukaryotic cells. They also enter the circulation. It is suggested that every day ~30 billion bacteriophage particles cross the gut epithelium and enter human tissues (Van Belleghem et al. 2018), and many phages can be identified in human blood. Therefore, phage intake from the natural environment must be massive, and likely to include phages of human gut-adapted bacteria and archaea. These phages could directly modify the composition of the gut microbiota (Fig. 18.2).

However, there is a second potential mechanism. Phages induce an immune response, and phage-neutralising antibodies are commonly found in the blood of humans and other animals (Fig. 18.2). Specific IgA in the gut, but also IgG and IgM can all inactivate phages and decrease the titre of active phages in the faeces (Van Belleghem et al. 2018). It is therefore possible that antibody-mediated disturbances of gut phage populations might be one way in which dysbiosis can occur. Could contact with the vast variety of phages in the natural environment affect control of gut phages by the immune system? It is at least possible that intake of phages, especially during early life, might modify the immune response to phages, and

therefore the composition of the microbiota. I am not aware of any studies that address this issue, which remains a speculation.

18.5 Is the Microbiota of the Natural World Still "Natural"?

Can we benefit from our evolved dependence on the microbiota of the natural environment if that microbiota is not natural anymore? The influence of mankind on the biosphere has been massive. The issue of the effects of industrial and agricultural pollution, including antibiotics, herbicides, insecticides, other agrochemicals, plastics, and monoculture has been reviewed elsewhere (Flandroy et al. 2018; Jin et al. 2017; Hallmann et al. 2017), though it is worth pointing out that glyphosate was identified as a microbicide before it was found to be a useful herbicide (Abraham and Monsanto Technology LLC 2010), and glyphosate was recently detected in the urine of more than 90% of a cohort of pregnant American women, so it is now ubiquitous (Parvez et al. 2018). We do not know whether this matters or not.

Rather than expanding on the issue of chemical damage, I will mention two other interrelated microbially relevant issues. First, we are rapidly replacing the naturally evolved biomass of our planet. At least 90% of mammal biomass is now human or domesticated livestock. Similarly the chicken, first domesticated around 8000 years ago, is now the most numerous terrestrial vertebrate, and at least 70% of all bird biomass is now domesticated poultry (Bar-On et al. 2018). This replacement of vertebrates with chickens, humans, and livestock also changes the faecal matter deposited on the planet. The production of faeces from all cattle, sheep, goats, pigs, and chickens has been estimated at about 14×10^{12} kg/year (Waltner-Toews 2013). Individual humans produce about 145 kg of faeces each year, so the human population of about 7 billion produces about 1×10^{12} kg/year. Does this have a significant effect on the types of gut-adapted strains in our environment? It can perhaps be argued that the biomass of bacteria (about 15% of total biomass on the planet) is about 1000× the biomass of humans, and about 700× the biomass of livestock, so the contribution that our faeces make to the microbiota of the planet must be modest (Bar-On et al. 2018). However, in the places where humans live the effect may be large. We have little understanding of the impact of our faecal output, factories, chemicals (industrial and in the home), and industrialised agriculture on the microbiota to which we are exposed.

18.5.1 Organic vs. Non-organic

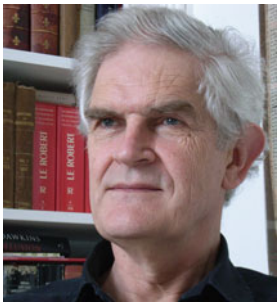
Organic food production might be one way to mitigate the effects of environmental pollution by industrial and agrochemicals. A recent meta-analysis of 56 studies and 149 paired comparisons of organic and conventional farming systems based on global data accumulated over 20 years, demonstrated that organic farming increases the biomass and activity of the soil microbiota (Lori et al. 2017). This must affect our exposure to environmental microbiota in air and dust, but what about the organisms we take in with organically grown food? The answer is unclear.

A recent study compared conventionally grown fruit and vegetables to products that were labelled organic, and purchased from the same grocery stores in the USA. The findings were different for different products. Organic spinach, lettuce, and tomatoes had more diverse microbiota than conventionally grown products, whereas the opposite was true for peaches and grapes, where the conventionally grown products had richer microbiota (Leff and Fierer 2013). There were also differences in the composition of the microbiota, the most consistent being a greater relative abundance of Enterobacteriaceae on conventional spinach, lettuce, tomatoes, and peaches when compared to organic produce (Leff and Fierer 2013). This study found little difference between conventional and organic apples. However, a more detailed study that looked separately at the microbiota of the stem, peel, fruit pulp, seeds, and calyx found a different microbiota at each site, and significantly increased diversity at each site in organic apples (Wassermann et al. 2019). Thus, according to this study, consuming an apple corresponds to an intake of about 10^8 bacteria, and these are more diverse, and include more lactobacilli, if the apple is organic.

18.6 Conclusions

It is now generally accepted that populations in developed countries are suffering from striking increases in disorders that involve faulty regulation of the immune system, and that this faulty immunoregulation is at least partly attributable to diminished or distorted exposure to the microorganisms with which we coevolved, because these play a crucial role in setting up immunoregulatory mechanisms. Much attention has been focussed on the obvious ways in which the modern Western lifestyle impedes mother-to-baby transmission of the microbiota, and on the further distortion of the child's microbiota caused by unvarying diets and antibiotics. Much less attention has been applied to the issue of contact with the microbiota of the natural environment, although contact with this was inevitable during our evolution, and has therefore become a necessity. The coining of the misleading expression "hygiene hypothesis" has led to an unproductive obsession with home hygiene practices, whereas the most careful studies reveal that what matters is not how you clean your toilet, but whether you are exposed directly to the natural environment, and whether the microbiota of the home resembles that of the natural environment

(Weber et al. 2015; Kirjavainen et al. 2019). This essay seeks to list some of the ways in which microorganisms from nature might be exerting health benefits. Some of the suggested mechanisms are speculative and require much more research, but the purpose of this essay is to encourage such research, and to emphasise the need for an evolutionary approach as suggested in the Old Friends Hypothesis.



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Chapter 19
Biochemistry and Molecular Biology of the Enzyme ACC Deaminase

Shimaila Ali and Bernard R. Glick

Abstract Throughout their development processes over many millions of years, plants have adopted a number of mechanisms whereby they could either modify themselves (genotypically and/or phenotypically), or their interaction with their surrounding environment. The utilization of such strategies, which is a direct consequence of plant–microbe interactions, can help plants to grow and adapt better in searching and utilizing potential energy resources, in overcoming various environmental abiotic stresses, and in fighting against plant pathogens. The functioning of the microbial enzyme 1-aminocyclopropane-1-carboxylase (ACC) deaminase is believed to be one of the key mechanisms that is provided by soil microbes that plants have benefitted from under a variety of environmental conditions. The 1-aminocyclopropane-1-carboxylase deaminase is a multimeric enzyme that belongs to the tryptophan synthase beta superfamily of enzymes that requires pyridoxal phosphate as a cofactor and acts to cleave ACC, the immediate precursor of ethylene in all higher plants. ACC deaminase is particularly important in lowering inhibitory plant stress ethylene levels that form as a consequence of various environmental stresses, both abiotic and biotic, thereby significantly facilitating plant growth, especially under adverse conditions. The enzyme ACC deaminase has been reported to be present in various groups of *Biota* including all three domains of life, i.e., Archaea, Bacteria, and Eukarya. The activity of the enzyme is primarily organism specific, but environmental factors also affect its activity. Here, the phylogeny of organisms encoding this enzyme and the biochemistry of ACC deaminase from various sources is documented and compared. The possible transcriptional regulatory mechanisms of this enzyme in various bacteria are also described herein, with the best-studied and most common mechanisms elaborated in detail. The fundamental information summarized here provides an important step toward understanding

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_19

one of the key mechanisms of plant growth promotion by bacteria and fungi. Thus, the work described here is central to effectively using plant growth-promoting bacteria and fungi as biological tools in agricultural practice.

19.1 Introduction

In the absence of beneficial soil microorganisms, i.e., plant growth-promoting bacteria (PGPB) and mycorrhizal fungi, the growth of plants would be severely limited, especially during periods of environmental stress. However, by various estimates, these plant-helper soil microorganisms have been interacting with plants and facilitating their growth for somewhere between fifty and several hundred million years. The environmental stresses that these soil microorganisms help the plants to overcome include, but are not limited to, abiotic stresses such as drought, flooding, and high salt concentrations; extremes of temperature and sunlight; the presence of inhibitory metals and organic compounds; and the paucity of certain nutrients in the soil such as fixed nitrogen, iron, and phosphorus. These soil microorganisms help plants to overcome biotic as well as abiotic stresses; these include fungal and bacterial plant pathogens, and nematode and insect predation. Given the effectiveness of certain soil microorganisms in promoting plant growth, it is not surprising that the scientific literature is filled with countless examples describing PGPB and mycorrhizae, both separately and together, facilitating plant growth under laboratory, greenhouse, and field conditions (Glick 2012; Reed and Glick 2013).

While PGPB utilizes a range of different mechanisms to promote plant growth, arguably, one of the key mechanisms utilized by PGPB to facilitate plant growth is the use of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Importantly, ACC is the immediate precursor, in all higher plants, of the plant hormone ethylene. Ethylene is involved in seed germination, tissue differentiation, root development, lateral bud development, flowering, anthocyanin synthesis, fruit ripening, aroma production, leaf senescence, leaf and fruit abscission, and maintenance of plant–microbe interaction in Rhizobia nodule formation and mycorrhizae–plant interactions (Ali et al. 2017; Glick 2015). Moreover, ethylene is also a plant stress hormone, with its level rising as a consequence of various environmental stresses. While this hormone is typically required in very low concentrations for normal plant growth and development, the much higher levels of ethylene that plants produce when they are subjected to either abiotic or biotic stresses are generally deleterious to plant growth and development. Thus, by lowering the amount of ACC, and hence the level of ethylene, in plant tissues, ACC deaminase can ameliorate many of the harmful inhibitory effects of stress ethylene.

It has been widely demonstrated in laboratory experiments that many different plants exhibit a much higher level of resistance to a wide range of environmental stresses, especially abiotic stresses, when those plants have first been inoculated with ACC deaminase-containing PGPB. These laboratory observations are consistent

with the notion that ACC deaminase-containing PGPB probably also provide an advantage to uncultivated plants growing in the natural environment. In fact, in one experiment, researchers isolated PGPB from the rhizosphere (the area around the plant roots) of wild barley plants growing in a region of northern Israel termed "Evolution Canyon" where the two slopes of this canyon are around 250 m apart at their bases (Timmusk et al. 2011). The south-facing slope of this canyon is quite arid, receives an excessive amount of sunlight and has only very sparse plant growth compared to the north-facing slope where the plant growth is relatively lush. Both slopes contained similar genera of bacteria in the barley rhizospheres. However, nearly all of the bacteria that were examined from the much more highly stressed (water and light stress) south-facing slope included ACC deaminase activity that allowed the plants on this slope to withstand the harsher conditions encountered. Thus, in this natural environment, the drought conditions on the south-facing slope selects for bacteria that contain traits, such as the presence of ACC deaminase, that allow both the PGPB and the host plants to better survive these harsh conditions. Moreover, under the more moderate conditions on the north-facing slope, the same bacteria are largely devoid of those traits that facilitate bacterial and plant survival under harsh conditions.

Enzyme ACC deaminase is a common component of a large number of PGPB, enabling these bacterial strains to be highly efficacious in facilitating plant growth under a wide range of stressful conditions. ACC deaminase (EC: 3.5.99.7) belongs to the enzyme superfamily entitled tryptophan synthase beta subunit-like pyridoxal phosphate (PLP)-dependent enzymes and is quite ubiquitous in nature (Singh et al. 2015) occurring in all three domains of life, i.e., Archaea, Bacteria, and Eukarya, having been observed in bacteria, fungi, Stramenopiles, Archaea and plants. In the material that follows, the biochemistry and molecular biology of this key microbial enzyme are examined in some detail.

19.2 Prevalence of ACC Deaminase in *Biota*

The ACC deaminase was initially purified and characterized from members of the γ-Proteobacteria, mainly *Pseudomonas* (Honma and Shimomura 1978; Klee et al. 1991). Subsequently, ACC deaminase and its homologues were purified and characterized from other microbial sources (Fujino et al. 2004; Jacobson et al. 1994; Hontzeas et al. 2004; Minami et al. 1998). To date, the active enzyme has been reported in α, β, and γ Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Archaea, various fungi, and yeast (Ekimova et al. 2018; Fujino et al. 2004; Hontzeas et al. 2004; Marques et al. 2010; Minami et al. 1998; Nascimento et al. 2014; Singh et al. 2015). Moreover, ACC deaminase has also been reported in plants including *Arabidopsis*, poplar, tomato, and corn (McDonnell et al. 2009; Singh et al. 2015). Despite the fact that bacterial genera Chlorobi, Bacteroidetes, and Firmicutes have been documented to contain ACC deaminase activity, the genes (*acd*S) responsible for such activity have yet to be reported (Nascimento et al. 2014). This apparent

contradiction may reflect the presence of noncanonical ACC deaminase enzymes that are members of the amino hydrolase superfamily and are also able (albeit sometimes inefficiently) to cleave ACC (Li et al. 2001). On the other hand, microbial genera including *Meiothermus* and *Phytophthora* have been found to possess sequences similar to *acd*S genes in their genomes; however, no experimental evidence of enzyme activity has yet been documented (Nascimento et al. 2014). Similarly, genomic and metagenomic database search-based studies revealed that sequences similar to the *acd*S gene locus of *Pseudomonas* sp. UW4 are the most prevalent in bacteria (485 individual strains) and include *Acidovorax, Bordetella, Brenneria, Burkholderia, Collimonas, Cupriavidus, Curvibacter, Dickeya, Herbaspirillum, Halomonas, Lonsdalea, Methylibium, Pantoea, Phytophthora, Polaromonas, Pseudomonas, Ralstonia, Serratia, Tatumella, Variovorax,* and *Xenophilus*. Within the domain Archaea, strains of *Archaeoglobus fulgidus, Pyrococcus abyssi, Pyrococcus furiosus,* and *Thermococcus nautili* were found to contain ACC deaminase genes; likewise, the fungal phyla Ascomycota and Basidiomycota, and soybean, potato, maize, and castor oil in the kingdom Plantae all appear to contain the *acdS* gene (Singh et al. 2015). However, some misidentification of the presence of this enzyme in various bacteria has also been reported. To avoid this sort of overestimation of the ACC deaminase positive strains in any environment, Li et al. (2015) proposed a molecular tool for differentiating true ACC deaminase-containing bacteria from its structural homologs. This tool is based on consensus-degenerate hybrid oligonucleotide primers and is specific for picking up key differences between ACC deaminase and its structural homologs, which are found in amino acid sequences at E295 and L322 residues that are unique for ACC deaminase, whereas ACC deaminase homologs can have any other amino acids at those two positions (Todorovic and Glick 2008; Li et al. 2015). Using this approach, some genes belonging to the bacterial genera *Enterobacter*, *Klebsiella*, and *Bacillus* that were previously reported to be putatively positive for the presence of ACC deaminase were found to be false positives (and are likely to be D-cysteine sulfhydrase) based on their genomic structures (Nascimento et al. 2014; Li et al. 2015).

19.3 Phylogenetic Origin of ACC Deaminase

The structural gene responsible for ACC deaminase, *acd*S, is predominantly found on bacterial chromosomal DNA, with a few bacteria containing the gene on their plasmids. Protein sequence analysis of ACC deaminase from diverse bacterial genera found that more than 60% of the amino acid residues were identical, even for the most phylogenetically distinct sequences. It has been proposed that *acd*S originated from a bacterial/Eukaryote ancestor (Nascimento et al. 2014) and most likely is the product of mutation from some ancestral gene. Based on analysis of a limited number of bacterial ACC deaminase protein sequences and 16S *rRNA* gene analysis of the same bacterial genera (mainly *Pseudomonas*), it was hypothesized

that many of the ACC deaminase genes have been transmitted through horizontal gene transfer (HGT) (Hontzeas et al. 2005; Blaha et al. 2006). Furthermore, when the evolution of *acd*R (the bacterial gene responsible for the regulation of *acd*S) was studied, it was suggested that *acd*R may have also evolved through HGT, however, these two genes may have been evolved separately from one another (Prigent-Combaret et al. 2008). More recently, Nascimento et al. (2012) proposed that the *acd*S gene is transferred between many strains of *Mesorhizobium* spp. via symbiotic island exchange. A more extensive study based on protein structure and phylogeny analyses performed on a vast number of diverse bacterial and fungal genera, suggested that current ACC deaminase genes share a common ancestor and likely evolved from site-specific mutations within the ancestral enzyme gene (Nascimento et al. 2014). Additional mutations in the parental genes over time may justify the lack of stringency in terms of substrate specificity among similar proteins. It was also proposed that the vast majority of *acd*S and *acd*R genes evolved through vertical inheritance although HGT may explain the presence of these genes in organisms that are not involved in interacting with ACC in the environment (Nascimento et al. 2014).

19.4 Protein Biochemistry

Biochemical studies on ACC deaminase reveal that this is exclusively a cytoplasmically localized enzyme and is not secreted (Jacobson et al. 1994). The enzyme from various microbial origins share amino acid similarities (Prigent-Combaret et al. 2008), encoded by a single gene namely *acd*S, and contain 325–345 amino acid residues with a subunit molecular mass of 33–42 kDa (Tables 19.1 and 19.2). The ACC deaminase is a multimeric enzyme and has been suggested to be functional in either of homodimer, homotrimer, or of homotetramer forms (Fedorov et al. 2013). Based on its three-dimensional structure, ACC deaminase enzyme belongs to the tryptophan synthase beta superfamily and essentially acts as hydrolase (Karthikeyan et al. 2004). ACC deaminase requires pyridoxal phosphate (PLP) as a cofactor and deaminates ACC, an immediate precursor of the plant hormone ethylene, into ammonia and α-ketobutyrate. The cofactor PLP is tightly bound to the enzyme and the amount of cofactor that is required for the enzyme to become active is a 1:1 enzyme subunit to PLP ratio, for example, 2 moles PLP is needed to make a mole of dimeric ACC deaminase active and 3 moles of PLP is required to make a mole of trimeric ACC deaminase active (Honma 1985).

Despite the fact that this enzyme shares at least 60% of its amino acid residues between diverse bacterial genera (Nascimento et al. 2014), the secondary structures (mainly alpha helices) of enzymes isolated from various sources are found to be somewhat different (Hontzeas et al. 2004). At present, the ACC deaminase enzyme has been well characterized from at least four diverse phylogenetic origins namely *Pseudomonas* spp. (Hontzeas et al. 2004; Jacobson et al. 1994; Karthikeyan et al. 2004), yeast (Minami et al. 1998), *Methylobacterium* spp. (Fedorov et al. 2013), and

Table 19.1 Biochemical comparison of ACC deaminase enzymes purified from different sources

Strain	K_m (mM)	k_{cat} (m^{-1})	k_{cat}/K_m	Optimum pH	Optimum temperature, °C	Structure	Reference
Pseudomonas putida GR12-2	n.d.	n.d.	n.d.	8.5	30	Homotrimer	Jacobson et al. (1994)
Pseudomonas sp. strain APC	1.97	70	35.5	8.5	30	Homotrimer	Honma and Shimomura (1978); Karthikeyan et al. (2004)
Pseudomonas putida UW4	3.4 ± 0.2	146 ± 5	43.9	8	22	Homotrimer	Hontzeas et al. (2004)
Methylobacterium nodulans ORS2060	0.80 ± 0.04	111.8 ± 0.2	139.8	8	50	Homotetramer	Fedorov et al. (2013)
Methylobacterium radiotolerans JCM2831	1.8 ± 0.3	65.8 ± 2.8	36.5	8	45	Homotetramer	Fedorov et al. (2013)
Cyberlindera saturnus	2.6	n.d.	n.d.	9.0	37	Homodimer	Honma and Shimomura (1978); Minami et al. (1998)
Penicillium citrinum	4.8	n.d.	n.d.	8.5	n.d.	Homodimer	Jia et al. (2000)

n.d., not determined

Table 19.2 Functional ACC deaminase gene containing microbes

UniProt No.	Protein name	Gene name	Organism	Number of amino acids
A1TVP2	ACCD	*acd*S Aave_4493	*Acidovorax citrulli*	338
B9MGI8	ACCD	*acd*S Dtpsy_3150	*Acidovorax ebreus*	338
B9JJB7	ACCD	*acd*S Arad_8832	*Agrobacterium radiobacter*	337
B9K206	ACCD	*acd*S Avi_5856	*Agrobacterium vitis*	337
Q89XR6	ACCD	*acd*S blr0241	*Bradyrhizobium diazoefficiens*	337
A4YUJ5	ACCD	*acd*S BRADO3803	*Bradyrhizobium sp.*	339
Q0B569	ACCD	*acd*S Bamb_5155	*Burkholderia ambifaria*	338
B1K774	ACCD	*acd*S Bcenmc03	*Burkholderia cenocepacia*	338
Q390Z5	ACCD	*acd*S Bcep18194	*Burkholderia lata*	338
Q62CE3	ACCD	*acd*S BMAA0952	*Burkholderia mallei*	338
A9AQJ3	ACCD	*acdS* Bmul_5215	*Burkholderia multivorans*	338
A3P669	ACCD	*acd*S BURPS1106A	*Burkholderia pseudomallei*	338
Q2T6A1	ACCD	*acd*S BTH_II1101	*Burkholderia thailandensis*	338
A4JKV8	ACCD	*acd*S Bcep1808	*Burkholderia vietnamiensis*	338
Q5KMX3	Putative ACCD	CNB00190	*Cryptococcus neoformans*	345
Q0K1H0	ACCD	*acd*S H16	*Cupriavidus necator*	338
Q7M523	ACCD	*acd*S	*Cyberlindnera saturnus*	341
Q9ZHW3	ACCD	*acd*S	*Enterobacter cloacae*	338
Q98AM7	ACCD	*acd*S mlr5932	*Mesorhizobium japonicum*	337
A2SLW2	ACCD	*acd*S Mpe_A3598	*Methylibium petroleiphilum*	338
B8IP05	ACCD	*acd*S Mnod_5479	*Methylobacterium nodulans*	337
B2TBV3	ACCD	*acd*S Bphyt_5397	*Paraburkholderia phytofirmans*	338
Q13ME5	ACCD	*acd*S Bxeno_B1776	*Paraburkholderia xenovorans*	338
Q51813	ACCD	*acd*S	*Pseudomonas fluorescens*	338
Q5PWZ8	ACCD	*acd*S	*Pseudomonas putida*	338

(continued)

Table 19.2 (continued)

UniProt No.	Protein name	Gene name	Organism	Number of amino acids
Q48KS9	ACCD	*acd*S PSPPH_1761	*Pseudomonas savastanoi*	338
P30297	ACCD	*acd*S	*Pseudomonas* sp.	338
Q87YW7	ACCD	*acd*S PSPTO_3675	*Pseudomonas syringae*	338
Q9V2L2	Putative ACCD	PYRAB00630	*Pyrococcus abyssi*	330
Q8U4R3	Putative ACCD	PF0010	*Pyrococcus furiosus*	329
O57809	Putative ACCD	PH0054	*Pyrococcus horikoshii*	325
B2UGM5	ACCD	*acd*S Rpic_2004	*Ralstonia pickettii*	338
Q8XS35	ACCD	*acd*S RSp0646	*Ralstonia solanacearum*	338
Q93AG0	ACCD	*acd*S	*Rhizobium leguminosarum*	339
Q9AHF0	ACCD	*acd*S	*Rhizobium radiobacter*	337
Q9URX3	Probable ACCD	SPAC922.03	*Schizosaccharomyces pombe*	338
Q9WY68	Putative ACCD	TM_0225	*Thermotoga maritima*	312
Q6J256	ACCD	*acd*S	*Variovorax paradoxus*	338
C5CQC9	ACCD	*acd*S Vapar_5099	*Variovorax paradoxus*	338

This data was extracted from the Swiss-Prot database, which was reviewed and compiled from literature and curator-evaluated computational analysis. Here, ACC deaminase is denoted as ACCD

Penicillium (Jia et al. 2000) with the enzyme from *Methylobacterium nodulans* strain ORS2060 showing the highest catalytic efficiency (Table 19.1). It is also important to note that the levels of induction and expression of the *acd*S gene vary according to environmental conditions and most importantly from organism to organism.

A multiple sequence alignment of the amino acid sequence of ACC deaminase proteins from diverse microbial origins revealed that the positions of many amino acids are highly conserved over the course of evolution, and it has been proposed that these specifically localized amino acids have important roles in protein functional domains (Kanika et al. 2015). PLP-dependent enzymes have been proposed to have Tyr268, Tyr294, Lys51, and Glu295 (*Pseudomonas* sp. UW4 ACC deaminase numbering scheme; Duan et al. 2013) as conserved residues at their active sites. Several structural analysis studies of ACC deaminase including NMR and X-ray crystallography (Karthikeyan et al. 2004) and mutagenesis, mainly for *Pseudomonas* (Hontzeas et al. 2004; Ose et al. 2003) and yeast ACC deaminases (Minami et al. 1998) have shown that key residues found within the enzyme active site are Tyr269, Tyr295, Lys51, and Glu296 (yeast ACC deaminase numbering sequence) and,

similarly, Tyr268, Tyr294, Lys51, and Glu295 (*Pseudomonas* sp. UW4 ACC deaminase numbering scheme). The possible role of a reactive thiol group of Cys162, which is located between the two major domains of the molecule, has also been suggested (Glick 2005). Overall, it has been suggested that Lys51 within the active site of ACC deaminase is involved in proton extraction from the substrate (namely ACC), whereas Tyr294 has been proposed as a key catalytic residue that positions the cofactor PLP to be in the correct orientation within the active site, helping substrate to bind properly, and facilitating the formation of the external aldimine between PLP and the substrate by reacting with the amino group of the substrate (Karthikeyan et al. 2004; Ose et al. 2003).

19.4.1 Optimal Temperature and pH Profile of ACC Deaminase

The optimal activity of the ACC deaminase enzyme occurs at pH 8–9. Pure enzyme from bacterium *Pseudomonas* sp. UW4 exhibited optimal activity at pH 8.0 with a sharp loss of activity at acidic pH 6.5 and a complete loss of activity at values of pH above 10, with estimated pK_a values of 7.4 and 9.5 (Hontzeas et al. 2004). Similarly, *P. putida* GR12-2 ACC deaminase has optimal activity at pH 8.5 and pK_a values of approximately 7.7 and 9.2 (Jacobson et al. 1994) while the ACC deaminase from the genus *Methylobacterium* exhibited optimal activity at pH 8.0 (Fedorov et al. 2013). The enzyme optimal temperature varies hugely among different bacterial genera. Enzymes from *Pseudomonas* spp. generally perform optimally at 30 °C, with the exception of strain UW4, which exhibits optimal activity at ~25 °C. Nevertheless, pseudomonad ACC deaminases are thermodynamically stable enzymes ($T_m = 60$ °C) based on their melting curve analysis (Hontzeas et al. 2004). On the other hand, ACC deaminases from *Cyberlindera saturnus*, *Methylobacterium nodulans* ORS2060, and *Methylobacterium radiotolerans* JCM2831 were found to be optimally active at 37 °C, 50 °C, and 45 °C, respectively (Table 19.1).

19.4.2 Effect of Heavy Metals on ACC Deaminase Activity

ACC deaminase-containing bacteria have been documented to promote host plant growth and development in the presence of heavy metals (Burd et al. 2000) and the effect of heavy metals on the activity of ACC deaminase has been investigated (Carlos et al. 2016). In this study, the ACC deaminase activity of ten different bacterial strains was measured in the presence and absence of heavy metals (Pb, As, Cu, Ni, Cd, and Mn). It was found that although all of the selected bacteria contain active ACC deaminase, there were differences in the way that metals affected those bacteria. For instance, the ACC deaminase activity of bacterial strains

Escherichia N16, *Enterobacter* K131, *Enterobacter* N9, and *Serratia* K120 was increased with the addition of Pb, As, and Cu compared to the control with no heavy metals. In addition, the presence of Ni, Cd, and Mn in the growth medium decreased the level of ACC deaminase in all bacteria tested except for *Klebsiella* Mc173, and the only bacterial strain that exhibited higher levels of activity in the presence of these heavy metals was *Serratia* K120, which also promoted *Helianthus annuus* plant growth in the presence of heavy metal contamination (Carlos et al. 2016).

19.5 Different Enzyme Assays

ACC deaminase enzyme activity may be assayed by monitoring the production of either ammonia or α-ketobutyrate, the end products of the ACC deaminase catalyzed reaction (Table 19.3). ACC deaminase is an inducible enzyme whose activity is induced by the presence of its substrate, namely ACC at levels as low as 100 nM, and the complete induction of the enzyme is a slow process that can take up to 10 h (Glick 2004). The screening of microbes for the presence of this activity is started by growing them in minimal media supplemented with ACC as the sole source of nitrogen (Penrose and Glick 2003) under growth conditions suitable for the microbe of interest. Briefly, ACC deaminase activity is measured in toluenized bacterial extracts, which are then incubated with the substrate, ACC. The final reaction is incubated with 2,4-dinitrophenylhydrazine that interacts with the newly released α-ketobutyrate and, after the addition of NaOH, the absorbance is read at 540 nm. The amount of α-ketobutyrate that is produced as a result of ACC deaminase activity is determined using a standard curve ranging from 0 to 1.0 μmol of α-ketobutyrate. Total protein is also estimated from the same toluenized bacterial extracts using bovine serum albumin as a standard over the range of 0.05–1 mg. Finally, the enzyme activity is determined in μmol of α-ketobutyrate produced per mg of total protein present in the bacterial extract in 1 h (μmol mg^{-1} h^{-1}). This method is generally used for determining the ACC deaminase activity by non-rhizobial bacterial strains (Penrose and Glick 2003) whereas, a modified method is used for Rhizobia (Duan et al. 2009). The above-mentioned enzymatic assay can precisely detect 0.1 μmol of α-ketobutyrate produced in the reaction. Nevertheless, a low level of ACC deaminase activity ($\geq$ 20 nmol mg^{-1} h^{-1}) is required to allow bacteria to grow on ACC and to lower stress ethylene levels. Interestingly, bacteria with higher enzymatic activity do not necessarily perform any better than the organisms that possess lower levels (<20 nmol mg^{-1} h^{-1}) of enzyme activity (Penrose and Glick 2003).

The ability of bacterial strains that produce ACC deaminase to consume ACC as a sole source of nitrogen has also been documented (Li et al. 2011). Nevertheless, identification of bacteria merely by growing them on minimal media containing ACC as the sole nitrogen source can overestimate the ACC deaminase prevalence in any environmental niche since nitrogen-fixing bacteria that do not contain ACC deaminase may also grow on this medium. In addition, when the presence of the

*acd*S gene is used to infer the presence of ACC deaminase in a population, nonspecific amplification of *acdS* homologs and mis-annotation of *acd*S genes in genomes may also lead to false positives (Li et al. 2015). Hence, explicit detection of the ACC deaminase structural gene (*acd*S) is important for predicting ACC deaminase activity and for identifying ACC deaminase producing bacteria and must be complemented by ACC deaminase activity measurements as described above.

Recently, a rapid method to screen ACC deaminase-producing bacteria has been reported (Patil et al. 2016). In this case, the bacteria are grown on minimal media supplemented with ACC and two indicator dyes, namely bromothymol blue and phenol red. Ammonia is produced as a consequence of the ACC deaminase catalyzed cleavage of ACC, which turns the media to a highly basic pH, a change that can be readily observed due to the presence of the pH indicator dyes. Unfortunately, this method, while rapid, is only semiquantitative and a detailed quantitative enzyme-based assay should be performed to confirm the presence and amount of active ACC deaminase.

Recently, the use of consensus-degenerate hybrid oligonucleotide primers for the specific amplification of bona fide *acd*S genes has been described (Li et al. 2015). More recently, a robust molecular tool was described for monitoring the size, transcription levels, and diversity of *acd*S genes in a variety of environmental niches (Bouffaud et al. 2018). This study is based on quantitative real-time PCR and is able to detect highly diverse alleles of *acd*S. As mentioned above, *acd*S genes are extant among Proteobacteria, Actinobacteria, Deinococcus-Thermus, and microeukaryotes. This method did not cover the thermophilic genus *Meiothermus* of the Deinococcus, however, all other phylogenetic groups were well represented, and no known overestimation has been found (Bouffaud et al. 2018).

The activity of purified ACC deaminase has also been measured using UV–Vis spectrophotometry where enzyme activity was monitored in a coupled reaction either with L-lactate dehydrogenase (Fedorov et al. 2013; Hontzeas et al. 2004), which is used to measure the amount α-ketobutyrate produced, or with glutamate dehydrogenase which measures the amount of ammonia produced (Fedorov et al. 2013) by monitoring the disappearance of the cofactor NADH at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). The K_M values for all of the three enzyme-catalyzed reactions were determined by monitoring the disappearance of ACC and plotting the reaction rate versus the substrate concentration and then fitting the data to the Michaelis–Menten equation (Fedorov et al. 2013; Hontzeas et al. 2004). The use of molecular tools for the detection of *acd*S genes has been widely reported (Caballero-Mellado et al. 2007; Govindasamy et al. 2008; Ma et al. 2003; Nikolic et al. 2011; Onofre-Lemus et al. 2009; Shah et al. 1998). However, due to the high similarity of the *acd*S genes to its homologs, primer biased, mis-annotation of *acd*S genes within genomes may occur, and most importantly, nonspecific amplification of *acd*S gene homologs may also occur. Therefore, care must be taken in documenting such results (Table 19.3).

Table 19.3 List of compounds that can serve as substrate and inhibitor for the enzyme ACC deaminase

Compound	Substrate/ inhibitor	References
1-Aminocyclopropane-1-carboxylic acid	Substrate	Honma and Shimomura (1978); Nascimento et al. (2014)
D-Vinylglycine	Substrate	Honma and Shimomura (1978); Nascimento et al. (2014)
β-Chloro-D-alanine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
D-Cysteine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
β-Fluoro-D-alanine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
1-Amino-2-vinylcyclopropane-l-carboxylic acid	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
O-Acetyl-D-serine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
β-Dichloro-D-alanine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
β-Difluoro-D-alanine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
3-Chloro-D-alanine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
D-Erythro-2-amino-3-chlorobutyrate	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
D-Threo-2-amine-3-fluorobutyrate	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
D-Serine	Substrate	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
L-Isomers of amino acids	Inhibitor	Fujino et al. (2004); Honma and Shimomura (1978); Nascimento et al. (2014); Walsh et al. (1981)
1-Amino-2-vinylcyclopropane-l-carboxylic acid	Substrate	Singh et al. (2015)
1-Aminocyclopropane-1-phosphonate	Inhibitor	Karthikeyan et al. (2004)
Iodoacetamide derivative 1, 5 N-iodoacetamidoethyl 1-aminonapthalene-5-sulfonic acid	Inhibitor	Singh et al. (2015)
Iodoacetamide	Inhibitor	Singh et al. (2015)

19.6 Levels of ACC Deaminase Activity Among Diverse Microbes

Generally, ACC deaminase activity of microbes is detected and quantified in vitro and the results of these assays vary from method to method. Table 19.4 presents the levels of enzyme activity quantified among various bacteria by different research groups; this table is representative of the data in the literature but is by no means exhaustive. At the outset, it should be noted that, perhaps surprisingly, ACC deaminase activity within one bacterial genus is not constant. For example, the enzyme activity for the bacterial genus *Pseudomonas* varies from 0.001 to 21.23 μmol mg^{-1} h^{-1}, and the same pattern of activity variation is true for the other genera (Table 19.4). Moreover, the level of ACC deaminase activity produced by nodule forming *Rhizobia* was observed to be considerably lower than for free-living bacteria when quantified under the same experimental conditions (Ma et al. 2003; Duan et al. 2009), (Table 19.4). Nodule forming *Rhizobia* produce only 2–10% of the amount of ACC deaminase activity compared to free-living bacteria but are up to 40% more efficient at functional nodule formation than ACC deaminase negative *Rhizobia* strains (Glick 2014). ACC deaminase produced by nodule-residing *Rhizobia* facilitates the process of nodulation, by decreasing the localized level of ethylene by breaking down the localized concentration of ACC, but does not lower the ethylene level throughout the plant and therefore does not protect the plant against various forms of environmental stress (Nascimento et al. 2012).

The other prominent microbial group that can produce ACC deaminase is fungi. For example, the levels of ACC deaminase activity were found to be as high as 9 μmol mg^{-1} h^{-1} in the fungal strain *Trichoderma estonicum* SKS1ACC (Saravanakumar et al. 2018) and 12.16 μmol mg^{-1} h^{-1} in the fungal strain *Trichoderma asperellum* T203 (Viterbo et al. 2010). Thereby, according to the levels of ACC deaminase produced in in vitro conditions, these three phylogenetic groups can be arranged as follows: free-living bacteria > fungi >*Rhizobia* (from the highest to the lowest levels of enzyme activity). These differences in activity may be a consequence of differences in enzyme catalytic activities among these three groups or may be due to the differences in the amounts of enzyme produced by one specific type of microbe. Interestingly, in the phylogenetic study carried out on the *acd*S gene, it was found that the various phylogenetic groups (fungi cluster with other fungi while *Rhizobia* cluster with other *Rhizobia,* very similar to the 16S/18S *rRNA* gene phylogram) cluster together based on the gene sequence that they carry (Nascimento et al. 2014). Despite the fact that microbes vary with respect to the levels of ACC deaminase that they produce, a low (minimal) level of ACC deaminase activity (20 nmol mg^{-1} h^{-1}) is required to allow them to grow on ACC as a sole nitrogen source and to lower stress ethylene levels. Interestingly, as mentioned earlier, microbes with higher levels of ACC deaminase enzymatic activity do not necessarily perform any better under laboratory conditions than do organisms that produce a moderate level (>20 nmol mg^{-1} h^{-1}) of enzyme activity (Penrose and Glick 2003).

Table 19.4 Reported levels of ACC deaminase activity in various bacteria

Bacterium	ACC deaminase activity (μmol mg^{-1} h^{-1} α-ketobutyrate)	References
Achromobacter xylosoxidans A551	0.4	Belimov et al. (2005)
A. facilis 4p-6	3.08	Belimov et al. (2001, 2005)
A. xylosoxidans AF288734	0.31	Dell'Amico et al. (2005)
A. xylosoxidans AF302096	0.56	Belimov et al. (2001)
A. xylosoxidans AF302097	0.15	Belimov et al. (2001)
A. xylosoxidans Bm1	0.09	Belimov et al. (2005)
Acinetobacter radioresistens CR4	1.235	Rashid et al. (2012)
Agarobacterium sp. AS5	1.43	Rashid et al. (2012)
A. tumefaciens D3	0.47	Hao et al. (2011)
A. tumefaciens GO-L6	0.98	Rashid et al. (2012)
A. vitis LL4	3.006	Rashid et al. (2012)
Alcaligenes sp. AF288728	1.17	Belimov et al. (2001)
Bacillus circulans V2	2.028	Rashid et al. (2012)
B. horneckiae YmS1	1.033	Rashid et al. (2012)
*B. idriensis*LR1	0.837	Rashid et al. (2012)
B. psychrosaccharolyticus UA-S1	0.438	Rashid et al. (2012)
B. pumilus AF288735	0.76	Belimov et al. (2001)
Bacillus sp. GPR2	1.052	Rashid et al. (2012)
Burkholderia caryophylli	0.6	Shaharoona et al. (2007)
Enterobacter aerogenes	0.34	Nadeem et al. (2007)
E. cloaceae	0.3	Nadeem et al. (2010)
Escherichia coli DH5a/p4U2	0.29	Shah et al. (1998)
Flavobacterium ferrugineum	0.41	Nadeem et al. (2007)
Methylobacterium sp.	0.09	Madhaiyan et al. (2006)
Microbacterium sp. AS1	2.02	Rashid et al. (2012)
Microbacterium sp. AS2	1.01	Rashid et al. (2012)
Microbacterium sp. AS3	0.84	Rashid et al. (2012)
Mycobacterium sp.	0.001	Dell'Amico et al. (2005)
Pseudomonas bathycetes	0.5	Nadeem et al. (2007)
P. brassicacearum AY007428	0.97	Belimov et al. (2001)
P. chlororaphis	0.46	Nadeem et al. (2007)
P. fluorescens	0.42	Nadeem et al. (2007)
P. fluorescens ATCC17400/ pRKACC	0.16	Shah et al. (1998)
P. fluorescens biotypeF	0.34	Zahir et al. (2008)

(continued)

Table 19.4 (continued)

Bacterium	ACC deaminase activity (μmol mg^{-1} h^{-1} α-ketobutyrate)	References
P. fluorescens biotypeG	0.49	Shaharoona et al. (2006)
P. fluorescens TDK1	0.35	Zahir et al. (2009)
P. oryzihabitans AF288732	0.89	Belimov et al. (2001)
P. putida UW4	21.23	Ma et al. (2003)
P. syringae	0.44	Nadeem et al. (2007)
P. tolaasii	0.001	Dell'Amico et al. (2005)
P. aeruginosa	0.15	Zahir et al. (2009)
P. fluorescens YsS6	12.5	Rashid et al. (2012)
P. fluorescens YsS7	11.5	Rashid et al. (2012)
Pseudomonas sp. 8R6	10.9	Rashid et al. (2012)
Pseudomonas sp. YsS2	9.88	Rashid et al. (2012)
Pseudomonas sp. YsS3	8.58	Rashid et al. (2012)
Pseudomonas sp. YsS4	10.79	Rashid et al. (2012)
Pseudomonas sp. YsS5	12.34	Rashid et al. (2012)
Rhizobium hedysari	1.78	Ma et al. (2003)
R. leguminosarum 99A1	0.43	Ma et al. (2003)
*R. leguminosarum*128C53K	0.01	Belimov et al. (2001, 2005)
R. gallicum PB2	0.08	Duan et al. (2009)
R. hedysari ATCC43676	0.2	Duan et al. (2009)
R.hedysari ATCC43676	0.02	Ma et al. (2003)
R. leguminosarum PB45	0.1–0.3	Duan et al. (2009)
Rhodococcus equi CL-S3	3.975	Rashid et al. (2012)
Rhodococcus sp. AF288731	0.83	Belimov et al. (2001)
Rhodococcus sp. Strain Fp2	7.32	Belimov et al. (2001, 2005)
Rhodococcus sp. strain4N-4	12.97	Belimov et al. (2001, 2005)
Serratia ficaria	0.33	Nadeem et al. (2010)
S. quinivirans SUD165	0.01	Belimov et al. (2001, 2005)
Trichoderma asperellum T203	12.16	Viterbo et al. (2010)
Trichoderma estonicum SKS1	9.32	Saravanakumar et al. (2018)
T. harzianum TSK8	8.18	Saravanakumar et al. (2018)
Uncultured *Devosia* sp. AS6	1.37	Rashid et al. (2012)
Uncultured Pseudomonas sp. YsS1	11.47	Rashid et al. (2012)

(continued)

Table 19.4 (continued)

Bacterium	ACC deaminase activity (μmol mg^{-1} h^{-1} α-ketobutyrate)	References
Variovorax paradoxus 2C-1	13.59	Belimov et al. (2001, 2005)
V. paradoxus 5C-2	4.32	Belimov et al. (2001, 2005)
V. paradoxus sp.	1.81	Belimov et al. (2005)
V. paradoxus 3P-3	3.7	Belimov et al. (2001, 2005)

19.7 Transcriptional Regulation

A number of studies have described the cloning of genes encoding ACC deaminase, and in all instances, the resulting clones have been shown to encode a functional ACC deaminase (Van Loon and Glick 2004). The expression of ACC deaminase is a highly regulated process and is affected by oxygen availability, substrate concentration, and product levels. Analysis of many bacterial genomes suggests that the transcription of *acd*S genes is not controlled by a single factor, and different phylogenetic groups may utilize different mechanisms to regulate the expression of this gene (Nascimento et al. 2014). We suggest that the *acd*S gene is most likely under the control of at least one of the following four factors: (a) a combination of LRP (leucine-responsive regulatory protein), CRP (cyclic AMP receptor protein), and FNR (fumarate–nitrate reduction regulatory protein), (b) *nif*A, (c) *Rpo*S, or (d) other mechanisms. Table 19.5 summarizes the mechanisms that have been to date found to regulate *acd*S gene expression in different bacteria.

19.7.1 Factor a: LRP, CRP, and FNR

ACC deaminase has been extensively studied in the *Pseudomonas* genus. The *acd*S genes from *Pseudomonas* sp. strains 6G5, F17, and UW4, and *Enterobacter cloacae* strain CAL2 have an open reading frame of 1014 nucleotides (Van Loon and Glick 2004). In *Pseudomonas* sp. UW4, which has been studied in great detail, several regulatory factors have been identified on the DNA region upstream of the *acd*S gene and include a CRP binding site (or box), an FNR binding site, an *acd*B gene (not immediately upstream of *acdS*), an AcdR binding site, and also an open reading frame encoding an *acd*R gene (Cheng et al. 2008; Grichko and Glick 2000; Li and Glick 2001; Van Loon and Glick 2004). These factors work in a coordinated manner and are actively involved in transcriptional regulation of the *acd*S gene (Fig. 19.1). The *acd*R gene is transcribed in the opposite direction of the *acd*S gene and encodes

Table 19.5 A summary of the *acdS* gene regulatory mechanisms that have been found in various phylogenetic groups

Bacteria	AcdR or similar protein	AcdB	FNR box	CRP box	nifA	σ70	σ54	GntR	LysR	MFS family	References
Pseudomonas sp. 6G5	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Li and Glick (2001)
Pseudomonas sp. F17	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Li and Glick (2001)
P. putida UW4	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Li and Glick (2001)
Enterobacter cloacae strain CAL2	Yes	Yes	Yes	Yes	No	No	No	No	No	No	Li and Glick (2001)
Bradyrhizobium japonicum USDA 110	Yes	No	No	No	No	Yes	No	No	No	No	Singh et al. (2015)
Rhizobium leguminosarum bv Viciae 128C53K	Yes	No	No	No	No	Yes	No	No	No	No	Singh et al. (2015)
Burkholderia xenovorans LB4000	Yes	No	No	No	No	No	No	No	Yes	No	Nascimento et al. (2014)
Burkholderia sp. CCGE1002	No	No	No	No	No	No	No	No	No	No	Nascimento et al. (2014)
Burkholderiaphymatum STM815	No	No	No	No	No	No	No	No	No	No	Nascimento et al. (2014)
Variovorax parrdoxus 5C2	No	No	No	No	No	No	No	No	No	No	Singh et al. (2015)
Achromobacter xylosoxidans A551	No	No	No	No	No	No	No	No	No	No	Singh et al. (2015)
Mesorhizobium loti MAFF303099	No	No	No	No	Yes	No	No	No	No	No	Nukui et al. (2006)
Many strains of *Meiothermus*	No	No	No	No	No	No	No	Yes	No	No	Nascimento et al. (2014)
Many strains of *Acinetobacter*	No	No	No	No	No	No	No	No	Yes	No	Nascimento et al. (2014)

(continued)

Table 19.5 (continued)

Bacteria	AcdR or similar protein	AcdB	FNR box	CRP box	nifA	σ70	σ54	GntR	LysR	MFS family	References
Brenneria sp. EniD312	No	No	No	No	No	No	No	No	Yes	No	Nascimento et al. (2014)
Dickeya spp.	No	No	No	No	No	No	No	No	Yes	No	Nascimento et al. (2014)
Pantoea sp. At-9B	No	No	No	No	No	No	No	No	Yes	No	Nascimento et al. (2014)
Saccharopolyspora erythraea NRRL 233	No	No	No	No	No	No	No	No	No	Yes	Nascimento et al. (2014)
Streptomyces hygroscopicus ATCC 53653	No	No	No	No	No	No	No	No	No	Yes	Nascimento et al. (2014)
Gluconacetobacter xylinus NBRC 3288	Yes	No	No	No	No	No	No	No	No	No	Nascimento et al. (2014)

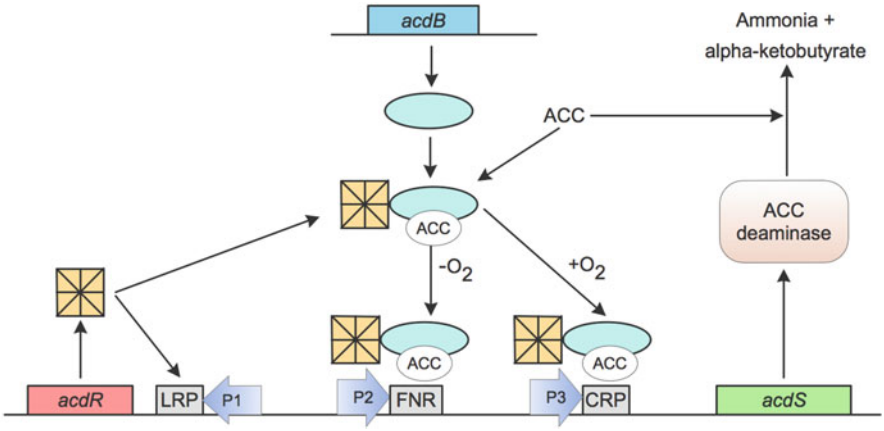

Fig. 19.1 Proposed mechanism of the regulation of *acd*S gene in *Pseudomonas* sp. UW4 through FNR, LRP, and CRP factors. Color key: orange box is LRP octamer, blue oval is GDPD (glycerophosphoryl diester phosphodiesterase); the product of the *acd*B gene

LRP. In addition, *acd*B from *Pseudomonas* sp. UW4 exhibits high similarity (90%) with the *P. fluorescens* pf0-1 glycerophosphoryl diester phosphodiesterase, GDPD (Cheng et al. 2008). The presence of ACC stimulates the transcription and expression of *acdR* (LRP). The active form of LRP is an octamer, which binds to a complex of ACC and GDPD, the product of the *acd*B gene (which is transcribed separately), and eventually forms a tripartite regulatory complex that binds to either an FNR box (under conditions of low oxygen concentration) or a CRP box (under conditions of high oxygen concentrations). The bound regulatory complex activates the promoter region of *acd*S (either P2 or P3), initiating the expression of this structural gene that encodes the enzyme ACC deaminase. It should be noted that not all bacteria contain an FNR or even a CRP box (Prigent-Combaret et al. 2008); in those instances, the regulatory complex is believed to bind directly to the (single) *acd*S promoter. The *acd*R gene constitutively promotes production of LRP from the P1 promoter; however, in the presence of excess amounts of the LRP octamer, the LRP octamer binds to an LRP box adjacent to the *acd*R promoter and prevents the expression of this gene. Following the synthesis of ACC deaminase, the ACC is cleaved to ammonia and α-ketobutyrate, and the later compound is eventually converted to a branched-chain amino acid such as leucine. In the presence of a large amount of leucine, this amino acid interacts with the LRP octamer (active form) and converts it to an inactive dimer–leucine complex. This complicated mode of regulation ensures that the *acd*S gene is only transcribed in limited amounts and when it is needed (Cheng et al. 2008).

19.7.2 Factor b: nifA

DNA sequence analysis indicates that some bacterial strains do not encode an *acd*R gene but are nevertheless still able to produce active enzymes. This observation led to the discovery of another possible mechanism of transcriptional regulation of this gene in some bacteria. For instance, in some strains of *Rhizobia* and *Mesorhizobium* the *acd*S gene is transcriptionally regulated under the control of a *nif*A promoter (the major function of this promoter is to control the transcription of all nitrogen fixation (*nif*) genes) (Nukui et al. 2006). In this case, the *nif*A promoter consists of *nif*A1 and *nif*A2 promoters that are located upstream of the *acd*S gene with a σ54 RNA polymerase recognition site. The proposed mechanism of regulation of *acd*S by *nif*A has been suggested to come from the *nif*A2 promoter that interacts with σ54 RNA polymerase and favors *acd*S transcription (Singh et al. 2015). The precise role of *nif*A1 in the expression of *acd*S is still an open question (Nukui et al. 2006). This mode of regulation may benefit the nodules that contain these *Rhizobia* and *Mesorhizobium* strains in preventing their premature senescence that is normally caused by excessive ethylene levels.

19.7.3 Factor c: RpoS

The sigma factor, RpoS, mainly regulates the genes that are expressed when bacteria enter the stationary phase of their growth or genes that are expressed in response to a number of stress stimuli (Hengge-Aronis 2002). In this way, RpoS is considered to be the main stress modulator in β and γ Proteobacteria (Osiriphun et al. 2009). Since ACC deaminase is mainly produced in the stationary phase of growth when bacteria face stress from the environment (Saleh and Glick 2001), the relationship between the expression of *acd*S and *rpo*S was investigated (Shah et al. 1998). In this study, it was found that the overexpression of *rpo*S affected two closely related bacterial strains differently. *Enterobacter cloacae* CAL2, an ACC deaminase positive strain, was genetically transformed with multiple copies of *rpo*S gene on a plasmid. The resulting overexpression of *rpo*S gene increased the ACC deaminase level by approximately 30% (Saleh and Glick 2001). On the other hand, when the same approach was used in bacterial strain *Pseudomonas* sp. UW4, the ACC deaminase levels decreased by 20% compared with the untransformed wild type (Saleh and Glick 2001). It is interesting to note that the *acd*S genes in these two bacteria exhibit 96% identity but are apparently controlled by different transcriptional regulators (Grichko and Glick 2001; Shah et al. 1998).

19.7.4 Factor d: Other Mechanisms

Many ACC deaminase positive bacteria possess *acd*R or similar genes in relatively close proximity to *acdS* (about 50 to a few hundred base pairs upstream of the *acd*S gene). However, some ACC deaminase positive bacteria have completely or partially lost the *acd*R gene but are nevertheless still able to produce active enzyme. In other cases, *acd*R or a similar LRP producing gene is located as far as 9 kb from the *acd*S gene, but is still able to control *acd*S regulation (Nascimento et al. 2014). In two strains from the genus *Burkholderia,* namely *Burkholderia* sp. CCGE1002 and *Burkholderia phymatum* STM815, no *acd*R gene has been detected, instead, these bacterial strains contain two copies of the *acd*S gene; one is on the bacterial chromosome and the other copy is located on the mega plasmid that these bacteria contain (Singh et al. 2015). In another member of the same genus, *Burkholderia xenovorans* LB4000*,* the LysR family of transcription regulatory elements has been found to be in close proximity to the *acd*S gene (Singh et al. 2015). Some members of *Acinetobacter* spp. and *Proteobacteria* spp. (e.g., *Brenneria* sp. EniD312, *Dickeya* spp., and *Pantoea* sp. At-9B) are also thought to be under the same (LysR) transcriptional regulation (Nascimento et al. 2014). Moreover, in many members of *Actinobacteria* and *Meiothermus*, another gene, responsible for the transcription of regulatory protein GntR, has been found in the neighborhood of the *acd*S gene and has been suggested to play a role in the regulation of the *acd*S gene in these bacteria (Nascimento et al. 2014). In *Saccharopolyspora erythraea* NRRL 233 and *Streptomyces hygroscopicus* ATCC 53653, the *acd*S gene is apparently a part of the MFS (major facilitator superfamily) proteins and may be regulated by the same operon regulatory elements, prominently the M20 peptidase (Nascimento et al. 2014). From the aforementioned, it is clear that the regulation of the *acd*S gene is a rather complex process with different microbes utilizing somewhat different strategies.

19.8 Future Prospects

While PGPB utilize a wide range of mechanisms to facilitate plant growth (Glick 2012), especially in the presence of many different biotic and abiotic environmental stresses, the use of the enzyme ACC deaminase to lower plant ethylene levels is probably one of the most important of those mechanisms. If the use of PGPB is to become a future cornerstone of a new paradigm in agricultural practice where the use of harmful chemicals is severely constrained, it is essential that scientists develop a detailed fundamental understanding of precisely how PGPB function. This understanding should enable scientists to more reproducibly employ the use of PGPB in horticulture, silviculture, and environmental clean-up as well as agriculture (Reed and Glick 2013).

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Chapter 20
The Diazotroph as an Endophyte and How a Diazotroph Interacts with Its Plant Host

Se-Chul Chun

Abstract The "diazotrophs" are dinitrogen-fixing (N_2-fixing) microorganisms that include members of the bacteria and archaea, and are functionally important microbes that convert gaseous N_2 to ammonia (NH_3). There are endophytic diazotrophic bacteria that live within plant tissues for at least part of the plants life cycle without causing apparent disease. Diazotrophs are ubiquitous and have been found in all species of plants studied to date. The diazotroph and plant relationships have been much studied. Some diazotrophs may enhance host growth, nutrient acquisition, and improve the plant's ability to tolerate abiotic stresses such as drought and salt. They may also induce plant resistance to insects, pathogens, and herbivores. Recently, plant microbiome studies have attracted much attention as we try to understand the dynamics underlying interactions of plants and microorganisms. The future is certain to bring even more genetic evidence on evolutionary points of view regarding the diazotrophic bacteria and their hosts.

20.1 Introduction

The microorganisms are almost everywhere in our ecosystem. They are in every form of life and every inorganic entity of its surrounding environment. The term "diazotroph" refers to dinitrogen-fixing (N_2-fixing) microorganisms that include members of the bacteria and archaea, and are functionally important microbes that convert gaseous N_2 to ammonia (NH_3) (Church and Böttjer 2013), playing critical roles of geo-biochemical elemental cycling in ecosystems. Diazotrophs can be found inside epidermal tissues of plants in which the microbes fix nitrogen from the air, or these microbes can fix nitrogen while free living in soil. What are they doing inside the plants? The diazotrophs reside in those environments where plant tissues offer support for them to have a living space, and where the diazotrophs can possibly receive nutrients such as carbohydrates. The plants benefit not only from the

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_20

microbially provided nitrogen supply, but also from other biologically advantageous effects due to indoleacetic acid production (IAA) and stimulations of plant responses that are triggered by the diazotrophs. Some diazotrophs may stimulate the plant in ways that promote cell growth and induce defensive systemic responses of the plant against pathogens. Diazotrophs include among their membership many species of bacteria. Notably, the *Rhizobia* are well known as symbiotic bacteria which often act by making nodules on the roots and even on the stems ~~on~~ of leguminous plants, fixing atmospheric nitrogen and providing it to the plants (Singh et al. 2006). However, it also has been reported that bacteria belonging to the beta-subclass of proteobacteria such as *Burkholderia* and *Ralstonia* similarly could induce nodules on legumes and can fix atmospheric nitrogen (Chen et al. 2001; Moulin et al. 2001; Vandamme et al. 2002; Verma et al. 2004). Many nodule-forming bacteria are collectively referred to as legume-nodulating bacteria (Zakhia et al. 2004).

Much of the atmospheric nitrogen fixed on earth is attributed to the symbiosis of *Rhizobia* with legumes (Chaintreuil et al. 2000). Although these genera of *Rhizobia* do not induce nodulation on the plants other than legumes, they can still fix nitrogen from air and plants can utilize this nitrate. All rhizobial genera including *Azorhizobium, Bradyrhizobium, Ensifer, Mesorhizobium, Methylobacterium,* and *Rhizobium* belong to the alpha-subclass of proteobacteria (http://www.rhizobia.co.nz/Rhizobia_Taxonomy.html). It has been much reported that rhizobia may occur as root endophytes (colonize intercellular spaces of roots) in nonlegumes such as rice, promoting the plants growth and crop productivity, in addition to those symbiotic associations which rhizobia have with legumes (Singh et al. 2006; Biswas et al. 2000; Matiru and Dakora 2004; Peng et al. 2002; Perrine et al. 2001; Yanni et al. 1997, 2001).

The rhizobia and other diazotrophs reside inside or outside of the root surface and fix nitrogen from the atmosphere, providing their product as nitrate that plants can utilize. It is obvious that many of these endophytes can promote plant growth and productivity. What has not been much studied is the possibility that these endophytic rhizobacteria could induce disease and drought resistance in addition to supporting plant growth promotion, producing IAA and nitrogen fixing (Ji et al. 2014a). The endophytic green fluorescent protein gene (*gfp*)-tagged *Bacillus subtilis* CB-R05 could colonize well on the root tissues of rice (*O. sativa* Ilpum cv.) for 16 days of growth after inoculation (Ji et al. 2014b). *B. subtilis* CB-R05 was originally isolated from endophytic tissues of rice and demonstrated to promote plant growth and induce disease resistance. In their works, *B. subtilis* CB-R05 was transformed with *gfp* gene and inoculated into rice seeds through immersion into the suspension of *gfp*-tagged *B. subtilis* CB-R05 for 1 h. The seeds were planted into the soil and seedlings had grown for 16 days, being observed for their colonization to root system with confocal fluorescent microscopy (Ji et al. 2014b).

Bowen and Rovira (1999) wrote a valuable review of research and described that proper management of the rhizosphere could improve plant growth. They reviewed studies on the increases in growth of tomatoes inoculated with *Azotobacter* (Brown et al. 1964). Similar results were also obtained by Rovira (1965) for wheat, following inoculation with *Azotobacter chroococcum*, *Clostridium pasteurianum,* and *Bacillus*

polymyxa. Bowen and Rovira (1999) discussed the possible mechanisms for plant growth-promoting rhizobacteria (PGPR) responses such as both positive and negative plant growth effects (by potential phytohormones), induced systemic resistance to phytopathogens, siderophore production, phosphate solubilization, and root associated biological nitrogen fixation (BNF) (Kennedy et al. 2004).

Dobbelaere et al. (2003) reported on the diazotrophic benefit of PGPR, focusing on the microbes mechanisms of action including BNF, plant growth promotion by plant hormone produced such as auxins, cytokinins, gibberellins and ethylene, and phosphate solubilization, increase in nutrient uptake, enhanced stress resistance, vitamin production, and biocontrol. In this review, I will be presenting some updated information on previously reported genetic studies on the diversity of diazotrophs, and discussion focusing on interactions.

20.2 Background of Diazotrophs

A bacterium that fixes nitrogen in root nodules was discovered by Beijerinck in 1888. He isolated the bacteria but did not have the experimental conditions which would allow the bacteria to fix nitrogen (Beyerinck 1888). He named the bacterial strain *Bacillus radicicola*, and we currently know it as *Rhizobium leguminosarum*. Beijerinck obtained the organism from root nodules of legumes that first had been discovered to fix gaseous nitrogen by German scientists Hellriegel and Wilfarth in the year 1888. Beijerinck (1901) also isolated an *Azotobacter* sp. that now is known to fix gaseous nitrogen. Diazotrophs were first suggested to penetrate all tissues of sugarcane (Dobereiner 1992), and subsequent studies have shown that these bacteria are not restricted only to this crop (Reis et al. 2000) but are in fact widely present in plants. Regardless of their non-endosymbiotic or endosymbiotic nature, many diazotrophs have the genes for nitrogen fixation (nif genes) but not nodule inducing (nod genes) like those of the leguminous *Rhizobia*.

Among the various N_2-fixing endophytic bacteria, two types have been suggested to classify these bacteria (Reis et al. 2000): the obligate endophytes *Acetobacter diazotrophicus*, which cannot survive in the soil, and the facultative ones that include the *Azospirillum* group (Baldani et al. 1997) and other many bacteria such as *Burkholderia vietnamiensis* (Gillis et al. 1995), *B. brasilensis* (Baldani et al. 2000; Hartmann et al. 1995), and *B. subtilis* (Ji et al. 2014a), which can survive in soil and plants. These diazotrophs are genetically diverse. The biological traits for host ranges, tissue colonized, and endosymbiosis are shown in Table 20.1 (Baldani et al. 1997; Reis et al. 2000).

The crops of family Poaceae, previously known by the name Gramineae, are the grasses such as rice and wheat, currently need to receive costly mineral fertilizer (Döbereiner et al. 1995; Triplett 1996). Studies on long-term N-balance and ^{15}N isotope dilution technique (Viera-Vargas et al. 1995) have shown that some of Brazilian sugarcane (Saccharum spp.) varieties may in fact obtain up to 70% of their N requirements by nitrogen fixation. This process seems to involve

Table 20.1 Obligate and facultative endophytic diazotrophs occurring in non-legume plants[a]

Diazotroph	Plant	Plant part
	Facultatives	
Azospirillum brasilense	Cereals Forage grasses Sugarcane	Roots, stems, seeds Roots, stems Roots, stems, leaves
Azospirillum lipoferum	Cereals Forage grasses Sugarcane Tuber plants Palm trees	Roots, leaves Roots, stems, seeds, xylem sap Roots, stems, leaves Tubers, roots Roots, stems, fruits
Azospirillum amazonense	Cereals Sugarcane Palm trees	Roots, stems, seeds Roots, stems Roots, stems, fruits
Burkholderia	Rice Sugarcane	Roots Stems, leaves
Azospirillum irakense	Rice	Roots
Bacillus subtilis	Rice	Stems, roots, leaves
Bacillus amyloliquefaciens	*Gossypium* sp. (cotton)	Roots
Paenibacillus sp.	Chinese hybrid poplar Larch Spruce	Tissue cultured shoots Tissue cultured shoots Tissue cultured shoots
Enterobacter ludwigii	*Vitis vinifera*	Planlets Roots
	Obligates	
Azorhizobium	*Sesbania*, rice, wheat	Roots
Herbaspirillum seropedicae	Cereals Sugarcane Forage grasses Palm trees	Roots, stems, leaves, seeds Roots, stems Roots, stems, leaves Roots, stems
Herbaspirillum rubrisubalbicans	Sugarcane *Miscanthus* spp.	Roots, stems, leaves Roots, stems, leaves
Herbaspirillum frisingense	*Miscanthus* spp. *Spartina pectinata* *Pennisetum purpureum*	Roots Roots Roots, stems
Gluconacetobacter diazotrophicus	Sugarcane *Pennisetum purpureum* Sweet potato	Roots, stems, leaves, trash roots, Stems Roots, stems, tubers
Azoarcus spp.	Kallar grass Rice Cereals	Roots, stems Plant Roots, stems, leaves
Burkholderia brasilensis	Sugarcane Tuber plants Palm trees	Roots, stems, leaves Roots, tubers Roots, stems
Burkholderia "tropicalis"	Sugarcane	Roots, stems

[a]After Reis et al. (2000)

participation by both rhizosphere and endophytic diazotrophs (Baldani et al. 1997). Several N_2 fixing bacteria such as *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella pneumoniae*, *Azotobacter vinelandi*, *Paenibacillus polymixa*, *Azospirillum* spp., *Herbaspirillum* spp., and *Gluconacetobacter diazotrophicus* colonize the sugarcane plant (Cavalcante and Döbereiner 1988; Olivares et al. 1996).

20.3 Biology of Diazotrophs and Interaction with Plants

Diazotrophic bacteria could fix gaseous nitrogen epiphytically or endophytically from air and could supply that needed resource to the plants. In addition, they could have other functions to benefit the plants such as not only producing IAA, gibberelline, and cytokinin-like substances (Fuentes-Ramírez et al. 1993; Bastián et al. 1998) but also promoting plant's growth and inducing resistance to pest and other abiotic stress like drought (Ji et al. 2014b). The genetics of diazotrophs are now much elucidated on the interaction of plants with advanced biotechnology. The biology of endophytes is summarized with the proposed mechanisms for endosymbiosis in Table 20.2 (Dent 2018 and Pinski et al. 2019).

Recently, plant microbiomes are a very hot topic to study. The microbiome of plants includes the epiphytes and endophytes in all parts of plant tissues. Endophytic bacteria primarily survive in the intercellular spaces due to an abundance of carbohydrates, amino acids, and other nutrients available from the plant. Some endophytes are capable of intracellular colonization. Endophytic strains colonize various parts of plants, including the roots, leaves, stems, flowers, and seeds. However, the roots of plants are the part that endophytes reside the most abundantly, both in terms of the microoganisms number and diversity (Pinski et al. 2019). Diazotrophic endophytes which have the functions of nitrogen fixation are of special interest for agricultural practice. Of them, the bacteria that have other benefits such as phosphate solubilities, IAA and siderophore production, and antagonistic effects against pathogens are very promising for the crops. The antagonistic effects of endophytes against phytopathogens are associated with the endophytes production of chitinase, protease, and siderophores (Pinski et al. 2019). The enhanced resistance which endophytes provide to plants against environmental stresses might be due to the activity of deaminase 1-aminocyclopropane-1-carboxylate (ACC). This could decrease ethylene production by degrading ACC, which is known as the precursor of ethylene leading in the necrosis of cell (Kandel et al. 2017). Numerous studies have demonstrated that endophytic bacteria could induce the resistance of plants to cold and drought, and also stimulate plant immune systems in a process called priming (Liu et al. 2017; Gagne-Bourque et al. 2015).

In the study of Ji et al. (2014b), rice seeds were inoculated with *Bacillus subtilis* CB-R05 that possessed antagonistic effects against several fungal pathogens—it is a diazotrophic bacteria marked with the green fluorescent protein (*gfp*) gene. The pathogenesis-related (PR) proteins (PR2, PR6, PR15, and PR16) in rice inoculated with CB-R05 were generally more strongly expressed in the rice leaves inoculated

Table 20.2 Examples[a] of the genes associated with the endosymbiosis of diazotroph and plant

Genes involved in endosymbiosis and function	Implications and findings
Motility and chemotaxis – MCP gene in *Gluconacetobacter diazotrophicus*	MCP, a transmembrane sensor protein allows *G. diazotrophicus* to sense concentrations of molecules while *Che* proteins enable orientation and movement (Miter et al. 2013)
– potential MCP gene (Hsero_3720) in *Herbaspirillum seropedicae* SmR1	Reduction in chemotaxis towards the plant and attachment to the roots of *Zea mays* when the gene is inactivated (Balsanelli et al. 2016)
– *tlp1*: Transducer-like protein 1, chemoreceptor-like protein	Significant reduction of *Azospirillum brasilense* Sp7 in root of *Triticum aestivum* (Greer-Phillips et al. 2004)
– *CheA* (chemotaxis protein) in *Pseudomonas fluorescens* OE28.3, SBW25, F113 and WCS365	Ten- to 1,000-fold decrease in the ability to colonize the root tip of ten- to 1,000-fold decrease in the ability to colonize the root tip of *Solanum lycopersicum* (De Weert et al. 2002)
– *pilX* (azo2916): Type IV fimbrial biogenesis protein PilX	Reduced root colonization of *Azoarcus* sp. BH72 in *Oryza sativa* ssp. *Japonica* cv. Nipponbare (Shidore et al. 2012)
– *pilT* (azo3468): Type IV pilus retraction protein	Strong reduction in endophytic colonization. In *O. sativa* subsp. *Indica* cv. IR36, 50% reduction in surface colonization of *Azoarcus* sp. BH72 (Böhm et al. 2007)
– *mot3:* Bacterial flagellar motility	in *Triticum aestivum*, significant decrease in adsorption capacity of *A. brasilense* Sp7 to roots (Croes et al. 1993)
Plant polymer degradation (PPD) – *eglS*: Endo-beta-1,4-glucanase	In *Brassica rapa* subsp. *pekinensis* and *chinensis*, decrease of endophytic colonization of *Bacillus amyloliquefaciens* TB2 (Kerff et al. 2008)
– eglA(azo2236): Beta-1,4-glucanase (cellulase) (EC 3.2.1.4)	In *O. sativa* subsp. *Indica* cv. IR36, decrease of endophytic colonization of *Azoarcus* sp. BH72 (Reinhold-Hurek et al. 2006)
– GH gene: Glycoside hydrolases (GH)	GHs promote entry into plant, sugar, and cell wall metabolism of bacteria, and interaction of host-microorganism (Miethke and Marahiel 2007) *G. diazotrophicus* has specific cell wall-degrading enzymes (Adriano-Anayal et al. 2005).
Adhesion, biofilm formation – *lapA*: Surface adhesion protein,	In Z. mays, impaired root colonization *Pseudomonas putida* of KT2440
– *agpB*: Curli subunit	In *Medicago sativa*, reduced attachment of *Salmonella enterica* serovar Enteritidis capacity to the root
– *gumD* (YP_001602791): Polysaccharide biosynthesis glycosyltransferase, exopolysaccharide biosynthesis	In *Oryza sativa*, defective root surface attachment, reduced root epiphytic and endophytic colonization of *G. diazotrophicus* PAL5
– *gmsA* (CAK07156.1): Glucomannan production protein	In *Pisum sativum*, defective attachment and biofilm formation of *Rhizobium*

(continued)

Table 20.2 (continued)

Genes involved in endosymbiosis and function	Implications and findings
	leguminosarum biovar viciae 3841 on the root hairs
– Hsero_1294 and *fhaB*: Filamentous hemagglutinin proteins.	In *Triticum aestivum,* upregulation of two genes of *Hsero_1294* and *fhaB* of *H. seropedicae* SmR1 suggests involvement of root attachment.
Lipopolysaccharide, membrane proteins – *rfbB* (Hsero_4410): dTDP-D-glucose 4,6-dehydratase, biosynthesis of rhamnose.	In *Zea mays*, 100-fold lower attachment to root surface, reduced efficiency in endophytic colonization of *H. seropedicae* SmR1
– *rfbC* (Hsero_4411): dTDP-4-keto-6-deoxy-D-glucose 3,5-epimeras, biosynthesis of rhamnose.	In *Z. mays*, 100-fold lower attachment to root surface, reduced efficacy in endophytic colonization of *H. seropedicae* SmR1
– *ampG* (YP_003773531.1): Muropeptide permease of the major facilitator superfamily, recycling of the cell wall peptidoglycan.	In *Z. mays*, ten-fold reduction in endophytic population in roots at one and 3 days after inoculation of *H. seropedicae* SmR1
Detoxification	Endophyte survival requires the ability to detoxify or manage movement of xenobiotics using efflux pumps. *G. diazotrophicus* has poor survival in the rhizosphere (Saravanan et al. 2008; Baladani and Baldani 2005; Matiru and Thomson 1998).
– *gor* (GDI_2216): Glutathione reductase (EC 1.8.1.7)	Reduction in number of tightly root-associated endophytic colonization of *G. diazotrophicus* PAL5 in *O. sativa* subsp. *Indica* cv. IR42 (Alqueres et al. 2013)
– *sodB* (GDI_2168): SOD	Reduction of number of tightly root-associated endophytic colonization of *G. diazotrophicus* PAL5 in *O. sativa* subsp. *Indica* cv. IR42 (Alqueres et al. 2013)
– *smeE* (SMD_3657): RND efflux system, inner membrane transporter, part of the SmeDEF efflux pump involved in quinolone resistance	Impaired colonization of roots of *Stenotrophomonas maltophilia* D457 in *Brassica napus* cv. Californium (Garcia-Leon et al. 2014)
Fe uptake – Dicitrate ton-B dependent receptors – Siderophore biosynthesis	Biologically available Fe is limited in plants and endophytes. Uptake of ferric siderophore complexes is achieved via TonB dependent receptors (Galperin 2005). Endophytes with large numbers of these receptors may compete with plants or fungi for iron acquisition (Miter et al. 2013)
Degradation of organic compounds	*G. diazotrophicus* is at the extreme low end of the ability to degrade complex plant metabolites (Dent 2018).
– *alkB* (Bphyt_5401): Alkane monooxygenase – alkane hydroxylase (Bphyt_1856) – dioxygenases	In *Burkhoderia phytofirmans* PsJN, alkane monooxygenase (alkB, Bphyt_5401) and cytochrome P450 alkane hydroxylase (Bphyt_1856). AlkB enzymes are required for break-down of aliphatic hydrocarbons.

(continued)

Table 20.2 (continued)

Genes involved in endosymbiosis and function	Implications and findings
	At least 15 dioxygenase genes were found in the genome of *B. phytofirmans* PsJN (Miter et al. 2013)
– *oxc*: Oxalate decarboxylase	In *Lupinus albus* L., cv. *Amiga*, *Z. mays* subsp. *mays*, cv. Birko, impaired early colonization of *B. phytofirmans* PsJN (Kost et al. 2014)
– *thuA*: Trehalose utilization-related protein	In *Medicago sativa*, impaired colonization of *R. meliloti* 1021 to roots (Jensen et al. 2005)
Transporter (SUM transporter, Porin, putrescin)	*G. diazotrophicus* PA15 has a relatively high number of transporter genes enabling transport of nutrients and excretion of toxins (Miter et al. 2013). The high number of ABC family in *G. diazotrophicus* PAI5 and *Azospirillum* sp. B510 (Miter et al. 2013). Low numbers of the ABC family of transporters, porin genes and the lack of putrescine transporters perhaps suggests poor rhizosphere competence (Dent 2018).
– (Hsero_4782): ABC multidrug transporter	20-fold lower endophytic and epiphytic populations of *H. seropedicae* SmR1 at three days after inoculation in *Zea mays* (Balsanelli et al. 2016)
– (2553408008): Outer membrane efflux transporter	Four- to six-fold decrease ability of *Paraburkholderia kururiensis* M130 to colonize root of *Oryza sativa* var. BALDO (Levy et al. 2018)
Secretion systems (type I & IV, type II, III, Va, Vb, VI)	*G. diazotrophicus* in common with many other endophytes has available key secretion systems (Dent 2018)
– *hrcN* (Hrubri_2444): T3SS ATPase	100-fold decrease in endophytic population in roots at nine days after inoculation of *Herbaspirillum rubrisubalbicans* M1 on *Oryza sativa* (Schmidt et al. 2012)
– *ppkA* (azo3888): T6SS serine/threonine protein kinase	In *O. sativa* ssp. *Japonica* cv. Nipponbare, increased colonization capacity of Azoarcus sp. BH72 (Shidore et al. 2012)
– *cesT* (2553406074): Tir chaperone protein	In *O. sativa* var. BALDO, four- to six-fold decrease in the ability of *Burkholderia kururiensis* M130 to colonize roots (Levy et al. 2018)
Signaling and transcription regulator	Signaling systems are more complicated with the genome size, phylogeny, ecology and metabolic activities of the bacteria (Cases and de Lorenzo 2005). Bacteria in diverse habitats encode more ECF sigma factors compared with stable niches (Gourion et al. 2009)

(continued)

Table 20.2 (continued)

Genes involved in endosymbiosis and function	Implications and findings
– *rpfF* (Smal_1830): Enoyl-CoA hydratase, synthesis of quorum sensing molecule—diffusible signal factor (DSF)	In *Brassica napus* cv. Californium, decreased colonization efficiency of *Stenotrophomonas maltophilia* R551–3 and plant growth promotion (Alavi et al. 2013)
– *pcoI:* Acyl-homoserine-lactone synthase, quorum sensing molecules biosynthesis	In *Triticum aestivum*, deficiency in colonization of *P. fluorescens* 2P24 (Wei and Zhang et al. 2006)
– *bpI.1* (Bphyt_0126): AHL synthase of chromosome 1 QS system	In *Arabidopsis thaliana* Col-0, decreased root colonization of *B. phytofirmans* PsJN (Zuniga et al. 2013)
– (azo2408): GGDEF domain-containing protein	In *O. sativa* ssp. *Japonica* cv. Nipponbar, decreased root colonization of *Azoarcus* sp. BH72 (Shidore et al. 2012)
– *rpoS*: Stationary-phase sigma factor, regulating biofilm formation, *agfD* and other adhesins	Reduced colonization of *Staphylococcus enterica* serovar Newport to the sprout of *M. sativa* (Barak et al. 2005)
Plant cell wall modification – *yoaJ*: Expansin, causes loosening and extension of plant cell walls by disrupting the non-covalent bonding between the cellulose microfibrils and matrix glucans	In *Z. mays*, significant reduction in ability to colonize of *Bacillus subtilis* 168 to roots (Kerff et al. 2008)

[a]Adapted from Dent (2018) and Pinski et al. (2019)

with CB-R05 compared with the untreated control. Under the confocal laser scanning microscope (CLSM), the *gfp*-tagged CB-R05 bacterial cells were observed to penetrate the rhizoplane, especially in the elongation and differentiation zones of the rice roots and colonize inside the root tissues (Ji et al. 2014b).

20.3.1 Genetics on the Interaction of Diazotrophs and Plants

At present, there are more than 800 complete microbial genomes in databases. However, only nine are endophytes (*Azoarcus* sp. BH72, *Burkholderia phytofirmans* PsJN, *Enterobacter* sp. 638, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Serratia proteamaculans* 568, *Klebsiella pneumoniae* 342, *Stenotrophomonas maltophilia* R551–3, and *Gluconacetobacter diazotrophicus* Pal5) (Bertalan et al. 2009).

The complete genomes of endophytic bacteria reveal remarkably few mobile elements in their genome. Of these, the genomes exhibit a range in size from 3.9 to 7.6 Mb (Dent 2018), with *G. diazotrophicus* having the smallest genome (Miter et al. 2013) of 3.9 Mb (Bertalan et al. 2009). *Gluconacetobacter diazotrophicus* is firmly in the facultative intracellular colonizer category (Cocking and Dent, 2019) and was first demonstrated in 2006 (Cocking et al. 2006). Certain strains of *G. diazotrophicus* are capable under the right conditions of intracellularly colonizing a range of crop species. Similarly, this character of wide host range has been demonstrated for a

variety of other phytosymbiont bacteria and their host plants (De Almeida et al. 2009; Thomas and Reddy 2013; White et al. 2014; Thomas and Sekhar 2014).

Facultative intracellular symbionts show their adaptive flexibility by possessing a relatively greater number of mobile genetic elements as compared with obligate intracellular symbionts (Toft and Anderson 2010). For example, *G. diazotrophicus* has 109 transposases, which is four to five times more mobile elements than possessed by many other endophytes (Bertalan et al. 2009; Miter et al. 2013), reflecting a high degree of adaptive flexibility on the part of *G. diazotrophicus*. Such flexibility might be needed to overcome host-related constraints such as the requirement for bacteria to attach to host cells, subsequently enter the cytoplasm, multiply, exiting, and then being transmitted to new host individuals. All that activity must occur without the bacteria being recognized by the host immune system (Toft and Anderson 2010) or at least not targeted for destruction even if immune recognition does happen.

Genetic diversity and adaptive flexibility can also be achieved though bacteria possessing plasmids that carry genes responsible for necessary functions such as nitrogen fixation, sulfur utilization, and hydrocarbon degradation. Nitrogen-fixing genes could, in particular, be conserved in chromosomal DNA and within plasmids (Banu and Prasad 2017).

The symbiotic bacteria of genus *Rhizobium* carry high molecular weight plasmids ($90–350 \times 10^6$ daltons) and it is important to note that the plasmids of *R. leguminosarum* have a role in nodule formation (*nod* genes) symbiosis as well as carrying nitrogen fixation (*nif*) genes (Nuti et al. 1979).

Plasmids do occur in *G. diazotrophicus* but their numbers and sizes vary between strains, with for example *G. diazotrophicus* UAP8070 and UAP5665 each having three plasmids of 93, 22, and 22 kb in size (Caballero-Mellado and Martínez-Romero 1994), PR2 has two plasmids one of which is particularly large at 170 kb and a smaller one at 24 kb, whereas Pal5 has two plasmids of 38.8 and 16.6 kb, and strain UAP5541 has no plasmids at all (Dent 2018; Fuentes-Ramírez et al. 1993, 1999).

The colonization of plant hosts by endophytic bacteria is described as a complex process that could be distinguished into five steps as described by Pinski et al. (2019): (1) recognizing root exudates and motility towards the plant, (2) adhering to the surface of roots, (3) biofilm formation, (4) root surface penetration, and (5) colonization of the internal parts of a plant (Kandel et al. 2017; Hardoim et al. 2015). Each of these steps is mediated by various biomolecules which drive dynamic changes in the expression of the bacterial genes as well as in the colonized host (Pinski et al. 2019). Colonizing the roots by endophytic bacteria starts with the chemotaxis of planktonic bacteria towards the roots followed by attachment to the rhizoplane. It was demonstrated that methyl-accepting chemotaxis proteins (MCPs) could play a key role in these first stages (Scharf et al. 2016). These are transmembrane sensors that result in the signal compounds surrounding the bacteria being detected to either direct the bacteria towards attractants or away from repellents (Scharf et al. 2016).

The involvement of the MCPs in plant colonization was demonstrated by the inactivation of MCP genes in *Herbaspirillum seropedicae* SmR1. Of the 66 genes

encoding for MCPs in *H. seropedicae* SmR1, nine were differentially expressed in the cells associated with roots. Inactivation of one of these, the gene (accession number Hsero_3720), resulted in a two-fold reduction in the ability of the mutant strains to attach compared to the wild-type strain (Balsanelli et al. 2016; Pinski et al. 2019) (Table 20.2). This MCP is a key transducer required to sense rhizosphere compounds and to direct bacteria towards the host-secreted compounds. However, there was no difference in epiphytic and endophytic colonization by the SmR1 mutant strain compared with the wild-type strain (Balsanelli et al. 2016).

The inactivation of another MCP chemotaxis-like protein encoded by *tlp1* gene has resulted in the impairment of chemotaxis to several terminal electron acceptors (oxygen and nitrate) and redox active chemicals, indicated by studying a mutant of the rhizospheric strain *Azospirillum brasilense* Sp7 that displayed impaired colonization of the plant roots (Greer-Phillips et al. 2004; Michiels et al. 1991; Pinski et al. 2019) (Table 20.2).

Defining the precise function of flagella is complicated because their protein constituents are microorganism-associated molecular patterns (MAMPs) that elicit response by the plant against phytopathogens. A lack of flagellum synthesis in *Azoarcus* sp. BH72 has yielded mutants that were not motile, although the mutants' attachments to the root surface remained the same (Buschart et al. 2012). The impaired *Azoarcus* sp. BH72 flagella could not be recognized by the plant, resulting in failure to induce a plant defensive response (Buschart et al. 2012). However, in *A. brasilense* Sp7 the flagellum was found critical to achieve adherence to wheat roots (Croes et al. 1993) (Table 20.2).

The pili produced by *Azoarcus* sp. BH72 are also involved in colonizing the surface and interior of roots. The inactivation of the pilus-associated *pilX* gene has resulted in a greatly reduced root colonization that could be due to the impaired twitching motility of the mutant (Shidore et al. 2012). In another study, pili mutants with no twitching motility also showed a significant decrease in colonization of the surface and interior of roots (Böhm et al. 2007). A microscopy study on the type IV pili of *H. seropedicae* SmR1 during the colonization of wheat roots offered further suggestion for the involvement of this structure in attachment to host cells (Pankievicz et al. 2016) (Table 20.2). Some contradictory information on the involvement of type IV pili was presented by Cole et al. (2017). In their study, mutations in the pilus locus increased the colonization fitness of the rhizospheric strain *Pseudomonas simiae* WCS417r. However, this study may imply that the mutation promotes a planktonic lifestyle, possibly resulting in a reduction in cell-to-cell communication and cell-to-surface interactions, leading to increased motility and colonization efficiency (Cole et al. 2017).

Biofilm formation could be a possible factor for attachment of bacteria to the root surface (Pinski et al. 2019). The biofilm typically contains water, proteins, polysaccharides, extracellular DNA (eDNA), RNA, and ions (Żur et al. 2016). A series of mutants representing a range of species has suggested that additional polymers contribute to bacterial attachment and colonization. In *Gluconacetobacter diazotrophicus* PAL5, exopolysaccharide (EPS) is also involved in forming the biofilm. The inactivation of the *gumD* gene responsible for the first step of EPS biosynthesis resulted in a decrease in the rhizospheric and endophytic colonization

of rice roots by PAL5 (Meneses et al. 2011) (Table 20.2). Also, a similar deficiency in the formation of biofilm and attachment was reported in mutants of *Rhizobium leguminosarum* biovar *viciae* 3841 that were not able to produce EPS and glucomannan (Williams et al. 2008; Pinski et al. 2019). In addition, cellulose is another major component of the biofilm in some species. The study of a mutant that lacks cellulose production (*wssD* gene mutation in *Herbaspirillum rubrisubalbicans* M1) showed a decrease in attachment to the root surface of plants (Monteiro et al. 2012).

The surface-associated protein LapA of *Pseudomonas* functions in biofilm production and contributes to cell-to-cell attachment by regulating cell hydrophobicity (Ainelo et al. 2017). In a *lapA* mutant, initial attachment to the roots was similar to that observed for the wild type, but the formation of a microcolony and the subsequent development of a mature biofilm was impaired, resulting in poorer root colonization (Martinez-Gil et al. 2010). Only those bacteria that can adhere to the surface of roots will be able to colonize the inside of a plant, although root exudates can attract both groups of bacteria. Adhesion to roots can be mediated by the previously described flagella and pili, as well as by specialized proteins such as curli and hemagglutinins (Kandel et al. 2017; Hardoim et al. 2015) (Table 20.2). In *Salmonella enterica*, the *agfA* gene encodes the secreted curli protein subunit, while *afgB* encodes the surface-exposed nucleator around which the curli amyloid fibers form. The knockout mutants of the *agfB* gene have shown a decrease in both the initial attachment as well as the progress of attachment and colonization over time, whereas inactivation of the *agfA* gene did not influence on the initial attachment and colonization (Barak et al. 2005). Although hemagglutinins are well known for their role in both plant and human pathogenesis, genes encoding for hemagglutinins are also frequently reported in the genomes of endophytic bacteria (Taghavi et al. 2010; Miter et al. 2013; Pedrosa et al. 2011) and upregulation of two genes encoding the filamentous hemagglutinin proteins (Hsero_1294 and *fhaB*) in *H. seropedicae* SmR1 when the mutants were attached to plant roots has suggest their involvement in attachment to the root surface (Pankievicz et al. 2016) (Table 20.2).

In Gram-negative bacteria, lipopolysaccharide (LPS) consists of three components: (1) lipid A, (2) a core region, and (3) O-antigen. Of those three, core region usually consists of no more than five sugar units (Steimle et al. 2016). Rhamnose is a monosaccharide that is frequently detected as part of LPS.

The O-antigen and its biosynthesis requires four genes—*rfbABCD*. In general, the genes involved in LPS biosynthesis have been reported to be upregulated during early stages of colonization (Shidore et al. 2012; Camilios-Neto et al. 2014). The mutation of one of the genes (in the mutation of either *rfbB* or *rfbC*) related to rhamnose biosynthesis resulted in a 100-fold lower level of attachment to the root surface and a lower endophytic colonization of maize (*Z. mays*) by *H. seropedicae* SmR1 (Balsanelli et al. 2010) (Table 20.2).

The membrane protein in bacteria is also critical for the successful plant and endophyte interactions. Of these, the muropeptide permease is necessary for peptidoglycan recycle of bacterial cell wall (Pinski et al. 2019). The sensitivity to SDS and alterations in the LPS biosynthesis increased when the gene of this permease in

H. seropedicae SmR1was inactivated. Even if there was no structural change in LPS as in the wild-type strain, LPS quantity reduced leading into a ten-fold reduction in the endophytic colonization of maize (Tadra-Sfeir et al. 2011).

A proteomic analysis of *G. diazotrophicus* PAL5 in response to plantlets indicated a higher amount of the outer membrane lipoprotein (Omp16) (Dos Santos et al. 2010). The OprF membrane protein is a trait for pseudomonads and was also reported to play a role in the attachment to the root surface. This protein is a major porin and facilitates movement of polar solutes across the outer envelope and is a part of membrane integrity as it maintains stability of membrane as integral structural protein.

The inactivation of the *oprF* gene of *Pseudomonas fluorescens* CHA0 resulted in a significant decrease in its attachment to cucumber and tomato roots (Crespo and Valverde 2009). Plant cell walls are composed of cellulose and pectin, and could be degraded by some bacterial endophytes (Reinhold-Hurek et al. 2006). For example, rice root colonization by an *Azoarcus* sp. BH72 mutant significantly decreased and was unable to spread to the aboveground parts of the plant when the microbes endoglucanase activity that degrades cell wall was lost. Furthermore, the endoglucanase was greatly expressed in the time when the bacterial cell contacted with the rice roots (Reinhold-Hurek et al. 2006). Also, in *Bacillus mycoides* EC18, the genes encoding hydrolases, pullulanase, and a chitin-binding protein are upregulated when exposed to root exudates.

Plants are well known for producing expansin compounds that play a role in cell enlargement and other developmental events requiring cell wall loosening (Sampedro and Cosgrove 2005). The expansins seem limited to bacteria from *Bacillus*, *Xanthomonas*, *Xylella*, *Ralstonia,* and *Erwinia* genera (Kerff et al. 2008). Expansins can facilitate the loosening of the cell wall components during division which could also be features of endophytic interactions (Irizarry and White 2018). In their study, upregulation of expansin gene expression was observed in the cotton seedling roots following inoculation with *Bacillus amyloliquefaciens* (Irizarry and White 2018). However, in rare cases, endophytic bacterial genomes, containing genes for expansins, could facilitate cell wall extension by the plants (creep) without any actual breakdown or covalent modification of the cell wall polymers. Thus, it could be well understood that an expansin deficient mutant of *Bacillus subtilis* 168 did not colonize roots of maize efficiently, although the extension activity expressed by the parental wild-type strain was weak but existent when assessed in vitro (Kerff et al. 2008).

Despite all these results that demonstrate that cell wall-degrading enzymes and bacterial expansins are involved in attachment, cell wall-degrading enzymes and expansins are not prerequisite for most successful colonizations. This lack of enzyme requirement may relate to the fact that many endophytes penetrate through wounds and natural openings such as the stomata, particularly on the leaves and young stems (Wallace and May 2018). In addition, genes for plant cell wall-degrading enzymes have not been discovered in most of the genomes of endophytes (Ali et al. 2014; Martin-Moldes et al. 2015).

In addition to serving as chemotactic attractants for bacteria, the root exudates of plants also facilitate microbial attachment and internal colonization of the plant roots (Pinski et al. 2019). The secretion systems of endophytes are thus pivotal for successful colonization by endophytes (Pinski et al. 2019). Bacteria must have adequate transporters and enzymes for root exudates that primarily consist of sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenolics, plant growth regulators, secondary metabolites, proteins, and enzymes (Badri et al. 2009). In general, plant root secretion systems are also considered to have a role in bacteria successfully avoiding elimination by the plant defense system (Pinski et al. 2019). Oxalate is one of the root exudate compounds found in maize and lupin. Bacteria can utilize oxalate through the activity of oxalate decarboxylase. The gene *oxc* encode for oxalate decarboxylase in *Burkholderia phytofirmans* PsJN. The inactivation of *oxc* in *Burkholderia phytofirmans* PsJN showed reduction in the early colonization of maize and lupin, even if the effect was less significant in maize. Kost et al. (2014) suggested that this might be due to the fact that maize roots produce less oxalate than lupin (five-fold less per g of root fresh weight at three days after inoculation).

There are eight different types of secretion systems used for Gram-negative bacteria respectively designated as type I secretion system to type VII secretion system, plus Sec and Tat. For gram-positive bacteria, there are six types (Sec, Tat, SecA2, Sortase, Injectosome, and type VIII secretion system) (Liu et al. 2017). A type III secretion system (T3SS) in Gram-negative bacteria transports effector proteins across the inner cell membrane, through the periplasmic space and the outer membrane of the bacteria into the cytoplasm of the plant host cells (Table 20.2). The translocated effector proteins could modulate the hosts metabolism and the defense system response (Pinski et al. 2019). M1 mutants of *H. rubrisubalbicans* have impaired T3SS and have been found not to achieve successful endophytic colonization (Baetz and Martinoia 2014). Interestingly, endophytic colonization may also be delimited by secretion systems as suggested by the discovery that the T6SS mutant of *Azoarcus* sp. BH72 had even a higher colonization capacity than the wild-type strain did (Shidore et al. 2012). The quorum sensing molecule DSF (diffusible signal factor) in the well-studied endophytic *S. maltophilia* R551–3 regulates chemotaxis, cell motility, biofilm formation, and multidrug efflux pumps. A mutant lacking of DSF production could not form well-organized cell aggregates properly, and also did not colonize efficiently resulting in the bacteria not promoting host plant growth (Alavi et al. 2013). Similarly, in a mutant of *P. fluorescens* 2P24 that could not produce a quorum-sensing molecule of acyl-homoserine-lactone, colonizing efficiency of the rhizosphere was significantly reduced (Wei and Zhang 2006).

Other more general transcriptional regulators are also involved in plant-endophytic bacteria interactions. For example, a deficiency mutation of *rpoS*, which is a general stress response regulator and stationary-phase sigma factor, resulted in decrease in the root attachment of *Salmonella enterica* (Barak et al. 2005) (Table 20.2).

In the study of Arsene et al. (1994), the root surface of wheat was predominantly colonized with *Azospirillum brasilense* Sp7 whereas a spontaneous mutant Sp7-S generated from Sp7 was hardly found to be attached to the root surface and did not bind Congo red (Katupitiya et al. 1995a, b). In enriched media, there was little difference between a spontaneous mutant Sp7-S and its parental strain Sp7 (Pereg-Gerk et al. 1997). The only difference between two strains was that the Sp7-S did not show flocculation and swarming in specialized growth media. The spontaneous mutation occurred in a regulatory gene for flocculation (flcA) (Levy et al. 2018). As a result, production of exopolysaccharide significantly reduced in the mutant Sp7-S (flcA-) and surface colonization reduced. However, it could colonize between cortical cells and in crevices where lateral roots emerge and para-nodules, formed responding to the amendment of 2,4-D (Deaker and Kennedy 2001). The colonization of the mutant Sp7-S (flcA-) in this way shows higher rates of nitrogenase activity than is appeared by Sp7. In the study explained here, a laboratory model of para-nodulation in wheat was applied as several researchers had conducted (Tchan and Kennedy 1989; Katupitiya et al. 1995a, b; Sriskandarajah et al. 1993).

The application of synthetic auxin has been shown to arrest normal lateral root development (Sriskandarajah et al. 1993) and provides numerous modified lateral root structures from a greater number of primordia, in turn enabling a more extensive colonization by the Sp7-S strain of *A. brasilense* (Deaker and Kennedy 2001). Deaker and Kennedy (2001) confirmed that by using nifH-lacZ fusions, *A. brasilense* Sp7-S, a mutant capable of more endophytic colonization of wheat roots than the wild type Sp7, fixed more N_2 than the wild type Sp7 strain. In their study, the nifH nitrogenase gene was strongly expressed in the wheat rhizosphere of para-nodulated wheat inoculated with *A. brasilense* and grown in different hydroponic systems, and apparently this effect was attributed to the bacteria having improved access to carbon compounds and a more favorable microaerobic concentration.

20.3.2 Genetics of Plants on Interactions with Endophytic Bacteria

Not only the genetic elements of endophytes, but also those of plants, are important and interact for successful achievement of colonization and endosymbiosis. Plants have various receptors and symbiosis can involve changes in plant hormone signalling pathways. In addition, small RNAs (sRNAs) could also be involved in symbiosis (Carvalho et al. 2016; Thiebaut et al. 2014).

Plant receptors play a role in recognizing bacterial pathogen signals, and that is mainly mediated by the family of receptor-like kinases (RLK), which include a leucine-rich repeat LRR-RLKs), wall-associated kinases (WAK), lectin receptor-like kinases (LecRLKs), and Lys-motif receptors (LysM). However, only a few studies

have reported their comparative relevance in identifying beneficial endophytes (Carvalho et al. 2016).

Plant hormone-signalling pathways are critical in plant defense, and they are well understood to be also involved in interactions with endophytic bacteria (Lebeis et al. 2015). Studies have reported the functions of ET (ethylene), SA (Salicylic acid), and JA (Jasmonic acid) not only in regulation of endophytic bacterial colonization but also the diversity of endophytic bacterial populations. It has been found that a typical endophytic colonization of *Medicago truncatula* by *Klebsiella pneumoniae* 342 led to activation of the ET signalling pathway, although in contrast, an ET-insensitive mutant of *M. truncatula* was hyper-colonized by that endophytic strain (Iniguez et al. 2005). These results were in agreement with a study that found sugarcane colonized by both the diazotrophic endophyte of *Gluconacetobacter diazotrophicus* PAL5 and *Herbaspirillum rubrisubalbicans* HCC103 showed an increased expression of a putative ET receptor (SCER1) at 24 h as well as seven days after the inoculation.

The Small RNAs (sRNAs) also affect plant growth and development as post-transcriptional regulators of gene expression (Pinski et al. 2019). They additionally influence plant responses to abiotic stresses and phytopathogens (Carvalho et al. 2016). Recent studies have also indicated the involvement of sRNAs during interactions of plants with endophytic bacteria. For example, when *T. aestivum* was inoculated with endophytic *Rhizobium* and *Azorhizobium caulinodans* ORS571, an altered miRNA (microRNA) expression was observed. The peak response appeared at 12–24 h after inoculation and the responses were different in the roots and shoots. The roots seemed more sensitive to the inoculation than the shoots, possibly because this strain colonizes roots (Qiu et al. 2017). Thiebaut et al. (2014) reported the response of maize sRNAs to inoculation with the diazotrophic *H. seropedicae* and *A. brasilense* which indicated that there to have been upregulation of the copper-miRNAs (Cu-miRNAs) coupled with an inhibition of their targets. The names for these copper miRNAs originated from the fact that they target the genes for proteins with a Cu cofactor such as laccases, superoxide dismutases, and cupredoxins. These enzymes are known to be involved in rapidly generating an oxidative burst and response signaling during pathogen attack (Thiebaut et al. 2014; Pilon 2017). In the study of Thiebaut et al. (2014), Cu-miRNAs' target genes were downregulated, suggesting that both diazotropic strains suppress the early plant defense response (Thiebaut et al. 2014).

The endophytic colonization process is also influenced by the up or downregulation of genes related to cell wall modifications (Pinski et al. 2019). These genes encode for hydroxyproline-rich glycoproteins (HRGPs), expansins, and pectinesterases. The HRGPs implicated in biological functions are usually grouped into three complex multigene families, i.e., (i) arabinogalactan proteins (AGPs), (ii) extensins, and (iii) proline-rich proteins (Johnson et al. 2017).

The extensins of plants are well-studied in relation to their interactions with pathogenic and beneficial bacteria. Extensins play an important role in plant defense by strengthening the cell wall following a phytopathogen inoculation (Pinski et al. 2019). Interestingly, there have been studies which found that extensins also increase in the nodules colonized by symbiotic *R. leguminosarum*. Extensins form part of the

root mucilage along with arabinogalactan-proteins, pectic polysaccharides, secondary metabolites, antimicrobial compounds, and extracellular DNA, and play a key role on root defense through the formation of a root extracellular trap. The expansins are also involved in the development of the plant, loosening the cell wall constituents during division.

Pectinesterases catalyse the de-esterification of polygalacturonans, which might also be characteristics of endophytic interactions (Irizarry and White 2018). This enzyme was associated with the lateral root development in cucumber seedlings (Zhang et al. 2014). In the study of Xie et al. (2012), the gene of predicted enzyme pectate lyase was upregulated. Pectate lyase degrades pectin and has been previously reported to contribute to the nodule-formation of rhizobacteria to colonize roots during early stages of symbiosis (Xie et al. 2012). Xie et al. (2012) described that a legume pectate lyase (LjNPL) was essential for normal initiation of infection of *Mesorhizobium loti* in the plant *L. japonicus.*

The increase of lignin in cell walls is a key reaction to plant pathogens and is a barrier against the spread of bacterial cells (Liu et al. 2018; Pinski et al. 2019). Also, greater lignification decreases the infiltration of fungal enzymes and toxins through plant cell walls (Liu et al. 2018). Considering these protective responses associated with lignification, it is perhaps unexpected that endophytic colonization would trigger an accumulation of lignin, i.e., a higher lignin content was observed in the roots of cotton following a *B. amyloliquefaciens* pb1 challenge (Irizarry and White 2018). An increase in the expression of cell wall bound peroxidase that is coinvolved in lignification of cell walls was found in *A. thaliana* in response to an endophytic colonization by *P. putida* BP25 (Sheoran et al. 2016). An increased expression of cinnamyl alcohol dehydrogenase (CAD), which is known to be involved in lignin biosynthesis, was reported in *M. sinensis* followed by an endophytic colonization by *H. frisingense* GSF30T (Straub et al. 2013). However, the expression of CAD was downregulated in wheat inoculated with *A. brasilense* FP2 (Camilios-Neto et al. 2014). The patterns and types of lignin produced with inoculation of endophytes seem distinctive (Pinski et al. 2019). An analysis of the profile for miRNAs expression of maize challenged with diazotrophic bacteria indicates decrease in lignin biosynthesis, because miR408 was induced followed by the downregulation of its targets, which are laccases (Thiebaut et al. 2014). Therefore, miRNA expression seems likely that these endophytes affect lignification in the host (Pinski et al. 2019).

20.3.3 Diversity of Diazotrophs

20.3.3.1 *Azotobacter*

Azotobacter species (perhaps most notably *Azotobacter vinelandii* and *A. chroococcum*) are free-living bacteria appearing round and oval with flagella (Yamagata and Itano 1923). *Azotobacter* are aerobic heterotrophic organisms that act as diazotrophs under either aerobic or microaerobic conditions, depending on an

adequate supply of reduced C compounds such as sugars for energy (Kennedy et al. 2004). When wheat was inoculated with *Azotobacter* under aseptic (gnotobiotic) conditions in the greenhouse, 50% customary requirement for urea-N was replaced (Soliman et al., 1995; Hegazi et al. 1998). Kennedy et al. (1998) reported that strains of this bacterial genus was epiphytes of wheat rhizoplane but not the endophytic root invaders. It was reported that cotton yield could increase by 15–28% following inoculation with *Azotobacter* (Iruthayaraj 1981), possibly due to biological nitrogen fixation (BNF), production of antibacterial and antifungal compounds, growth regulators, and siderophores (Pandey and Kumar 1989). Also, *Azotobacter* has been reported to produce plant growth hormones, improved nutrient uptake, and display antagonistic effects against plant pathogens (Parmar and Dadarwal 1997).

20.3.3.2 *Azospirillum*

Azospirillum is a Gram-negative, microaerophilic heterotroph, non-fermentative, and nitrogen-fixing bacterial genus from the family of Rhodospirillaceae (Roper and Ladha 1995), and it is often associated with roots of cereals and grasses (Grifoni et al. 1995).

Azospirillum grow robustly in the rhizosphere of gramineous plants such as rice and wheats (Kennedy and Tchan 1992; Ladha et al. 1982; James et al. 2000). They can also penetrate the root to grow endophytically in intercellular crevices (Sumner 1990), although they are usually considered as epiphytes growing close to or on root surfaces (Kennedy et al. 2004). Both *Azospirillum lipoferum* and *Azospirillum brasilense* have been isolated from roots and stems of rice plants (Ladha et al. 1982; James et al. 2000) while *Azospirillum amazonese* has been isolated from the roots of wheat (Pereira et al. 1988).

In addition to biological nitrogen fixation (BNF) capability, *Azospirillum* also produce plant growth factors (auxins) that cause the plant to produce more roots (Khan et al. 2010). *Azospirillum* was also associated with the rhizosphere of ginger followed by the treatment with that bacterium (Govindan et al. 2009). They also reported the stimulation of root growth for inoculated as well as uninoculated but with a great difference as evident from the fact that the mean root length for inoculated plants was 16.9 cm while the corresponding figures for non-inoculated plants was only 2.1 cm (Govindan et al. 2009).

Azospirillum diazotrophs could not induce nodulation in their associated plants unlike Rhizobium-like symbiotic associations. However, BNF can occur inside epidermal tissue (apoplast) as endophytes or in the rhizosphere as free-living bacteria (Tejera et al. 2004). There have been many reports that tropical soils of Brazil often contain approximately 10^4 to 10^6 cells of *Azospirillum* per g of soil. Yield of wheat inoculated with two *A. brasilense* strains 245 and 107 that were isolated from surface sterilized wheat roots resulted in repeatable yield increases (Baldani et al. 1983; Baldani et al. 1987 and Boddey et al. 1986). The favorable effect of strains Sp 245 and Sp 107 on wheat, in contrast to a lesser reaction achieved by strain Sp 7, could be due to the more successful establishment of the Sp 245 and Sp

107 inoculants in the internal parts of wheat roots (Baldani et al. 1986). Their results (Baldani et al. 1986 and 1987) suggest that the *Azospirillum* could penetrate to the inside of epidermal tissues, although it also could reside as free-living bacteria in the soil and rhizosphere of many crops. Baldani et al. (1986, 1987) suggested that the *Azospirillum* could colonize inside epidermal tissues although these bacteria could exist as free-living bacteria in the soil and rhizosphere of many crops. Obligate endophytes such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. could be a very promising group for nitrogen fixation in sugarcane compared to *Azospirillum*. *Azospirillum* isolates can colonize perhaps endophytically with sugarcane (Tejera et al. 2004). However, obligate endophytes of *G. diazotrophicus* were not detected in the apoplastic fluid of the stems (Tejera et al. 2004).

Field experimental results in India have shown that application of *A. diazotrophicus* by inoculating setts (cuttings) increased sugarcane yield for four varieties significantly when the microbe was applied in association with vesicular arbuscular mycorrhiza (Muthukumarasamy et al. 1999a, b). Those authors claimed that this practice completely substituted for the recommended dose of 275 kg urea-N ha^{-1}. This is an ambitious claim, but the result could involve a PGPR effect.

20.3.3.3 *Acetobacter*

Acetobacter as gram negative produce carbon dioxide and water from lactate and acetate called acetic acid bacteria (Cleenwerck et al. 2002). Of this genus, *Gluconacetobacter diazotrophicus* has a rod-like shape and circular ends (Eskin et al. 2014) and is well known obligate endophyte which grows best on sucrose-rich medium (James et al. 1994) such as sugarcane sap. This bacterium also produces much IAA, and this is related to its endosymbiosis. They do not survive in the surface of root of sugarcane. Boddey et al. (1991) conducted a ^{15}N dilution/N balance study, confirming that up to 60–80% of sugarcane plant N (equivalent to over 200 kg N ha^{-1} $yr.^{-1}$) was derived from BNF, and *A. diazotrophicus* apparently played a role for much of this BNF (Boddey et al. 1991). Many scientists now apply Acetobacter-sugarcane system as an effective experimental model for BNF study.

Treating seedlings with this bacterium results in greater growth rates while *nif*$^-$ mutants of this genus are significantly less effective in increasing plant growth, indicating that the diazotrophic character (*nif*$^+$) is important for this system (Lee et al. 2002).

A field study of co-inoculation of *A. diazotrophicus* with vesicular arbuscular mycorrhiza has shown increased sugarcane yield for four varieties of sugarcane (Muthukumarasamy et al. 1999a, b). Those researchers insisted that this practice should completely substituted for the recommended dose of 275 kg urea-N ha^{-1} (Muthukumarasamy et al. 1999a, b).

20.3.3.4 *Azorhizobium*

Azorhizobium is gram-negative and fix nitrogen in symbiosis with plants of *Sesbania rostrata* of Leguminosae (Dreyfus et al. 1988). *A. caulinodans* ORS571 has been fully sequenced (Lee et al. 2008). The species of rhizobia responsible for nitrogen fixation in *Sesbania rostrata* is *Azorhizobium caulinodans*. Matthews et al. (2001) reported that *A. caulinodans* could increase the dry weight of wheat in a greenhouse experiment. In a following experiment, they used a N-fixing (nif^+) and non-N-fixing (nif^-) strains of *A. caulinodans*. However, both strains promoted wheat growth compared with the non-inoculated plants. This result indicates that the beneficial effect of inoculating wheat plants with *A. caulinodans* was attributed to plant growth promotion (PGP) substance production rather than by BNF. Also, Saleh et al. (2001) said that its inoculation could save up to 50% of the recommended rate of urea-N in greenhouse trials under gnotobiotic (or sterile) conditions.

20.3.3.5 *Azoarcus*

The endophytic gram negative *Azoarcus* sp. BH72 was originally isolated from Kallar grass (*Diplachne fusca*) growing in the typical saline-sodic soils in Pakistan (Reinhold-Hurek et al. 1993). *Azoarcus* spp. also colonize grasses, such as rice, in both the laboratory and the field (Hurek et al. 1994). In rice roots, the zone behind the meristem was most intensively colonized. The rhizoplane in the zones of elongation and differentiation is the preferential entry sites of the bacteria followed by the colonization of the root interior both inter- and intracellularly. These bacteria were found not only in the root cortex but also in the xylem. However, it is not known yet that *Azoarcus* resided intracellularly in living plant cells. The influence of inoculation with *Azoarcus* sp. BH72 in aseptic systems on rice roots was cultivar-dependent (Reinhold-Hurek et al. 2002).

20.3.3.6 *Clostridium*

Clostridium is a strictly anaerobic endospore-forming gram-positive that produces butyric acid (Seki et al. 2003). *Clostridium* can be commonly found in soil. This species was first reported for its contribution to the growth of winter wheat by providing extra soil nitrogen in West Australian soils of light structure (Parker 1953). *Clostridia* are obligately anaerobic heterotrophs only capable of fixing N_2 in the absence of oxygen (Saralov and Babanazarov 1983; Kennedy and Tchan 1992). *Clostridia* can usually be isolated from rice soils (Khamas et al. 1994; Elbadry et al. 1999) and their activity has been observed to increase after returning straw to rice fields, to raise the C to N ratio (Kennedy et al. 2004). Inoculation with clostridia can increase rice yields significantly in favorable conditions when C containing compounds are incorporated to the soil (Mishustin et al. 1983).

20.3.3.7 Enterobacteriaceae

Several genera of the enterobacteriaceae identified from soil include diazotrophs, particularly those from the rhizosphere of rice (Kennedy et al. 2004). It has been speculated that the long agricultural use of organic matters such as manure fertilizer for crops might have developed an enteric flora adapted to an animal-soil-plant rhizosphere nutritional cycle (Kennedy et al. 2004). These enteric genera contain some examples of diazotrophs with PGP activity including *Klebsiella*, *Enterobacter*, *Citrobacter,* and probably several others not yet identified. *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Citrobacter freundii* may pose concern for human safety, or at least a need to show that no risk is present, when inoculant bacteria even remotely related to others negatively associated with human health that are produced in large quantities (Kennedy et al. 2004). This fact was recently noted for *C. freundii* (Nguyen et al. 2003), although the level of risk is probably quite slight.

20.3.3.8 *Burkholderia* and *Pseudomonas*

These genera may also pose concern for human safety (Kennedy et al. 2004). A concern exists on the use of *Burkholderia vietnamensis* in biofertilizer products because its relative *Burkholderia cepacia* has been linked to cystic fibrosis. We must consider the safety issues to develop biofertilizer of these bacteria, although these bacteria commonly exist in most soil, and crop rhizospheres does not seem to have a significant safety risk in relation to human health. The typical species of these bacteria are *Pseudomonas putida* and *P. fluorescens* that are plant-associated bacteria.

20.3.3.9 *Herbaspirillum*

These bacteria are gram negative, in general vibrioid, and sometimes helical with one to three polar flagella. The *Herbaspirillum* are isolated from herbaceous seed-bearing plants. Notably, *Herbaspirillum* colonize the roots of sugarcane, rice, maize, sorghum, and other cereals as an endophyte (James et al. 2000). BNF with this bacterium could provide 31–54% of total nitrogen of rice plants (30-d-old rice seedlings) from the atmosphere (Baldani et al. 2000).

Herbaspirillum could fix nitrogen at 48–63 mg of dry weight per tube, and 95–107 mg of total nitrogen in rice plant per tube containing 12.5% atoms of ^{15}N in a form of $(NH_4)_2SO_4$ (Baldani et al. 2000 and Reis et al. 2000). Rice yield increased significantly when inoculated with *Herbaspirillum* in a greenhouse experiment ($p = 0.05$) up to 7.5 g per plant (Mirza et al. 2000). Shoot and root length, 1,000-grain weight and grain yield of rice can also increase when inoculated with *Herbaspirillum seropedicae* in field conditions (Arangarasan et al. 1998). Seed

germination of rice can be enhanced significantly when inoculated with *Herbaspirillum* (Pereira et al. 1988).

20.3.3.10 *Rhizobium*

Rice roots can be colonized endophytically with *Rhizobium leguminosarum* bv. *trifolii* in fields where rice crop is rotated with Egyptian berseem clover (*Trifolium alexandrinum*), substituting 25–33% of the amount of N-fertilizer recommended for rice in field (Yanni et al. 1997). Field trials showed that rice yield increased with the inoculation of this bacterium by 3.8 t ha^{-1} (Yanni et al. 2001). This bacterium is also able to colonize endophytically inside rice roots grown under gnotobiotic conditions. Grain yield and agronomic N-fertilizer efficiency was significant including shoot and root growth promotion. However, the population size was not higher showing the low numbers of the order of 10^5 cells (colony-forming units) per gram dry weight, too low for significant BNF.

Laboratory and greenhouse studies conducted at the International Rice Research Institute (IRRI) showed that when rice is inoculated with this strain the expression levels of both the growth and yield reporter genes (e.g. lacZ or gusA) connected to rhizosphere associations improve. Other diazotrophs including *Azospirillum*, *Herbaspirillum,* and *Burkholderia* are closely associated with the rhizosphere of rice plants (Baldani et al. 2000; Balandreau 2002; Malik et al. 2002). All these diazotrophs can provide urea-N by BNF as supplementary amount to the crops but only in the right condition for expression of N_2-fixing activity and subsequent transfer of N to plants (Kennedy et al. 2004). Watanabe et al. (1987), and Roger and Ladha (1992) concluded that diazotrophs can supplement 20–25% of the total N needs of rice, considering all the results of many works conducted at IRRI at Los Baños in the Philippines.

20.3.3.11 *Bacillus*

Bacillus subtilis is a gram-positive bacterium, rod-shaped, and catalase-positive, and belongs to phylum Firmicutes, class Bacilli, order Bacillales, Family Bacillaceae (Turnbull, 1996). The *B. subtilis* strain CB-R05 was isolated and studied from the rice plants, and is a diazotrophic endophytic plant growth-promoting bacterium (Ji et al. 2014a, b). In their studies, a *gfp*-tagged *B. subtilis* CB-R05 was transformed by electroporation, and then rice seedlings were inoculated with the *gfp*-tagged CB-R05 bacteria. Localization and colonization of the *gfp*-tagged CB-R05 bacteria was confirmed on the tips and xylems of rice roots in a hydroponic culture and soil culture system using confocal laser scanning microscopy (CLSM) until the 16 days after inoculation during experiments (Ji et al. 2014b).

20.3.3.12 *Anabena*

Anabena belonging to cyanobacteria also called blue green algae that fix atmospheric nitrogen are well known for their symbiotic relationship with *Azolla*, which is a heterosporous aquatic fern (Ladha and Watanabe 1984). The symbiont *Anabena azollae* habits in specialized cavities formed in the dorsal leaf lobes and the apex of the stem of the fern (Ladha and Watanabe 1984). Also, many other cyanobacteria are reported to cohabit with rice, proliferating as planktonic cells on the soil-water surface. The inoculation studies of *Anabena* to rice had been also conducted to exploit nitrogen supplement in paddy fields, specifically in several countries of South East Asia (Prasanna et al. 2009). There are two ways to input nitrogen supply based microbes in wetland rice cultivation. The first one includes indigenous organisms of heterotrophic aerobic, microaerophilic and anaerobic bacteria in soil that associated with rice, photosynthetic bacteria, and cyanobacteria (Bray 1986). The other one uses amending green manures of fern *Azolla* and legumes. Traditional cultivation of wetland rice has been highly sustainable because biological N_2 fixation has allowed a moderate but stable rice yield in practice to be maintained for years without amending N fertilizer (Bray 1986). It is well known that the exchange of nitrogen from the cyano-symbiont for carbohydrates of the host plant had been attractive for scientists to have intensive studies mainly because of the potential for *AzoIla* and *Anabaena* to be in practice as organic fertilizer in wetland rice soils, and even as nitrogenous food sources for various animals (Van Hove et al. 1987; Van Hove 1989; Watanabe et al. 1989). However, the molecular studies on the specific genes associated for interaction of *Anabena* and *Azolla* seems not to be much conducted. However, update advances in molecular genetic techniques would allow us to understand genetic relationship of *Anabena* and *Azolla* which are symbiotically competent in near future.

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Chapter 21
The Hologenome Hypothesis and Its Application to Plant-Microbe Interactions on an Evolutionary Scale

S. Kouas, N. Khan, and A. M. Hirsch

Abstract When roots are pulled from the ground, one cannot ignore the soil particles with their rhizosphere microbes still attached to the root surface and root hairs. The rhizosphere is home to a vast variety of microorganisms that associate with plants and either positively or negatively influences plant growth, while the rhizosheath is defined as the weight of soil strongly attached to the plant root surfaces. Root hairs, epidermal extensions that increase surface area, are a critical component of rhizosheaths. Within the root and in continuation with the aerial portion of the plant is the endosphere, providing both intra- and extracellular locations for microbial habitation. Endophytic microbes often enter plant roots through cracks in the epidermis or in areas where lateral roots emerge, or through stomata, openings for gas exchange, in the aerial parts of the plant. Microbial pathogens as well as symbionts and commensals enter into roots, stems, or leaves employing these "doorways" to enter plant tissues—sources of nutrients and carbon for microbes.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_21

21.1 Roots and Their Microbiomes

When roots are pulled from the ground, one cannot ignore the soil particles with their rhizosphere microbes still attached to the root surface (the rhizoplane) and root hairs (Fig. 21.1a, b). The rhizosphere is home to a vast variety of microorganisms that associate with plants and either positively or negatively influences plant growth, while the rhizosheath is defined as the weight of soil strongly attached to the plant root surfaces (Brown et al. 2017). Root hairs, epidermal extensions that increase surface area, are a critical component of rhizosheaths. Within the root and in continuation with the aerial portion of the plant is the endosphere, providing both intra- and extracellular locations for microbial habitation. Endophytic microbes often enter plant roots through cracks in the epidermis or in areas where lateral roots emerge, or through stomata, openings for gas exchange, in the aerial parts of the plant. Microbial pathogens as well as symbionts and commensals enter into roots, stems, or leaves employing these "doorways" to enter plant tissues—sources of nutrients and carbon for microbes.

Plant microbiomes are sometimes compared to human gut microbiomes (Note: The gut-like appearance of the root in Fig. 21.2 is solely an artist's interpretation of the similarities between the two). Indeed, knowledge of human microbiomes has had a positive influence on studies of plant-associated microbes in various plant tissues and organs. The ecologist, D.H. Janzen, is often quoted by biologists who study

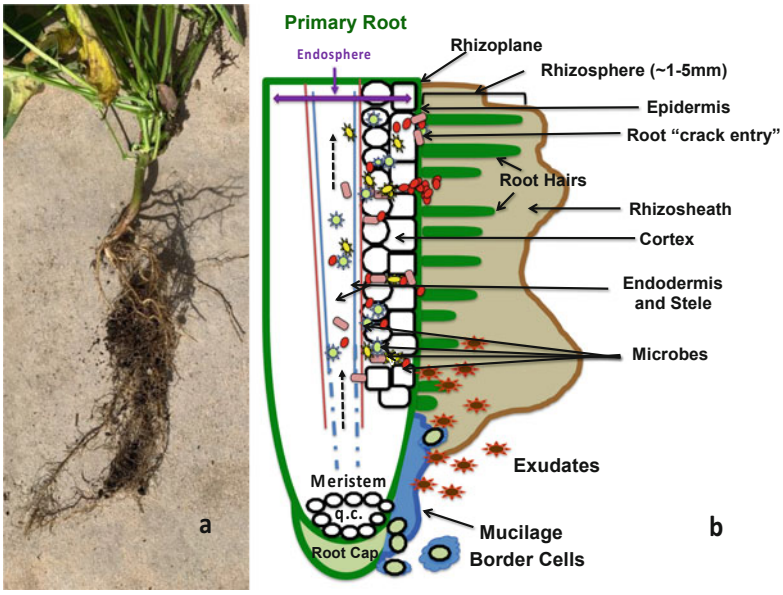

Fig. 21.1 Diagrammatic representation of plant-rhizobacteria interactions (**a**) A a photo of a bean plant root with adhering soil (**b**) Representation of a longitudinal root section showing the root tissues and the avenues of bacterial entry into roots

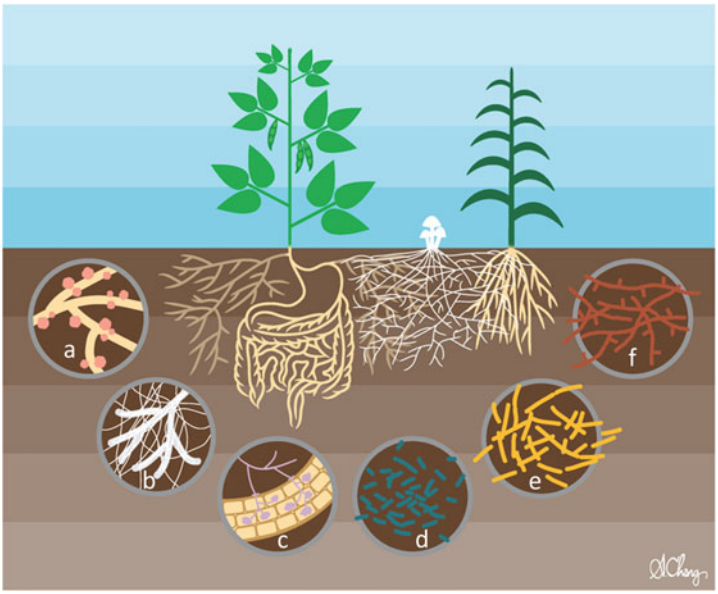

Fig. 21.2 Legumes (l.) and non-legumes (r.) interact with microbes. Legumes establish N_2 fixing nodules with rhizobia (**a**) while non-legume nodules are populated by *Frankia* (actinomycetes) (**f**). Both interact with ecto-mycorrhizae and may develop mushrooms external to the plant (**b**) or (**c**) arbuscular mycorrhizal (internalized) fungi). Gram-negative (**d**) and Gram-positive bacteria (**e**, **f**) promote plant growth Drawing: Alan Chong, UCLA

plant microbiomes. Janzen (1985) in "The Biology of Mutualism" wrote: "Plants wear their guts on the outside." Like the gut, root microbiome bacteria interact with the outside surface of the tissue. In animals, gastrulation results when the blastula folds into itself giving rise to a tube consisting of ectoderm, mesoderm, and endoderm, the ectoderm being analogous to the plant epidermis. However, Janzen's full quote is "Plants wear their guts on the outside but have practically the same kind of diffuse and often obligatory mutualism with bacteria, nematodes, fungi and other litter decomposers." The keyword is "mutualism:" a quid pro quo that takes place between more than one organism involving nutrition, protection from pathogens, and development. This is especially true for roots, which associate with myriad organisms in the soil (Fig. 21.2). Leaves have their own microbiomes, the phyllosphere. Both external and internal microbes inhabit the cuticle-covered leaf made up of photosynthetic tissues. The microbes obtain carbon compounds by inhabiting the endosphere following entry via the stomata of both leaves and young stems. Microbiomes inhabiting the external and internal tissues of seeds make up the spermosphere, while the external microbial environment is known as the anthosphere (Compant et al. 2019). In this chapter, we will describe the microbes diagrammatically depicted in Fig. 21.2: (a) bacteria that convert N_2 to NH_3^+, of which some induce nitrogen-fixing nodules on roots; (b) ectomycorrhizal and (c) arbuscular mycorrhizal (AM) fungi; and (d) plant-growth promoting (PGP)

Gram-negative bacteria, e.g., *Azospirillum* and *Pseudomonas* spp.; (e) Gram-positive PGP bacteria such as *Bacillus* and *Paenibacillus*; and also (f) Gram-positive PGP actinobacteria, e.g., *Micromonospora* and *Streptomyces* spp. Fungi that associate with roots are essential for phosphate (P) nutrition especially in the absence of added fertilizer. The bacteria that support plant growth are commonly known as Plant Growth Promoting Bacteria (PGPB; Bashan and Holguin 1998), and PGPB are found as both free-living or symbiotic organisms and also as endophytes that colonize plant tissues (Glick 2012). Bacteria and fungi together are often called PGPM (Plant Growth Promoting Microbes). This chapter focuses on the bacteria, which are mostly isolated from soil. However, PGPB have been isolated from many plant organs including leaves, flowers, stems, and seeds as well as legume root nodules, which house diverse PGPB in addition to the dominant rhizobia (Martínez-Hidalgo and Hirsch 2017). Mycorrhizal fungi are essential for plant growth promotion as well and were probably one of several critical forces leading to plant life on land.

Bacteria with PGP properties exert their effects on plants both directly and indirectly. Microbial inoculants for phosphate biofertilizers in agriculture include both P-solubilizing bacteria (PSB) and mycorrhizal fungi, both of which promote P nutrition, while N_2-fixing organisms provide fixed nitrogen (ammonia) for plants. These two essential plant nutrients are usually not retained in soil and accumulate in groundwater, resulting in N and P accumulation in waterways and lakes, a common consequence of the overuse of commercial fertilizers. By contrast, microbial inoculants are cost effective, eco-friendly, and renewable sources of plant nutrients. Besides P-solubilization and N_2 fixation, soil bacteria sequester iron via siderophores, produce plant hormones, for example, indole acetic acid (IAA), and cytokinins, and other growth factors. They also suppress pathogenic microorganisms that have deleterious effects on plant growth via induced systemic resistance (ISR) (Glick 2012) and produce various defense compounds. Mycorrhizal fungi perform many of these same functions but are especially known for their effects on P and Fe nutrition.

Nitrogen fixation ability is present in several bacterial and archaeal groups. However, the most studied are the rhizobia, which establish an intimate developmental and physiological relationship with plants to establish nitrogen-fixing nodules. Many soil bacteria and archaea fix N_2 to ammonia, but do not establish novel structures on plant roots. This type of N_2 fixation is termed free-living or associative nitrogen-fixation and is an ancestral strategy. An environmental source of fixed N is extremely important for sustainable agriculture because it ensures that an eco-friendly source of N-fertilizer, which reduces production costs and mitigates the negative environmental impacts of chemical fertilizers (Smercina et al. 2019), is employed. The best-known nitrogen-fixing species that associate with non-legumes belong to many diverse bacterial genera including *Azospirillum*, *Azotobacter*, *Paraburkholderia*, *Herbaspirillum*, and some *Paenibacillus,* with species belonging to *Azotobacter* and *Azospirillum* being some of the most significant (Goswami et al. 2016). Of the archaea, the methanogens such as *Methanococcus* fix atmospheric nitrogen (N_2) to ammonia, while other archaea are involved in the soil N cycle. The

symbiotic nitrogen fixers belong to either the alpha Proteobacteria (*Rhizobium*, *Bradyrhizobium*, *Microvirga*, etc.) or the beta-Proteobacteria (*Paraburkholderia* and *Trinickia,* Estrada-de los Santos et al. 2018) and others.

Bacteria with PGP traits are also known for their abilities to provide plants with nutrients, especially the macro-elements P, K, and iron. N, P, and K are the most limiting elements for plant growth, in part because negatively-charged nutrients such as NO_3^- and PO_4^- are often limiting in soils, which themselves are negatively charged. On the other hand, cations are usually not limiting in soil. Under nutrient deficiency conditions, inoculation with PGPB enhances the plant's acquisition of the "big three" nutrients, NPK. Several bacteria are known to increase the availability of P and K for plant uptake. P-solubilizing bacteria (PSB) mineralize insoluble phosphate, which leads to improved P uptake by plant. As reviewed by Alori et al. (2017) and Kalayu (2019), PSB belong to different bacterial groups including *Pseudomonas*, *Bacillus*, *Paraburkholderia*, *Rhizobium*, *Bradyrhizobium,* and others (Ruzzi and Aroca 2015). The mechanisms of microbial P solubilization rely principally on the release of organic acids that acidify the soil and also facilitate the mineralization of organic P through enzymes such as phytases (Kalayu 2019).

P-solubilizing bacteria and mycorrhizal fungi in association with symbiotic nitrogen fixation are critical for ensuring soil fertility, sustainability, and food security in many parts of the world. However, most research is still limited in terms of screening the best performing strains of bacteria and fungi for agriculture. *Bacillus, Pseudomonas, Rhizobium,* and the fungi *Aspergillus* and *Penicillium*, are the most efficient P solubilizers to increase P bioavailability in soil. In addition, many PS microbes function as biocontrol agents against plant pathogens via the production of antibiotics and antifungal metabolites.

Other soil bacteria solubilize potassium (K), and these are known as potassium-solubilizing bacteria (KSB). The mechanisms of K solubilization include lowering soil pH via proton extrusion and production of organic acids, acidolysis, chelation, and bacterial oxidation-reduction reactions (Uroz et al. 2009; Sattar et al. 2019).

PGPB also influence plant growth via several indirect mechanisms. As reviewed in many studies, the indirect actions include: biocontrol via the production of siderophores, enzymes, and antibiotics to suppress pathogen growth as well as ISR, and the stimulation of stress hormones in plants grown under abiotic stress conditions (Glick 2012; Goswami et al. 2016; Backer et al. 2018; Ferreira et al. 2019).

PGPB are found as free-living organisms in soil or as symbiotic partners associated with a particular plant species (Glick 2012). Bacteria with PGP traits have been isolated from different plant tissues including seeds, roots, stems, and leaves. Many of these endophytes have the potential to be used as PGPB. The combination of beneficial endophytes and soil bacteria has considerable potential for being used worldwide because inoculation with them is considered an environmentally safe way to meet the nutritional demands of plants to achieve high productivity, and at the same time reduce the use of chemical fertilizers.

For legume plants, co-inoculation of rhizobia with PGP microorganism strains is a promising practice that leads to improved plant growth and productivity.

Co-inoculation of soybean with *Bradyrhizobium* and PGPB resulted in an increase in nodule number and shoot dry weight (Zeffa et al. 2020). Co-inoculation of a *Bacillus* strain with *Rhizobium leguminosarum* enhanced plant growth and nodulation of pea and lentil (Mishra et al. 2009; Schwartz et al. 2013). A *Bacillus* strain increased nodulation of *Phaseolus vulgaris* in combination with *Rhizobium tropici* CIAT 899 and this beneficial effect occurred under controlled as well as under field conditions (Camacho et al. 2001). Co-inoculation of chickpea with nitrogen-fixing and phosphorus-solubilizing bacteria improved nodulation, plant growth, and yield (Elkoca et al. 2007), and co-inoculation of PGPR and rhizobia was shown to have a synergistic effect on bean growth (Korir et al. 2017).

21.1.1 What About Potential Pathogens That May Affect Animals or Humans?

The most prevalent microbes in the soil are likely to be those associated with plants because plants are sources of carbon, which is needed for heterotrophic bacteria and fungi. However, depending on the land's use, soil can also be "home" to animal and human pathogens. Land that is used for crop production and pasture for various animals often houses potential pathogens such as *Bacillus anthracis*, *E. coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and many others. However, at the same time, many of these isolates positively affect plant growth. Strategies for determining the potential pathogenicity of microbial isolates can be found in the following reference (Martínez-Hidalgo et al. 2018) and the references therein.

21.2 Symbiosis as an Evolutionary Driving Force

All plant roots have microbiomes, but the larger question is: how did this interaction become established between soil microbes and plant roots at the very beginning of land plant evolution? For an answer, we need to go into the earliest records of the evolution of plants, their roots, and in some cases, their associated microbes, which were all present at least 420 to ca. 360 million years ago (Mya), in the Devonian. Early on, the land masses were clustered together and surrounded by shallow seas. They were not arranged at that time on Earth the way we are accustomed to seeing them now from satellite photos and maps. It was the Age of the Fishes and the beginnings of vascular plant root evolution on land. Evidence of bacterial presence in oceans dates back at least 3.5 billion years ago (Gya), but data remain controversial with respect to land colonization. Support had also come from isotopic signatures dating from 2.6 to 2.7 Gya based on Watanabe et al. (2000). These authors reported that land colonization was likely based on the presence of soil-surface microbial mats, consisting of sheathed cyanobacteria and thin filaments (i.e., lichen-

like) in 2.7 Gya fossilized marine shales. Evidence for an early Silurian (440 Mya) origin, based on a study of a consortium of bacteria and cyanobacteria as the photosynthetic partner in a microbial mat, was also published (Tomescu et al. 2008), while other reports indicate that lichen fossils (with both cyanobacteria and green algae) were present from the Lower Devonian (Honegger et al. 2013a, b). The connections linking the lichen-like fossils of the Watanabe et al. studies and other microbial mat-like consortia have not been made. In any cases, the increased O_2 brought about by cyanobacterial and unicellular green algal photosynthesis in the ancient world most likely led to the quicker formation of an ozone shield, thereby reducing ultraviolet irradiation and accelerating the rate of evolution.

Strong evidence for land plant evolution is based on fossils from the middle Ordovician to the early Silurian. In mid-Ordovician (460 to 455 Mya) shales from Saudi Arabia and Argentina, spores similar to bryophyte (mosses and liverwort) spores (Strother et al. 1996; Rubenstein et al. 2010) were found. A plant similar to *Cooksonia*, a leafless, rootless plant with terminal sporangia, might be a possible candidate. That fungi in the Ordovician became associated with plants was demonstrated by finding fossilized non-septate hyphae and spores of Glomalean-like fungi within bryophyte cells (Redecker et al. 2000). Additional evidence for the evolution of land plants comes from the Rhynie Chert (Scotland) in the fossils of *Algaophyton* (formerly *Rhynia*) *major*. Edwards (1986) described this plant based on a study of fossils that had characters of both bryophytes and tracheophytes (vascular plants). The dichotomously branched vertical axis of the plant was ca. 183 mm tall and rootless, but on one side of the fossilized underground rhizomes, root hair-like structures (rhizoids) were found. The vertical axis was externally characterized by stomata on the epidermis and terminal sporangia, and sections of the axis show what appears to be conducting cells, but no true vascular tissue, leading Edwards (1986) to decide that the plant was a bryophyte. In addition, Edwards noted that cell walls of the outer cortex contained fungal hyphae. Later, Remy et al. (1994) observed that highly branched hyphae (arbuscules) were present in cortical cells, indicating that even the most ancient bryophytes harbored mycorrhizal fungi. The correlation between land colonization and mycorrhization suggests that the AM fungal association may have been the driver for plant survival in environments low in nutrients, especially P. Indeed, Pirozynski and Malloch in their 1975 publication hypothesized that the interaction with fungi enabled the evolution of land plants by opening a completely new environment as early as 460 Mya.

Although early Devonian plants did not have true roots or leaves, true roots are found in fossils from the Middle Devonian (410.8 to 407.6 Mya) (Fig. 21.3) on the ventral surface of horizontal stems of *Bathurstia denticulata* (Zosterphyllopsida) (Kotyk and Basinger 2000). The roots were unbranched, had a central vascular strand, and were several cm long (1.7 mm average in width), but longer roots were wider and reached lengths of 5 cm. Based on extant plants and fossils, it is also highly likely that many microbes in the soil, especially AM fungi, were associated with plants early on. We cannot be certain of the identities of these other microbes, but many cyanobacteria, which in addition to undergoing photosynthesis fix

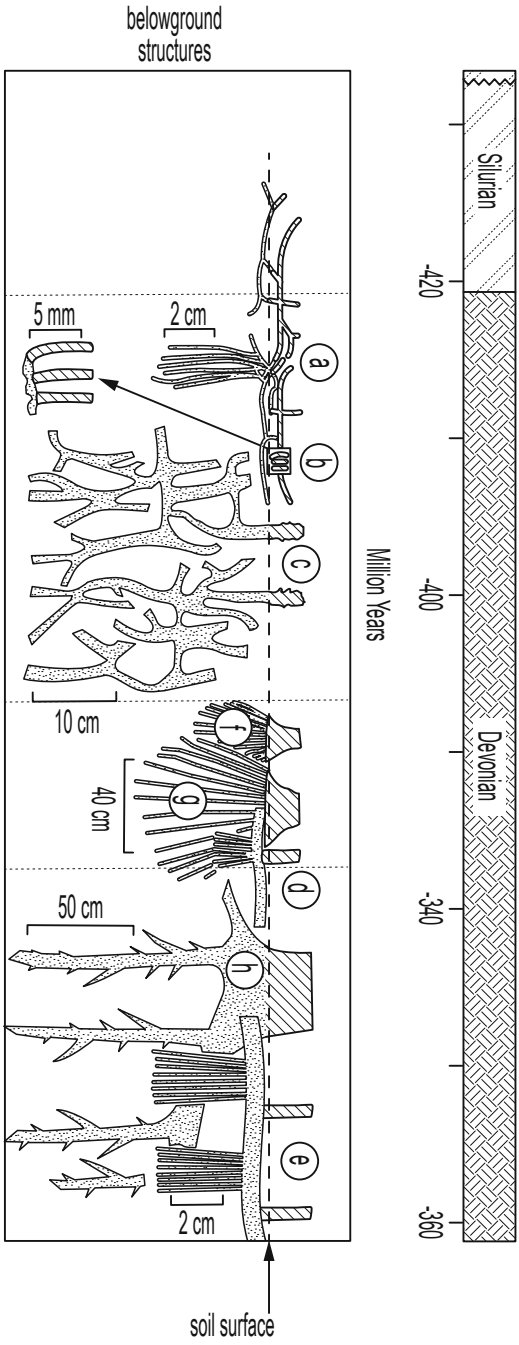

Fig. 21.3 Conceptualized changes in above- (cross-hatched) and below-organ modifications during the Silurian transition to the Devonian Period. (**a**) *Zosterophyllum* (**b**) *Rhynia* (**c**) *Drepanophycus* (**d**) Aneuophytes (fern-like plants) (**e**) Other fern-like plants, some tree-like and deep-rooted (**f**, **g**) (**h**) *Archaeopteris* and related plants with deeply penetrating roots. Modified from Xue et al. (2016)

nitrogen, as well as various heterotrophic bacteria, were likely to be a part of the ancient plant microbiome.

With the development of an ozone shield to keep out damaging ultraviolet irradiation and enable changes in soil structure that are probably correlated with plant colonization and decay, rhizomes grew larger and deeper over time. In *Zosterophyllum* (Fig. 21.3a), the rhizomes were both vertically and horizontally oriented, which is also true of the Rhynie Chert plants, but no obvious root-like structures extend from the horizontal rhizome of *Rhynia* (b). The lycopsid *Drepanophyus* (c) developed deeper (10 cm), vertical and highly branched rhizomes that were longer than those found on the other two fossilized plants in Fig. 21.3. By the Middle-Late Devonian, fossils of various progymnosperms (d), including aneurophytes (fern-like organisms) (e), as well as rooted trees (f, g) have been reconstructed (Fig. 21.3) from fossils (Xue et al. 2016). From the Mid-Devonian onwards, reconstructed fossils of *Archaeopteris* (h) strongly resembled seed-plants in that they produced an elaborate root system and also formed secondary vascular tissue, well-developed leaves, and exhibited endogenous development of lateral roots (Stein et al. 2019). The roots also penetrated more deeply into the soil, at least 50 cm. The net impact of all these developmental changes eventually led to forests that are likely to have resulted in profound changes in climate as well as other environmental factors.

21.3 Microbiome-Mediated Evolution

Plant evolution, like many profound changes in behavior, is influenced by quid pro quo. Photosynthates produced by green plants provide carbon for heterotrophic microbes, which in turn fix nitrogen to ammonia, solubilize rock phosphate to P, etc. to promote plant growth via photosynthesis, which promotes the transfer of carbon compounds to the heterotrophs. The symbiotic microbes also protect plants from pathogen attack and promote their growth by synthesizing various plant hormones. We and others hypothesize that this interaction is a strong co-evolutionary stimulus for the evolution of plant microbiomes and their effects on plant evolution. As plants elaborated leaves, roots, other plant organs, as well as secondary tissues, the plant microbiome also evolved because each plant organ had a specific environment (root vs. leaf) or biochemistry (mineral nutrient/water acquisition vs. photosynthesis) to which the microbes needed to adapt.

The hologenome concept of evolution (HCE), as defined by Zilber-Rosenberg and Rosenberg (2008), explains the outcome of the interaction between plants and microbes over time. HCE is a biological hypothesis that explains the evolution of both plants and animals. Briefly sketched in Rosenberg et al. (2007), the hypothesis was further developed in the 2008 publication (Zilber-Rosenberg and Rosenberg 2008). HCE proposes that biological individuality is due to a multispecies assemblage that results from the symbiotic union between an animal and/or a plant plus its symbiotic microbiota. This union results in a biological individual that the authors

call a "holobiont" (from the Greek, *holos*, all; *biont*, life). To summarize, HCE states that the holobiont is a unit of selection in evolution, such that its hologenome (sum of the genetic material from the animal/plant plus the genetic material of the members of the microbiome) is transmitted from one generation to the next pending no changes in the environment (Suárez Díaz 2019). Thus, the holobiont with its hologenome is the unit of natural selection upon which evolution works, and microbial symbionts have a critical role in the adaptation and evolution of higher organisms. In short, microorganisms are essential not only for the health and maintenance of individual higher organisms, but they are also a significant factor in species survival and genetic variation (Rosenberg et al. 2007). Thus, the holobiont (Margulis 1993; Rohwer et al. 2002) (the host and its symbiotic microbiota) along with its hologenome, acting together, should be considered a unit of selection in evolution. The result is that relatively rapid variation in diverse microbial symbionts plays an important role in the adaptation and evolution of the holobiont (Zilber-Rosenberg and Rosenberg 2008).

Considerable support exists for the hypothesis that holobionts and their hologenomes should be considered levels of selection in evolution (Rosenberg and Zilber-Rosenberg 2018), see also (Suárez Díaz 2019). First, all animals and plants harbor a diverse microbiota, which is supported by abundant data. The second principle is that the holobiont functions as a distinct biological entity, and that interactions between microbiomes and their hosts affect the fitness of their holobionts. However, the extent to which the microbiota contributes to holobiont fitness and survival varies enormously. The third principle, whereby the genomes of both hosts and a significant fraction of their microbiomes are transferred between generations, is the most contentious but evidence exists in the presence of microbes in plant seeds (Nelson 2018) and microbial transmission from mother to child in vaginal birth. The fourth principle, genetic variation in holobionts, occurs when new symbiotic microorganisms are incorporated in the microbiota. Because microorganisms respond more rapidly and can use multiple mechanisms (including horizontal gene transfer) to respond to changing environmental conditions, a completely entire new set of possibilities exists for holobionts to adapt to dramatic environmental shifts, which are not limited to genetic changes in the host's genome. Zilber-Rosenberg and Rosenberg acknowledge that HCE requires not only the existence of interactions between the host and the symbionts, but that these interactions: (1) are reliably transmitted transgenerationally and (2) affect the fitness of the holobiont. If there are host-symbiont interactions that do not exhibit any (or only one) of these two properties, then the holobiont cannot be considered a unit of selection, but a conglomerate of independent units of selection interacting ecologically with each other (Suárez Díaz 2019). In their original paper, Zilber-Rosenberg and Rosenberg (2008) presented evidence to support the claim that the symbionts that comprise a holobiont are intergenerationally transmitted with sufficient fidelity to support the claim that holobionts are units of selection. In their words: "The hologenome theory of evolution relies on ensuring the continuity of partnerships between holobiont generations."

The hologenome hypothesis explains a great deal of how the evolution of plants may have proceeded as the Earth itself evolved. One could ask the same question of any organisms, including lichens, for which the hologenome hypothesis was originally described (Frank 1877). The cooperation of multiple microbiomes to make a complex organism had its roots in earlier times and it also led to major changes in Planet Earth, i.e., not only in the variety of organisms present, but also in the changes in soil, atmosphere, and other climatic factors for making an environment more hospitable to these organisms.

21.4 Engineering Microbiomes

In the last decade, climate related disasters have had a direct impact on factors that influence agroecosystems and compromise the global food security (Cavicchioli et al. 2019). Many studies have supported the use of microbes and their metabolites in improving overall plant growth and also in mitigating plant stress responses (Rodriguez and Durán 2020; de Vries et al. 2020). However, to fully realize the potential of microbial technology in enhancing crop production and improving plant resilience, current research effort is focused on breeding "microbe-optimized plants." Accordingly, microbiome engineering has emerged as an alternative to modify and promote positive interactions between microbes and plants to improve plant fitness. The goal is to pursue modifications of microbial community compositions that result in improved plant phenotypes and benefit ecosystems. Microbial strains with desired functions can be selected and combined to form synthetic microbiomes. For examples, Lebeis et al. (2015) demonstrated that microbiomes can be optimized for disease resistance in plants by applying phytohormones that activate plant immune signaling responses.

Advancements in high-throughput "multi-omics" technologies and computational developments have also led to a greater understanding and manipulation of plant holobionts. Indirect approaches to this research include the use of soil amendments, while direct approaches involve the use of specific microbes, modified microbial consortia, microbiome breeding, and transplantation. Recently, a method used typically in human metagenomic studies, MWAS (metagenome-wide association study) has been proposed by Beilsmith et al. (2019) to find associations between host plant genes and microbial species. MWAS could prove helpful in unravelling functional associations interplaying in plant-microbe interactions. Finally, microbiome engineering is an interesting option to improve and enhance the biological capabilities of the plant using a combination of desired selected strains that prove to be effective, compatible with each other and the soil conditions, and non-pathogenic. As indicated by Martínez-Hidalgo and Hirsch (2017) in their review on plant microbiomes for sustainable agriculture, it is essential to test the long-term persistence and effectiveness of the transfer of microbial technology from lab to field, especially in fluctuating environmental conditions.

21.5 Epilogue

The statement put forth by Pasteur (1885) is worth reviewing in the context of microbes and their hosts: "Life would not long remain possible in the absence of microbes." Gilbert and Neufeld (2014) addressed Pasteur's statement in an exercise to describe the evolutionary changes that would occur after eliminating microbes from the rest of contributors to life on Earth but at the same time retaining mitochondria and chloroplasts, without which life would not exist. They then asked: "Or would it?" The answer is that life might be feasible, but it would be very different than it is now on Earth, but how different is difficult to say since so much of life on Earth as we know it depends on the microbial partners that live in symbiosis with their larger eukaryotic associates. In any case, it is highly unlikely that the diversity seen in life on Earth described herein would ever have come about, much less been maintained if any parameter crucial for hologenome survival had been different.

The processes whereby plants and their microbiomes adapt to their environments, whether past, present, or future, depends on not only on microbes and their plant hosts, but also on external factors such as geology, climate, water, temperature, and serendipity to some extent, several of which are influenced by living organisms. To rephrase L. Pasteur's quote: "Life *as we know it* would not long remain possible in the absence of microbes." Microbes via their interactions with other organisms in an environment that facilitated symbiotic interactions have given us an evolutionarily diverse and for us, livable world. Recognizing how long it took for life to evolve, diversify, and create Earth's complex symbiotic environment may help us preserve it—by alerting us to the fact that maintaining as much of the symbiotic world as we can is not a trivial task, especially in our ever changing world.

Acknowledgements We thank the Fulbright Foundation for supporting S. Kouas' stay in the Hirsch lab, and also acknowledge the long-term grant support from the Shanbrom Foundation to study arid soils and their microbes. Thanks to S.J. Kirchanski for his helpful comments on the manuscript, and we also extend our gratitude to J.W. Schopf, J.F. Basinger, W.E. Stein, T. Algeo, D. Murphy, and J. Shen-Miller for their helpful comments, suggestions of papers, and answers to various questions sent by email. Allan Chong (UCLA) did the artwork for Fig. 21.2. We also thank Christon J. Hurst for his support and helpful comments.

Finally, we appreciate P. G. Gensel and D. Edwards for writing the book "Plant Invade the Land. Evolutionary and Environmental Perspectives", which was an important reference for this chapter.

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Chapter 22
Beneficial Role of Plant Growth-Promoting Rhizobacteria in Bioremediation of Heavy Metal(loid)-Contaminated Agricultural Fields

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Abstract The synergy of plants and microbes is one of the most interesting parts of holobiont research that yet have to be unwrapped before we can understand its implications in agriculture. Environmental stresses on plant ecology have further added to our curiosity in this context. Microorganisms are key players in benefitting plant health. This chapter mainly covers heavy metal and metalloid (HM)-induced phytotoxicity in different crops. We will be describing the role of soil-dwelling plant growth-promoting rhizobacteria (PGPR) in the mitigation of HM-induced damages in plants. We will also consider more generally the influential role of these microbes in biotic stress tolerance and the agricultural adoption of PGPR-involved strategies to combat HMs, which will help us provide adequate food for the world's human population and the animals on which we depend for food, labor and companionship. Our starting point will be PGPR collected directly from the crop rhizosphere and associated with the lessening of HM content in crops, but excluding those intracellular endophytic microbes and those involved in PGPR-assisted phytoremediation. The principal rationale for these research efforts is to reduce the consumer's health risks that are directly associated with the mobilisation or immobilisation of HMs inside plant cells. These microbes are possibly the best candidates for bioremediation because of their resilience and ability to withstand high HM levels, their mediation of the limiting effects that recalcitrant metals exert upon plant's health, our successes of collaboration with the plants and microbes for biocontrol activities and microbial phytostimulation. This elaborative study covers the effect of 10 HMs (viz. Arsenic,

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Cadmium, Chromium, Cobalt, Copper, Lead, Manganese, Mercury, Nickel and Zinc) on crops and the HM-resistant PGPR discovered since 20 years. In addition, a general account of fundamental principles behind bacterial heavy metal resistance has been elaborated. Hence, this chapter will be of great interest especially to environmental microbiologists.

22.1 Introduction

The global food crisis is one of the discernible situations that necessitate substantial attention. Due to high population growth (especially in China and India, the top two populated countries in the world) with a proportionate decrease in cultivable land, this catastrophe is becoming more acute daily. Apart from natural sources, several unplanned anthropogenic activities are known to generate an additional burden that jeopardises the environment and its ecosystem, contaminating its different components including soil and groundwater (Sharma and Archana 2016; Liu and Ma 2020). Heavy metal(loid)s (HMs) are one of the recalcitrant contaminants in agricultural fields that degrade the soil quality affecting the growth and crop yield, causing severe to chronic phytotoxicity. This might be due in part to the selection pressure that HMs impose on the soil-dwelling microbiome involved in phytostimulation and maintaining soil-biogeochemical cycling. However, certain microorganisms with their unequivocal properties combat HMs, developing an array of active or passive resistance mechanisms to survive in such a harsh environment (Chen et al. 2016; Tiwari and Lata 2018; Kotoky et al. 2019). There are successful candidates among them that have been found to colonise the soil area around the rhizosphere and rhizoplane (root surface) in response to enriched soil nutrients including the attractants released as root exudates from host plants. Host root exudates provide nutrients and act as signaling molecules to the colonisers to establish effective plant-microbe interactions. These exudates take the foremost part in controlling the diversity and composition of plant-associated soil microbial communities (Steinauer et al. 2016).

Plant growth-promoting rhizobacteria (PGPR) are group of free-living rhizobacterial communities that competitively colonise around the root surfaces stimulating plant growth by secreting a variety of phytostimulating substances and preventing some causes of host's diseases in a sustainable manner (Kloepper 1978). Rhizobacterial plant growth-promoting (PGP) traits include 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, phosphate solubilisation, indole-3-acetic acid (IAA) production, nitrogen fixation, siderophore production and many more. PGPR also protect plants from invading phytopathogens by secreting antibiotics, antifungal compounds, hydrocyanic acid (HCN), chitinase, etc. The PGPR strains with remarkable HM-withstanding property assist their immobile host to develop HM-tolerance for their combined survival in their contaminated habitat. These microbes are known as HM-resistant PGPR (HMR-PGPR). For several years, these PGPR strains have been isolated from the metal-contaminated rhizosphere of

different crops including vegetables (Mitra et al. 2018a, b; Pramanik et al. 2017, 2018a, b; Khanna et al. 2019).

So, to ensure food security, the development of environmental cleanup methods is urgently needed to accomplish the reclamation of contaminated agricultural lands. Unlike the issue of organic pollutants, which sometimes seemed easier to resolve, mitigation of heavy metal contamination has been proving to be one of the more difficult tasks ever undertaken. Organic contaminants can be degraded. The metal pollutants are instead non-degradable in nature, and these contaminants can only be transformed into less toxic forms or removed by means that include accumulation and adsorption. Most of the conventional methods for remediation of heavy-metal-contaminated soil are physicochemical in nature which is expensive, ineffective, creates secondary pollutants and unsuitable for large areas (Quartacci et al. 2006). In this context, HM-resistant PGPR-induced bioremediation is one such approach which is inexpensive, effective, sustainable and ecofriendly. Unlike some non-PGPR microbial strains (Hu et al. 2007; Rehman et al. 2008; Muneer et al. 2009; Shakya et al. 2012; Liu et al. 2013; Davolos and Pietrangeli 2013) isolated from contaminated soil and groundwater, HM-resistant PGPR play a dual role in heavy metal bioremediation as well as plant growth promotion. Some of the non-PGPR strains have also been proven promising as potent bioremediators.

This chapter encompasses heavy metal and metalloid resistant plant growth-promoting rhizobacteria (HMR-PGPR), which are a functionally defined group of microorganisms, discovered during the last two decades that have been found to improve the growth of different crops across the world under different levels of HMs contamination. It covers latest information on diverse HMR-PGPR that exhibited various degrees of HM-resistance, different levels of release of plant growth-promoting substances and different capacities to accelerate plant growth by reducing HM stress-induced morpho-biochemical changes in the affected plants. A brief account of how biotic stress tolerance is facilitated by plant growth-promoting bacteria (PGPB), general HM resistant mechanisms, signaling cascades and genetically modified PGPR are also presented and discussed. Furthermore, we will provide some conclusions about the major obstacles to the application in HMR-PGPR in the field and future prospects of these strains. We will also discuss the times and places where non-HM resistant PGPR, metal-resistant plant growth-promoting bacteria (PGPB) and rhizobia have been advocated. Overall, this chapter is a substantial collection of information on heterogeneous microbial communities (especially HMR-PGPR) interacting with diverse hosts working in different soil types for crop improvement in a sustainable manner.

22.2 Heavy Metal(loid)-Induced Phytotoxicity in Crop Plants

The incessant spread and increasing levels of HMs in agricultural soils have caused severe impairment of crops which not only results in reduced yield but also a serious toxic threat to the crop consumers. Plants, being immobile, are unable to escape from this stressful environment and uptake bioavailable non-essential HM cations into their plant cells along with essential soil nutrients. These HMs, upon surpassing certain threshold levels, impose severe cellular damages with various unusual morphological manifestations. The threshold level of HMs to induce phytotoxicity highly depends on plant species or even a particular cultivar. The uptake, translocation and cellular compartmentalisation of heavy metals may be governed by perhaps only one or just a few genes (Ernst 1996). Moreover, this also depends on the cationic forms of HMs. The observable external changes include reduction of seed germination, changes in root-shoot length and changes in root-shoot fresh and dry weight that ultimately decrease plant biomass (Table 22.1). As the root is directly exposed to the soil HMs, the root is the first organ encountered by toxic HMs, and the toxic effects follow into the shoots and other aerial parts of the plants. Affected root growth results in the poor acquisition of essential nutrients, and thereby an insufficient supply of nutrients to the photosynthetic cells in the aerial parts. To date, the members of Poaceae are the most studied crops on which the phytotoxic effects of different HMs have been investigated (Fig. 22.1). The phytotoxic consequences of all the ten HMs (viz. arsenic, cadmium, chromium, cobalt, copper, lead, manganese, mercury, nickel and zinc) discussed here have been studied on Poaceae (Fig. 22.1). After Poaceae, the HM-phytotoxicity studies have focused mainly on members of Fabaceae, Solanaceae and Brassicaceae, as predominant crops (Fig. 22.1). The less-studied families in the context with HM phytotoxicity are Amaryllidaceae, Euphorbiaceae, Amaranthaceae, Rosaceae, Linaceae, Malvaceae, Asteraceae and Cucurbitaceae (Fig. 22.1).

Among HMs, arsenic (As) is considered as an analog of phosphate (P) that competes with P-transporters in the root plasma membrane (Meharg and Macnair 1992). Although As-tolerance has been identified in a number of plant species (Meharg and Macnair 1992), elevated As-level has been found to negatively affect rice, maize, black gram, soybean, mung bean, cucumber, sorghum, barley, mustard, broccoli, pea and Chinese cabbage (Table 22.1). Biochemical changes identified in these crops include a reduction in photosynthetic pigments (chlorophyll, carotenoids), increased accumulation of reactive oxygen species (ROS), membrane lipid peroxidation, inhibition of ATP formation, enhanced proline and protein content and increased abscisic acid (ABA) synthesis (Table 22.1). Furthermore, altered activities of various cellular enzymes including RuBisCO, amylase, protease, catalase, peroxidase and other antioxidant enzymes are evident (Stoeva et al. 2005; Srivastava et al. 2017; Ghosh et al. 2018; Dong et al. 2020; Chauhan et al. 2020). Besides, As-mediated induction of cell death in root tips, proteomic alteration and disruption

Table 22.1 Heavy metal(loid)-induced phytotoxicity in different crops

HMs	Crop	Phytotoxic effects[a]	References
As	*Oryza sativa* (Rice)	• Reduced root-shoot length, biomass and root hair • Increased accumulation of ROS and MDA • Damaged cortical cells and cellular structure • Reduction in RuBisCO activity, photosynthesis • Increased ABA synthesis and growth inhibition	Chauhan et al. (2020)
	Oryza sativa (Rice)	• Decreased rice biomass • Inhibition of root growth • Inhibition of RuBisCO and photosynthesis	Dong et al. (2020)
	Oryza sativa (Rice)	• Reduced seed germination • Decreased root-shoot elongation • Decreased amylase and protease activity • Increased antioxidant enzymes, MDA and proline	Ghosh et al. (2018)
	Vigna mungo (Black gram)	• Catalase activity decreased • Increased amount of lipid peroxidation • Peroxidase increased tremendously • Superoxide dismutase increased • Ascorbate peroxidase also increased • Reduction of photosynthetic pigments	Srivastava et al. (2017)
	Glycine max (Soybean)	• Inhibition of leaf development • Cell death in root tips • Decreased root-shoot biomass • Reduction in chlorophyll content • Increased membrane lipid peroxidation	Armendariz et al. (2016)
	Oryza sativa (Rice)	• Inhibition of ATP formation • Lowered the yield of rice grain • Increased oxidative stress	Syu et al. (2015)
	Phaseolus radiatus (Mung bean), *Cucumis sativus* (Cucumber), *Triticum aestivum* (Wheat), *Sorghum bicolor* (Sorghum), *Hordeum vulgare* (Barley), *Brassica campestris var. chinensis* (Chinese cabbage), *Brassica oleracea* (Broccoli),	• Inhibition of seed germination • Decreased seedling growth	Yoon et al. (2015)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
	Brassica nigra (Mustard), *Pisum sativum* (Pea)		
	Oryza sativa (Rice)	• Stimulation of antioxidant enzymes • Increased accumulation of stress-responsive amino acids	Dave et al. (2013)
	Oryza sativa (Rice)	• Reduced seed germination • Stunted root-shoot growth • Inhibition of root formation at higher concentration	Shri et al. (2009)
	Zea mays (Maize)	• Proteomic alteration • Disruption of normal cellular function	Requejo and Tena (2006)
	Phaseolus vulgaris L. (Mung bean)	• Reduced growth, leaf gas exchange, water potential • Decreased protein and chlorophyll content • Root-shoot significantly reduced • Increased peroxidase activity and lipid peroxidation	Stoeva et al. (2005)
Cd	*Pisum sativum* (Pea)	• Reduced root-shoot length • Decreased fresh, dry weight, biomass • Increased proline, glycine betaine and soluble proteins, sugar content decreased • Chlorophyll 'a', 'b', carotenoid content decreased • Activities of antioxidant enzymes increased • Accumulation of phenols decreased	Sager et al. (2020)
	Oryza sativa (Rice)	• Reduced seed germination • Decreased root-shoot length • Decreased fresh and dry weight • Decreased amylase, total sugar, chlorophyll • Protease activity decreased • Increased total protein, antioxidant enzymes • Increased proline ad ethylene content	Mitra et al. (2018a)
	Oryza sativa (Rice)	• Reduced seed germination • Decreased root-shoot length • Decreased fresh and dry weight • Decreased amylase, total sugar, chlorophyll • Increased protease activity and total protein • Increased total protein, antioxidant enzymes • Increased proline ad ethylene content	Pramanik et al. (2018a)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
	Solanum lycopersicum (Tomato) *Cucumis sativus* (Cucumber)	• Decreased root-shoot dry weight, decreased number of leaves • Total content of organic acid decreased • Activities of SOD and GR were depressed • CAT, APX activities, H_2O_2 were increased	Wu et al. (2015)
	Solanum tuberosum (Potato)	• Increased MDA content • Decreased chlorophyll content	Xu et al. (2013)
	Lactuca sativa (Lettuce)	• Decreased plant dry weight • Strong reduction of the maximum photochemical efficiency of PS II • Impairment of net CO_2 assimilation rate • Decrease in RuBisCO activity • Decreased efficiency of nutrient uptake and carbohydrate assimilation	Dias et al. (2013)
	Oryza sativa (Rice)	• Decreased root-shoot dry weight and biomass • Decreased chlorophyll content • Increased oxidative stress	Chou et al. (2011)
	Triticum aestivum (Wheat)	• Inhibition of root elongation	Cao et al. (2007)
	Phaseolus vulgaris (Mung bean)	• Decreased root-shoot length • Reduced dry weight and chlorophyll	Tripathi et al. (2005)
Co	*Triticum aestivum* (Wheat)	• Decreased growth, water content, osmotic potential • Reduced carbon assimilation rate, stomatal conductance, intercellular CO_2 concentrations, transpiration rate, photosynthetic capacity	Ozfidan-Konakci et al. (2020)
	Hordeum vulgare (barley) *Brassica napus* (Oilseed rape) *Lycopersicon esculentum* (Tomato)	• Decreased plant growth • Inhibition of plant shoot biomass	Li et al. (2009)
	Lycopersicon esculentum (Tomato)	• Decreased biomass, decreased concentration of Fe in different parts, chlorophyll, Hill reaction activity, catalase activity • Increased peroxidase, acid phosphatase, ribonuclease • Increased carbohydrate and phosphorus fractions in leaves	Chatterjee and Chatterjee (2003)
	Brassica oleracea (Cauliflower)	• Chlorosis on young leaves, a decrease in chlorophyll concentration • Restriction of translocation of P, S, Fe, Mn and Zn from roots to tops.	Chatterjee and Chatterjee (2000)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
Cr	*Brassica napus* L. (Oilseed rape)	• Accumulation of reactive oxygen species, malondialdehyde • Antioxidant enzyme activities enhanced • Damaged the leaf and root ultrastructures	Gill et al. (2015)
	Vicia faba (Faba bean)	• Abberation of mitosis • Cr(VI)-induced disturbances of mitotic microtubules	Eleftheriou et al. (2015)
	Zea mays (maize)	• Decreased mitotic index, genomic template stability and soluble protein levels • Decreased growth-promoting hormones	Erturk et al. (2014)
	Pisum sativum (Pea)	• Growth inhibition, root deformations • DNA damage, cell cycle arrest and polyploidisation	Rodriguez et al. (2011)
	Oryza sativa (Rice)	• Root-shoot growth, leaf area, fresh and dry weight decreased • Grain weight and paddy yield decreased • Reduction in levels of nutrients in root and shoot	Sundaramoorthy et al. (2010)
	Lycopersicon esculentum (Tomato)	• Stunted growth, brownish, necrotic shoot and plant bending • Lethality observed in higher doses	Goupil et al. (2009)
	Pisum sativum (Pea)	• Chlorosis and wilting in leaves • SOD activity increased at lower Cr supply, decreased at higher Cr. • Significant reductions in Chl a and b • Monodehydroascorbate reductase activity significantly decreased	Pandey et al. (2009)
	Vigna mungo (Blackgram)	• Decreased germination percentage, root-shoot length, fresh & dry weight • Decreased total chromosome length, absolute chromosome length and average chromosome length of seedlings • Significant mutagenic effect on the root tip cells	Chidambaram et al. (2009)
	Brassica napus (Rapeseed)	• Plant growth inhibition • Genetic damage and DNA methylation	Labra et al. (2004)
Cu	*Linum usitatissimum* (Flax)	• Reduced plant height, diameter, fresh and dry biomass • Reduced chlorophyll contents in the leaves • Excess generation of reactive oxygen species • Increased activities of superoxide dismutase, peroxidase in the roots and leaves	Saleem et al. (2020)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
	Brassica campestris ssp. *chinensis* Makino (Chinese cabbage)	• Decreased mineral nutrients, chlorophyll content • Increased MDA content and DNA methylation level	Zhou et al. (2017)
	Withania somnifera (Indian ginseng)	• Reduced leaf fresh weight, shoot length • Reduction in chlorophyll and carotenoid concentration • Increased lipid peroxidation, high $O_2^{-\bullet}$ and H_2O_2 content • Increased Ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione-S-transferase, guaiacol-peroxidase activities in leaves	Khatun et al. (2008)
	Oryza sativa (Rice)	• Rice growth reduced • Grain yields decreased	Xu et al. (2006)
	Prunus cerasifera (peach rootstock)	• Reduced relative growth rate for both fresh and dry weight • Severe browning and necrosis • Increased total catalase, superoxide dismutase activity with the induction of *Sod* and *Cat* gene expression	Lombardi and Sebastiani (2005)
	Cucumis sativus (cucumber)	• Young expanding leaves exhibited a reduction in leaf area, while mature leaves showed reduced photosynthesis • Sucrose, starch content increased in both types of leaves • Net CO_2 assimilation decreased in mature leaves	Vinit-Dunand et al. (2002)
Hg	*Avena sativa* (Common oat)	• Decreased yield of aerial mass and roots • Increased contamination of Hg in soil increased N and K, but decreased P	Sadej et al. (2020)
	Triticum aestivum (Wheat)	• Roots of the plant were more affected as compared to the shoot • The malondialdehyde content increased in the roots • Significant decrease in root and shoot growth, content of chlorophyll and total soluble protein • Enzymatic antioxidants decreased	Sahu et al. (2012)
	Jatropha curcas (Physic nut)	• Loss of biomass, leaf area and growth • Reduction of net photosynthesis	Marrugo-Negrete et al. (2016)
	Brassica juncea (Indian mustard)	• Significant reduction in biomass, relative water content in leaves • Alteration of leaf cellular structure • Decreased number of palisade and spongy parenchyma cells • Reduced cell size and clotted depositions	Shiyab et al. (2009)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
	Lycopersicon esculentum (Tomato)	• Decreased root-shoot growth • Decreased chlorophyll content in leaves • Enhancement of antioxidant enzyme activities, malondialdehyde formation, H_2O_2 content.	Cho and Park (2000)
Mn	*Triticum aestivum* (Wheat)	• Inhibited the uptake of other elements • Affected antioxidant enzymes	Faria et al. (2020)
	Glycine max (Soybean)	• Reduced CO_2 assimilation rate, stomatal conductance • Increased antioxidant enzymes in roots • Calcium travelled dramatically from the healthy to necrotic tissue under high Mn	Santos et al. (2017)
	Vigna unguiculata (Cowpea)	• Formation of brown spots in sensitive cultivars • Induction of callose formation and an enhanced release into the apoplast of phenols, peroxidases and other stress-related proteins • Proteins related to CO_2 fixation, stabilisation of the Mn cluster of the photosystem II, pathogenesis-response reactions were affected	Führs et al. (2008)
Ni	*Solanum lycopersicum* (Tomato)	• Inhibition of growth, biomass, impairment of photosynthesis, photosystem function, mineral homeostasis, root activity and osmotic balance • Increased ROS production in leaves and roots of tomato seedlings as compared with control plants	Jahan et al. (2020)
	Oryza sativa (Rice)	• Reduced the growth and yield of rice plants compared to the plants grown in normal soil without Ni stress • Reduced nutrient (NPK) content in rice straw and grain	Nazir et al. (2016)
	Zea mays (Maize)	• Seedling mortality at high Ni concentration • Inhibition of seedling growth and development • Leaves exhibited chlorosis and yellow spotting • Decreased the amount of soluble sugars in leaves	Nie et al. (2015)
	Vigna cylindrica (Catjang) *V. mungo* (Black gram) *V. radiata* (mung bean)	• Reduction in seed germination, fresh biomass • Drastic decline was observed for the formation of nodules and chlorophyll a and b contents	Ishtiaq and Mahmood (2012)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
	Cicer arietinum (Chickpea)	• Decline in the seed germination, biomass and plant growth • Suppression of root nodules, roots and lateral roots • Reduction in chlorophyll content and development of chlorosis	Khan and Khan (2010)
	Hordeum vulgare (Barley)	• Decreased dry weight, which was more prominent in roots than in shoots • Interveinal chlorosis of younger leaves, necrosis of mature leaves and browning of the root system	Rahman et al. (2005)
Pb	*Lactuca sativa* (Lettuce)	• Decrease in shoot growth • Disturbed lettuce growth and net photosynthesis	Xiong et al. (2018)
	Vicia faba (Faba bean)	• Induction of lipid peroxidation and H_2O_2 generation in leaves • Overproduction of ROS resulting in bimolecular damage • Decreased chlorophyll content	Shahid et al. (2014)
	Glycine max (Soybean)	• Inhibitory effect on carbohydrate content • Starch was more reduced as compared to other carbohydrates • Carotenoids were less affected as compared to total chlorophyll • Reduction of protein content	Imtiyaz et al. (2014)
	Triticum aestivum (Wheat)	• Increased lipid peroxidation, enhanced soluble protein concentrations, accumulation of proline in roots • Enhanced Esterase activity • Inhibition of α-amylase activity • Antioxidant enzymes activities	Lamhamdi et al. (2011)
	Solanum lycopersicum (Tomato)	• Decreased calcium, magnesium, potassium phosphorus concentration in shoot and leafs • Decreased Na content in roots, shoots and leafs • Reduction in chlorophyll biosynthesis • Decreased root, shoot and leaf water contents	Akinci et al. (2010)
	Allium sativum (Garlic)	• Antioxidant enzymes increased in roots and shoots • Root-shoot growth were significantly inhibited	Liu et al. (2009)
	Phaseolus vulgaris (Mung bean)	• Decreased root-shoot length • Reduced dry weight and chlorophyll	Tripathi et al. (2005)
	Oryza sativa (Rice)	• Reduced chlorophyll in leaves, carotene, sugars, phenols, nonprotein	Chatterjee et al. (2004)

(continued)

Table 22.1 (continued)

HMs	Crop	Phytotoxic effects[a]	References
		nitrogen, protein, iron, manganese, copper, zinc, Hill reaction activity, peroxidase activity • Decreased plant dry weight and inhibition of root growth	
Zn	*Hordeum vulgare* (Barley)	• Reduction in the chlorophyll content • Decreased root-shoot biomass	Mossa et al. (2020)
	Solanum lycopersicum (Tomato)	• Generation of H_2O_2 and induction of oxidative stress • Reduction of stress-controlling enzymes (APX and SOD) in the root • Reduction in contents of Chl-a and T-Chl	Akanbi-Gada et al. (2019)
	Carthamus tinctorius (Safflower)	• Stunted growth, brownish roots, chlorosis on the leaves • Roots and shoots biomass production reduced significantly	Namdjoyan et al. (2017)
	Beta vulgaris (Sugar beet)	• Inward-rolled leaf edges, damaged and brownish root system, with short lateral roots • Decreased N, Mg, K and Mn concentrations in all plant parts • Significant decrease in the root/shoot ratio	Sagardoy et al. (2009)

[a]*ROS* Reactive oxygen species, *MDA* Malondialdehyde, *RuBisCO* Ribulose-1,5-bisphosphate carboxylase/oxygenase, *ABA* Abscisic acid, *SOD* Superoxide dismutase, *GR* Glutathione reductase, *CAT* Catalase, *APX* Ascorbate peroxidase

of normal cellular function have also been identified (Requejo and Tena 2006; Armendariz et al. 2016).

Likewise, phytotoxicity of other HMs reported almost parallel kinds of morpho-biochemical dysfunctions (Table 22.1). Studies of cadmium (Cd)-induced phytotoxicity have focused mainly on rice, wheat, tomato, potato, cucumber, pea, lettuce and mung bean (Table 22.1). An upsurge of ethylene content in rice seedlings has been noticed in response to Cd stress (Mitra et al. 2018a; Pramanik et al. 2018a) that is linked to increased accumulation of H_2O_2, leading to cell apoptosis (Chmielewska-Bak et al. 2014). Cobalt (Co), one of the naturally occurring HMs in the earth's crust, spreads through human activities as well, and that element is taken up by plants from the contaminated soil. However, information on Co-phytotoxicity is less available in the literature compared to As and Cd. Wheat, barley, oilseed rape, tomato and cauliflower have been studied so far to elucidate Co-induced phytotoxicity (Chatterjee and Chatterjee 2000, 2003; Li et al. 2009; Ozfidan-Konakci et al. 2020). Co was found to decrease plant growth, photosynthetic rate, water content, osmotic potential, stomatal conductance, transpiration rate and cause chlorosis that ultimately

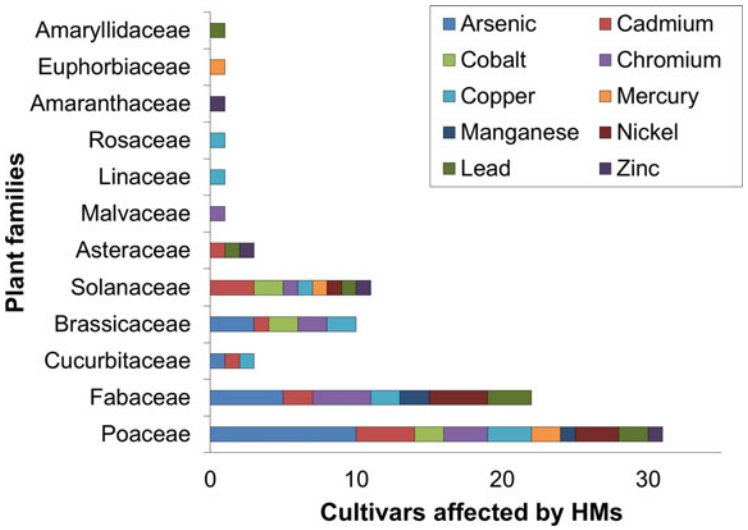

Fig. 22.1 Families of studied agricultural crops affected by heavy metal(loid)s

manifested as decreased plant biomass (Table 22.1). An exogenous application of $CoCl_2$ was shown to decrease plant ethylene levels compared to controls (Pramanik et al. 2017, 2018a). The number of phytotoxicity studies on chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) on the more common crop plants is also impressive, with reporting of various morpho-biochemical malfunctions in plants.

22.3 Role of Heavy Metal(loid) Resistant Plant Growth-Promoting Rhizobacteria in Crop Improvement

Soil, being the sink of nutrients for plants, is also the chief source of contaminants. The information summarised in Table 22.1 provides an idea of observed intensification of heavy metal contamination and consequences of the major HM contaminants on some common crops. Plants have developed their own natural mechanisms to regulate the uptake, translocation and accumulation of HMs, which is known as natural phytoremediation. In reality, plants are not the only warriors that are exposed to and affected by soil HMs, and indeed there similarly exist some close neighbors like the rhizospheric microbial community that also have direct or indirect influences on plant growth. Phytoremediation is one of the safest, eco-friendly technologies and is often triggered by plant growth-promoting bacteria (PGPB) as a response to accelerated HM uptake and accumulation in the plant cells (Ullah et al. 2015). This concept of designing and promoting bacteria-assisted phytoremediation

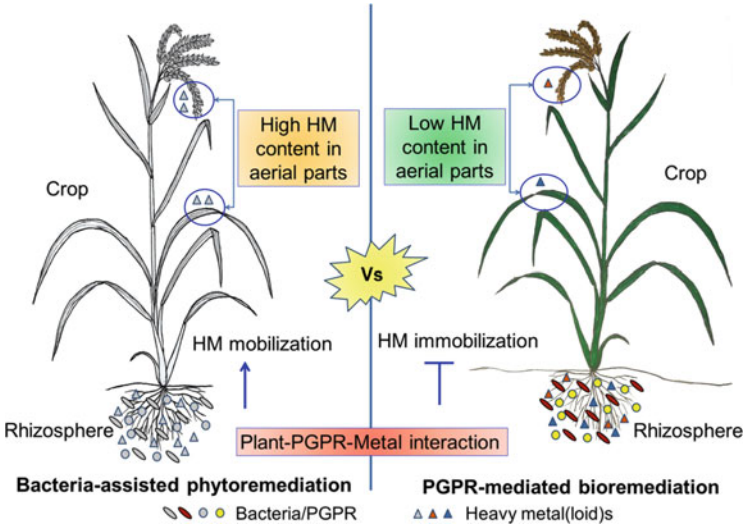

Fig. 22.2 Bacteria-assisted phytoremediation and PGPR-mediated bioremediation of heavy metal (loid)s

technology is not intended to be applied only in the case of agricultural crops that are consumed by humans, cattle or other animals to reduce the high chances of HM toxicity in the food chain (Fig. 22.2). Rather, the preferred usage of PGPR-mediated bioremediation would be in such cases where some specific group of PGPR reduce both the HM-induced phytotoxic effects and HM-uptake as well (Fig. 22.2). PGPR fall under a special group of fast-growing microorganisms which are a good instance of phytostimulating biological agents of natural occurrence. Since many years, soil microbiologists and environmentalists have been devoting their tireless efforts to isolate PGPR strains with greater efficiency of bioremediation and plant growth promotion, and to apply their discoveries about HM-contaminated soil for the benefit of sustainable agriculture (Table 22.2). Here, in this review, we will largely examine HM-resistant PGPR (involved in PGPR-mediated bioremediation) publications from the last two decades and present their results in brief (Table 22.2). We have considered only those HM-resistant PGPR strains which were tested for their plant growth-promoting activities on selected crops, with those microbes having been applied as bioinoculants either in laboratory conditions or in the field. It is evident from Table 22.2 that the phytotoxic effects mentioned in Table 22.1 have been significantly reduced by the use of HM-resistant PGPR.

One of the most vital and key representations of this chapter is the documentation of culture media for the isolation of HM-resistant PGPR. Proteobacteria seem to have been the most commonly isolated group from all the stated culture media. Yeast extract mannitol (YEM) medium has been the most preferable isolation medium, followed by Davis Mingioli (DM) medium with Cd (Fig. 22.3). From a critical

Table 22.2 Heavy metal(loid)-resistant PGPR including rhizobia discovered in the last two decades and their applications

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Enterobacter bugandensis TJ6	*Lactuca sativa* (Lettuce)	Urease screening agar plates	Urease, IAA, siderophore	(MIC-mg L^{-1}) Cd-400 Pb-1700	*L. sativa*	Hydroponic experiment in a glasshouse	• Compared with controls, inoculation with both strains significantly improved the root and shoot dry weight • Soluble protein and vitamin C content enhanced by both strains • Reduced Cd and Pb content in edible tissue. • Strain HD8 found more proficient in reduction of Cd and Pb uptake in lettuce than TJ6	Wang et al. (2020)
Bacillus megaterium HD8				Cd-700 Pb-2100				
Serratia marcescens SNB6	*Chrysopogon zizanioides* (Vetiver grass)	NM	Phosphate, IAA, siderophore	(MIC- ND) Cd	*C. zizanioides*	Pot experiment conducted in green house	• Improved the genes expression of (low molecular weight organic acids) LMWOAs, PGP traits, biomass • Improved the activities of antioxidant enzymes	Wu et al. (2020)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Agrobacterium fabrum SDW_6	*Zea mays* (Maize)	Dworkin and Foster (DF) nutrient 100 mg L^{-1} Cr	Phosphate, siderophore, IAA, potassium	(MIC- ND) Cr	*Z. mays*	Pot experiment	• Improvement in intake of N, P, K in leaves and in roots of maize after combined application of SDW_6 with 500 μM Fe • SDW_6 had better efficiency than SDW_{10} • Significantly enhanced of chlorophyll content • Enhancement of root-shoot dry weight, plant height, roots-shoot length in maize by SDW_6 strain	Danish et al. (2019)
Leclercia adecarboxylata SDW_{10}								
Serratia liquefaciens CL-1	*Amaranthus tricolor* (Elephant-head amaranth)	Luria–Bertani's (LB) supplemented with 3mM Cd	IAA, siderophore, ACCD	(High degree of resistance-mM) Cd-6.2 Pb-12.0 Cu-6.3	*Brassica napus* (Oil-seed rape)	Pot experiment	• Cell adsorption of Cd increased by strain CL-1 compared to strain X30 • Biomass, pH, polyamine content relative abundance of	Han et al. (2018)
Bacillus thuringiensis X30			IAA, ACCD	Cd-3.6 Pb-8.7 Cu-3.9				

							arginine decarboxylase-producing bacteria (ADPB) of rhizosphere soils, increased by strains • Both bacteria colonized oilseed rhizospheric soil • Both strains reduced Cd content of root • Cd translocation factor reduced significantly strain CL-1 than strain X30	
Azotobacter chroococcum CAZ3	*Capsicum annum* (Chilli)	Ashby's mannitol medium	IAA, siderophore, ACCD, ammonia	(Maximum tolerance-μg mL^{-1}) Cu-1400 Pb-2000	*Zea mays* (Maize)	Pot experiment	• CAZ3 strain improved growth, maize yield, presence of both metals • Levels of proline, malondialdehyde and antioxidant enzymes in foliage significantly reduced • Accumulated greatest quantities of metals in roots than other organs • In roots, shoots, kernels metal concentrations reduced	Rizvi and Khan (2018)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Enterobacter aerogenes K6	*Oryza sativa* (Rice)	Davis Mingioli (DM) medium with 1000 µg/mL Cd	Phosphate, IAA, siderophore, N_2, ACCD, HCN, ammonia	(High degree of resistance-µg/mL) Cd-4000 Pb-3800 As-1500	*O. sativa*	*In vitro* PGP experiment, *In vivo* root colonisation study	• Revealed growth promotion of rice seedling under Cd stress • Reducing oxidative stress (through antioxidants), stress ethylene by combined outcome of Cd resistance and PGP activities • 100% seed germination • Improved root length, shoot length, root weight, shoot weight • Strain K6 increased Chl-a, Chl-b and total chlorophyll content • In seedling tissues Cd uptake reduced	Pramanik et al. (2018a)

							• Colonisation of numerous bacterial cells around root surface	
Enterobacter K2	*Oryza sativa* (Rice)	Davis Mingioli (DM) medium with 500 µg/mL Cd	Phosphate, IAA, siderophore, N_2, NH_3, ACCD, HCN	(MIC- µg/mL) Cd-4000 Pb-4000 As-1200 Ni-600 Hg-40	*O. sativa*	*In vitro* PGP experiment in glass beaker	• Enhancement of germination percentage • Growth parameters (root length, shoot length and root-shoot biomass) increased significantly • Reduction of stress ethylene • Elongation of root increased by preventing senescence • Total chlorophyll content increased	Pramanik et al. (2018b)
Enterobacter asburiae S2	*Oryza sativa* (Rice)	Davis Mingioli (DM) medium with 1000 µg/ml Cd	Phosphate, IAA, N_2, ACCD	(MIC- µg/mL) Cd-3500 Pb-2500 As-1050	*O. sativa*	Pot experiment in growth chamber	• Germination percentage increased • Improvement in root-shoot length, fresh weight, dry weight • Significant reduction of Cd uptake, comparison to Cd treated	Mitra et al. (2018a)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/MTC/MRL/Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
							un-inoculated seedlings • Reduction of oxidative stress, stress ethylene	
Klebsiella michiganensis S8	*Oryza sativa* (Rice)	Davis Mingioli (DM) medium supplemented with 1000 µg/ml Cd	Phosphate, IAA, N_2, ACCD	(MIC- µg/mL) Cd- 3500 Pb- 3000 As- 1000	*O. sativa*	*In vitro* PGP experiment in glass beaker	• Revealed cadmium elimination proficiency • Good effect in seed germination percentage • Growth parameters (root-shoot length, fresh weight, dry weight) improved • Enhanced chlorophyll a, b and total chlorophyll content	Mitra et al. (2018b)
Klebsiella pneumoniae K5	*Oryza sativa* (Rice)	Davis Mingioli (DM) medium supplemented with Cd^{2+} (100–1000 µg/mL)	Phosphate, IAA, N_2, ACCD, NH_3, Siderophore	(MIC—µg/mL) Cd-4000 Pb-4000 As-1500	*O. sativa*	*In vitro* PGP experiment, *In vivo* root colonisation study	• Enhanced germination percentage, root-shoot length, root-shoot dry weight, seedling vigor index • Improved	Pramanik et al. (2017)

							antioxidant enzymatic activities • Increased α-amylase, total sugar, total protein, chlorophyll and proline content • Decreased stress ethylene and Cd content in seedlings	
R. leguminosarum bv. *viciae* SV15	Nodule of *Vicia faba* (Fava bean)	YEM medium	ND	(MIC -mM) Cu 0.5	*V. faba*	Hydroponic culture	• Co-inoculation treated sets with Cu increased root length, shoot dry weight • Reduced copper uptake in roots • in co-inoculated Cu treated plants, decrease antioxidative enzymes	Fatnassi et al. (2015)
Rhizobium sp. SV20				0.5				
Pseudomonas sp. SV23				1				
E. cloacae SV27				2				
Photobacterium sp. strain MELD1	*Phragmites australis* (Reed)	Luria Bertani (LB) and M9 minimal media plates	IAA	(MIC—mg. kg^{-1}) Hg—33	*Vigna unguiculata* ssp. *sesquipedalis* (Long yard bean)	In vivo experiment	• Significant improvement in biomass, root length, seed number • Helped in mercury uptake restricted to roots	Mathew et al. (2015)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Pseudomonas brassicacearum ssp. *brassicacearum* strain DBK11	*Brassica napus* (Indian mustard plant)	Medium 72- for tryptone casein soya agar Or Liquid medium 1 (DSMZ Medium 1a), 30°C	ND	(MIC- ND) Zn	*Brassica juncea* (Indian mustard plant)	Glasshouse pot experiment	• Root biomass increased after WSM1325 *(BRo)* inoculation compared to control • Plant growth higher with both bacterial strains inoculation • Co-inoculation of isolates, decreases metal toxicity, in plant growth • More Zn bioaccumulation, translocation showed significantly • B*RP*Zn set reduces Zn from soil	Adediran et al. (2015), Achouak et al. (2000)
Rhizobium leguminosarum bv. *trifolii* strain WSM1325	*Clover* sp. (Clover)							
Bradyrhizobium sp. YL-6	*Glycine max* (Soybean)	Modified YEM agars with 20 mg L^{-1} of Cd	IAA, siderophore, ACCD, phosphate	(MIC- mg L^{-1}) Cd -100	*G. max*, *Lolium multiflorum* (Italian ryegrass)	Pot experiment in glasshouse	• Enhanced shoot dry weight compared to uninoculated control in *L. multiflorum*	Guo and Chi (2014)

							• Increase photosynthetic pigments, mineral nutrients in both plants • Cd accumulation increased in *L. multiflorum* root, decreased in *G. max*	
Serratia sp. MSMC541	*Lupinus luteus* (Yellow Lupin)	YEM Agar 25 µg/mL congo red	ND	(MTC- mM) As-13.3 Cu-2.3 Pb-9.0 Zn-30 Cd-2.2	*L. luteus*	Pot experiment	• Increased shoot biomass • Plant tolerance to metals • Repressed metal translocation to shoot • Plant biomass improved	Aafi et al. (2012)
Agrobacterium radiobacter D14	*Pt. vittata* L. (Chinese brake)	Chemically defined medium with 800 (µmol/L) As (III)	IAA, siderophore	(MIC-mmol/L) As(III)-14 As(V)-150	*Populus deltoides* (Poplar)	Greenhouse pot experiment	• As uptake and translocation improved • Root-shoot growth enhanced • Plant dry weight increased • Chlorophyll, soluble protein content increased • Superoxide dismutase, catalase activity enhanced, malondialdehyde activity reduced	Wang et al. (2011)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Bacillus sp. PSB10	*Brassica campestris* (Mustard), *Lycopersicon esculentum* (Tomato)	Nutrient agar	Phosphate, siderophore, IAA, HCN, ammonia	(MIC-µg/mL) Cr-550	*Cicer arietinum* (Chickpea)	Open field experiment in pot	• Improved root-shoot length, nodule numbers, nodule dry weight, total dry weight • Enhanced seed yield, grain protein • Chromium uptake decreased	Wani and Khan (2010), Wani et al. (2007)
Bradyrhizobium sp. 750	*Lupinus* (Yellow Lupin) D.M.A.C. (Uppsala, Sweden)	NM	ND	(MTC-mM) As-2 Cd-<0.5 Cu -1.5 Pb-2 Zn-<1	*L. luteus*	In situ field experiment	• Zn accumulation higher than other HMs in roots, shoots • Strain 750 increased biomass, fixed nitrogen in soil • All strains improved biomass • Reduced metal accumulation in root shoot	Dary et al. (2010), Rodrı´guez-Llorente et al. (2010), Zurdo-Pineiro et al. (2007)
Pseudomonas sp. Az13	Rhizospheric region of legume plant	NM		MTC-mM As-4 Cd-1 Cu-4.5 Pb-5 Zn-3				
Ochrobactrum cytisi Azn6.2	*Medicago polymorpha* Nodules (Alfaalfa)	Tryptone yeast (TY) extract medium		MTC-mM As-8 Cd-1.5 Cu-3.5 Pb-6 Zn-10				

Burkholderia sp. RX232	*Salix caprea* (Goat willow)	Tryptic soy agar with $ZnSO_4$ (2 mmol l^{-1})	Siderophore, ACCD	(MIC-mmol/L) Cd-4 Zn-16	*S. caprea*	Greenhouse Pot experiment	• Helped in root growth • Significant reduction of HMs in roots	Kuffner et al. (2010)
Rhizobium sp. RP5	*Pisum sativum* (Pea plant)	Yeast extract mannitol (YEM) with $NiCl_2$ (0–350 µg. ml^{-1}) & $ZnCl_2$ (0–1500 µg.ml^{-1})	N_2, IAA, siderophore	(Tolerance-µg/mL) Ni-350 Zn-1500	*P.sativum*	Pot experiment	• Increased dry matter, nodule numbers, root-shoot N, leghemoglobin, seed yield, grain protein • Reduced HMs toxicity in plant organ	Wani et al. (2008a)
Rhizobium species RL9	*Lens esculenta* (Lentil)	Yeast extract mannitol medium	Siderophore, IAA	(Up to-µg/mL) concentration of Zn-400	*L. esculenta*	Pot experiment	• Enhanced dry weight, nodule numbers, nodule dry mass, growth, seed yield respect to uninoculated plants • Improved leghaemogloblin content, grain protein greater than uninoculated sets • Metal reduction noticed in plant tissue	Wani et al. (2008b)

(continued)

Table 22.2 (continued)

PGPR	Associated crop	Media[a]	PGP traits[b]	MIC[c]/MBC/ MTC/MRL/ Highest tolerance	Plant growth promotion study performed on (plant)	Mode of study	Improvements in crops after PGPR inoculation	Reference
Pseudomonas aeruginosa MKRh3	Rhizosphere samples of different plant species	King's B agar	IAA, siderophore, ACCD, phosphate	(MIC-mM) Cd-7	*Vigna mungo* (Black gram)	Pot experiment	• Significantly developed root-shoot length, root-shoot weight, plant growth, root branching • HM toxicity decreased • Cd accumulation lowered after inoculation	Ganesan (2008)
Bacillus subtilis strain SJ-101	Composite samples of rhizospheric soil	T-medium with (HMs) 1.0 mM each of Pb, Ni, Zn, Cr, Co, Cu	IAA, phosphate	(Maximum tolerance upto-mM) Ni-3.4	*Brassica juncea* (Indian mustard plant)	Pot experiment in open-field condition	• Plant growth promotion increased • Strain reduced phytoxicity by biosorption and bioaccumulation	Zaidi et al. (2006), Zaidi and Musarrat (2004)
Sinorhizobium meliloti strains Alf12 *S. meliloti* Alf 2L4 *S. meliloti* Med4D	*Trifolium subterraneum* cv. *Nuba* (Sub clover), *Lotus corniculatus* (Birdsfoot trefoil), *Medicago sativa* (Alfalfa), *Vicia sativa* (Garden vetch)	Yeast Extract-Mannitol (YEM)	N_2	(MIC- ND)	*Medicago sativa* (Alfalfa)	Leonard jars were used for plant growth in growth chamber	• Healthier plants and N content increased • Symbiotic good effect noticed in contaminated soil • Nodulation and N_2 fixation similar to control	Carrasco et al. (2005)

Mesorhizobium huakuii subsp. *rengei* B3	*Astragalus sinicus* (Chinese Milk Vetch)	NM	N_2	(MIC- ND) Cd	*A. sinicus*	Hydroponic experiment	• Nodule formation noticed • Synergetic relationship enhanced to accumulate Cd^{2+} in nodules 1.5-fold	Sriprang et al. (2003)
Proteus mirabilis T2Cr *P. mirabilis* CrP450	Composite surface soil collected	Luria–Bertani (LB) media	IAA, phosphate, ACCD, siderophore	(MIC-ppm) Cr-90 Cr-110	*Zea mays* (Maize)	Pot experiment under room conditions	• Enhanced plant height, fresh weight, leaf area greater than control • Chlorophyll content improved by both strains compared to control • Isolates with salicylic acid application increased Cr tolerance by reducing metal uptake from root to shoot • Oxidative stress decreased by both strains with SA	Islam et al. (2016)

[a]*NM* Not mentioned

[b]*Urease* Urease activity, *IAA* Indole-3-acetic acid, *Siderophore* Siderophore production, *Phosphate* Phosphate solubilisation, *Potassium* Potassium solubilisation, *ACCD* 1-aminocyclopropane-1-carboxylic acid deaminase activity, N_2 Nitrogen fixation, *HCN* Hydrocyanic acid production, *Ammonia* Ammonia production, *ND* Not determined

[c]*MIC* Minimum inhibitory concentration, *MBC* Minimum bactericidal concentration, *MTC* Maximum/maximal tolerance/tolerable concentration

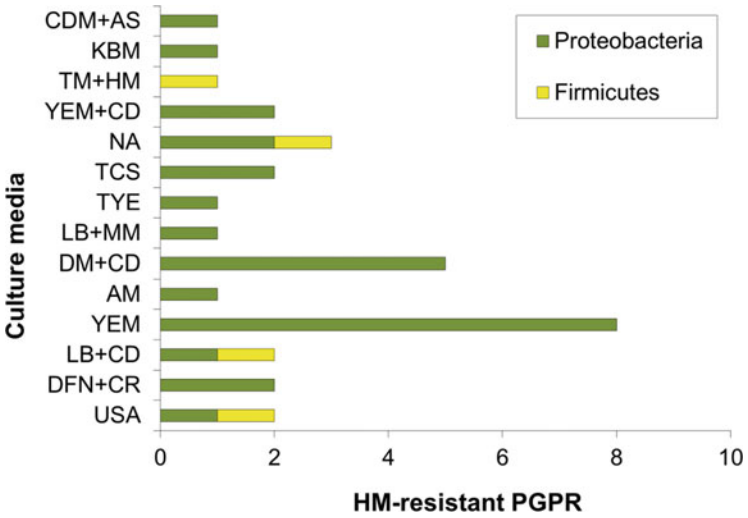

Fig. 22.3 Medium used for isolation of heavy metal(loid)-resistant PGPR. (*CDM* Chemically defined medium, *KBM* King's B medium, *TM+HM* T-medium with HM, *YEM+CD* Yeast extract mannitol with Cd, *NA* Nutrient agar, *TCS* Tryptone casein soya, *TYE* Tryptone yeast extract, *LB +MM* Luria–Bertani minmal media, *DM+CD* Davis Mingioli with Cd, *AM* Ashby's mannitol, *YEM* Yeast extract mannitol, *LB+CD* Luria–Bertani with Cd, *DFN+CR* Dworkin and Foster nutrient with Cr, *USA* Urease screening agar)

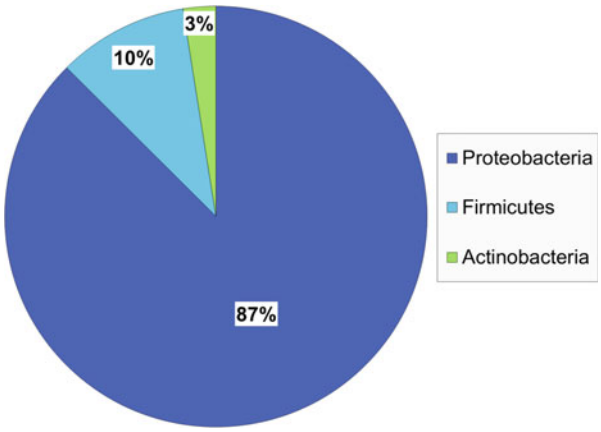

Fig. 22.4 Diversity and distribution of heavy metal(loid)-resistant PGPR

analysis of the information presented in Table 22.2, we find that the diversity of the HM-resistant PGPR community covers only three bacterial groups, i.e. proteobacteria, firmicutes and actinobacteria, and it is prominantly dominated by proteobacteria (Fig. 22.4). Furthermore, proteobacteria is the most abundant

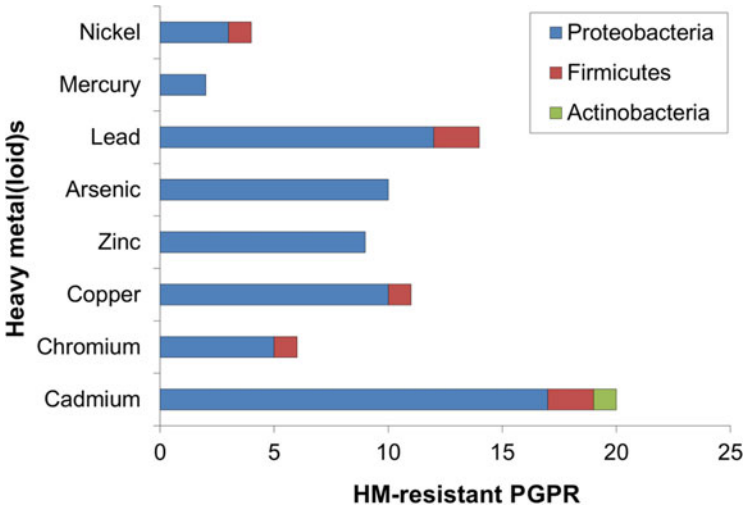

Fig. 22.5 Diversity and abundance of heavy metal(loid)-resistant PGPR

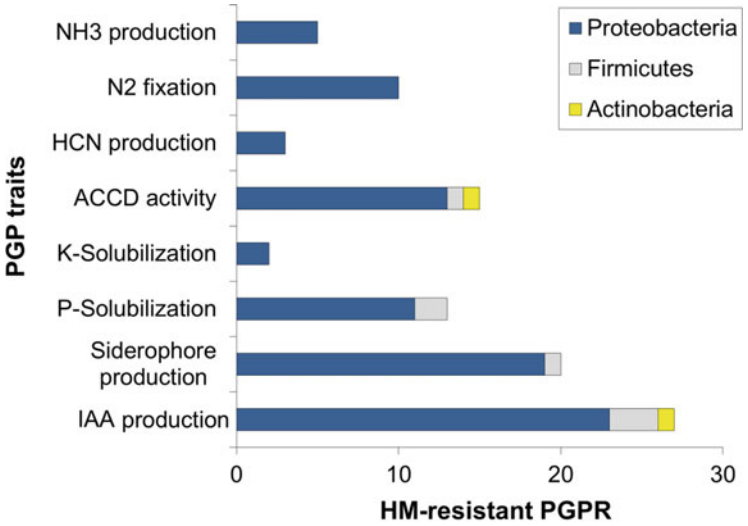

Fig. 22.6 Plant growth-promoting traits in heavy metal(loid)-resistant PGPR

PGPR member responsible for resistance to all the studied heavy metal(oid)s. Actinobacteria exhibit their remediational property only against Cd. The firmicutes are a set of PGPR sensitive to As, Hg and Zn (Fig. 22.5). Additionally, among the PGPR members, all the documented phenomenal PGP traits are mainly portrayed by the proteobacterial representatives, and actinobacterial agents are accountable only for their IAA and ACC deaminase producing capabilities (Fig. 22.6). Moreover, in

case of firmicutes, they are the silent member in case of N_2 fixation, potassium solubilisation, ammonia and HCN production. However, the firmicutes have exhibited ACC activity, P-solubilisation, siderophore activity and IAA production (Fig. 22.6).

22.4 Genetically Modified Plant Growth-Promoting Rhizobacteria for Crop Enhancement

Natural components like the PGPR play an indispensable role in the advancement of sustainable agriculture and also serve as an imperishable treasure box for the environment. Considering the limitations of these natural bio-agents, the idea of using genetic modification approaches has attracted the attention of scientists with the goal of attaining greater desired efficiency. With the improvements achieved by genetically engineering PGPR, the heavy metal accumulating gene and the biocontrolling genes can be assembled to conduct enhanced bioremediation and potentially achieve biocontrol in the rhizospheric soil. In this context, for superior cadmium (Cd^{2+}) bioaccumulation purpose, the phytochelatin synthase gene (PCS_{AT}) from *Arabidopsis thaliana* was introduced into *Mesorhizobium huakuii* strain B3 and then set up as a symbiosis with *M. huakuii* strain B3 and *Astragalus sinicus,* whereupon a desired activity was noted accordingly (Sriprang et al. 2003). It was possible to carry out that project because the peptides like phytochelatins (PC) and metallothioneins (MT) exhibit high affinity towards a variety of heavy metals (Chaudhary and Shukla 2019). Furthermore, genetically transformed rhizobacterial strains demonstrated significant biocontrol potentiality over fungal phytopathogens (Sattiraju et al. 2019). In such cases, incorporation of a mini-Tn5 vector containing the complete operon for the biosynthesis of an antifungal metabolite phenazine- 1-carboxylic acid (PCA), within *Pseudomonas fluorescens* has been documented to accelerate the suppression of fungal diseases by the genetically engineered bacterial strain in comparison to the natural bacterial strain (Timms-Wilson et al. 2000). Similar kinds of approaches were reported from several studies where genetically engineered PGPR strains showed enhanced PGP traits as well as biocontrol efficiency (Bloemberg and Lugtenberg 2001) and can be exemplified by the integration of Cry-toxin-encoding *cry1Ac7* gene from *Bacillus thuringiensis*, chitinase-encoding *chiA* gene from *Serratia marcescens* and ACC deaminase-producing gene from *Enterobacter cloacae* into rhizobacterial strains like *Pseudomonas* sp. (Sattiraju et al. 2019). The relocation of *sss* gene from biocontrol strain *P. fluorescens* WCS365 to other *P. fluorescens* rhizobacterial strains was found to improve the competitive root colonising efficiency (Dekkers et al. 2000). Apart from the genetically modified PGPR, transgenic plants also display greater PGP traits, especially higher ACC deaminase activity and heavy metal accumulation (Zhuang et al. 2007; Stearns et al. 2005; Nie et al. 2002). However, genetically modified PGPB are considered less effective in terms of their survival and

proliferation as compared to non-transformed versions of the same organisms; and this decreased fitness may be due to overburden of metabolic load by the expression of foreign genes (Glick 2020).

22.5 Plant Growth-Promoting Rhizobacteria in Biotic Stress Tolerance

The rhizosphere is a phenomenal environment where the plant-beneficial microbes especially the bacteria renowned as rhizobacteria, colonise and steadily perform several plant growth-promoting activities by means of facilitating nutrient availability and assimilation, and help conquer over disease-instigating microbes (Pérez-Montaño et al. 2014). The plant growth-promoting activities of these beneficial rhizobacteria include nitrogen fixation, solubilisation of minerals like phosphorus, production of ACC-deaminase and other plant growth regulators like auxins, gibberellins and cytokinins. Biocontrol properties are one of the key characteristic features of these PGPR (Kloepper 1978). Their antagonistic potentiality against phytopathogens is mainly categorised according to activities like the production of siderophores, lytic enzymes, antibiotics, bacteriocins, volatile organic compounds (VOC), hydrogen cyanide (HCN) and their ability to obstruct bacterial quorum sensing (Aloo et al. 2019; Pérez-Montaño et al. 2014; Kumar and Dubey 2012). Apart from these capabilities, PGPR also induce systemic resistance (ISR) proficiency which can help suppress pathogenicity that other microbes exhibit against host plants, and PGPR do as well improve the sustainability of agricultural systems (Beneduzi et al. 2012). Among the reported PGPR genera, *Pseudomonas* sp., *Bacillus* sp. and *Streptomyces* sp. are the warhorses in the avenue of biocontrol of phytopathogens (Table 22.3; Arrebola et al. 2019). Moreover, the rhizobacterial phyla involved in this job are dominated by proteobacteria, firmicutes and actinobacteria (Fig. 22.7). The bio-protecting efficiency of PGPR are not only restricted to countering the pathogenic microbial members of the rhizosphere community like fungi and bacteria, but are also promising as agents against metazoan phytopathogens like insects and nematodes (Table 22.3; Fig. 22.8).

The biological control of phytopathogens by the PGPR group of organisms does in many ways strengthen both plant and soil health. Rhizobacterial secretion of siderophores is among the mechanisms exhibited by the PGPR members that are antagonistic against other microoganisms. The actions of siderophores are based upon their chelation of iron which inhibits iron-dependent nutritional or energetic processes in those other microbes (Chaiharn et al. 2009). In iron-limiting soil environments, the binding of iron by siderophore-producing rhizobacteria can also boost up the availability of iron to those plants that are able to accumulate siderophore-bound iron (Tank et al. 2012). Apart from iron chelation, siderophores can bind with other heavy metals like Cd, Cu, Pb, Al and Zn which in turn diminishes the stress to plants that may be imposed by those other heavy metals

Table 22.3 Biocontrol activities of different PGPR

PGPR	Phylum	Pathogen	Reference
Fungi as phytopathogen			
Streptomyces sp.	Actinobacteria	*Fusarium oxysporum* *Fusarium* sp. *Gaeumannomyces* sp. *Phomopsis* sp. *Ulocladium* sp. *Rhizoctonia solani* *Colletotrichum* sp.	Suarez Moreno et al. (2019)
Pseudomonas aeruginosa	Proteobacteria	*Rhizopus microsporus* *Fusarium oxysporum* *Aspergillus niger* *Alternaria alternata* *Penicillium digitatum*	Uzair et al. (2018)
Azotobacter sp. *Pseudomonas* sp.	Proteobacteria Proteobacteria	*Helminthosporium* sp. *Fusarium* sp.	Bjelić et al. (2018)
Bacillus sp.	Firmicutes	*Fusarium culmorum* *F. oxysporum* *Monographella nivalis*	Przemieniecki et al. (2018)
Bacillus subtilis	Firmicutes	*Puccinia striiformis*	Reiss and Jørgensen (2017)
Burkholderia cenocepacia *Pseudomonas poae*	Proteobacteria Proteobacteria	*Alternaria alternata*	Ghosh et al. (2016a)
Burkholderia tropica *B. unamae* *B. cepacia*	Proteobacteria Proteobacteria Proteobacteria	*Alternaria alternata* *Rhizopus stolonifer* *Helminthosporium compactum*	Ghosh et al. (2016b)
Pseudomonas fluorescens	Proteobacteria	*Fusarium oxysporum*	Selvaraj et al. (2014)
Bacillus subtilis	Firmicutes	*Colletotrichum gloeosporioides*	Ashwini and Srividya (2014)
Bacillus simplex *B. subtilis*	Firmicutes Firmicutes	*Fusarium* sp.	Schwartz et al. (2013)
Bacillus sp.	Firmicutes	*Rhizoctonia solani*	Selva Kumar et al. (2013)
Brevibacillus laterosporus	Firmicutes	*Fusarium equiseti*	Prasanna et al. (2013)
Pseudomonas chlororaphis	Proteobacteria	*Fusarium oxysporum* *Rosellinia necatrix*	Calderón et al. (2013)
Pseudomonas chlororaphis	Proteobacteria	*Sclerotinia sclerotiorum* *Pythium aphanidermatum* *Macrophomina phaseolina* *Rhizoctonia solani* *Sclerotium rolfsii* *Fusarium oxysporum* *Alternaria solani* *Botryodiplodia theobromae*	Kumar and Dubey (2012)

(continued)

Table 22.3 (continued)

PGPR	Phylum	Pathogen	Reference
Rhizobium leguminosarum *Bacillus subtilis* *Pseudomonas* sp.	Proteobacteria Firmicutes Proteobacteria	*Macrophomina phaseolina* *Fusarium oxysporum* *F. solani* *Sclerotinia sclerotiorum* *Rhizoctonia solani*	Kumar (2012)
Bacillus antiquum	Firmicutes	*Macrophomonia phaseolina*	Gopalakrishnan et al. (2011)
Pseudomonas aeruginosa	Proteobacteria	*Aspergillus niger* *Helminthosporium* sp. *Fusarium oxysporium*	Hassanein et al. (2009)
Bacillus licheniformis	Firmicutes	*Gibberella saubinetii* *Aspergillus niger*	Xiao et al. (2009)
Rhizobium spp.	Proteobacteria	*Fusarium oxysporum*	Mazen et al. (2008)
Bacillus amyloliquefacines	Firmicutes	*Fusarium oxysporum*	Chen et al. (2007)
Rhizobium leguminosarum	Proteobacteria	*Pythium* spp.	Huang and Erickson (2007)
Pseudomonas fluorescens	Proteobacteria	*Pythium ultimum* *Rhizoctonia solani*	Andersen et al. (2003)
Rhizobium sp.	Proteobacteria	*Macrophomina phaseolina*	Deshwal et al. (2003)
Myxococcus sp.	Proteobacteria	*Cylindrocarpon* sp. *Fusarium oxysporum* *Phytophthora capsici* *Pythium ultimum* *Rhizoctonia* sp. *Sclerotinia minor* *Verticillium albo-atrum* *V. dahliae*	Bull et al. (2002)
Streptomyces sp.	Actinobacteria	*Pythium ultimum* *Fusarium oxysporum*	Castillo et al. (2002)
Pseudomonas fluorescens	Proteobacteria	*Fusarium oxysporum* f.sp. *ciceris*	Rangeshwaran and Prasad (2000)
Pseudomonas fluorescens	Proteobacteria	*Rhizoctonia solani*	Ligon et al. (2000)
Bacteria as phytopathogen			
Pseudomonas stutzeri *P. alcaligenes* *P. aeruginosa* *P. denitrificans* *P. syringae* *P. fluorescens*	Proteobacteria Proteobacteria Proteobacteria Proteobacteria Proteobacteria Proteobacteria	*Ralstonia solanacearum*	Mohammed et al. (2020)
Streptomyces sp.	Actinobacteria	*Burkholderia glumae*	Suarez Moreno et al. (2019)
Bacillus amyloliquefaciens	Firmicutes	*Ralstonia solanacearum*	Etesami and Alikhani (2017)

(continued)

Table 22.3 (continued)

PGPR	Phylum	Pathogen	Reference
Nematode as phytopathogen			
Pseudomonas aeruginosa *Burkholderia gladioli*	Proteobacteria Proteobacteria	*Meloidogyne incognita*	Khanna et al. (2019)
Pseudomonas fluorescens *Rhizobium leguminosarum*	Proteobacteria Proteobacteria	*Meloidogyne javanica*	Tabatabaei and Saeedizadeh (2017)
Bacillus velezensis *B. mojavensis*	Firmicutes Firmicutes	*Heterodera glycines*	Xiang et al. (2017)
Bacillus tequilensis *B. flexus*	Firmicutes Firmicutes	*Meloidogyne incognita*	Tiwari et al. (2017)
Bacillus sp. *Lysobacter* sp.	Firmicutes Proteobacteria	*Meloidogyne incognita*	Zhou et al. (2016)
Pseudomonas fluorescens *Bacillus Subtilis*	Proteobacteria Firmicutes	*Meloidogyne graminicola*	Priya (2015)
Pseudomonas fluorescens	Proteobacteria	*Helicotylenchus multicinctus*	Selvaraj et al. (2014)
Pseudomonads putida *P. fluorescens* *Serratia marcescens* *Bacillus amyloliquefaciens* *B. subtilis* *B. cereus*	Proteobacteria Proteobacteria Proteobacteria Firmicutes Firmicutes Firmicutes	*Meloidogyne incognita*	Almaghrabi et al. (2013)
Insect (Pest) as phytopathogen			
Pseudomonas protegens	Proteobacteria	*Galleria mellonella*	Bensidhoum et al. (2016)

(Ahemad and Kibret 2014). PGPR additionally produce various defensive lytic enzymes such as chitinase, glucanase, cellulase, protease, chitosanase, peroxidase, catalase, phenolic lyase, superoxide dismutase, etc. (Aloo et al. 2019) which can act to protect plants from the pathogens. Pathogens responsible for several plant diseases are directly liable for plant growth inhibition and these are mainly fungi and insects (Banerjee and Mandal 2019). The lytic enzymes like chitinase, chitosanase, glucanase and cellulases produced by PGPR act in a straight line biocontrol mechanism against the chitin and glucan cell wall components of those fungi and insects. Disease control management by the PGPR is additionally accomplished not only by means of antibiotics produced like zwittermicin, mycosubtilin, gramicidin S, polymyxin B, bacilysin, rhizocticins, etc. but also by bacteriocins (Saraf et al.

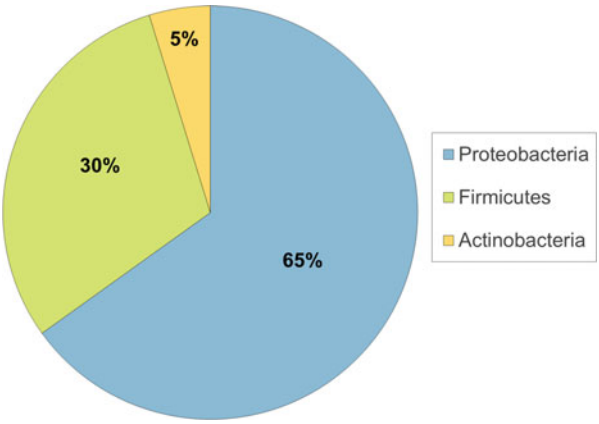

Fig. 22.7 Diversity and abundance of PGPR with biocontrol potentiality

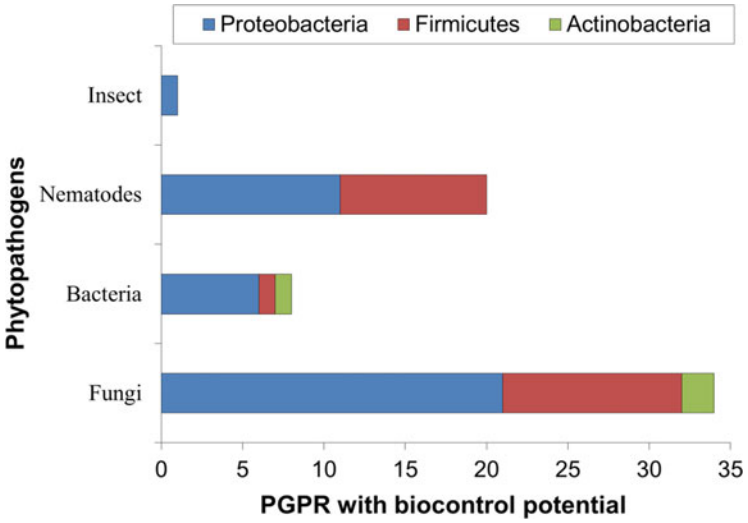

Fig. 22.8 Biocontrol proficiency of various PGPR against different phytopathogens

2014; Haggag 2008; Leclere et al. 2005; Chin-A-Woeng et al. 2003). Enhancement of plant defense mechanisms by a combination of ISR plus biocontrol ability was also validated by studies of several PGPR that produce VOCs (Shafi et al. 2017; Cao et al. 2011). The occurrence of such dual potentiality can be exemplified by VOCs like 2, 3-butanediol, isoprene and acetoin that are produced by different PGPR (Lee et al. 2015; Ryu et al. 2004). Plant pathogens can also be controlled by many PGPR via HCN production, a recognised VOC which disrupts the electron transport system that leads to blocking the energy supply of the pathogens (Patel and Minocheherhomji 2018).

In recent years, biocontrol has become an emerging and promising technological approach in developing sustainability in agriculture with optimism both for its comprehensive potentiality against various types of plant pathogens as well as its being an efficient alternative resource over chemical fungicides and pesticides. In addition, several PGPR have been documented for their ability to remediate heavy metals in agricultural fields. There are indeed many published reports on heavy metal remediation by the PGPR (Table 22.2); although reporting on the combinational effect of HM bioremediation cum biocontrol activity by PGPR is very scarce. Two such examples of combined activity by PGPR are *Alcaligenes* sp. and *Pseudomonas aeruginosa*, where nickel and manganese bioremediations were testified along with aptitude for biocontrol of phytopathogens like *Aspergillus niger, A. flavus, Fusarium oxysporum, Alternaria alternata, Cercospora arachichola* and *Metarhizium anisopliae* (Sayyed and Patel 2011). There is some justifiable optimism that the application of this kind of heavy metal remediating cum biocontrolling PGPR in agricultural fields will replace the usage of chemical pesticides and fertilisers, which in turn will decrease the bioaccumulation of hazardous chemicals into agronomic plants and passage of these contaminants further up the biological chain, leading to a more environmentally safe and affordable agriculture in terms of human welfare. However, the effective biocontrol property of PGPR against invading phytopathogens is subject to the considerations of soil type, host plant species and influential holobiont microbial community in the rhizosphere (Subrahmanyam et al. 2020).

22.6 Mechanism of Heavy Metal(loid) Resistance by Plant Growth-Promoting Rhizobacteria

Plant-associated HM-resistant PGPR are more profoundly present in heavy-metal-contaminated soil, as evidenced by many earlier publications (Pandey et al. 2010; Chen et al. 2016; Treesubsuntorn et al. 2018; Pramanik et al. 2017, 2018a, b; Mitra et al. 2018a, b). Such PGPR strains are known to develop resistance mechanisms in adaptation to the different HM ions present in their habitats (Table 22.4). The various known survival strategies which metal tolerant species have used to combat HMs are summarised in Table 22.4. These include active transport of metal ions (efflux/influx) by the presence of a group of specific membrane bound, cytoplasmic or periplasmic metal transporters (Nies 2003; Yang et al. 2019), production of biodegradable metal chelators like siderophores (Sinha and Mukherjee 2008; Dimkpa et al. 2008), intracellular bioaccumulation and biosorption (Chen et al. 2016; Treesubsuntorn et al. 2018; Pramanik et al. 2017, 2018a, b; Mitra et al. 2018a, b; Pal and Sengupta 2019), enzymatic oxidation and reduction metal transformations (Chatterjee et al. 2009; Pramanik et al. 2016; Ghosh et al. 2018; Kamaruzzaman et al. 2019), extracellular complexation by the secretion of extracellular polysaccharides (EPSs) (Gupta and Diwan 2017), etc. (Table 22.4). The genetic determinants of

Table 22.4 General mechanism of heavy metal(loid)-resistant PGPR including rhizobia

PGPR and Rhizobia	Heavy metal resistance	Proposed mechanism	References
Serratia marcescens S2I7	Cd(II)	Detoxification of Cd(II) by glutathione S-transferase (GST) mechanism and *czcD* gene-mediated protein	Kotoky et al. (2019)
Lysinibacillus varians KUBM17 *Pseudomonas putida* KUBM18	Cd(II), Pb (II)	Bioaccumulation of Cd(II) and Pb(II)	Pal and Sengupta (2019)
Caulobacter flavus RHGG3^T	Co(II), Cd (II), Zn(II)	Export of Co(II), Cd(II), Zn(II) metal cations from both cytoplasm and periplasmic space to outside of cell by efflux transporter protein encoded by several *czc* genes such as *czcA, czcB, czcC* and *czcD*. Another gene *znt* found to be involved in Cd(II) resistance encoded a Cd(II) exporting ATPase	Yang et al. (2019)
	Cu(II)	Cu(II) resistance by several efflux proteins encoded by different *cop* genes and also by multicopper oxidase protein encoded by *cueO*. Another gene system *cut* also found to be involved in Cu(II) resistance	
Bacillus cereus, *Bacillus aerius, Exiguobacterium profundum*	Cr(VI)	Reduction of Cr(VI) into Cr(III) and by adsorption of Cr(VI)	Kamaruzzaman et al. (2019)
Curtobacterium sp. GX_31, *Sphingomonas* sp. GX_15	Cd(II)	Biosorption of Cd(II) by physical entrapment, ion exchange and complexation on cell surface	Li et al. (2018)
Cupriavidus necator GX_5	Cd(II)	Bioaccumulation of Cd(II)	
Enterobacter sp. S2	Cd(II)	Bioaccumulation of Cd(II)	Mitra et al. (2018a)
Klebsiella michiganensis S8	Cd(II)	Cytosolic accumulation of cadmium	Mitra et al. (2018b)
Enterobacter aerogenes K6	Cd(II)	Bioaccumulation of Cd(II)	Pramanik et al. (2018a)
Bacillus aryabhattai MCC3374	As (III) and As (V)	Bioaccumulation, Biotransformation of As(V) to As(III) by arsenate reductase respectively	Ghosh et al. (2018)
Klebsiella pneumoniae K5	Cd(II)	Bioaccumulation of Cd^{2+} ions and biosorption of Cd^{2+} by negatively charged EPS	Pramanik et al. (2017)
Cellulosimicrobium funkei AR6	Cr(VI)	Bioreduction of Cr(VI) to Cr(III) without extracellular donor, immobilisation	Karthik et al. (2017a, b)

(continued)

Table 22.4 (continued)

PGPR and Rhizobia	Heavy metal resistance	Proposed mechanism	References
		of Cr(III) by cell wall, intracellular accumulation of Cr(III)	
Enterobacter sp. P36	Cu(II)	Cu(II) accumulation in bacterial cell	Sharaff et al. (2017)
Bacillus aryabhattai AB211	Cu(II)	Resitance by Cu(II) ion efflux system P-type ATPase (CopA), and copper resistance CopC/CopD protein	Bhattacharyya et al. (2017)
	Co(II), Zn (II), Cd(II)	Resitance due to Co(II)/Zn(II)/Cd (II) resistance protein CzcD and heavy metal resistance transcription regulatory protein HmrR. Zn(II) resistance also conferred by Sensor protein of zinc sigma-54-dependent two-component system and its regulatory protein	
	As(V) and As(III)	Arsenic resistance by arsenic efflux protein pump and arsenate reductase enzyme	
Enterobacter sp. EG16.	Cd(II)	Intracellular accumulation, biosorption by physical adsorption, ion-exchange and complexation on cell surface	Chen et al. (2016)
Bacillus flexus ASO-6	As (III) and As (V)	Oxidation of As(III) by arsenite oxidase encoded by *aoxB* gene	Das et al. (2016)
Rhizobium sp. ND2	Cr(VI)	Reduction of Cr(VI) to Cr(III), adsorption of chromium on cell wall	Karthik et al. (2016)
Raoultella sp. CrS2	Cr(VI)	Cr (VI) reduction by constitutive chromate reductase enzyme	Pramanik et al. (2016)
Bradyrhizobium japonicum	Pb(II), Ni (II)	Biosorption of Pb(II) and Ni(II) metal ions by amino, nitro functional groups present on bacterial cell wall	Seneviratne et al. (2016)
	Cu(II)	Biosorption of Cu(II) metal ions by alcoholic and amino functional groups present on bacterial cell wall	
Enterobacter cloacae HG 1 *Klebsiella pneumoniae* HG 3	Hg(II)	Mercury tolerance by EPS binding of mercury ions (hypothesised)	Gontia-Mishra et al. (2016)
Enterobacter ludwigii HG 2	Hg(II)	Mercury tolerance by *mer* operon (hypothesised)	
*Bacillus muralis*CA9 *B. muralis* CA16b *Bacillus simplex* CA15 *B. simplex* CA16a *B. simplex* CA22	Hg(II)	Reduction of Hg^{2+} into volatile Hg^0 by cytoplasmic mercuric reductase encoded by *merA* gene	Calzada Urquiza et al. (2016)

(continued)

Table 22.4 (continued)

PGPR and Rhizobia	Heavy metal resistance	Proposed mechanism	References
Bradyrhizobium japonicum E109	As (III) and As (V)	Bioaccumulation of As(III), reduction of As(V) to As(III) by arsenate reductase encoded by *arsC* gene and efflux by As(III) efflux pump encoded by *arsB* gene, oxidation of As(III), increased production of biofilm (possibly associated with resistance)	Armendariz et al. (2015)
Azospirillum brasilense Az39		Bioaccumulation of As(III), reduction of As(V) to As(III) by arsenate reductase encoded by *arsC* gene and efflux by As(III) efflux pump encoded by *arsB* gene, increased production of biofilm (possibly associated with resistance) Higher resistance to arsenic due to presence of two extra genes *arsH* and *Acr3* which encode NADPH:FMN oxide reductase and As(III) efflux protein respectively	
Rhizobium sp. CCNWSX0481 SV20, *Rhizobium leguminosarum* bv. *viciae* SV 15, *Pseudomonas* sp. SV23, *Enterobacter cloacae* SV27	Cu(II)	Bioaccumulation of Cu(II)	Fatnassi et al. (2015)
Pseudomonas spp. *Cronobacter* spp. *Bacillus* spp.	Hg(II)	Conversion of methyl mercury into Hg^{2+} ions in cell and conversion of toxic Hg^{2+} into less toxic form Hg_2S	Rafique et al. (2015)
Mesorhizobium amorphae 186	Cu(II)	Efflux of Cu(II) metal ions from cytoplasm to periplasmic space by P-type ATPase (CopA-6910), and CusAB detoxification of periplasm by exporting Cu(II) ions from periplasm to extracellular spaces	Hao et al. (2015)
Enterobacter cloacae AW1 *Pseudomonas fluorescens* AW2 *Pseudomonas putida* AW4 *Pseudomonas poae* AW5 *Pseudomonas poae* AW6	As (III) and As (V)	Bioaccumulation	Oller et al. (2013)

(continued)

Table 22.4 (continued)

PGPR and Rhizobia	Heavy metal resistance	Proposed mechanism	References
Pseudomonas aeruginosa OSG41	Cr(VI)	Bio-reduction of hexavalent chromium	Oves et al. (2013)
Rhizobium leguminosarum RL 9	Ni(II)	Metal adsorption/desorption	Wani and Khan (2013)
Pseudomonas aeruginosa WI-1	Pb(II)	Metallothionein (encoded by *bmtA* gene) mediated metal sequestration and intracellular bioaccumulation	Naik et al. (2011)
Sinorhizobium spp.	Zn(II), Cd (II), Pb(II), Cu(II)	Adsorption of heavy metal ions on cell surface, intracellular accumulation of heavy metal ions	Zribi et al. (2011)
Ochrobactrum cytisi Azn6.2	Cd(II), As (II), Zn(II), Cu(II)	Biosorption/ Desorption by lipopolysaccharides of cell wall	Rodríguez-Llorente et al. (2010)
Bacillus spp., *Achromobacter* spp., *Brevundimonas* spp., *Microbacterium* spp., *Ochrobactrum* spp. *Ensifer* spp. *Bosea* spp. *Sinorhizobium* spp. *Bordetella* sp. *Ancylobacter dichloromethanicum* As3-1b *Georgenia ferrireducens*As5-12 *Rhodococcus erythropolis*As5-4a	As (III) and As (V)	Reduction of As(V) into As(III) by arsenate reductase encoded by *ArsC* gene, efflux of As(III) by *ArsB* and *ArsA* genes which code for As(III) efflux pump and used proton motive force and AS(III) activated ATPase Another gene *ACR3* homologous to *ArsB* also codes for As(III) efflux protein in highly resistance strains Either one or both types of genes in combination confer resistance among these bacteria	Cavalca et al. (2010)
Mesorhizobium sp. RC1, *Mesorhizobium* sp. RC4	Cr(VI)	Reduction of Cr(VI)	Wani et al. (2009)
Cellulosimicrobium cellulans KUCr3	Cr(VI)	Reduction of Cr(VI)	Chatterjee et al. (2009)
Azotobacter chroococcum HKN-5 *Bacillus megaterium* HKP-1	Pb(II), Cd (II)	Adsorption of Pb^{2+} and Cd^{2+} on cell wall	Wu et al. (2009)
Enterobacter asburiae PSI3	Cd(II)	Complexation of metal by extracellularly secreted organic acids	Kavita et al. (2008)
Rhizobium sp. RP5	Zn(II), Ni (II)	Metal adsorption/desorption	Wani et al. (2008a)
Rhizobium leguminosarum RL 9	Zn(II)	Metal adsorption/desorption	Wani et al. (2008b)

(continued)

Table 22.4 (continued)

PGPR and Rhizobia	Heavy metal resistance	Proposed mechanism	References
Pseudomonas putida ARB86	Ni(II)	Absorption and accumulation of Ni in cells	Someya et al. (2007)
Bradyrhizobium sp. (vigna) RM8	Zn(II), Ni (II)	Metal adsorption/desorption	Wani et al. (2007)
Brevibacillus brevis B1	Zn(II)	Bioaccumulation and Biosorption	Vivas et al. (2006)
Pseudomonas aeruginosa sp. NBRI 4014 mutants	Cr, Cd(II), Ni	Bioaccumulation and internal sequestration by resistant enzymes	Gupta et al. (2004)
*Azospirillum lipoferum*137 *Agrobacterium radiobacter*10	Cd(II)	Accumulation of Cd	Belimov et al. (2004)
Azospirillum brasilense Sp245	Co(II)	Rapid adsorption of Co^{2+} on cell surface followed by rapid metabolic transformation	Kamnev et al. (2004)
Pseudomonas putida PNL-MK25	Cu(II)	Efflux of Cu(II) metal ions by P1-type ATPase (CueA)	Adaikkalam and Swarup (2002)
Serratia plymuthica Br-10	Cd(II)	Bioaccumulation	Carlot et al. (2002)

metal resistance can be localised either in chromosomal or extrachromosomal genetic elements.

Heavy metals most commonly exist in the form of cations which can form many unspecific complexes. Among all these, a few HM cations are important biological trace elements (such as Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Mo^{2+}, Co^{2+}) used in regulating several important biochemical reactions. The intracellular passage of different HMs is, in fact, governed by two opposite types of uptake systems. The first of these systems is constitutively expressed, fast, unspecific and uses a variety of substrates, while the second system is inducible, slow and highly specific for substrates (Nies 1999). The main driving force for the first system is an electrochemical gradient across the plasma membrane, and for the second system it is the energy generated by ATP hydrolysis (Nies and Silver 1995). The constitutive and unspecific nature of the first kind of system causes most of the HM-toxicity in bacteria as it continuously accumulates a heavy metal even if the cell already contains a high concentration of that same HM (Nies and Silver 1995). After a metal has been accumulated beyond threshold levels, HMs impart several toxic effects such as inhibition of enzyme actions due to the binding of Hg^{2+}, Cd^{2+} and Ag^{2+} to -SH groups, generation of oxidative stress and inhibition of the activity of sulphate and phosphate compounds by structurally related chromate and arsenate, respectively. Briefly, there are six widely known heavy metal resistance mechanisms in bacteria, they are: (1) exclusion of HMs by permeability barriers, (2) extracellular sequestration, (3) intracellular

sequestration, (4) enzymatic detoxification of HMs, (5) active transport or efflux system of HMs and (6) reduction in HM sensitivity of cellular targets.

However, the details of many heavy metal resistance mechanisms used by PGPR are still to be fully explored, and we will have to unravel the genetic mysteries behind metal-PGPR interactions to effectively apply them for HM-bioremediation.

22.7 Constraints in the Application of Plant Growth-Promoting Rhizobacteria

Although the PGPR strains far discovered have proven promising in controlled laboratory conditions, their efficacy in reality is contingent on how they act in field conditions. During the last few decades, a number of PGPR strains have been discovered around the world but few reached the ultimate goal of having utility for farmers. In contrast to the laboratory, the reality of field work is one of non-optimal conditions that may or may not be favouarbale for the survival and proliferation of the PGPR strains (Glick 2020). The existence and growth of field-applied PGPR strains indeed depends on a vast range of adverse environmental factors that need to be overcome so that the microbes take part in assisting plant growth-promotion activities in contaminated soil (Fig. 22.9). It is not an easy task to achieve successful application of such PGPR strains even if they hold a bunch of potentially beneficial

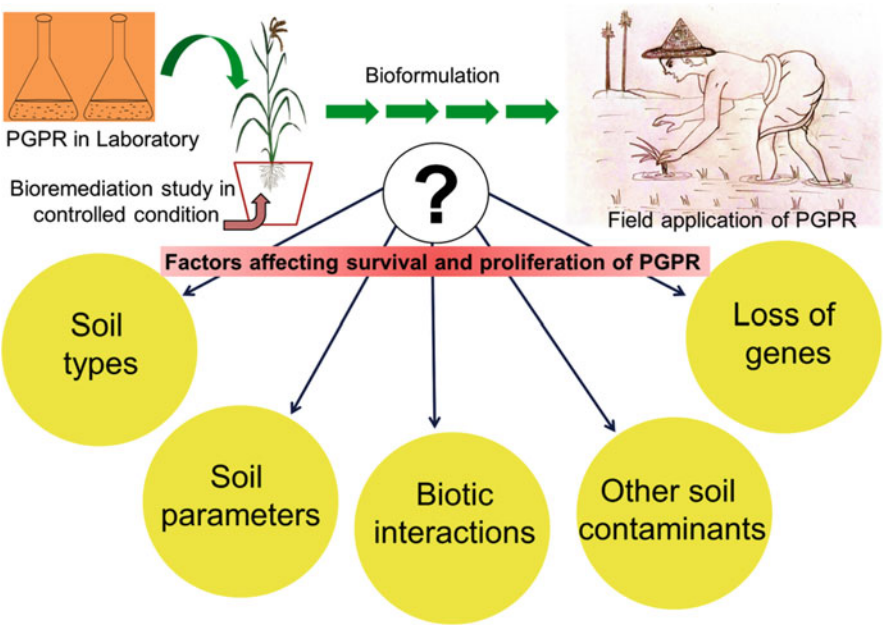

Fig. 22.9 Factors affecting survival and proliferation of PGPR

traits for the crop plants. Apart from following government-enforced guidelines, one of the major constraints in field application is soil type and it directly influences the survival and growth of the microbial communities (Fig. 22.9). To introduce a genetically engineered orgainsm, we need to give special attention to the fact that government legislation varies from country-to-country. Soil parameters such as compaction, oxygen content, pH and temperature are also crucial in this respect because they can affect the functioning of the microbes. In contrast to wild type indigenous strains, the genetically modified organisms are often less adaptive perhaps as a consequence of burdensome metabolic demands due to the expression and perhaps overexpression of foreign DNA (Glick 2020). In addition, PGPR strains often do not have equal abilities to compete with soil-borne phytopathogens and other antagonistic soil microbial communities, the PGPR strains sometimes do not have the capacities to tolerate a wide range of soil contaminants, and habituation to growing in nutrient-rich media under laboratory conditions may have resulted in functional loss of active genes that previously made the microbes suitable in contaminated rhizopshere environments (Glick 2020; Fig. 22.9).

22.8 Conclusion

Heavy metal(loid)-affected agricultural crops have benefitted for many years from the application of indigenous HM-resistant PGPR. Although there are a lot of constraints associated with the application of these microorganisms, their great diversity and natural abundance in contaminated soil offers a ray of hope as we explore their potential role in agriculture. Recent advancements in bioremediation strategies have given us cause for optimism. But, before field application, these PGPR should be verified for their degree of metal resistance, their level of plant growth-promoting traits, and obviously their ability to reduce HM-content in plant parts under controlled conditions. Henceforth, these PGPR are naturally dwelling microflora that should be isolated, enriched and applied for sustainable agriculture in HM-contaminated fields.

Contributing authors of this book chapter

Acknowledgements KP and SB are thankful for financial assistance from the University Grants Commission, India for UGC—Dr. D. S. Kothari Post-Doctoral Fellowship [No.F.4-2/2006 (BSR)/BL/19-20/0072 dated October 21, 2019] and Department of Biotechnology, India for granting DBT Twinning Project [No. BT/PR25738/NER/95/1329/2017 dated December 24, 2018], respectively. DM acknowledges DST-PURSE program, Visva-Bharati for financial support.

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Chapter 23
Defensive Microbiomes: A Widespread Phenomenon in Nature

Sarah Worsley

Abstract Microbes, such as bacteria and fungi, produce antibiotic compounds during competition with other species for resources such as space and nutrients. Such compounds have underpinned much of modern medicine as, in their purified form, they are widely prescribed by humans as antibiotics to cure bacterial and fungal infections. However, numerous other organisms have been using the antimicrobial products of microbes to protect themselves against disease for millennia, with many eukaryotic species forming close mutualistic interactions with defensive microbes which live on or within their host species. In addition to producing antibiotics, these microbes can inhibit infection by stimulating their host's immune system and by competing with, and thus excluding, pathogenic organisms. Developing an understanding of how interactions between hosts and protective microbes arise and are effectively maintained over time, despite the evolution of pathogenic resistance, could inform our own use of antibiotics as well as novel therapies. This essay will discuss the prevalence of defensive microbiomes in nature and how their assembly may inform future strategies to protect against disease.

23.1 Microbes Underpinning Modern Medicine

Humans have been widely using antibiotics to cure diseases caused by bacterial and fungal agents since the late nineteenth century. Almost all of the earliest antibiotics, and many of those used today, are the purified natural products of microbes which have been isolated from environmental samples, such as soil (Gould 2016; Hopwood 2007). In natural systems, microorganisms produce these antibiotic compounds to kill or inhibit the growth of other microbial species during competition for resources such as nutrients or space.

Even before it was known that microbes were the source of many antimicrobial compounds, ancient civilisations are thought to have used their products to prevent

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_23

and cure diseases. For example, there is evidence that societies in Egypt and Rome topically applied mouldy bread to wounds to prevent infections. Presumably, the fungal species that were growing on the bread were producing antimicrobials that inhibited the proliferation of pathogenic microbes (Gould 2016). Famously, Alexander Fleming serendipitously showed that fungi could make such compounds by leaving agar plates growing a bacteria, called *Staphylococcus,* uncovered whilst away on holiday. He noticed that a contaminating fungus, called *Penicillium notatum,* created bacteria-free zones wherever it was growing, suggesting that the fungus was producing a potent antibacterial which he named penicillin (Fleming 1929). Penicillin was the first modern antibiotic to be mass-produced and was hugely important during World War II when it was used to treat the infected wounds of injured soldiers (Dias et al. 2012). Around the same time that Fleming discovered penicillin, a scientist called Selman Waksman was studying a phylum of bacteria called Actinobacteria. Waksman observed that many actinobacterial species isolated from soil could selectively inhibit the growth of other microorganisms when they were grown together in the laboratory (Hopwood 2007). This finding led to a systematic search for actinobacterial isolates that could kill disease-causing bacteria and fungi. This, in turn, resulted in the isolation of several clinically useful antibiotics, including streptomycin which is produced by the bacterial species *Streptomyces griseus* and can be used to treat a variety of bacterial infections, including tuberculosis (Schatz et al. 1944). Currently, the phylum Actinobacteria is responsible for producing over half of all clinically useful antibiotics (Hopwood 2007; van der Meij et al. 2017; Devine et al. 2017). Genome sequencing has also revealed that many isolates have the genetic potential to produce a huge diversity of different natural products that may demonstrate novel activities and could also be exploited in the future.

Thus, microbially produced antibiotics have been instrumental in reducing human mortality throughout history, but particularly over the last century when they became widely prescribed in the clinic. However, it is not only humans that have exploited the products of microbial competition to prevent infection and disease. In fact, an increasing number of organisms with markedly distinct natural histories are being found to accumulate microbial species that produce antimicrobial compounds. These microbes, in turn, are being shown to protect their hosts against infections caused by parasitic and pathogenic microorganisms.

23.2 The Microbiome

Almost all organisms, at some stage during their lifecycle, interact extensively with a complex community of microorganisms which make use of host resources and are acquired from the host's environment. These diverse microbial assemblages, their collective genomes as well as the host habitat, are collectively referred to as an organism's microbiome (Marchesi and Ravel 2015). Advances in nucleic acid sequencing technologies have enabled us to investigate the composition and function

of these microbial communities in great detail and many studies have demonstrated that there are often consistent patterns in the microbial groups that associate with a particular host species. For example, in humans the early-life infant gut is almost always dominated by certain species of the bacterial genus *Bifidobacterium*, which are then superseded by members of the phyla Firmicutes and Bacteriodetes later in adult life (Matamoros et al. 2013). Furthermore, several microbial communities, for example those associated with plants and insects, have been found to be tightly linked to host phylogeny, suggesting that particular microbial assemblages are maintained and are changing over evolutionary time alongside their host (Brucker and Bordenstein 2012; Fitzpatrick et al. 2018; Sanders et al. 2014).

The non-random accumulation of microbial communities within the host microbiome suggests that host species may be able to influence which microorganisms they associate with. In fact, hosts are expected to experience strong natural selection to filter the enormous pool of microbial species available to them and evolve mechanisms that encourage the persistence of microbes that provide them with significant fitness benefits (Archetti et al. 2011; Foster et al. 2017; Scheuring and Yu 2012). Microorganisms can be advantageous to their host in a number of different ways. For example, many provide their hosts with nutritional benefits by breaking down complex, otherwise indigestible molecules, or by supplementing the host diet with essential nutrients through pathways such as nitrogen fixation or phosphate solubilisation. Other symbionts, which are the focus of this essay, can provide protective benefits to their host by inhibiting the growth and invasion of pathogenic and parasitic organisms.

23.2.1 Leafcutter Ants and Their Protective Microbes

Attine leafcutter ants represent a fascinating example of a defensive mutualism between antimicrobial-producing bacteria and a eukaryotic host. Leafcutter ants are indigenous to Central and Southern America and are renowned for their specialised agricultural activities. Worker ants collect fresh leaf material from their surrounding environment which is then taken back to their nests and used as a compost to grow a mutualistic food fungus, called *Leucoagaricus gongylophorous* (Worsley et al. 2018; Currie 2001). In return for a growth substrate, the fungus produces swellings called gongylidia which are rich in lipids and proteins. These are harvested by the ants and used as the sole nutrients source for the queen and her larvae (De Fine Licht et al. 2014; Currie 2001).

Although this system is effective, the clonal fungal cultivar is a rich food source and is therefore at risk from being parasitised by other organisms. Indeed, another fungus called *Escovopsis weberi* is highly specialised to grow on the food fungus and, if left unchecked, can cause ant colony collapse. This occurs when the ants are starved of their food source and eventually die or abandon their nest (Currie et al. 1999a; de Man et al. 2016). However, the ants have evolved several lines of defence against such invasions, including specialised behaviours that enable them to detect

and weed out pieces of infected garden (Currie and Stuart 2001). As an additional line of defence, leafcutter ants also interact extensively with antibiotic-producing bacteria in the phylum Actinobacteria. These bacteria grow as a visible white mass on specialised structures that are found on particular regions of the ants cuticle (Currie et al. 1999b; Andersen et al. 2013; Kost et al. 2007; Currie et al. 2006). Fueled by competition for the nutrients provided by the ant host, the bacterial mutualists produce a variety of antibacterial and antifungal compounds. These compounds have been shown to inhibit the growth of other microorganisms that might invade the fungus garden, including the parasite *E. weberi,* and have also been identified at active concentrations in the nests of leafcutter ants (Currie et al. 1999b, 2003; Barke et al. 2010; Haeder et al. 2009; Sen et al. 2009; Worsley et al. 2018; Schoenian et al. 2011).

However, there is an interesting evolutionary twist to this story—*E. weberi* has recently been shown to combat both of the ants two major lines of defence. The parasite has evolved to produce chemicals, called shearinine D and melinacidin IV, that not only prevent the weeding behaviours of the ants by causing paralysis and eventual mortality, but that also inhibit the growth of the actinobacterial symbionts (Heine et al. 2018). Despite this, widespread resistance does not seem to be the case in nature and the attine-actinobacteria mutualism is thought to have survived and enabled ants to farm their fungus for over 50 millions years (Currie et al. 2006). Instead, a constant coevolutionary arms race seems to be occurring within the leafcutter ant system, involving the *Escovopsis* parasite, the ants, and their protective symbionts. The evolution of resistance in *Escovopsis* drives the evolution of novel antimicrobial compounds in the mutualistic Actinobacteria and therefore prevents the dominance of resistant parasite strains (Currie et al. 2006; Pathak et al. 2019; Worsley et al. 2018). Additionally, different actinobacterial species on the ants' cuticle produce different types of antimicrobial compound, resulting in a form of multidrug therapy whereby parasites are faced with too many compounds to evolve resistance to all of them at once (Barke et al. 2010; Seipke et al. 2011).

23.2.2 Actinobacteria as Protective Symbionts

Leafcutter ants are not alone in recruiting antibiotic-producing Actinobacteria to protect against disease. In fact, Actinobacteria are thought to be involved in approximately half of all described examples of defensive mutualism (Kaltenpoth 2009) and interact with a range of terrestrial and marine invertebrates, as well as several plant host species (Kaltenpoth 2009; Seipke et al. 2012; Viaene et al. 2016). Apart from having a diverse secondary metabolism capable of producing many antimicrobial natural products, Actinobacteria are also characterised by a lifecycle involving filamentous growth and spore-forming stages. Members of this phylum are also capable of subsisting on a wide range of carbon sources and metabolic waste products that are often present at very low concentrations. Together, these characteristics may have enabled Actinobacteria to interact with a diverse range of hosts

and become so widespread as defensive symbionts (Kaltenpoth 2009). It is possible that many actinobacterial species began as commensals or mild parasites, competing with other microbial species for host resources. The production of antimicrobials during this microbial warfare may have, in turn, become beneficial to the host by preventing pathogenic infection. This may have driven the evolution of host mechanisms to ensure that Actinobacteria were consistently able to colonise the microbiome (Kaltenpoth 2009). The spore-forming capabilities of Actinobacterial species may have also aided this process by enabling species to resist environmental stressors that may be experienced in the absence of the host during inter-individual and inter-generational transmission (Kaltenpoth 2009).

The spore-forming capabilities of actinobacterial species may be particularly important during symbiosis with solitary 'Beewolf' digger wasps. Female solitary digger wasps (in the genera *Pilanthus*, *Trachypus* and *Philanthinus*) lay their eggs in burrows, which they dig into the soil and provision with a paralysed honey bee; the developing larvae feed on the bee before spinning cocoons (Kaltenpoth 2009; Kaltenpoth et al. 2005). The brood chambers are humid and damp providing optimal growth conditions for a variety of fungi and bacteria. However, to prevent developing infections, the mother wasp coats the brood chamber walls with secretions containing a species of Actinobacteria, called *Candidatus Streptomyces philanthi,* which the wasp cultures in specialised antennal glands (Kaltenpoth et al. 2005, 2006). These bacteria then become incorporated into the larval cocoon and produce antibiotics on the cocoon surface (Kroiss et al. 2010). Remarkably, the *Streptomyces* symbiont can remain viable on the cocoon wall as spores for up to nine months before the larva emerges, despite the cocoon surface being a very poor environment with limited nutrients availability (Kaltenpoth et al. 2010). This long-term survival also allows the *Streptomyces* bacteria to be vertically transmitted across wasp generations as they are then taken-up by the fully-developed females that emerge from the cocoons (Kaltenpoth 2009; Kaltenpoth et al. 2010).

23.2.3 Competitive Exclusion and Modulation of the Host Immune System

Actinobacteria are important symbionts for many organisms, however, defensive microbes are not limited to this bacterial phylum. In fact, a large number of other bacteria, across a wide range of phyla, are also known to provide their hosts with protection against disease. For example, hoopoe birds (*Upupa epops*) are known to culture high densities of *Enterococcus* bacteria in their uropygial (preen) glands; these bacteria produce volatile antimicrobial substances that are known to inhibit the growth of feather-degrading microorganisms (Martin-Platero et al. 2006; Martin-Vivaldi et al. 2010). Similarly, embryos of several species of crustacean are coated in a dense growth of Gram-negative bacteria that produce antifungal compounds

against the fungal pathogen *Lagenindium callinectes* (Gil-Turnes and Fenical 1992; Gil-Turnes et al. 1989).

Most described examples of defensive mutualisms involve bacterial partners. In comparison, far less is known about the role that fungal species can play in providing protective benefits to host organisms. This is partly because, relative to bacteria, fungi remain hugely understudied in the context of the microbiome. They can also be difficult to culture, making it hard to characterise their function. However, genomic studies as well as bioactivity assays using fungal isolates have demonstrated that many fungal species encode a diverse secondary metabolism capable of making a large arsenal of different antimicrobials (Rateb and Ebel 2011). Fungal species are also hugely abundant in the microbiomes of many species and in some cases, such as sponges, are transmitted maternally across generations suggesting that they can be closely associated with their host organisms (Maldonado et al. 2005). One of the few examples of a defensive fungal symbiont is the association between leaf rolling weevils (*Euops chinensis*) and the fungal species *Penicillium herquei*. The *Penicillium* symbiont is added to the leaves in which the weevils roll their eggs and larvae. Here, it has been shown to produce the antimicrobial scleroderolide which inhibits the growth of microbial pathogens (Wang et al. 2015). With greater study and the development of new techniques, further examples of defensive partnerships involving fungi may come to light.

Antibiotic production is a key mechanism by which microbes are able to protect their host against infection. However, this often also works in addition to, or in combination with, other mechanisms. By colonising a host and taking up resources such as space and nutrients, symbiotic microbes can also competitively exclude pathogenic or parasitic microorganisms which use the same resources. For example, in mice, a bacterial species called *Bacteroides thetaiotaomicron*, is known to consume carbohydrates that are required for the growth of *Citrobacter rodentium* (a mucosal pathogen of mice). By preventing access to a key resource, *B. thetaiotaomicron* is able to exclude the pathogenic species from the mouse intestinal lumen (Buffie and Pamer 2013).

Members of the microbiome can additionally contribute to the defence of their host by priming the host immune system so that it can efficiently respond to pathogenic attack. For example, evidence from mouse models suggests that commensal bacteria in the intestinal tract can enhance host immunity by directing the development of immune cell populations involved in both innate and adaptive immune processes. These bacteria also promote the production of antimicrobials and pro-inflammatory factors by cells in the gut (Buffie and Pamer 2013; Hooper et al. 2012). Similarly, bacterial symbionts that colonise plant roots, such as *Bacillus*, *Streptomyces* and *Pseudomonas* species, have also been shown to prime the plant immune system, resulting in an elevated and accelerated response to pathogenic infection (Pieterse et al. 2014; Kurth et al. 2014). The plant host recognises residues on the surface of these beneficial microbial species (called microbial associated molecular patterns, or MAMPs) which leads to the activation of signaling cascades involved in mounting an immune response. These pathways are then primed to respond to pathogenic invasion (Pieterse et al. 2014; Selosse et al. 2014).

23.3 Selecting Protective Microbes

In many cases, protective microorganisms appear to be a consistent part of, or even dominate, the host microbiome. For example, Actinobacteria massively outnumber other microbial species on the cuticles of leafcutter ants (Andersen et al. 2013) and Bifidobacteria dominate the infant human gut, where they inhibit the growth of pathogens and promote immune system development (Matamoros et al. 2013). A great challenge is to understand how a host can selectively associate with these microbial species when it is exposed to a huge environmental pool of microbes. Such knowledge could enable us to enhance the presence of beneficial microbes, for example in the human gut following a course of antibiotics, or in the roots of economically important crop plant species to reduce yield losses caused by disease.

23.3.1 The 'Partner Choice Problem'

The issue of how a host recruits specific microbes is often referred to as the 'partner choice problem'. This is because for a host to be able to accumulate beneficial species, it must be able to distinguish between different strains in its environment and limit its interactions to microbes that provide it with significant benefits (Archetti et al. 2011). For many instances of partner choice in nature individuals can distinguish between better or worse partners via costly phenotypes. For example, elaborate male ornaments, such as tail feathers, facilitate mate choice in many bird species, since only high-quality individuals can afford to invest in these (Archetti et al. 2011). However, with microbial-host interactions, it seems unlikely that such signals could exist and be detected by the host, allowing discrimination between thousands of microbial species. Instead other mechanisms are thought to enable hosts to indirectly bias the accumulation of beneficial microbial species from their environment (Archetti et al. 2011; Boza et al. 2019; Scheuring and Yu 2012).

One mechanism by which a host could encourage the colonisation of protective species within its microbiome is by giving certain microbes preferential access to resources in the host niche. The simplest way by which this can occur is by transmitting them vertically across host generations, rather than acquiring them horizontally from the environment (Boza et al. 2019). This is the case for leafcutter ants, which remain sterile before they hatch from the pupal stage (Marsh et al. 2014). Following hatching, they are inoculated with the antibiotic-producing Actinobacteria that grow on older worker ants, within a 24 h window (Marsh et al. 2014). These filamentous Actinobacteria then bloom over the ant cuticle in the absence of any competition, before receding to grow around specialised crypts which are thought to supply the cuticular microbiome with resources (Currie et al. 2006).

A second mechanism by which a host could drive the accumulation of beneficial microbial species is by providing its microbiome with specific nutrients that are

preferentially utilised by microorganisms with the desired metabolic capabilities, such as antibiotic production (Boza et al. 2019; Foster et al. 2017). This hypothesis can be extended, since resources can also drive competition between strains. Therefore, hosts could also provide their microbiome with resources that ensure beneficial microorganisms successfully outcompete other species (Archetti et al. 2011; Scheuring and Yu 2012). There are several examples of this occurring in corals which, along with their dinoflagellate symbionts, produce large quantities of the compound dimethylsulfoniopropionate (DMSP) (Raina et al. 2013). It is thought that bacteria that can degrade DMSP may have a nutritional advantage over non-degraders and that this compound could therefore be important in structuring the initial coral microbiome (Apprill et al. 2009; Raina et al. 2010). Interestingly, one bacterial coloniser, called *Pseudovibrio*, can also use DMSP as a precursor to produce antimicrobial compounds that inhibit the growth of coral pathogens, suggesting that DMSP may also play a role in fuelling competitive exclusion and host protection (Raina et al. 2016).

Finally, a host can also direct microbiome establishment by producing compounds or barriers that block the colonisation and survival of non- beneficial species, whilst still enabling or promoting colonisation by beneficial species (Boza et al. 2019). Plants exude a variety of toxic molecules, called allelochemicals, which inhibit a broad range of bacteria, fungi and invertebrates, as well as other plants growing in close proximity (Neal et al. 2012; Hartmann et al. 2009; Bais et al. 2006). Beneficial microbial species must be able to tolerate allelochemicals to colonise the root microbiome of the host plant. The compound DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is an antimicrobial allelochemical that is constitutively produced by maize seedlings and is toxic to many bacterial species (Neal et al. 2012). However, the plant-beneficial species, *Pseudomonas putida*, is able to degrade DIMBOA and additionally upregulates the production of a broad-spectrum antibiotic called phenazine in response to detecting the compound, allowing effective colonisation of the root microbiome and host protection against fungal pathogens (Neal et al. 2012).

23.4 Taking Inspiration from Defensive Microbiomes

As discussed, defensive microbiomes appear to be a widespread phenomenon in nature and in several instances, such as in the leafcutter ant and beewolf digger wasp systems, there is evidence to suggest that they have remained effective at suppressing pathogens over millions of years. On the flip-side, humans have been extensively using antimicrobials as medicine and in agriculture for around a century, but we have seen a rapid rise in pathogenic resistance and a concurrent decrease in the effectiveness of clinically available antibiotics. This contrast begs the question of whether there is anything to be learnt from the protective mutualisms that have evolved in nature between hosts and microbial species and if an understanding of how protective microbiomes evolve could lead to new ways to combat infections.

23.4.1 Novel Antimicrobial Compounds

With the rapid emergence of drug-resistant pathogens, there has been a renewed effort to search for novel antimicrobials in the environment. In the past, antimicrobials were isolated from soil samples, however, frequent re-discovery of the same compounds has encouraged scientists to explore other niches for new antimicrobials. Increasingly, it is thought that defensive microbiomes may yield structurally diverse and novel compounds that have a greater efficacy against human pathogens (Adnani et al. 2017; Chevrette et al. 2019; Seipke et al. 2012). Within the microbiome, microbial species face intense competition for host resources. This fuels the production of antimicrobial agents as species produce them to inhibit the growth of competing microorganisms. Symbiotic species that rely on their host for resources must also ensure continued host survival. Thus, coevolutionary dynamics between invading pathogens, symbionts and host organisms, are expected to result in the continued evolution of novel antimicrobial compounds with distinct activities. These may be able to target pathogen populations that are clinically relevant (Pathak et al. 2019; Adnani et al. 2017; Chevrette et al. 2019). For example, a group of compounds called the formicamycins have recently been isolated from a species of *Streptomyces* growing in association with African *tetraponera* plant-ants; these compounds were shown to inhibit multidrug resistant pathogens which showed no evidence of being able to evolve resistance, suggesting that the formicamycins had a highly effective mode of action (Qin et al. 2017). Looking within microbiomes for new antimicrobials may also prove advantageous, since compounds produced by beneficial microbes that interact with a eukaryotic host are likely to prove less toxic to human cells, or those of other animal and crop species (Adnani et al. 2017).

23.4.2 Safeguarding Our Antimicrobials

There is evidence to suggest that several defensive mutualisms have remained effective for millions of years, with little evidence of pathogenic resistance evolving in these systems. For example, Beewolf digger wasps are thought to have been using the same antibiotics (produced by their *Streptomyces* symbionts) since the Cretaceous period (Engl et al. 2018). It is thought that pathogen resistance is avoided in this system because the *Streptomyces* symbionts produce a large variety of antimicrobials at any one time. In fact, it has been shown that multiple antimicrobials, that vary slightly in their structure and activity, can be produced from the same gene in the *Streptomyces* symbiont's genome (Engl et al. 2018). This variable cocktail, in addition to the targeted application of antibiotics in the larval brood chamber, is thought to reduce the chances of pathogen resistance evolving, as multiple cellular processes are targeted at once. Such multidrug strategies are thought to be common across vertebrate and invertebrate species (Florez et al. 2015). Leafcutter ants are also known to use multidrug therapy to combat infections in their fungal gardens;

worker ants host slightly different actinobacterial communities on their cuticles and each bacterial specie is capable of making multiple different antimicrobials, which are also thought to be constantly evolving (Barke et al. 2010; Seipke et al. 2011; Worsley et al. 2018). Although the use of multidrug therapy in humans is controversial, varying our antibiotic usage and ensuring that their application is targeted (to bacterial or fungal infections only), may help to safeguard novel antibiotics into the future.

23.4.3 Manipulating Microbiomes

Developing an understanding of the host mechanisms and environmental factors that influence the assembly of a microbiome could inform strategies to manipulate their composition. For example, there is great interest in being able to enhance the presence of beneficial, protective microbial species within the microbiome to improve or restore host health. An increasing number of studies are demonstrating that the human infant gut microbiome can be profoundly disrupted by factors such as antibiotic treatment, birthing method (C-section versus vaginal birth) and formula feeding (Mueller et al. 2015; Tamburini et al. 2016). This in turn is linked to an increased risk of developing infections caused by pathogenic strains, such as *Clostridium difficile*, as well as immune and metabolic diseases later in life (Mueller et al. 2015; Tamburini et al. 2016; Matamoros et al. 2013). Thus, scientists are investigating ways to restore the healthy infant gut microbiota and increase its resilience to infection. It is known that human breast milk contains a high density of complex oligosaccharides and long-chain polyunsaturated fatty acids and that each of these is preferentially consumed by a single species of co-adapted gut bacteria (Tamburini et al. 2016; Matamoros et al. 2013; Zivkovic et al. 2011). For example, specific oligosaccharides are known to promote the proliferation of Bifidobacterium species which play an important role in inhibiting the growth of pathogens and directing immune system development (Tamburini et al. 2016; Matamoros et al. 2013; Zivkovic et al. 2011). Many public health organisations now recommend breast feeding over formula milk whenever possible. Several studies have also looked into whether formula milk could be modified to include important components of breast milk; these would act as prebiotics to restore or promote the defensive microbiome (Borewicz et al. 2019; Mueller et al. 2015; Tamburini et al. 2016).

Similarly, there is a lot of interest in manipulating the root microbiome of key food crop plant species to suppress disease and improve harvestable yields (Newitt et al. 2019; Zhang et al. 2015; Ryan et al. 2009). This method could act as a potential alternative to the application of environmentally damaging chemical pesticides and also provide a mechanism of protection when there are no resistance genes available to breed into the crop of interest (Newitt et al. 2019). Several potential methods to manipulate plant root microbiome composition are beginning to be explored. This includes the application of antibiotic-producing biocontrol agents as probiotic seed coatings before sewing the crop (O'Callaghan 2016); this ensures that beneficial

strains are delivered to the soil directly surrounding the germinating seed, enhancing their potential to colonise and compete on the emerging roots. This strategy mimics the vertical transmission of strains seen by other eukaryotic hosts, such as leafcutter ants. Plants also release approximately 20% of the carbon that they fix during photosynthesis into the soil via their roots (Bais et al. 2006; Chaparro et al. 2013). This root exudate contains a huge variety of carbohydrates, proteins and organic acids which can all act as substrates or inhibitors for different microbial species (Bais et al. 2006). For example, secretion of the tricarboxylic acid intermediate, malic acid, by the plant species *Arabidopsis thaliana* has been shown to enhance the recruitment of the bacterial species *Bacillus subtilis* to roots when plants are infected with a foliar pathogen (Rudrappa et al. 2008). An increased release of malic acid from the roots initiates the movement of *B. subtilis* towards *A. thaliana* and promotes the subsequent formation of biofilms by this bacterial species on the plant roots (Rudrappa et al. 2008). In turn, *B. subtilis* is capable of priming the plant host's immune system, reducing the severity of infections (Rudrappa et al. 2008). Understanding the role of individual root exudates, like malic acid, as well as the genetics underlying their production and release from roots could enable crop breeders to create new plant lines that are more effective at attracting protective bacteria (Zhang et al. 2015; Ryan et al. 2009).

Thus, understanding how organisms interact with protective microbes in natural systems could provide the inspiration to develop novel methods of disease control for the benefit of human health, agriculture and conservation. Major challenges include unpicking the complexity of microbiomes and understanding the factors that contribute to microbial community variation and stability over time. New technologies could further enable the exploration of defensive mutualisms involving underexplored microbes, such as fungi and viruses, which could also open up novel avenues for disease prevention into the future.

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Chapter 24
Coevolution of Molluscs and Their Microbes

Aide Lasa and Jesús L. Romalde

Abstract Symbiotic associations between multicellular hosts and microbes are widely distributed in nature, as they are essential for the ecological success and evolutionary diversification of multicellular organisms. These associations form the holobiont that, ultimately, represents the unit element for selection and evolution. The impact of such interactions may even lead to the development of complex structures in the host and microbe reprogramming based in intricated cell communications and signalling. However, the establishment and success of the holobiont may be influenced by the surrounding microbial environment. In molluscs, the environmental microbiome plays an important role in the establishment of microbe-host symbiosis, especially in bivalves due to their filter-feeding habit. One of the most studied symbiotic interactions between mollusc and marine bacteria is the *Euprymna scolopes-Aliivibrio fischeri* association. The Hawaiian bobtail squid, which is its common name, provides protection and nutrients to *A. fischeri* and, in return, the marine bacteria produces light that makes the squid virtually invisible to predators. The cooperation of these two organisms has been shaped and evolved through time to forge one of the most elegant interactions between animal and microbes. In bivalve molluscs, symbiotic bacteria have been found in the gills using inorganic chemical energy for the synthesis of organic compounds for the benefit of their hosts, such as the chemosymbiotic bacteria associated with *Bathymodiolus* mussels or lucinid clams. On the other hand, vibrios and bivalves have built an intimate association too, in which both partners have developed different strategies to survive. In this chapter, the most relevant aspects of these associations and the results of their coevolution will be summarised.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_24

24.1 Introduction

24.1.1 The Holobiont Concept

Multicellular organisms are a complex association of different organisms with diverse degrees of complexity, formed by the aggregation of a host organism plus its microbiome. This complexity has been shaped, in part, through the formation of beneficial associations with symbiotic microbes. The term symbiosis describes any long-term relationship between organisms of different species, no matter whether they are mutualists, commensalists or parasitic (Zilber-Rosenberg and Rosenberg 2008; Theis et al. 2016; Roughgarden et al. 2017; Suárez 2018). Permanent symbiotic associations form the so-called holobiont, which necessarily includes either a microbe or macrobe host (animal or plant) and its set of symbiotic microbes (bacteria, fungi, viruses, etc.). The sum of the genetic information of the host and its symbiotic microorganisms define the hologenome (Zilber-Rosenberg and Rosenberg 2008). The hologenome theory assumes the holobiont as a unit element for selection and evolution, thus natural selection can act to remove deleterious mutations in the host and the associated microbes, while selecting advantageous genetic variations (lRosenberg and Zilber-Rosenberg 2016). In evolving holobionts, symbiogenesis confers cellular-tissue and organ-level development and morphological complexity. In many organisms, microbially produced signals trigger the development of organs (Douglas 2010), as occurs with development of the light organ in the bobtail squid (McFall-Ngai et al. 2012) as an example. Therefore, host-microbe interaction within the hologenome context is a key factor for evolution, supported by an entangled network of communications between holobiont components that are founded on metabolic and genetic signals (Collens et al. 2019).

Recently, a new perspective has been introduced in which the environmental microbiome would play an important role in the formation and structure of the host-microbial complex (Singh et al. 2020). This eco-holobiont concept proposed that both biotic (animal and plants) and environmental (soil, water, air, etc.) microbiomes form a microbial loop that may impact the assembly of holobionts within an ecosystem (Fig. 24.1). For example, bivalve mollusc associated microbiota are highly impacted by the microbiome composition of the marine ecosystem. Indeed, their powerful feeding activity links the molluscs to the surrounding environment, allowing them to filter large volumes of water while concentrating on different microorganisms (Kueh and Chan 1985; Prieur et al. 1990), many of which are ingested as food.

In this chapter, we recapitulate and explore the most relevant symbiotic associations between marine molluscs and microbes and the coevolution of the host-microbe complex.

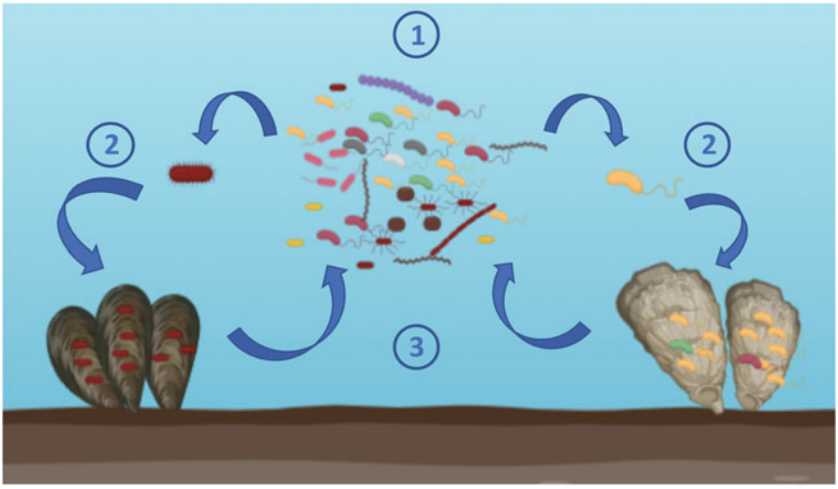

Fig. 24.1 Environmental microbiome impact the assembly of the holobiont complex and the microbial loop: (**1**) Microbial diversity in the environment; (**2**) Selection of the appropriate symbiont by the host; (**3**) Enrichment of the surrounding habitat with selected symbionts—Lasa & Romalde

24.2 Hawaiian Bobtail Squid and *Aliivibrio fischeri*, an Intimate Partnership

The Hawaiian bobtail squid (*Euprymna scolopes*) is characterised by its ability to produce light in his 'light organ' at night to make him virtually invisible to predators. However, the genome of the squid does not contain any gene encoding proteins that can produce light. So, how could an animal produce light if this ability is absent in his own nature? Hence, why not interact with an organism that has already solved this issue, such as the luminous bacteria *Aliivibrio fischeri*, and establish a permanent association? As a result, evolution has forged one of the most elegant and complex bacterial-animal interactions in nature between the Hawaiian bobtail squid and the marine bacteria *A. fischeri*. The nocturnal squid provides nutrient supplies and shelter to *A. fischeri* and in return the mollusc masks its shadow from predators by mimicking the moonlight using counter-illumination produced by the bacterial luciferase (Jones and Nishiguchi 2004). The acquisition of *A. fischeri* is horizontal and the colonisation of the light organ by this bacterium produces irreversible morphological changes in the squid (Koropatnick et al. 2014; Stubbendieck et al. 2019). This microbe-host specificity relies on mutual communication between the partners (Mandel and Dunn 2016).

This symbiotic association starts at the very beginning of the squid life cycle, when the squid emerges from the egg and swallows a tiny amount of seawater that contains millions of bacterial cells. Although *A. fischeri* constitutes a very low fraction of these planktonic cells, only this organism will colonise the nascent

organ that will develop as the 'light organ' of the squid. Selection upon the microbial community maintains partner fidelity and prevents non-bioluminescent bacteria from colonising the organ (Mandel and Dunn 2016). The microbial population interact with the ciliated epithelial fields, an organ coated by a mucus layer (peptidoglycan derivatives), which facilitates bacterial aggregation (Nyholm et al. 2000; Stubbendieck et al. 2019). Then, nitric oxide (NO) production by the eukaryotic cells appears to be a specificity determinant in the establishment and maintenance of the host-microbe association (Wang and Ruby 2011). Several studies have shown that the ability to sense and detoxify NO is important for symbiotic specificity that also acts as a temporal signal to modulate bacterial gene expression promoting successful colonisation (Wang et al. 2010; Tucker et al. 2010; Mandel and Dunn 2016). Only when the selected symbiont has colonised the ciliated appendages, does morphogenesis of the host light organ start (apoptosis, hemocyte infiltration and tissue regression) and recruiting of bacteria occur in the appendages. This process is promoted by the production of tracheal cytotoxin by A. *fischeri*, which, at the same time, diminishes the production of NO by the squid (Altura et al. 2011). Finally, the bacteria proceed to migrate through the pores in response to a gradient of chitibiose produced in the duct and into the deep crypts of the light organ (Mandel et al. 2012). In the organ crypts, proliferation and reprogramming of the bacteroid occurs which results in the loss of the flagella (Ruby and Asato 1993). A major determinant of squid colonisation specificity is biofilm formation of *A. fischeri* (Yip et al. 2005; Mandel et al. 2009), which is facilitated by the squid through specialised mucosal structures and generating environmental conditions to select *A. fischeri* over non-symbiotic bacteria (Nyholm et al. 2002; Nyholm and McFall-Ngai 2003).

An important aspect on the symbiosis is the selection of the appropriate partner, and the ability of individual bacteria to produce light seems to be important in successful persistence of the interaction. Several studies (Visick et al. 2000; Tong et al. 2009; Koch et al. 2013) have supported the idea that the host is detecting light production by bacterial cells and possibly altering physiological conditions to sanction the non-luminiscent strains. The host may even eject those light deficient strains allowing future recolonisation. The fact is that the squid influences the local *A. fischeri* populations by enriching the planktonic community with those strains that are more capable at symbiosis (Lee and Ruby 1992). The squid subjects its symbiotic partners to daily cycles of expulsion ('venting') and regrowth of nearly 95% of light organ populations to $>10^5$ bacteria, thus increasing the relative abundance of their light organ inhabitants in the surrounding environment (Lee and Ruby 1992). As a free-living bacteria, evolution while away from the host is likely to occur, which has the potential to impact microbial-host interaction (Soto et al. 2019). Increased microbial motility and biofilm capacity have been shown to be beneficial traits for host colonisation (Stabb and Visick 2013). In marine habitats, an increased biofilm capacity can facilitate attachment to suspended particulate matter or sediment, where nutrients might be concentrated (Thompson and Polz 2006; McDougald and Kjelleberg 2006). Thus, environmental adaptive evolution acting on these traits might have positive impact on host colonisation (Soto et al. 2019).

In summary, this cooperative partnership has evolved through time in a complex relationship in which both sides have deployed a sophisticated signalling communication network to ensure the success of the holobiont.

24.3 Gill Symbionts

An illustrative interaction between bivalve molluscs and microbes takes place in the molluscan gills. In this organ, environmentally or vertically transmitted symbiotic bacteria colonise by endocytosis the gill filaments, as the gills are developing, in a self-infection process (Wentrup et al. 2014). In this association, microbes are internalised by the host eukaryotic cells and contained inside specialised vesicles or bacteriocytes. Among the high bacterial diversity in water environments, chemosymbiotic bacteria have been associated with clam and mussel gills where they use inorganic chemical energy for the synthesis of organic compounds for the benefit of their hosts (Fig. 24.1).

For example, methanotrophs and thiotrophs have been reported to be the most common symbiotic bacteria in *Bathymodiolus* mussels (Fujiwara et al. 2000), a bivalve adapted to live in cold seeps and hydrothermal vents. The *Bathymodiolus platifrons* species depend on the organic carbon supplied by the symbionts inside their gills, and the establishment of the endosymbiosis is a crucial step to deep-sea adaptation in this mussel (Kellermann et al. 2012). However, the presence of these chemosyntetic symbionts is environmentally dependant in *Bathymodiolus* mussels (Duperron et al. 2007; Bettencourt et al. 2008; Yu et al. 2019), and when they are transferred from a vent to an atmospheric adapted environment the abundance of symbionts has been observed to decline significantly (Sun et al. 2017). Yu et al. (2019) found that the lysosomal system of the mussel plays an important role in controlling the abundance of endosymbionts in the host. They discovered a lysosomal-related gene, encoding a vesicle-associated membrane protein (VAMP), which expressed at a high level and presented exactly where the methanotrophs occurred. Its expression decreases in the absence of methane, thus the presence of methanotrophs is reduced too.

Gill endosymbionts, which are environmentally acquired, are also present in L*ucinidae* clams. Despite their filter-feeding habit, these clams complete their nutritional needs through obligate chemosymbiotic associations with gammaproteobacterial endosymbionts inside bacteriocytes in their gills (Taylor and Glover 2000). Different studies revealed that the main functions of the lucinid endosymbionts were denitrification, assimilation of nitrogenous compounds and inorganic carbon fixation for their hosts using energy obtained by the oxidation of reduced sulfur compounds (Cavanaugh et al. 2006; König et al. 2016; Petersen et al. 2016). A recent study analysed the associated symbionts to the lucinid clam *Phacoides pectinatus* by utilising 16S rRNA gene targeting, metagenomics and metatranscriptomics (Lim et al. 2020). Authors found that the major fraction of the endosymbionts (84%) was dominated by one gammaproteobacterial

Sedimenticola-like species. They also observed lower abundances of *Kistimonas*-like and *Spirochaeta*-like gammaproteobacteria (13% and 0.2%, respectively). Transcript clusters belonging to *Sedimenticola*-like species revealed genes involved in sulfur and hydrogen oxidation, and carbon fixation. Metatranscriptomics also revealed potential host-microbiota interactions involved in the establishment and maintenance of the symbiosis, including transfer of nutrients (carbon, vitamins and cofactors) from symbiont to host via host lysosomal digestion. Authors also detected high abundance of lysozyme-encoding transcripts that may indicate the presence of active lysosomes. Thus, the selection of symbiotic bacteria may be determined by the bactericidal lysozyme secretion and other related compounds. Symbiont related transcripts pointed out the expression of a hypothetical filamentous hemagglutinin responsible for adhesion to host tissues or genes encoding virulence and bacterial secretion. Despite the recent evidences, the significance of chemosymbiosis remains unclear and further studies are required to fully understand the role of bactericidal, adhesion and virulence factors in host selection and microbiome persistence.

24.4 Vibrios and Bivalve Molluscs, the Eternal Cold War

Vibrios are amongst the most common and widespread prokaryotes in temperate marine environments, representing the major culturable fraction of the marine microbial community (Ceccarelli et al. 2019). These marine bacteria possess high colonisation potential, so they are frequently found associated with marine animals, plants, algae and zooplankton (Takemura et al. 2014; Le Roux and Blokesch 2018) constituting a very good example of a microbial loop leading to the assembly of eco-holobionts (Fig. 24.1). Intimate associations, as the one presented above, with mutualistic interactions are well documented, however *Vibrio* associations with bivalves may also be neutral. Parasitic interactions are the most documented *Vibrio*-bivalve associations, as they cause important damages to the host (Romalde and Barja 2010; Goudenège et al. 2015; Lemire et al. 2015; Bruto et al. 2018; Dias et al. 2018), thus lead to significant economic loses to shellfish farming industry (Travers et al. 2015).

Bivalve molluscs accumulate exogenous bacteria as a result of their filter-feeding habit. But these bacteria do not always colonise their tissues, instead they are expelled from the mollusc, and as such, this bacterial fraction is also known as transient microbiota. The stable fraction or resident microbiota is, in general, composed of complex *Vibrio* populations (Romalde et al. 2014; Chen et al. 2016; Bruto et al. 2017) found both in healthy and diseased animals, where the microbes can reach concentrations close to 100-fold higher than those in seawater (Shen et al. 2009). It is still not clear whether the presence of vibrios in bivalves results from stable associations or from non-specific uptake from the surrounding environment. Hunt et al. (2008a, b) found similar diversity of *Vibrio* populations in mussels as compared to water samples, suggesting that the host tissues do not represent a high selective habitat. In contrast, Bruto et al. (2017) showed that some populations were

positively associated with oyster tissues, including *V. crassostreae* and *V. splendidus* populations. A recent study based on 16S rRNA gene-based analysis (Vezzulli et al. 2018) investigated the different susceptibility of oysters and mussels to *Vibrio* colonisation. The authors showed that in hemolymph and the digestive gland of *Crassostreae gigas*, vibrios accounted the larger fraction of the microbiota compared to *Mytilus galloprovincialis*, suggesting that oysters may provide better conditions for their survival.

Indeed, a study characterising the structural variation of the microbiota of *M. galloprovincialis* at the tissue scale (Musella et al. 2020) showed specific alterations in the different tissues (gill, digestive gland, foot, stomach and hemolymph) analysed. The dominant microbial families were different in each tissue reflecting an adaption to the respective tissue. For instance, the digestive gland was characterised by the presence of bacteria that ferment complex polysaccharides of dietary origin (*Ruminococcacea* and *Lachnospiraceae*), stomach and foot were dominated by anaerobic microorganisms (*Spirochaetaceae* and *Mycoplasmataceae*, respectively), while gills and hemolymph were colonised mainly by aerobic marine bacteria, such as Alteromonadales and *Hahellaceae* in gills and *Flavobacteriaceae* in hemolymph. This study (Musella et al. 2020) supports the idea of there being an active role of hemolymph and gills in the selection of microbial symbionts composing the microbiota of internal tissues. Interestingly, vibrios were present at low relative abundances (3% to 5%) being a subdominant genera and they were more represented in the surrounding seawater (10.6%) of the mussel farm reflecting those hosts having less affinity to *Vibrio* species when compared with other marine organisms, such as oysters.

As a result of their nature, a persistent contact between the bivalve host and potential *Vibrio* pathogens over several generations can lead into an arm race like host parasite interaction with fast coevolutionary changes on both sides. On the one hand, bivalves possess a powerful innate immune system that relies mainly on circulating hemocytes, which can infiltrate tissues as well, plus soluble factors such as reactive oxygen species (ROS) and antimicrobial peptides (AMPs), and tissue-mediated immune responses, creating a hostile environment for vibrios (Destoumieux-Garzón et al. 2014; Canesi and Pruzzo 2016; Gerdol et al. 2018). These defence mechanisms seem to be shared by the hemocytes of different molluscs, although comparative studies are not available. An essential question arises on how the host discriminates commensal vibrios from pathogenic strains when they face an encounter with *Vibrio* populations. The study carried out by Rubio et al. (2019) demonstrated that vibrios from the Splendidus clade were recognised by the oyster immune system, but only nonvirulent strains were controlled and virulent *Vibrio* species use different mechanisms to evade host cellular defences. In general, hemocytes have been found to play a key role in *Vibrio*-bivalve interactions by controlling infections through phagocytosis and by producing antimicrobial compounds. However, differences in susceptibility to *Vibrio* infections between oysters and mussels, the latter of which are less affected, appear to be related to the expression of AMPs and soluble plasma factors.

On the other hand, and similarly to other microbes adapted to metazoan hosts, vibrios either infecting or simply colonising bivalves have developed

countermeasures against host innate immunity which include dampening immune defences. Mechanisms of virulent strains that allow vibrios to evade host cellular defences have been shown to be microbial species-specific (Rubio et al. 2019). Among these mechanisms, resistance and tolerance to host antimicrobials compounds (ROS and AMPs) and heavy metals have been described in different *Vibrio* pathogens including *V. cholerae* and *V. tasmaniensis* LGP32 (Mathur and Waldor 2004; Mathur et al. 2007; Duperthuy et al. 2011; Destoumieux-Garzón et al. 2014; Vanhove et al. 2016). Mechanisms to escape from hemocyte control were also identified in *V. tasmaniensis* and *V. crassostreae* (Vanhove et al. 2016; Piel et al. 2019; Rubio et al. 2019). For instance, hemocyte evasion has been shown to be dependent on different molecular effectors, including the T6SS active against eukaryotic cells in *V. tasmaniensis*, or the ancestral R5–7 virulence factor together with a T6SS carried by a plasmid in *V. crassostreae* (Piel et al. 2019; Rubio et al. 2019). Besides, modifications of the LPS or the peptidoglycan have been linked in some bacterial species with the ability to escape from host recognition by becoming less immunogenic or by reducing the host immunity response (Charroux et al. 2018; Destoumieux-Garzón et al. 2020), although these mechanisms are poorly studied in *Vibrio* species. A recent study (Wegner et al. 2019) explored the molecular targets of coevolutionary interactions between Pacific oysters and their local *Vibrio* communities, by analysing the genomic traits of nine *Vibrio* strains from the Splendidus-clade with opposite virulence patterns. Those authors were able to identify two genes that could explain the host specific virulence patterns that represent local adaptation and may constitute targets for coevolutionary interactions, and which are involved in functions very different from other previously described virulence genes.

Despite our increasing efforts to completely unravel the mechanisms underlying the complex interactions and balance between vibrios and bivalves there is still a long way to go before we fully understand this partnership.

24.5 Concluding Remarks and Future Trends

Permanent associations between a macrobe host and microbe symbionts are widespread in nature involving mutualistic, commensal or parasitic relationships. Assuming these complex associations as a unit, termed the holobiont, natural selection acts by removing disadvantageous mutations in both partners and thereby promoting successful coevolution of the combined entity.

These associations have been less studied in marine organisms than in terrestrial animals, although representative examples do exist for marine environments. The *A. fischeri*-squid system has proven to be an excellent opportunity to study the principles of microbe-host interactions and the coevolution of these two organisms. Selection of the more suitable symbiont, based upon the availability of a high microbial diversity, is a crucial step to establish the permanent mutualistic association.

Vibrios are of special importance too in the life cycle of bivalve molluscs, representing constant and also intermittent associate partnerships as commensals and sometimes as pathogens. Vibrios have shown a multifaceted nature when associated with mussels or oysters, with several pathogenic species in the second case while only a limited number of species are pathogenic for mussels. Oysters and mussels have developed different mechanisms to control *Vibrio* infections and, in return, vibrios possess countermeasures to avoid the bivalve immune system.

Bivalves also display selectivity in their organ-associated microbiota reflecting an adaptation to the respective tissue. Gill symbionts are commonly found in molluscs that survive inside the eukaryotic cells in specialised vesicles. This association provides benefits to the host in the form of organic compounds in otherwise harsh and potentially poor environments, such as deep-sea marine vents, allowing the adaptation of the mollusc to these habitats.

The holobiont and hologenome concepts redefine what constitutes the animal individuality by stating that host and their symbiotic microbes are complex units of biological organisation susceptible to ecological and evolutionary pressures (Bordenstein and Theis 2015). From our understanding of their complexity, the study of ecological interactions and implications in the lifestyle of host and symbiotic organisms represents a milestone for scientists. The integration of new sequencing technologies, such as metagenomic and transcriptomics techniques, and genetic tools will provide more accurate answers and new perspectives to get the whole picture of these natural cooperative interactions.

Acknowledgements Dr. Jesús L. Romalde acknowledges the support of MINECO (Ministerio de Economía Industria y Competitividad, Spain) (Projects AGL2016-77539-R, AGL2013-42628-R, and AGL2010-18438) that funded his research on molluscan microbiota.

Aide lasa

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Chapter 25
Invisible Interactions between Microorganisms

Kenji Ueda

Abstract Interactions between microorganisms are fundamental to the constitution of community life, but the details have not yet been known due to their invisibility. Here, various modes in such interactions are enumerated to deepen our insights into the natural principles directing the development of ecosystem resilience.

25.1 Introduction

On March 11, 2011, a huge earthquake struck the northeast part of Japan. This earthquake generated tsunami tidal waves that destroyed many cities and took the lives of many people. Furthermore, the tsunami that then struck caused the breakdown of the Fukushima atomic power plant. Unfortunately, the damaged power plant dispersed a huge amount of radioactive material over the peaceful land.

Resilience is a word that became widely used after the catastrophe in Japan. Resilience is an academic term originally used in physics. While stress is defined as an impact of external forces, resilience refers to the ability to recover from stress damage. In recent years, the use of this term has been expanded, especially in the field of psychology. Psychological resilience is defined as the ability to restore one's mental state to that before a crisis.

During the last 9 years, the remains of most buildings and roads that were destroyed due to the earthquake and tsunami have been removed. Some of those material items lost have been reconstructed in different styles, but many others remain lost. In contrast, the ocean looks the same as before, and the fields once swept by the strong flow of seawater were soon covered with green (Fig. 25.1). The revival of plants in turn supports the life of many animals. Such natural restoration can be observed everywhere, demonstrating that nature intrinsically retains the will and power for directing self-recovery.

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_25

Fig. 25.1 Landscapes in Onagawa city (Miyagi, Japan). This place was destroyed by the tsunami tidal waves reaching 30 meters height on March 11, 2011. The pictures photographed from the same site on Aug 2011 show the serious breakage of buildings and roads (upper, Aug 2) as well as the effective growth of plants (lower, Aug 27). Courtesy, Otoshiro Hiratsuka

Presumably, resilience of ecosystems is supported by the activity of microorganisms. Great advances in experimental techniques in biochemistry and molecular biology have progressed our understanding of the physiological properties of microorganisms. However, it has not yet been explained in detail how the development of resilience is achieved by these small organisms. A key to the natural principle should reside in the function of microbial cells and their invisible interactions.

25.2 Structures Supporting Rigid Communities

25.2.1 Primary Structure: Self-Restoration

25.2.1.1 Self-repair

Early studies on bacterial genetics revealed the existence of sophisticated mechanisms of self-repair (Baharoglu and Mazel 2014). The enzyme DNA polymerase not only catalyzes replication but also exerts proofreading activities; the enzyme forms a molecular complex that can find its own mistakes, eliminate the wrong nucleotide base, and reintroduce the correct one. Bacteria have developed other mechanisms to screen for mismatches in base pairing, eliminate incorrect ones in the newly synthesized strand, and eventually redirect polymerase and ligase activities to fill in the gap using the proper base. In eukaryotes, much more complex mechanisms maintain the high reliability of copyediting.

Bacterial cells can also mend damaged DNA structures caused by exposure to injurious environmental factors. For example, the widespread enzyme DNA photolyase acts on pyrimidine dimer structures formed by UV illumination and resolves them to restore the original form by utilizing light energy. Maintenance is also carried out for proteins. Molecular chaperones prevent their misfolding, aggregation, and unusual accumulation. Bacterial cells can also repair damage to the cell wall by inducing enzymes involved in peptide glycan synthesis.

Another way of survival under a threat to DNA stability is known with regard to unique genetic features of the radio-tolerant bacterium *Deinococcus*. This extremophile group retains multiple copies of chromosomal DNA, which allows for growth even under high doses of irradiation (Ishino and Narumi 2015). This survival strategy of *Deinococcus*, which is based on the retention of spare genetic information, may support the hypothesis that rapid growth of many bacteria, which is accompanied by the formation of large DNA copy numbers, serves as a self-defense mechanism.

25.2.1.2 Adaptive Response

Bacteria have developed defensive functions against various environmental stresses, including exposure to harmful substances, physicochemical conditions, and

nutritional deprivations (Baharoglu and Mazel 2014). The expression of a defensive mechanism is linked to the sensing of the corresponding stress factor by a specific signal transduction cascade. Bacterial genetic studies have progressed our understanding regarding how bacteria sense a stress signal and induce a specific function that makes adaptation of the cells to the stress condition possible.

The major mode of an adaptive response occurs at the level of transcription. Various kinds of transcriptional regulators and cognate sensors have been identified and studied for the purpose of understanding their underlying signal transduction pathways. Typically, two-component regulatory systems include a signaling module that transmits the environmental signal to their specific gene expression regulation partner (Groisman 2016). An example would be stress-response sigma factors which specify the transcription of genes regulated by stress-response promoters and direct the expression of stress-responsive genes. The adaptive response also occurs at translational levels. This is mediated partially by the function of a specific RNA structure called a riboswitch, whose change in conformation affects the efficiency in translation of the genetic information encoded downstream from the switch.

25.2.1.3 Homeostatic Control

Adaptive responses contribute to cellular homeostasis. The sensing of damage and starvation induces the expression of repair and nutritional uptake systems to restore a healthy cellular structure and physiological state, respectively. Inner cellular homeostatic control involves second messengers, such as cyclic adenosine monophosphate (cAMP), which is formed due to the cyclization of AMP by adenylate cyclase (Green et al. 2014). The cAMP level serves as an indicator of the cellular metabolic state. Feedback mechanisms induced by a change in the cAMP level function until the cAMP level returns back to normal, hence restoring the original metabolic state and maintaining it.

25.2.2 Secondary Structure: Cellular Interaction

The ability of inducing stress responses and subsequent self-restoration is critical to the persistence of each organism. Hence, these mechanisms support the rigid structure of an ecosystem at its most basic level. However, these are essentially the conservative maintenance mechanisms within each organism that have no positive effect on the structuring of the life community. Thus, other mechanisms should also exist in the background of the structuring of rigid ecosystems. In this context, we can reasonably speculate that intercellular associations play an important role. However, knowledge on these associations has not been appropriately collected and analyzed, especially regarding interactions between invisible microbial cells.

25.2.2.1 Antibiosis

Ever since the discovery of penicillin, humans have successfully developed effective therapeutic techniques against pathogens based on the use of antibiotics. However, adaptation of this miracle weapon has immediately caused the emergence of antibiotic-resistant causative agents. A detailed study on resistance mechanisms has revealed the occurrence of surprising strategies that permit the growth of the specific organism targeted, even in the presence of the antibiotic (Blair et al. 2015). Such resistance mechanisms include degradation, modification, and elimination of the corresponding antibiotic and the formation of a backup mechanism for circumventing the pathway inhibited by the antibiotic. The genes directing the expression of those resistant mechanisms are even transferred between different taxonomic groups by mobile genetic elements, such as conjugative plasmids and transposons.

The occurrence of the abovementioned serious problem in the clinical area is an artifact. This is caused by human activity, which is administering a great amount of antibiotics to stop the growth of a specific bacterium. However, this phenomenon demonstrates that the natural microbial community retains the potential to respond even to such an unusual impact and recovers eventually.

Compounds exhibiting bactericidal activity are widely produced by actinomycetes, a group of filamentous bacteria represented by the genus *Streptomyces*. Since Selman Waksman's discovery of streptomycin, an effective agent against tuberculosis, many biologically active substances have been isolated from cultures of this group of bacteria and used for medicinal treatment. The term "antibiotics" was defined by Waksman as "a microbial product" that has the ability to inhibit the growth of certain bacteria (Davies 2006).

Although there is a discussion whether antibiotics actually serve as antibiotics in the natural environment, one major possibility is that these compounds actually serve as weapons that protect producer organisms from invasion by other microorganisms (Ueda and Beppu 2017). It may also make the producer dominant in nutritional competition by killing or inactivating competitors.

Competition is one of the major modes of interactions. It appears to have a negative effect on the persistency of a diverged community, because it makes organisms exclude each other. However, competition stimulates the development of various abilities and strategies to survive. The toughness of living organisms is established and maintained, at least in part, by being exposed to a tensed situation, as observed for various human activities, such as business, sports, and even arts. Thus, competition supports a rigid community structure.

25.2.2.2 Symbiosis

Symbiosis usually refers to an association that benefits at least one of the interacting organisms. Symbiotic interactions have been widely observed within the mutualistic

associations between higher organisms, including animals and plants. Interesting examples of microbial symbionts associated with a specific higher organism are also known. There, surprisingly significant roles are played by the relatively invisible microbial partner providing insights into the significance of partnerships even though the microbes often are presumed to be "lower" organisms.

In contrast to cases involving higher organisms, the details of interactions between microorganisms have not yet been fully understood. This is simply because the participants often are not visible to the naked eye. Furthermore, the manipulation techniques typically are based on pure cultivation of the partnering species, which prevents observing associations between different microorganisms. However, studies focusing on the physiology of syntrophic microorganisms, whose growth depends on an interaction with other microbes, have progressed the understanding of the mechanism underlying this mutualism.

A well-known instance of syntrophic interaction is the nutritional connection in methanogenic microbial communities (Schink 1997). This complex consortium consists of anaerobic bacteria digesting polysaccharides, oligosaccharides, and organic acids, as well as methane-producing archaea. These constituents are nutritionally interlinked; hence, pure isolation of each constituent either cannot be performed or requires highly specialized manipulation conditions.

Thus, each constituent of the methanogenic microbial community is physically challenged. In addition, they are highly sensitive to oxygen. This makes it likely for these weak organisms not to survive by themselves and would become extinct. However, the community widely occurs in the natural environment. The versatility of the complex community implies that the consortium constituents are intimately linked not only by mutual dependence in terms of nutritional supply but some multidimensional mechanism that keeps the rigid community structure intact, ensuring every constituent's survival by active involvement in the organization of the community.

25.2.2.3 Communication with Signals

The discovery of surprising social behavior in prokaryotic cells was triggered by the identification of the *N*-acyl-homoserine lactone (AHL) auto-inducer molecule (Horinouchi et al. 2010; Schuster et al. 2013). Originally, the compound was identified in a culture of *Vibrio* spp., a group that contains bioluminescent marine bacteria associated with marine animals, as a chemical factor essential for the expression of its autoinducible bacterial luciferase that catalyzes light emission. It became clear that the ability to synthesize and respond to this type of autoinducer is widely distributed among gram-negative bacteria. The AHL receptor protein serves as a transcriptional regulator that induces specific functions, including light emission, antibiotic production, and biofilm formation. Also, AHL is presumed to serve as an indicator of cell density. Accordingly, an increase in the number of cells in a specific bacterial group turns on the social behavior mode via elevated AHL

concentrations and subsequent induction of specific gene expression. This cell density-dependent control of the bacterial community is termed quorum sensing.

Another group of autoregulatory molecules precisely characterized for their actions are the gamma-butyrolactones (GBLs) produced by *Streptomyces* species (Horinouchi et al. 2010). Despite being prokaryotes, *Streptomyces* and related bacteria exhibit a complex life cycle resembling that of filamentous fungi. In the vegetative growth phase, the organism extends the projecting "substrate mycelium"—those mycelia that maintain contact with the substrate being used to sustain the fungus. Then, the substrate mycelium forms an aerial mycelium, which culminates in a spore chain. The cellular development from substrate to aerial mycelium is genetically linked to the production of secondary metabolites, including those that have antibiotic activity. In this life cycle, GBLs serve as a switch for the initiation of secondary metabolism and cell differentiation during the change from producing substrate to aerial mycelium. Probably, GBL-mediated signaling causes the cells within a colony to synchronize and simultaneously initiate drastic cellular development. Without such harmonization, antibiotics produced by some cells may kill others. In the case of streptomycin whose production is controlled by a GBL called A-factor, the self-resistance gene located in the center of the biosynthetic gene cluster is induced prior to the expression of the adjacent biosynthetic genes. [I would guess the rest of a fungal colony intrinsically is resistant to its self-produced antifungal agents unless resistance to its own antimicrobial compounds must be induced].

In addition to lactones, structurally diverged small molecules, including those with peptidic and furanosyl backbones, are known to direct social behavior of specific groups of bacteria (Horinouchi et al. 2010). The original identification of these cell-cell signaling molecules occurred in a study focusing on the expression of a specific phenotype, such as antibiotic production and biofilm formation in a given bacterial species, which is clearly observed in laboratory conditions. This, in turn, suggests that similar communication to control microbial functions or phenotypes invisible to researchers using conventional manipulation techniques likely also exists in other microbial groups but remains unknown.

25.2.2.4 Interspecies Communication

The above mentioned lactone signals retain structural divergence in terms of their alkyl side chains. The strict ligand selectivity of its cognate receptor distinguishes the true signal from other structurally similar derivatives, thus establishing species-specific communication. On the other hand, some strains or species share an autoinducer of identical chemical structure. In such a case, crosstalk between organisms affiliating with different species may take place.

The possible occurrence of interspecies communication is also inferred from the distribution of genes involved in the cell-cell signaling as observed with regard to those encoding AHL synthase and receptor. Usually, the AHL synthase and receptor are encoded by sequences adjacent to each other. However, some bacteria retain only

the gene coding for the receptor (Churchill and Chen 2011). This suggests that these bacteria do not send the command but rather respond to the one sent from other species.

The occurrence of a different mode of interspecies communication can be deduced from the activity of *Streptomyces* metabolites. As mentioned previously, this group of soil bacteria produces a great variety of secondary metabolites exhibiting antibiotic activity. However, *Streptomyces* species also produce substances that assist the growth of other organisms. One such substance is cobalamin (vitamin B12), which is a cobalt-containing tetrapyrolle that serves as an essential cofactor for several enzymes, such as methyltransferases (Sokolovskaya et al. 2020). Despite its essential role in a wide range of organisms, including mammals, the ability to synthesize this cofactor is distributed only among some prokaryotes. This indicates that prokaryotic cobalamin producers support the life of a vast variety of living organisms. Evidence from classic screening studies suggests that *Streptomyces* and related bacteria are effective suppliers of cobalamin.

Another example of *Streptomyces*-derived products of public benefit are desferrioxamines, which belong to a group of iron-chelating cage compounds termed siderophores (Yamanaka et al. 2005). The ferric iron-bound form of desferrioxamine is incorporated into the cell via a specific translocator protein and supplies ferric iron to ferric-dependent cellular functions. While the ability to synthesize desferrioxamines is specifically distributed to *Streptomyces* and some other bacterial groups, genes encoding membrane translocator proteins are widely distributed even among those lacking the ability to synthesize the compound. The latter group of organisms probably utilizes the siderophores produced by the formerly mentioned supplier group of bacteria.

25.2.2.5 *Communication* via *Non-Specific Metabolites*

Microbial interaction is mediated not only by specific bioproducts but also by non-specific ones. For example, classic works on diverged microorganisms demonstrated that CO_2 triggers their change into growth mode (Ueda and Beppu 2016). In *Streptomyces* and *Clostridium*, high CO_2 levels induce spore germination. In *Mucor*, pseudohyphal growth is activated in a high CO_2 atmosphere. Some bacteria require elevated CO_2 for their growth. Genetic evidence displayed in some model microorganisms has revealed that such high CO_2-dependent growth occurs in knockout mutants for carbonic anhydrase (Ueda and Beppu 2016). The enzyme carbonic anhydrase catalyzes the interconversion between CO_2 and bicarbonate (HCO_3^-) and is hence supposed to have a role in supplying bicarbonate to bicarbonate-dependent enzymes, such as acetyl-CoA carboxylase and carbamoyl-phosphate synthase that respectively are essential for the synthesis of fatty acids and nucleotides. Bacteria-lacking carbonic anhydrase cannot grow under normal atmospheric conditions but can grow in a high CO_2 atmosphere by consuming bicarbonate generated from CO_2 by natural equilibrium.

Since CO_2 is an effective indicator of metabolic activity, eliciting a response to its environmental concentration can be considered a general mechanism for adaptation. Similarly, other end products of energy metabolism, such as organic acids and nitrogen oxides, may also have similar roles as global indicators of environmental conditions to which microorganisms respond and adapt.

25.2.3 Higher-Order Structure: Autonomous Distribution and Self-Organization

Social behavior is broadly observed in the world of animals and is a major subject in animal ethology. Many animals form a flock and perform group action. In the case of animals of high intelligence, the group activity is directed by a leader. On the other hand, other animals of low intelligence, such as birds and fish, also form flocks

Fig. 25.2 Schooling behavior of the arabesque greenling *Pleurogrammus azonus*. The picture was reprinted from Kitagawa et al. (2011)

(Fig. 25.2), but the group action is not directed by a specific leader. Each individual has the ability and possibility to play the leading role, and who actually plays the role is determined by "chance." Once triggered, a great number of fish form a collective entity, moving in the same direction.

25.2.3.1 *Self-Organization*

The collective behavior of flock occurs in a self-organizing manner, in which each individual is programmed to place itself next to another one and swim in the same direction. Construction of a functional complex due to self-organization can be widely seen in the world of molecular apparatuses. An example of such sophisticated architecture is the bacterial flagellum (Thomson et al. 2018). Some bacteria swim toward an attractant or away from a repellent by flagellar rotation. This rotation transforms the fine fiber into a coiled structure that serves as a screw. The rotation is reversible, and the change in orientation allows the switching between swimming and tumbling modes. This surprising performance is achieved because of the ultrastructure of the flagellum fiber, which exerts both stiffness and suppleness, making the screw rigid and flexible, respectively.

The flagellum is predominantly a polymer of the flagellin protein (Thomson et al. 2018). Flagellin is synthesized inside the cell and subsequently transported across the membrane(s) by the flagellar basal body that functions as a specific membrane translocator. The exported flagellin molecules form the flagellum due to its ordered assembly that occurs in a self-organizing manner aided by a chaperone protein. The autonomously developed protein fiber serves as a highly functional apparatus that enables the active movement of prokaryotic cells. Similar self-organizing development occurs widely in cellular macromolecules including proteins, nucleic acids, and lipid bi-layers.

25.2.3.2 *Autonomous Distribution*

Another mystery in the aforementioned flock formation is the mechanism of its initiation. It appears to be determined occasionally and randomly, which triggers social action without the process of active selection. This kind of social behavior is observed not only with animals but also with microorganisms. *Dictyostelium*, a slime mold, is known for its surprising life cycle based on an autonomous distribution program (Huber 2016). In its vegetative phase, each *Dictyostelium* cell behaves as a free-living amoeba searching for and eating bacteria independently of other cells. However, food depletion causes the transformation of one cell into a sender of an aggregation signal. By sensing the concentration gradient of the signal molecule (i.e., cAMP), other cells migrate in the direction of the sender cell. Thus, aggregated cells form a moving body, which moves toward an appropriate place where the moving body settles and differentiates into a fruiting body containing dormant

spores. Amazingly, there is a group of bacteria, that is, *Myxococcus* and related gram-negative bacteria that undertake a similar life cycle (Kroos 2017).

Another kind of slime mold, i.e., *Physarum polycephalum,* attracts interest due to its seeming use of intelligence to solve maze puzzles. This type of mold forms tube-like structures termed pseudopodia (Alim 2018). If the organism is placed at one feeding site, it starts to grow pseudopodia cells in various directions from the feeding site, searching for more food supplies. Once the extended cell meets another feeding site, the plasmodium body starts to contract, leaving the tube that connects the two feeding sites in the shortest path in the maze. This surprising phenomenon implies that the mold has a program that makes decisions based on the integration of diverged inter- and intra-cellular signals.

Thus, the major subject in animal ethology has a significant connection to microbiology. The mechanism underlying autonomous social behavior may not be clarified only by the conventional analytical methods developed in microbiology and related research areas. Recently, researchers in the field of computational technology have successfully developed a robot that mimics natural social behavior. Integration of different research fields may progress a deeper understanding of the principles underlying the multilayered structures of microbial interactions.

25.3 Insights into the Framework of Resilient Ecosystems

In "Learning the Language of Addiction Counseling" (Miller 2015), the author Dr. Geri Miller proposes 10 ways to build psychological resilience. Of these, the first five overlap precisely with the mechanisms observed in microbes.

1. Make connections.
2. Avoid seeing crises as insurmountable problems.
3. Accept that change is a part of living.
4. Move toward your goals.
5. Take decisive actions.

Thus, the lessons we learn from the microbial community provide deep insights into the principle fundamental to the sustainable life of all life forms.

Despite the probability that the constitution of a healthy ecosystem is based on invisible interactions between microorganisms, our current knowledge does not fully explain how those interactions constitute effective organizations of life forms. If explained by an analogy to building construction, the mechanical strength of each building block is important, but possessing high rigidity is not sufficient for the successful construction of a building. It is also important that the building blocks be assembled in a harmonious manner. Furthermore, buildings resistant to large earthquakes should retain a flexible structure that absorbs strong physical power and gradually releases it in the course of restoration. In addition, for persistence, construction of such a special structure should be renewed constantly and reproducibly.

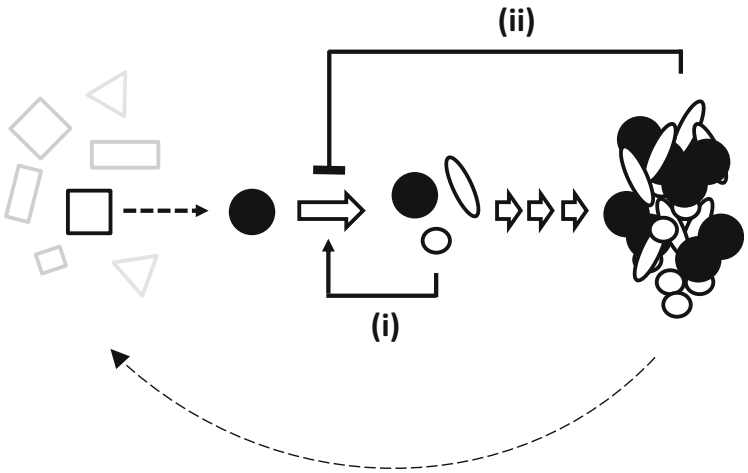

Fig. 25.3 Simple representation of microbial community development based on positive and negative feedback. The dashed arrow indicates an autonomous distribution system directing the initiation of development. Boxed arrows indicate the maturation steps, including those that proceed in a self-organizing manner

There are no artificial materials which meet this condition of self-renewability. In contrast, natural systems have already been developed in this manner.

Figure 25.3 represents a hypothetical model for the process of microbial community development. The positive feedback principle serves as the backbone for the growth of the community by stimulating and accelerating the adaptation of specific members of the community. Positive feedback is based on various events, including both symbiotic and antibiotic interactions, and these interactions are mediated by nutritional supply and deprivation, attack by inhibitory compounds, and supply of signals. The latter not only include specific chemical compounds but also non-specific small chemical molecules, such as CO_2, nitrogen oxide, and sulfides, as well as physicochemical factors, such as osmolarity, turbidity, and humidity. By sensing and adapting to a complex environment containing many factors, some organisms become dominant, thereby affecting direction of the community, which can result in either the representational stabilization or elimination of other microbes from the community. Such dynamic flow directs the formation and maturation of a community in a self-organizing manner.

The other framework component which is fundamental to social development is negative feedback, which maintains the homeostatic state of the community. Although arguments for the occurrence of such a mode of community control have not yet been actively collected, negative feedback regulation is a key principle in homeostatic control, as observed with the intracellular mechanisms. Factors that trigger such an opposite mode of regulation may also include the roles played by both specific and non-specific compounds and physicochemical elements.

Fig. 25.4 Stone wall of the Ueda castle (Ueda, Nagano) consisting of various size of stones, including a large stone called Sanada stone

Thus, a developed and maintained microbial community can easily and occasionally collapse due to various accidents caused by both naturally occurring environmental changes and artificial activities. Under such circumstances, resilience of the microbial community can be assessed based on the potential to start a re-establishment of the structure. Such a restart happens spontaneously, and it is very likely to occur. The ability to initiate a restart is potentially widely distributed to many microbial cells. Autonomous distribution is another aspect fundamental to the persistence of the microbial community and pertinent to survival of the ecosystem supported by the community.

Network formation based on self-organization and autonomous distribution offers both great rigidity and simultaneous flexibility to the organization. In the case of an organization based on the leadership of a specific leader, the accidental loss of the leader causes great damage to the order of the community. In contrast, an autonomous distribution system, which does not depend on a specific leader, adapts rapidly to any accidental situation.

An additional, but basic feature of microorganisms which contributes to strengthening the community is their huge diversity. Including a wide variety of building blocks can make the building more rigid and yet in some ways also flexible (Fig. 25.4). Although our information regarding the phylogenetic diversity of

microbes has increased remarkably due to technical developments in nucleotide sequencing of environmental DNA, most of the constituents of microbial populations have not yet been cultivated hence we still do not know the true diversity of microorganisms. Probably, the occurrence of complex and invisible interactions significantly correlates with the unculturability of these microorganisms. The wide variety in terms of their functional, physiological, and taxonomic properties is also an important aspect of rigid community structure.

Finally, the initial part of "Ho-job-ki, "the famous literature written by the Japanese essayist Kamo-no-Chomei on 1212, is cited to end the chapter.

The flowing river never stops
ゆく河の流れは絶えずして、
and yet the water never stays the same.
しかも、もとの水にあらず。
Foam floats upon the pools,
淀みに浮かぶうたかたは
scattering, re-forming,
かつ消えかつ結びて、
never lingering long.
久しくとどまりたるためしなし。
So it is with man
世の中にある人と
and all his dwelling places
栖とまたかくのごとし。
here on earth.

Acknowledgments The author would like to thank Mr. Otoshiro Hiratsuka and Dr. Takashi Kitagawa for their permission to use pictures presented in Figs. 25.1 and 25.2, respectively.

Kenji Ueda

Dedication The author dedicates this chapter to the people who died due to the Great East Japan earthquake on Mar 11, 2011 and their families.

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Part VI
Microbial Symbiosis as a Driving Force in Evolution

Chapter 26
The Game of Evolution Is Won by Competitive Cheating

Christon J. Hurst

Abstract Evolution is a competitive game and cheating wins against fair competition. If one group cheats successfully, then that group gains an advantage and the other groups need to find a similar strategy in order to survive. Examples of evolutionary cheating include having learned aerobic respiration at a time when biology perceived molecular oxygen to be a metabolic waste product. The full range of symbiotic associations from cooperation to predation can also be perceived as representing efforts to gain a competitive advantage because, after all, if you cannot successfully work together with your potential competitors then either stealing what they have created or simply eating your competition may prove to be the more successful route. It also is important to understand that the process of natural selection does not act upon individual species as if they were independent entities. Instead, natural selection acts upon the homobium, which is a term suggested by Bernhard Frank in 1877 to describe the combination of a species and its associated symbionts. This essay presents a summary of mutualistic symbioses including the adoption of mitochondria which are the aerobic power house of eukaryotic life, chloroplastids in their many forms among which are algal and bacterial symbionts that provide fixed carbon from photosynthesis, and bacteria as well as cyanobacteria that serve as nitrogen fixing symbionts of plants. The biological activity of endogenous viruses also qualifies them as mutualistic symbionts, and they too are part of the homobium that defines their host.

26.1 Introduction

When I was an undergraduate student studying biology at the University of Cincinnati, in Cincinnati, Ohio, one of my professors was Alex S. (Stewart) Fraser. Alex very enthusiastically taught the course on evolution. During one of our lectures he asked our class the question "How do you win at evolution?" and he then

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_26

immediately answered "By cheating!". Alex explained that it is successful cheating which brings evolutionary victory rather than trying to win by level competition. Anyone who watches young children playing a competitive game will notice this rule in action because the children make continued efforts at bending the rules, changing the rules, and just simply cheating. In terms of evolution, Alex told us that examples of successful evolutionary cheating were learning to use oxygen at a time when other species considered oxygen to be a metabolic waste product, and learning how to just simply eat your competitors.

My goals in writing this essay are both to appreciate Alex's instruction and to provide examples which might help with understanding this philosophical perspective. The contributions that symbiotic microbial interactions have made to evolution are numerous and their effects have been profound. I will consider the development of aerobic respiration and add to that several other examples of microbial symbiosis which have become fundamental. One of the rules which might be used to describe symbiosis is the old expression "If you can't beat them, join them". I would create an additional rule and state that "From mutualism to antibiosis, symbiotically speaking all's fair in love and war".

The principle concept is that evolutionary success comes from gaining a biologically competitive advantage by having found a way to do something differently. Once a successful means of cheating has been developed, then all of the other competitors must either rebalance the competition by accomplishing something that is equivalent or else the competitors will face a loss in the sense of becoming marginalized and possibly extinct.

26.1.1 The Pathway Leading from Ingested Microbes to Mitochondria and Chloroplasts

Mitochondria, which now are organelles, would have begun as engulfed aerobically respiring bacteria that somehow were not digested. Those bacteria became biologically inherited as internal passengers, termed inherited endosymbionts, within the descendants of their initial host cell. At that point, the host cell and its internal passengers began a common biological destiny and they were subjected to evaluations of competitive fitness as a combined entity. This combination of host and bacterial symbiont could have been perceived as cheating against the competition and eventually evolution resulted in the inherited bacterial symbionts becoming fixed organelles. The descendants of that first organism, whose inherited bacteria were uniquely destined to become mitochondria, successfully bested their competitors so extensively that mitochondria now are ubiquitous in eukaryotic organisms. Degli (2014) has presented a suggestion that methylotrophic proteobacteria could be among the closest living relatives of the microbes that endosymbiotically became mitochondria. Interestingly, in absence of oxygen, mitochondrial functioning seems to no longer be retained (Yahalomi et al. 2020) and that also may be a way of trying a

alter the competitive balance by not expending unnecessary energy to maintain metabolic activities that have become unuseful.

Chloroplasts, considered as inherited photosynthetic organelles, would have begun as undigested cyanobacteria, and since that event of indigestion the cyanobacteria have followed an evolutionary pathway similar to that of the mitochondria. The combination of chloroplasts plus mitochondria has meant that aerobic respiration associated with mitochondria may help to carry the hosts through periods when there is no sunlight available for photosynthesis. It is a combination which has enabled plants to reach a tremendous level of aquatic and terrestrial dominance.

Although we often think of chloroplasts as being a phenomenon associated with algae and vascular plants, there additionally are animal groups which interestingly have utilized ingested endosymbiotic algae, and sometimes adopted only chloroplasts that have been scavaged from ingested algae, as a photosynthetic source of energy and fixed carbon.

Howe et al. (2008) addressed the concepts of primary photosynthetic plastids having initially arisen by non-photosynthetic eukaryotic hosts engulfing photosynthetic prokaryotes, and those photosynthetic eukaryotes in turn having been ingested by non-photosynthetic eukaryote hosts to form secondary plastids.

Martin et al. (2015) have presented their perspective, and a set of suggestions, regarding the processes which may have led to evolution of a cell nucleus, mitochondria and chloroplasts, and successive gene transfer from intracellular organelles to the host cell genome.

26.1.2 A Fixation with Nitrogen

All of these concepts represent ways of cheating, after which other groups must try to at least level the competition or hopefully best the competition by trying something new. I will address these pathways to mitochondria and to chloroplasts later in this essay. I also will consider the question of what might have come next after the competitors had evolved to the point that all plants had both mitochondria and chloroplasts. Perhaps the next idea was finding a microbial symbiosis with organisms that had nitrogenase enzymes and therefore could provide a source of fixed nitrogen. The oldest of these symbioses could have been an association with the cyanobacteria whose descendants we know as *Anabaena*. There have been competing symbioses with the proteobacteria whose present descendants include *Rhizobium*, and the actinobacteria whose present descendants include *Frankia*.

26.1.3 Some Other Symbioses that Have Become Vital

During the late 1800s, it was possible to fully address the subject of microbial symbiosis in a single lecture. Today, not even a very large book could suffice to

present all that is known. I have summarized a few of the highlights in this essay, and hopefully you will enjoy reading it. At least, the photographs will seem nice!

The other symbioses that I have included are: fungal symbionts of plant roots, gastrointestinal symbiont microbes, luminescent bacterial symbionts, and endogenous viruses.

26.1.4 Ultimately, Evolutionary Fitness Is a Competition for Habitat and Niche

For a biological group at any taxonomical level, there will be both a broadly defined potential habitat within which that group is restricted to a more narrowly defined operational habitat, and a broadly defined potential niche within which that group is restricted to a more narrowly defined operational niche. Those restrictions which limit the habitat and niche from their broader potential levels to narrower operational levels are largely delineated by other species (Hurst 2016a).

The increased competitive fitness which derives from successful cheating may allow an expansion of either habitat, or niche, or both. That expansion might be represented in the claiming of a greater operational habitat and operational niche, or become even more significant by representing an inrease in the potential habitat and potential niche.

If competitive failure reduces a biological groups operational habitat or operational niche too severely, then that biological group faces possible extinction.

26.2 The Basic Concepts of Symbiosis

We think of symbiosis as being the 'living together' of two organisms which may represent different species, and each organism is termed to be a symbiont. Bernhard Frank seems to have been the person who initially named this concept when he included the term 'Symbiotismus' in a landmark publication (Frank 1877). Figure 26.1 is a photograph of Albert Bernhard Frank. He was born January 17, 1839 in Dresden, Germany and died September 27, 1900 in Berlin, Germany.

When Frank wrote his landmark publication (Frank 1877) he was considering lichens, which most typically are bipartite composite organisms consisting of a fungus partnered with either an algae or cyanobacteria. There also are tripartite lichens that consist of a fungus partnered with both an algae and a cyanobacteria. I will again mention the lichen symbiosis later in this essay.

Frank (1877) suggested that there were stepwise levels of symbiotic association from the most loose to the deepest possible necessary connection. Frank called the lowest stage pseudoparasitism 'Pseudoparasitismus' and suggested that was represented by epiphytes. The next higher level would be parasitism 'Parasitismus'

Fig. 26.1 A photograph of Albert Bernhard Frank. Bernhard Frank was born January 17, 1839 in Dresden, Germany and died September 27, 1900 in Berlin, Germany. Frank created the term 'Symbiotismus' (Frank 1877). Frank later described what he thought represented a parasitic attack by a fungus acting upon the roots of a tree, and he created the term mycorrhiza to represent that discovery (Frank 1885). We now understand that mycorrhizal relationship to be a mutually beneficial symbiosis, and term it to be an ectomycorrhiza as contrasted with an endomycorrhiza. This picture is considered to be free of copyright due to its age

in which one member only takes and does not give, and even destroys. Frank (1877) believed that his term symbiosis, which he actually stated in German as 'Symbiotismus', would represent the highest level of association of two beings, with their cohabitation relationship being a connected existance that provides one-of-a-kind services to each other. Frank believed that in such a relationship the two organisms would seem to lose the concept of being separate individuals. Frank suggested that the term 'Homobium' could be used to describe the combination of those two symbiotic organisms.

In reality, a species usually exists in close symbiosis with more than one additional species, and so we need to think of the homobium as being a partnering of more that just two species. We should understand that evolution acts not upon an individual species, but upon the homobium of a host and its associated symbionts.

I have summarized the early history of philosophy and research into symbiosis as the topic of a companion essay titled "Discovering the Symbiotic Nature of Microbial Life: Summarizing Milestone Publications from 1866 through 1947" and that appears on pages 213–241 of this same volume. Our biosphere is in fact an interconnecting network of symbiotic associations (Hunter 2006). My colleagues and I previously have presented in this series a volume on the mechanistic benefits of microbial symbiosis (Hurst 2016b) and a volume on the opportunistic pathogenicity which occurs when normally benign commensal or mutualistic symbionts turn against their host (Hurst 2016c).

Marshall Ward (Ward 1899) in Fig. 26.2 offered a belief that the term symbiosis should be used to describe mutualistically beneficial relationships between two species that could not survive without the support of one another. The opposite would be antibiosis, which Ward defined as one species existing in lethal antagonism against the other. Ward thought that both symbiosis and antibiosis began as random encounters with no lasting committment to the interaction. He termed those random encounters to represent disjunctive associations, as indicated in Fig. 26.3. Ward

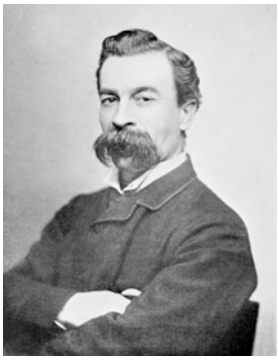


Fig. 26.2 A photograph of Harry Marshall Ward. Marshall Ward was born March 21, 1854 in Hereford, England and died August 26, 1906 in Torquay, England. Ward gave us the interpretation that chance encounters between members of two species can, over time, follow two pathways (Ward 1899) with one of those pathways eventually resulting in antibiosis and the other pathway resulting in symbiosis. This picture is considered to be free of copyright due to its age

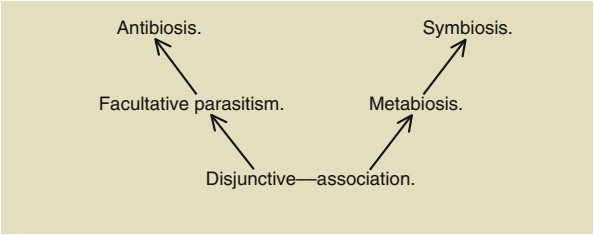


Fig. 26.3 The developmental pathway of symbiotic associations. This figure shows a diagram published by Marshall Ward (Ward 1899) in which he explained his perspective about the pathway which led to symbioic associations. Ward considered that associations between the members of two species first occur by happenstance, as unexpected random events and are disjunctive interactions that involve no requirement for continued association. Ward believed that if a disjunctive association was continued, then two pathways led onward. One of those pathways resulted in antibiosis and the other pathway resulted in symbiosis. Ward suggested the term antibiosis to represent antagonism. Ward talked about metabiosis as representing instances when one organism prepares a more suitable environment for another. We now consider metabiosis to be a form of commensalism. Commensalism is a relationship in which one species gains benefit while the other is not necessarily harmed. Ward suggested that metabiotic associations might be a 'half-way house' to symbiosis. Wards concept defined symbiosis as including associations in which neither species could survive alone. Ward suggested that symbiosis could be considered in a broad sense, and should not be used to describe interactions between species that only represented either temporary associations or transient encounters. Ward also suggested that there might be mutualistic cooperations in which either species could 'carry on' alone in a given situation although they did better when acting together. We now define mutualism as representing relationships from which each organism derives a net benefit

thought that the pathway to antibiosis had an intermediate stage which be called facultative parasitism. Ward believed that the opposite pathway, which resulted in symbiosis, had an intermediate stage of metabiosis.

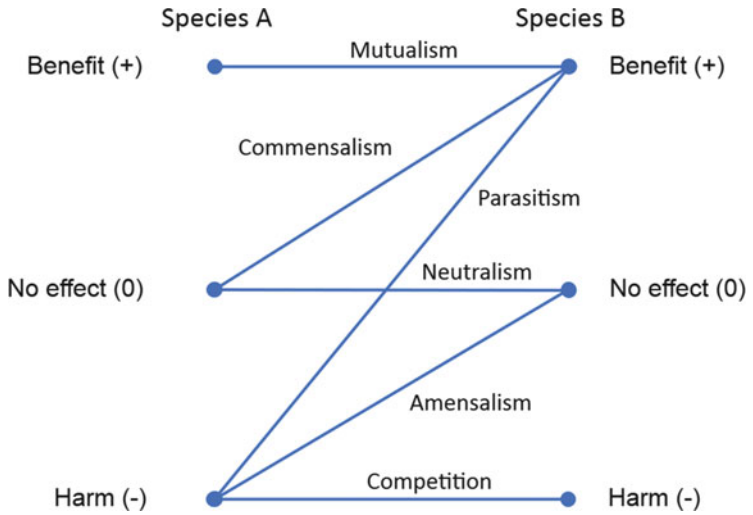


Fig. 26.4 Symbiotic relationships diagram. We now often define antibiosis, symbiosis, and many other types of relationships that occur between two species as all representing symbiosis. Those distinctions which we use for categorizing symbiotic associations represent whether the impact of one species on the other species results in either a benefit, no effect, or harm. Six types of symbiotic relationship, from mutual benefit to mutual harm, are diagrammed in this figure. This image is titled "Symbiotic relationships diagram.svg" by author Ian Alexander and it is being used under a Creative Commons Attribution-Share Alike 4.0 International license

We now often use the term symbiosis to represent many types of relationships that occur between two species. The distinctions which we use for categorizing symbiotic associations represent whether the impact of one species on the other species results in either a benefit, no effect, or harm. These variations on the general theme of symbiosis are represented in Fig. 26.4.

Endosymbionts represent one of the more interesting concepts in evolutionary biology. After all, it is perhaps easier to carry your symbionts around with you, and even inside of you, than to chance that the symbionts otherwise would always be nearby whenever you needed them. In the following sections of this essay I will present mitochondria first, because their adoption as endosymbionts presumably preceeded all of the other mutualistic symbioses that I will mention.

26.3 Aerobic Respiration and the Adoption of Mitochondria

One of the defining characteristics for many eukaryotes is the possession of mitochondria whose aerobic metabolism facilitates movement. Mitochondria are the powerhouse for organisms that variously swim, pull, crawl, walk, climb and fly. The most widely accepted hypothesis is that mitochondria originated as bacteria

from a proteobacterial lineage that were ingested by a eukaryotic organism. Somehow, the ingested proteobacteria managed to survive inside of the cell rather than being digested. That survival would have been an example of primordial success at evolutionary cheating. Mitochondria have a double membrane and part of the mitochondrial genome has been transferred to the host cell genome. The adoption of mitochondria as endosymbionts began a path of evolution that has left a trail from simple forms such as amoeba upward through both the 'animal' and 'plant' sides of our planets tree of life. Figure 26.5 shows the mitochondria inside of human cells.

Understanding the development of eukaryotic biology has in itself proven to be an interesting topic. Gould et al. (2016) have suggested that the eukaryotic endomembrane system might have originated from bacterial membrane vesicles released by the mitochondrial ancestor within the cytosol of its archaeal host at the time of eukaryotic origin. Hendrickson and Poole (2018) have presented their philosophical efforts at trying to understand evolutionary development of the eukaryotic cell nucleus.

It is not necessary that all of the cell types within an organism will contain mitochondria. An example of this would be the fact that mature mammalian erythrocytes lack mitochondria, because those cells eject their mitochondria during erythropoiesis after which the erythrocytes derive their energy via anaerobic fermentation. Interestingly, there are instances when mitochondrial functioning seems to no longer have been retained by organisms (Yahalomi et al. 2020) and mitochondria have even been lost (Karnkowska et al. 2016). These abandonments of a committment to mitochondria may be ways of trying to alter the competitive balance by not expending unnecessary energy to maintain metabolic capabilities that have become unnecessary.

26.4 Photosynthesis and the Evolution of Chloroplasts

Oxygenic photosynthesis, which I will simply refer to as photosynthesis for the remainder of this essay, uses water as its terminal reductant and generates molecular oxygen as a product. Bacterial anoxygenic photosynthesis is a different process and it oxidizes hydrogen sulfide to generate elemental sulfur. The chlorophyll to which I refer in this essay is that originated by cyanobacteria.

The development of oxygenic photosynthesis by cyanobacteria occurred perhaps 2.3 billion years ago, it was a major event in evolution and represented a cheating against the idea of purely existing by chemoautrophy. Cyanobacteria, which are prokaryotes, probably were the first photosynthesizers. When a eukaryotic organism ingested some cyanobacteria, but did not digest those cyanobacteria, the pathway which led to what we now call eukaryotic algae began to unfold (Fields 2020; Sibbald and Archibald 2020). Constantly being in close proximity to one another can lead to interesting encounters. Eating your neighbors would be a form of competitive cheating! Causing indigestion after you have been eaten could lead

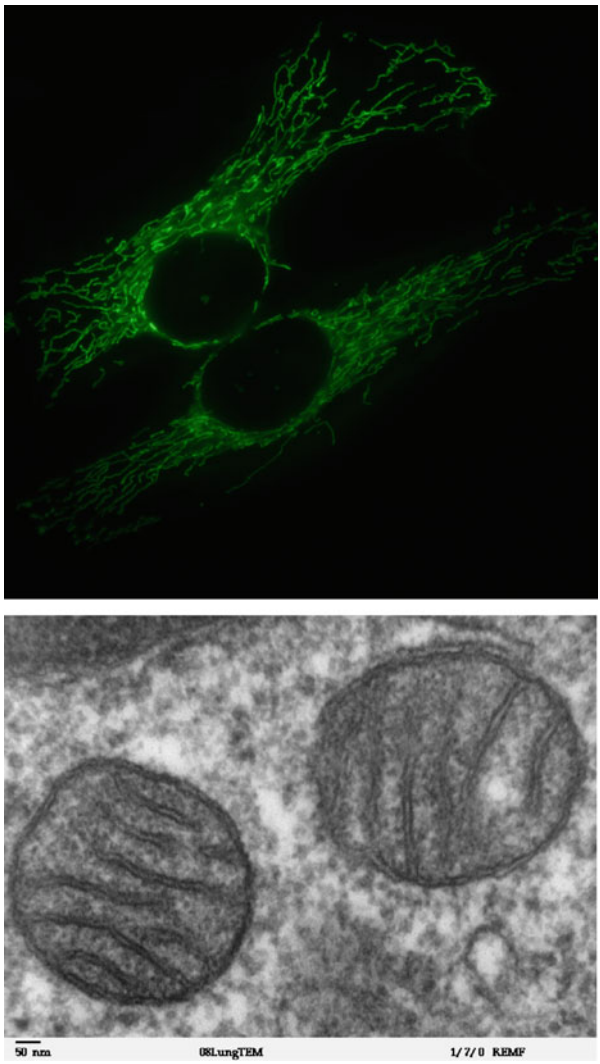


Fig. 26.5 Mitochondria. The most widely accepted hypothesis is that mitochondria originated as bacteria from a proteobacterial lineage that were ingested by a eukaryotic organism. Mitochondria have a double membrane and part of the mitochondrial genome has been transferred to the host cell genome. Mitochondria are the powerhouse of aerobic organisms. The upper image reveals the mitochondrial network (green coloration) of two cultured mammalian cells. The description for the upper image is "Fluorescent microscopy picture of HeLa cells expressing a mitochondrially targeted version of green fluorescent protein (mtGFP)" HeLa cells normally range in diameter from 20 to 40 μm. The large dark area in the center of each cell is its nucleus which has a diameter of approximately 10 μm. The upper image is titled "HeLa mtGFP.tif" by author Simon Troeder and is being used under a Creative Commons Attribution 4.0 International license. The lower image shows two mitochondria and the very small horizontal bar in the lower left underscript area indicates 50 nanometers. By my estimation the cross sectional diameter of these mitochondria thus would be 450–500 nanometers. The description provided for this lower image is "Transmission electron microscope image of a thin section cut through an area of mammalian lung tissue. The high magnification image shows a mitochondria. JEOL 100CX TEM". The title of this lower image is

Fig. 26.6 A Cyanobacterial Bloom. This image shows a bloom of freshwater cyanobacteria and the description is "Bloom of cyanobacteria in a freshwater pond. This accumulation in one corner of the pond was caused by wind drift. It looked as if someone had dumped a bucket [of] color into the water." The image is titled "Cyanobacteria Aggregation1.jpg" by author Christian Fischer and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

down another pathway of cheating. The result of that indigestion may be life as an organelle.

Figure 26.6 shows a cyanobacterial bloom. There are many diatoms, such as *Epithemia turgida* shown in Fig. 26.7 that obviously and recognizably still retain cyanobacteria as endosmybionts. Those cyanobacterial endosymbionts of *Epithemia* can improve the competitive fitness of their host by providing both fixed carbon and fixed nitrogen. Unfortunately, that type of enhanced competitive fitness cannot protect the host from being eaten. Figure 26.8 shows an image of *Amoeba proteus*, which is a eukaryote with its own endosymbiotic mitochondria, and its ingested meal of diatoms.

The evolutionary change of endosymbiont cyanobacteria into the organelles that we now know as chloroplasts must have represented a huge change in competitive fitness, second only to the establishment of mitochondria. The evolution of chloroplasts would have begun in aquatic environments with eukaryotic green algae. Figure 26.9 shows a genus of filamentous eukaryotic green algae with its very obvious chloroplasts. Figure 26.10 shows a floating colony of filamentous eukaryotic algae. When most of us think of chloroplasts, we image the kind that evolutionarily carried their successfully cheating ancestors upward to create moss and vascular plants as shown in Fig. 26.11.

Fig. 26.5 (continued) "Mitochondria, mammalian lung—TEM.jpg" by author Louisa Howard and it is a public domain image

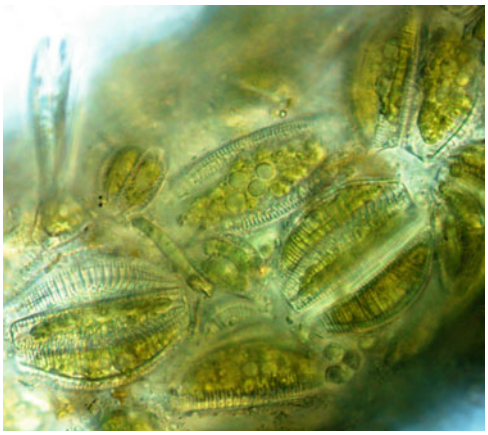


Fig. 26.7 Live *Epithemia turgida* showing endosymbiotic cyanobacteria. *Epithemia turgida* is a freshwater diatom species that often contains endosymbiotic cyanobacteria, as do many of the *Epithemia*. Those endosymbionts supply fixed nitrogen and carbohydrates. This portable supply of nitrogen enables the *Epithemia* to live in waters of low N/P ratio. In this image, the endosymbionts are visible as round bodies within the *Epithemia* cytoplasm. *Epithemia turgida* do in turn often live as epiphytes on algae and fungi. This image is being used with the kind permission of its author Rex L. Lowe

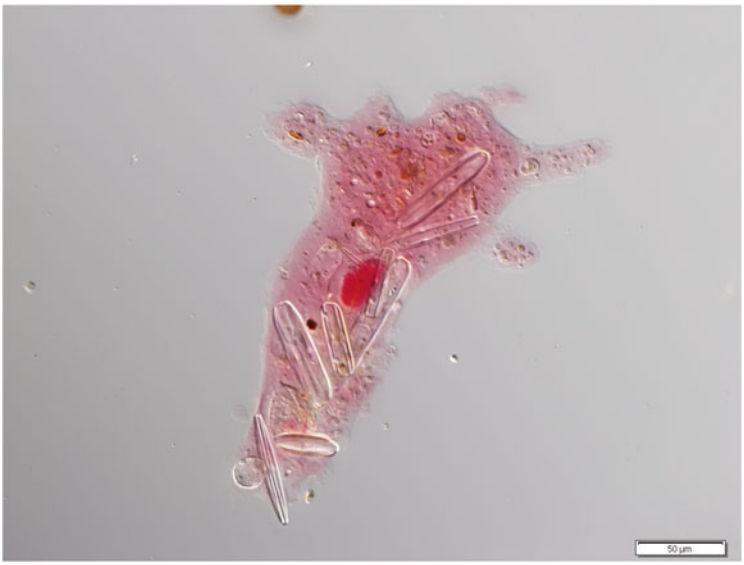


Fig. 26.8 Eating the neighbors. This photograph shows an amoeba that has ingulfed diatoms as an example of parasitism. This image is titled "Collection Penard MHNG Specimen 05bis-1-1 Amoeba proteus.tif" by author Dalinda Bouraoui and is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

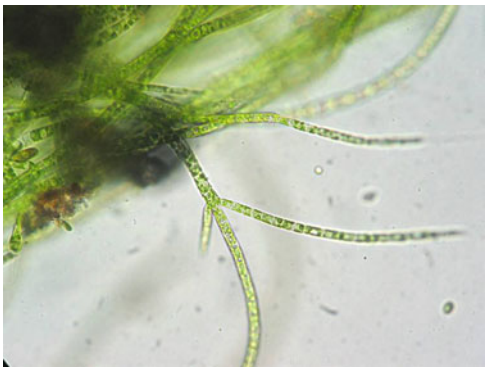


Fig. 26.9 *Stigeoclonium*, a fresh water algae. This figure shows *Stigeoclonium*, a fresh water algae that can grow free floating but most commonly is attached to either plants or hard surfaces. The title of this image is "Stigeoclonium sp zugespitzte seitenzweige.jpeg" which translates into English as [Stigeoclonium sp pointed side branches] by author Kristian Peters and the image is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

Fig. 26.10 The underside of floating algal mats. This figure shows the underside of floating algal mats. Such mats have contributed to the oxygenation of earth's atmosphere, facilitating in turn the evolutionary development and survival of aerobic life forms. The title of this image is "Floating Algal Mats (7634604112).jpg" by author Ian Sutton from Oberon, Australia and the image is being used under a Creative Commons Attribution 2.0 Generic license. This image was made along Tea Tree Creek Walk, which is in Mornington Peninsula National Park, Flinders, Victoria, Australia. I have modified the original image by performing an auto-correction

The chloroplasts which power the photosynthetic way of life depicted in Figs. 26.9, 26.10, and 26.11 are related and bounded by two membranes. Much of the genetic material for these chloroplasts has been transferred to the host cell genome, as earlier occurred during the development of mitochondria, by a process which Nancy Eckardt interestingly described as 'Genomic Hopscotch' (Eckardt 2006). And yet, there are other types of chloroplasts which seem to have either arisen independently or differently evolved when competitors needed to square off for evolution's challenge of competitive fitness.

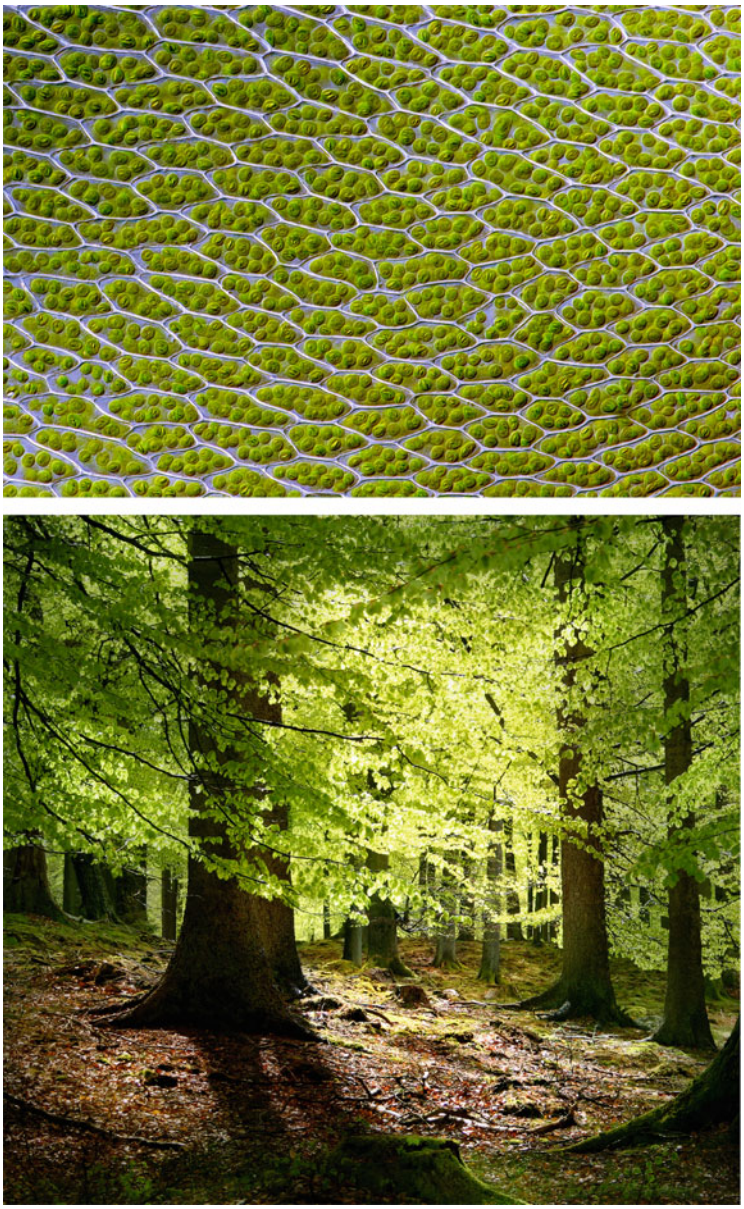


Fig. 26.11 Chloroplasts and upward mobility in the terrestrial photosynthetic way of life. Choloroplasts are a category of photosynthetic organelles. They presumably originated as cyanobacteria that had been ingested by a eukaryotic organism. Chloroplasts have a double membrane and are endosymbionts that have seen part of their genome transferred to the host cell genome. The chloroplasts are photosynthetic and provide fixed carbon for the cell. Chloroplasts are in a sense the powerhouse of the plant world. The upper image is titled "Bryum capillare leaf cells.jpg" by author Des_Callaghan and it is being used under a Creative Commons Attribution-Share Alike 4.0 International license. The description provided with the upper image was "Live leaf cells of the moss *Bryum capillare*, showing abundant chloroplasts (green spherical bodies) and their

Fig. 26.12 *Glaucocystis* algae showing its chloroplasts. The chloroplasts of *Glaucocystis* have a peptidoglycan layer which makes them different from the more typical chloroplasts. Typical chloroplasts lack a peptidoglycan layer. The peptidiglycan layer is presumed a relic representing an origin of these chloroplasts as endosymbiotic cyanobacteria. Glaucophytes have mitochondria which are double-membrane bound organelles. The description provided with this image is "*Glaucocystis* sp./from Kanazawa, Ishikawa Pref., Japan/Microscope:Leica DMRD (DIC)". This image is titled "Glaucocystis sp.jpg" by author ja:User:NEON/commons:User:NEON_ja and it is being used under a Creative Commons Attribution-Share Alike 2.5 Generic license

Glaucophytes are one variation on the theme of establishing chloroplasts from ingested cyanobacteria. Figure 26.12 shows the chloroplasts of *Glaucocystis*, which have a peptidoglycan layer that makes them different from the more typical chloroplasts. Typical chloroplasts lack a peptidoglycan layer. This peptidoglycan layer in the chloroplasts of *Glaucocystis* is presumed a relic representing the origin of these chloroplasts as endosymbiotic cyanobacteria. In case you are curious about asking the question, the answer is that 'yes', the mitochondria of glaucophytes do have the more typical double-membrane.

Chlorarachniophytes are amoeboid algae whose chloroplasts are surrounded by four membranes rather than the two membrane structure customary for chloroplasts. Figure 26.13 shows *Chlorarachnion*, and the unique four membrane structure of its chloroplasts suggests that these are secondary chloroplasts, acquired by ingestion of a eukaryotic algae rather than ingestion of a cyanobacteria. Thus, it is believed that the chloroplasts in *Chlorarachnion* were created by a two stage process. An ancestral, traditionally double membraned, chloroplast would have been created by primary symbiosis when some unknown eukaryote ingested a photosynthetic bacterium. After that eukaryote was in turn ingested by yet another eukaryote, through

Fig. 26.11 (continued) accumulated starch granules (elongated bodies within chloroplasts). Lighting = Differential Inference Contrast. Objective = Olympus UPlanFL N 60x". The lower image is titled "Grib skov.jpg" by author Malene Thyssen and is being used under a Creative Commons Attribution-Share Alike 2.5 Generic license. The description provided with the lower image was "New beech leaves, Gribskov Forest in the northern part of Sealand, Denmark." Moss can be seen growing on the bases of these beech trees

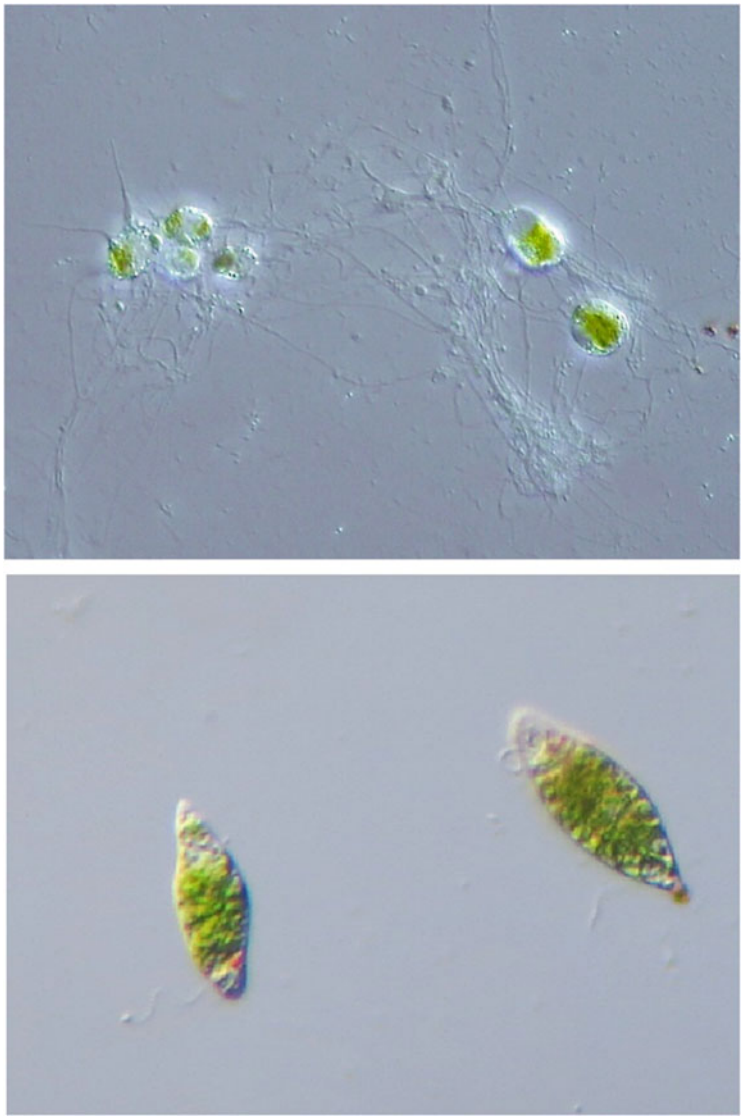


Fig. 26.13 *Chlorarachnion* with its quadruple membraned chloroplasts and *Euglena* with triple membraned chloroplasts. *Chlorarachnion* are amoeboid algae which have chloroplasts that are surrounded by four membranes. A two membrane structure is customary for chloroplasts. This unique four membrane structure suggests that the chloroplasts in *Chlorarachnion* were acquired by ingestion of a eukaryotic algae. Thus, it is believed that the chloroplasts in *Chlorarachnion* were created by a two stage process. An ancestral, double membraned, chloroplast would have been created by primary symbiosis when some unknown eukaryote ingested a photosynthetic bacterium. After that eukaryote was in turn ingested by yet another eukaryote, through secondary symbiosis the plastid would have been retained in a form that was surrounded by the quadruple membrane. The upper image is titled "Chlorarachnion reptans.jpg" by author ja:User:NEON/commons:User: NEON_ja and it is being used under a Creative Commons Attribution-Share Alike 2.5 Generic license. *Euglena* have chloroplasts that are bounded by three membranes, as do some

Fig. 26.14 *Paulinella.* This composite image shows *Paulinella* with its chromatophores. The image at left is of *Paulinella micropora* and appears courtesy of Hwan Su Yoon, the size bar is 10 µm. The image at right is titled "Paulinella chromatophora.jpg" by authors Luis Delaye, Cecilio Valadez-Cano, and Bernardo Pérez-Zamorano, it has been auto-corrected, and is being used under a Creative Commons Attribution 2.5 Generic license

secondary symbiosis the plastid would have been retained in a form that was surrounded by the existant quadruple membrane. *Euglena*, as also shown in Fig. 26.13, have secondary chloroplasts that interestingly are bounded by three membranes. Some dinoflagellates similarly have chloroplasts that are surrounded by three membranes.

Paulinella, shown in Fig. 26.14, has chromatophores that are different from the chloroplasts mentioned above. The chloroplasts of most eukaryotes seem to have derived, perhaps beginning a billion years ago, from a single incidence of a eukaryote ingesting but not digesting a cyanobacterium. *Paulinella* instead may have more recently taken on its endosymbiont cyanobacteria, with a guess of that having occurred 90–140 million years ago (Lhee et al. 2019). The chromatophores of *Paulinella* have undergone a genomic reduction but still are far larger than most plastid genomes, and some of this endosymbionts genetic information has been transferred to the host cell nucleus. Not all species of *Paulinella* possess these chromatophores. It is possible that the chromatophores of *Paulinella* represent a attempt to create a more balanced competition against the other above mentioned biological groups that contain chloroplasts. It also is possible that the

Fig. 26.13 (continued) dinoflagellates. The lower image is titled "Euglena—400× (8999902391).jpg" by author Picturepest, it has been cropped, and is being used under a Creative Commons Attribution 2.0 Generic license

chromatophores of *Paulinella* represent an attempt to achieve an evolutionary benefit which allows competitive success against the biological groups mentioned below, in Sect. 5. Microalgae such as *Paulinella* dynamically interact with bacteria in the phycosphere, which is a zone surrounding the phytoplankton cell (Lee and Jeon 2018), and those interactions may have evolutionary consequences for all of the participants.

26.5 Other Ways of Benefitting from Chloroplast's Photosynthetic Activity

There are many ways in which species have cheated, or at least tried to level the competition, by adopting photosynthetic cyanobacteria and algae without those adopted photosynthesizers becoming organelles. Or, the photosynthesizing symbionts have at least NOT YET become organelles.

26.5.1 Lichens Are a Mutualistic Association with Fungi in Which the Photosynthetic Symbiont Is Not Intracellular

Lichens are one of these examples, and Fig. 26.15 presents what is called a Wolf lichen. Lichens are typically bipartite composite organisms consisting of a photobiont and a mycobiont. The photobiont may be either a cyanobacteria or a green algae. There also are tripartite lichen symbioses which may contain both cyanobacteria and green algae in addition to the fungus. Many fungal genera contain members that form lichens. The mycobiont of most lichens is an ascomycete, although a few lichens are formed by basidiomycetes. The photobiont does not reside as an intracellular symbiont. Lichens contain their photobiont cells as a layer enclosed by hyphae of the partnering mycobiont. The cyanobacteria and green algae are photosynthetically active and contribute carbohydrates to this symbiosis. The cyanobacteria additionally are capable of contributing fixed nitrogen. The fungal hyphae serve by providing a protective shelter for their photosynthetic partner. Lichens generally are found in terrestrial environments although there are a few aquatic lichens. In terrestrial lichen symbioses, the fungus may serve by providing a surface area that can capture and retain rainwater thus supplying the photobiont with necessary moisture. The original lichen symbiosis might have been cheating, and all of the other lichen symbioses could represent efforts to level the competition. I used this image of a Wolf lichen when I created the artwork "Volvox reimagined" that appears on the cover of this book series.

Du et al. (2019) have demonstrated that cocultivation of mutualistic algae and fungi could result in eventual internalization of the photosynthetic algal cells. The

Fig. 26.15 An example of the wolf lichen, *Letharia vulpina*. Lichens are typically bipartite composite organisms consisting of a photobiont and a mycobiont. The photobiont cells variously are either a cyanobacteria or a green algae, those cells are photosynthetically active and contribute carbohydrates to this symbiosis. Lichens contain their photobiont cells as a layer enclosed by hyphae of the partnering mycobiont. The mycobiont will be either an ascomycete or a basidiomycete. There also are tripartite lichen symbioses which contain both cyanobacteria and green algae in addition to the fungus. This image is titled "Letharia vulpina JHollinger crop.jpg" and was photograghed on Mount Gleason in the San Gabriel Mountains near Los Angeles, California, USA. The author is Jason Hollinger and the image is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license. I incorporated Hollingers image of *Letharia vulpina* into the photomontage "Volvox reimagined" that appears as cover art for this book series

internalized algae cells then persisted in growing, dividing and functioning within the fungal hyphae. Perhaps lichens will follow this evolutionary path as a way of changing their competitive balance. Alternatively, the algal and fungal symbionts that form lichens may have tried internalization and found the idea to not have offered a competitive advantage. The consequences of an unsuccessful effort at evolutionary cheating likely would be a waste of energy and could be even worse than never having tried.

26.5.2 Mutualistic Associations by Which Animals Have Acquired Photosynthetic Symbionts

There are two approaches which animals have used to cheat the system by becoming photosynthetic. One of these approaches is to establish an intracellular symbiosis with intact, viable algal cells which the animals either have inherited or acquired by ingestion. The other approach is termed kleptoplasty, a process by which the animals ingest algae and then temporarily retain only the chloroplasts in a functional form.

Hydra viridissima has a symbiotic association with *Chlorella vulgaris*. The symbiont algae generally are inherited although, at least experimentally, they also

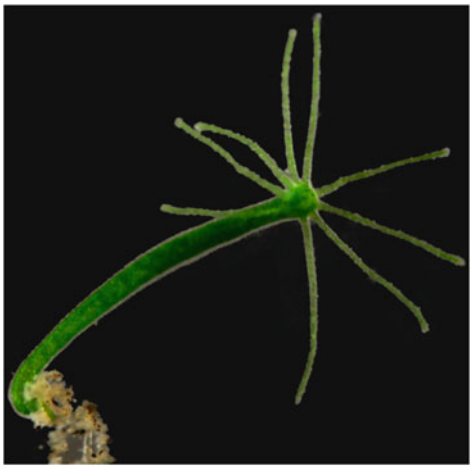


Fig. 26.16 *Hydra viridissima* showing the green color from its endosymbiont *Chlorella.* This image is titled "Mikrofoto.de-Hydra 15.jpg" by author Frank Fox www.mikro-foto.d and is being used under a Creative Commons Attribution-Share Alike 3.0 Germany license. The image has been cropped. *Hydra viridissima* has a symbiotic association with *Chlorella vulgaris*. The *Chlorella* cells are endosymbiotically present and viably reside intracellularly as individual algal cells within symbiosomes. Symbiosomes are intracellular vacuoles which protect the symbiont from being enzymatically attacked and digested by the host cell. For this partnering association, the symbiosomes are located within the endodermal epithelial cells of the *Hydra*. Each of those cells may contain approximately 20–40 symbiosomes. The algae provide products of photosynthesis in exchange for protection against predators

may be acquired from the environment. The *Chlorella* are endosymbiotically present and viably reside individually within symbiosomes. These symbiosomes are intracellular vacuoles which protect the algae from being enzymatically attacked by the host cell. For this association, the symbiosomes are located within the endodermal epithelial cells of the host. Each of those endodermal epithelial cells may contain approximately 20–40 symbiosomes, and each symbiosome typically contains a single *Chlorella* cell. The algae provide products of photosynthesis in exchange for protection against predators. *Hydra viridissima*, shown in Fig. 26.16, is the only *Hydra* species that has this type of association with *Chlorella* and the relationship can be perceived as a means of evolutonarily cheating against the other *Hydra* species. Hamada et al. (2018) have presented information on the metabolic co-dependence of the *Hydra-Chlorella* symbiosis.

Corals have developed symbioses with photosynthetic partners that have enabled tremendous evolutionary success (Frankowiak et al. 2016). Each of these partnerships competes against the others for predominance. Figure 26.17 shows one example of this symbiosis, *Porites porites*, including the presence of its intracellular symbionts which are viable *Symbiodinium* dinoflagellates sheltered within their protective symbiosomes. The symbiont algae can be either inherited or each generation of coral may newly acquire symbionts from the environment. The coral starve without their symbiont algae and research efforts have been progressing to develop

Fig. 26.17 The coral *Porites porites*. The figure shows the coral *Porites porites* and the location of its mutualistic endosymbionts, which are dinoflagellates of the genus *Symbiodinium*. The *Symbiodinium* are housed intracellularly inside symbiosomes within the endodermal tissues of corals. The *Symbiodinium* undergo mitotic division within those endodermal cells. These symbionts provide products of photoshythesis in exchange for being sheltered by their host. The symbionts also provide the typical coloration of their host. The upper image is titled "Porites porites French Bay.jpg" by author Jstuby at the Wikipedia project and is in the public domain. It shows a small colony of *Porites porites* in French Bay, San Salvador Island, Bahamas and the image has been cropped. The lower image is a scanning electron micrograph of freeze-fractured internal mesentery from a reef coral polyp of *Porites porites*. The lower image is titled "HostTissue section.png" by author Allisonmlewis and is being used under a Creative Commons Attribution-Share Alike 4.0 International license

greater thermotolerance in this vital symbiotic association (Buerger et al. 2020). I would be remiss if I did not mention the types of photosymbiotic relationships that similarly exist between dinoflagellates and anemones such as *Exaiptasia* (Foo et al. 2020), and the photosymbiotic relationships that exist between dinoflagellates and *Cassiopea* jellyfish. *Hydra*, the anemones, corals, and jellyfish are, of course, all members of the phylum Cnidaria which suggests that members of this animal phylum may have been cheating with their photosymbionts for a long while.

Sponges that live in shallow water often contain viable photosynthetic intracellular endosymbionts that variously may be either eukaryotic algae, or cyanobacteria (Erwin and Thacker 2007), or dinoflagellates. These endosymbionts can be inherited and they provide the host animal with products of photosynthesis. The fact that these types of photosynthetic symbionts found in sponges vary so greatly suggests that the sponges may have been trying to level the competitive playing field when they partnered with their photosynthetic symbionts. Figure 26.18 shows examples of shallow-water sponges. Deep water sponges may contain bacterial symbionts that provide nutritional benefits to their host.

Molluscs have cheated by using photosymbiosis in several ways! There are groups of bivalve molluscs that house photosynthetically active dinoflagellates (Armstrong et al. 2018; Li et al. 2018). Their molluscan relatives the sea slugs use kleptoplasts, which is an interesting story. The molluscan clade Sacoglossa contains many species of sea slugs that feed upon algae and retain only the algal chloroplasts, which are kept as organelles, within the animals intestinal cells. These captured chloroplasts can continue to function for a few months, providing a photosynthetic benefit to their host. This method of acquiring endosymbiont chloroplasts is called kleptoplasty (Laetz and Wägele 2017) meaning the chloroplasts have been stolen. The green pigment additionally may provide camouflage to the slugs. These animals sometimes are described as being 'solar powered' slugs. Figure 26.19 shows four species representing three genera of kleptoplastic sea slugs. *Elysia chlorotica*, shown upper left in Fig. 26.19, has been described as containing a constellation of stars in reference to its chloroplasts. *Costasiella kuroshimae*, shown upper right in Fig. 26.19, has a strange cuteness that resembles a child who has discovered green hair dye. There are several additional genera of seas slugs including *Bosellia* that form kleptoplasts but are not represented in Fig. 26.19. Evolutionarily, the first slug which learned to form kleptoplasts would have been cheating. The other kleptoplastic sea slugs might be perceived as either descendants of that first kleptic sea slug, or competitors that kleptically have leveled the playing field.

Flat worms also can form kleptoplasts! There are marine flatworms, including *Baicalellia solaris* and *Pogaina paranygulgus*, that accumulate kleptoplasts while feeding upon their diet of diatoms. Which cheated first? Was it the flat worms or the slugs?

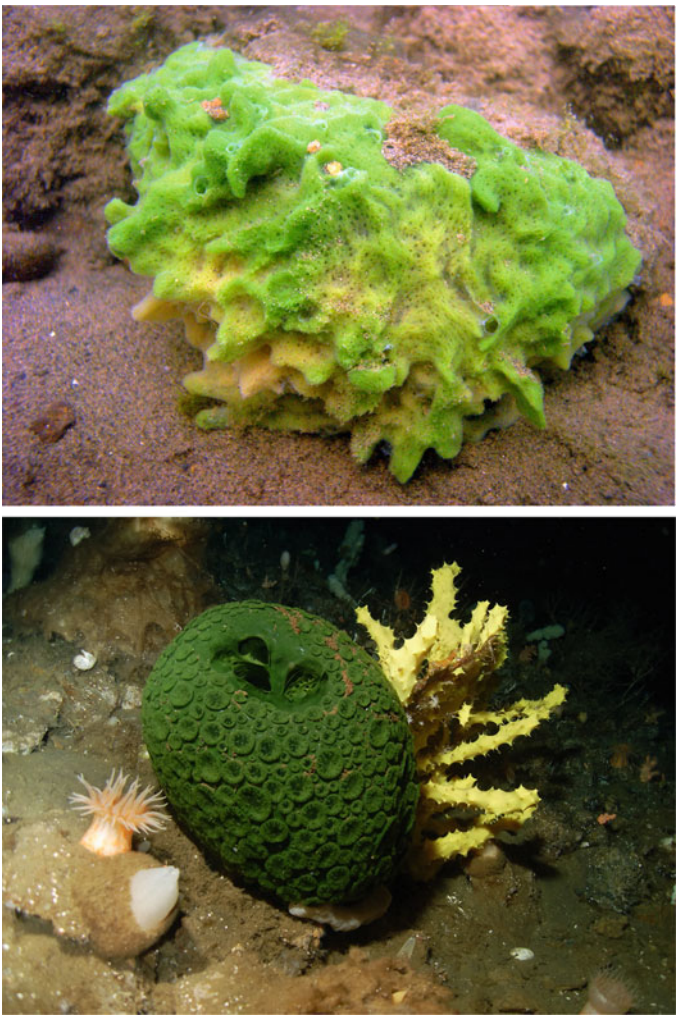


Fig. 26.18 Sponges containing photosynthetic endosymbionts. Many species of sponge contain intracellular photosynthetic symbionts. Those sponges typically house either *Chlorella*, cyanobacteria which often are *Synechococcus* including Candidatus Synechococcus spongiarum, or diatoms. The upper image shows a freshwater sponge, *Spongilla lacustris*, whose green coloration comes from its endosymbiotic *Chlorella*. That sponge was gowing in the Hanford Reach of the Columbia River in Washington State, USA. The image is titled "Spongilla lacustris.jpg" by author Kirt L. Onthank and is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license. The lower image is titled "Green and yellow sea sponges, Antarctica.JPG" by author Steve Rupp, National Science Foundation, and it is in the public domain. This lower photograph was made in McMurdo sound, Antarctica. Shallow water marine sponges often contain cyanobacteria or diatoms as photosynthetic symbionts. The dark green color of this sponge could suggest that it contains cyanobacteria. However, Robert Thacker very kindly has helped me to understand that in this case it is not possible to identify the symbiont from examining the photograph since many antarctic sponges host diatoms which might also be parasites on the host sponge

Fig. 26.19 Kleptoplastic Sea Slugs. This figure shows four species representing three genera of kleptoplastic sea slugs. The slugs feed upon algae but meanwhile phagocytize and then temporarily retain the chloroplasts as intracellular photosynthesizing symbionts. Those retained chloroplasts are termed 'kleptoplasts'. Clockwise from the upper left these animals are: *Elysia chlorotica*, *Costasiella kuroshimae*, *Oxynoe* [species uncertain], and *Elysia pusilla*. The upper left image is titled "Elysia-chlorotica-body.jpg" by author Karen N. Pelletreau et al., it is being used under a Creative Commons Attribution 4.0 International license and has been auto-corrected. The description which accompanies this image is "Anatomy of the sacoglossan mollusc Elysia chlorotica. Sea slug consuming its obligate algal food Vaucheria litorea. Small, punctate green circles are the plastids located within the extensive digestive diverticula of the animal." *Elysia chlorotica* typically grow to between 20 and 30 mm in length but can attain a length of 60 mm. The upper right image is titled "Costasiella Kuroshimae (19080120525) (2).jpg" by author alif_abdulrahman and is being used under a Creative Commons Attribution-Share Alike 2.0 Generic license. The image has been cropped. *Costasiella kuroshimae* grow to be 1 cm in length. The lower right image is titled "Oolivacea.Mgiangrasso.jpg", the author is anonymous although the description states the animal was photographed in the reef aquarium of aquarist Mike Giangrasso. This image is being used under a Creative Commons Attribution-Share Alike 2.5 Generic license. I have performed an auto-correction on the image. This species of slug, which grows to between 3 and 5 cm in length, was identified as Oxynoe olivacea. Oxynoe olivacea is not an officially recognized taxonomic name. Other members of the genus *Oxynoe*, including *Oxynoe antillarum*, also contain kleptoplasts. This specimen of *Oxynoe* was shown feeding upon *Caulerpa racemosa*. The lower left image is titled "Elysia pusilla.png" by authors Katharina Händeler, Yvonne P. Grzymbowski, Patrick J. Krug & Heike Wägele, and it is being used under a Creative Commons Attribution 2.0 Generic license. This particular specimen of *Elysia pusilla* was described as being approximately 1 cm in length

26.5.3 Cilliates and their Photosynthetic Endosymbionts

Ciliates have acquired photosynthetic capablility by ingesting and then retaining either intact viable algae as endosymbionts or keeping just the chloroplasts. Four of these ciliate groups are represented in Fig. 26.20.

Paramecium bursaria (He et al. 2019) and *Stentor polymorphus* (Wagner 2015) are examples of ciliates that ingest *Chlorella* algae and then retain those algae as viable endosymbionts. These two species of ciliate are presented on the right side of Fig. 26.20. Kodama and Fujishima (2015) have determined that infectivity of the *Chlorella* symbiont for *Paramecium* differs based upon the growth phase of the *Chlorella*.

Laboea strobila and *Myrionecta rubra* are examples of ciliates that eat algae but internally retain only the chloroplasts, and whether these accumulated chloroplasts can be called kleptoplasts is a technicality still being debated. These two species of ciliate are presented on the left side of Fig. 26.20.

Stoecker et al. (1988) helped to define our understanding of how *Laboea strobila* utilizes its accumulated chloroplasts. The chloroplasts of *Myrionecta rubra* are in turn ingested, and presumably used, by dinoflagellates which feed upon the *Myrionecta* (Park et al. 2006).

26.6 The Provision of Nitrogen by Microbial Symbionts of Plants

With all plants possessing mitochondria and chloroplasts, the ecological competition between plants may have seemed more balanced. So, then, what might have come along next as a way of gaining a competitive edge? I would guess that finding ones own source of fixed nitrogen that could be delivered directly to their roots may have been the next effort that plants made for gaining an evolutionary benefit by cheating against their competitors.

It is hard to know where and when this round of competition began. Fixed nitrogen has become the common currency for many symbiotic interactions. The need for plants to gain symbiotic associations with microbes that could provide fixed nitrogen might have resulted in plants initially sharing their roots with cyanobacteria. For that reason, I will start this part of my essay by presenting examples of nitrogen being supplied to plant roots by symbiotic cyanobacteria, which likely also had been the source of chloroplasts. Today, we know those nitrogen-providing microbes as *Anabaena*, *Calothrix* and *Nostoc*, which are members of the phylum Cyanobacteria, order Nostocales.

But, there also are two additional options that have survived until the present day. The proteobacteria provided one of those options, we know these successful microbes as *Rhizobium* and their close relatives. The name *Rhizobium* represents a genus and also is the name of a more general taxonomic group, both of which belong

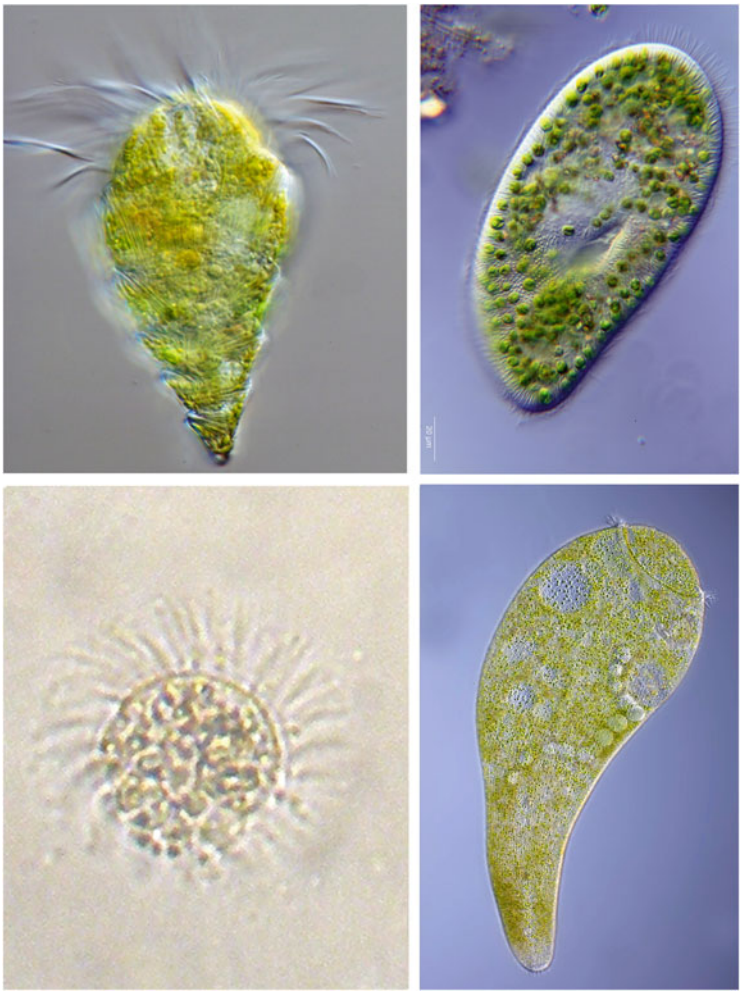


Fig. 26.20 Ciliates that acquire photosynthetic capability from their algal diet. Clockwise from the upper left, the images contained in this montage show the ciliates *Laboea strobila*, *Paramecium bursaria*, *Stentor polymorphus*, and *Myrionecta rubra*. All four of these species intracellularly accumulate photosynthetic capabilities from the algae which they have ingested. *Paramecium bursaria* and *Stentor polymorphus*, shown as the right half of this figure, consume *Chlorella* and retain those algae cells as viable, photosynthetically active endosymbionts. *Laboea strobila* and *Myrionecta rubra*, shown as the left half of this figure, ingest algae but then retain only the functional chloroplasts from their meals. The upper left image is titled "Live cell of the ciliate Laboea strobila from Formia Harbour, length 100 µm" by author Franz Neidl and is being used under a Creative Commons Attribution 3.0 License, the image has been adjusted and cropped. *Laboea strobila* feeds upon a variety of eukaryotic algae including *Chroomonas salina*, *Isochrysis galbana*, and *Pyramimonas*. The upper right image is titled "Paramecium bursaria.jpg" by author Anatoly Mikhaltsov, it is being used under a Creative Commons Attribution 4.0 International license and has been cropped. The size bar in the lower left corner of that image denotes 20 µm. *Paramecium bursaria* typically are 80–150 µm in length. The English translated description which accompanied this image is "Ciliate: Paramecium bursaria Her. It has a mutualistic endosymbiotic relationship with green algae called Zoochlorella. The algae are clearly visible in ciliate cytoplasm.

to the order Rhizobiales. Actinobacteria provided the other option and we call their descendants *Frankia*, a genus that was named in honor of Bernhard Frank. The *Frankia* are members of the phylum Actinobacteria, order Frankiales.

26.6.1 Cyanobacteria as Symbionts that Provide Fixed Nitrogen for Their Host Plants

Azolla is a genus of water ferns, also called mosquito ferns, that grow floating on the surface of freshwater. As shown in Fig. 26.21, these plants contain leaf pockets in which are housed symbiotic cyanobacteria of the genus *Anabeana*. The *Anabeana* provide fixed nitrogen to the plant. In warm climates where *Azolla* can overwinter, it has a long history of being used as a natural fertilizer in rice paddies. The density of *Azolla* plants also can block light which prevents competition from plants other than the rice. Rice plants are transferred to the paddies after growing tall enough to emerge above the *Azolla*. Cyanobacteria have a similar association with *Lemna* and *Spirodela*, both of which are floating freshwater plants commonly called duckweed.

Cycads (class Cycadopsida) are an ancient group of terrestrial plants which produce lateral roots that contain coralloid structures, meaning that those structures are coral-like in appearance. Figure 26.22 shows the difference between coralloid roots and more typical plant roots of cycads. Coralloid roots can be visible on the soil surface and they can extend to approximately 30 cm beneath the surface. Figure 26.23 shows a large mass of coralloid roots growing on a larger cycad. There is a diverse group of microorganisms associated with these coralloid roots. Importantly, cyanobacteria enter the coralloid roots of cycads and there the cyanobacteria become endosymbiotic. These symbiotic microbes typically are identified as *Anabaena cycadae*, also recognized as *Nostoc cycadae*. The coralloid roots similarly may contain members of the cyanobacterial genus *Calothrix*. The role of these symbiotic cyanobacteria includes fixing nitrogen and additionally producing beneficial amino acids among which are asparagine and citrulline. Figure 26.24 shows the ring shaped band of cyanobacteria that is located inside of a cycad coralloid root.

I want to thank Prashant Singh for very kindly helping me to understand the state of knowledge and questions that still exist regarding the taxonomy of those

Fig. 26.20 (continued) Light microscopy, differential interference contrast (DIC)". The lower right image is titled "Mikrofoto.de-Stentor-1.jpg" by author Frank Fox, it is being used under a Creative Commons Attribution-Share Alike 3.0 Germany license, the image has been adjusted and cropped. *Stentor polymorphus*, the species shown in this image, typically can be 500–1500 μm in length. The lower left image is titled "Myrionecta rubra.jpg" by author Minami Himemiya, it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license and has been cropped. *Myrionecta rubra* can be up to 100 μm in length and 75 μm in width. *Myrionecta rubra* feed upon algae of the class cryptophyceae and form blooms that are called Red Tide. All of these images have been rotated 90 degrees to the right so that the combined figure fits better on a printed page

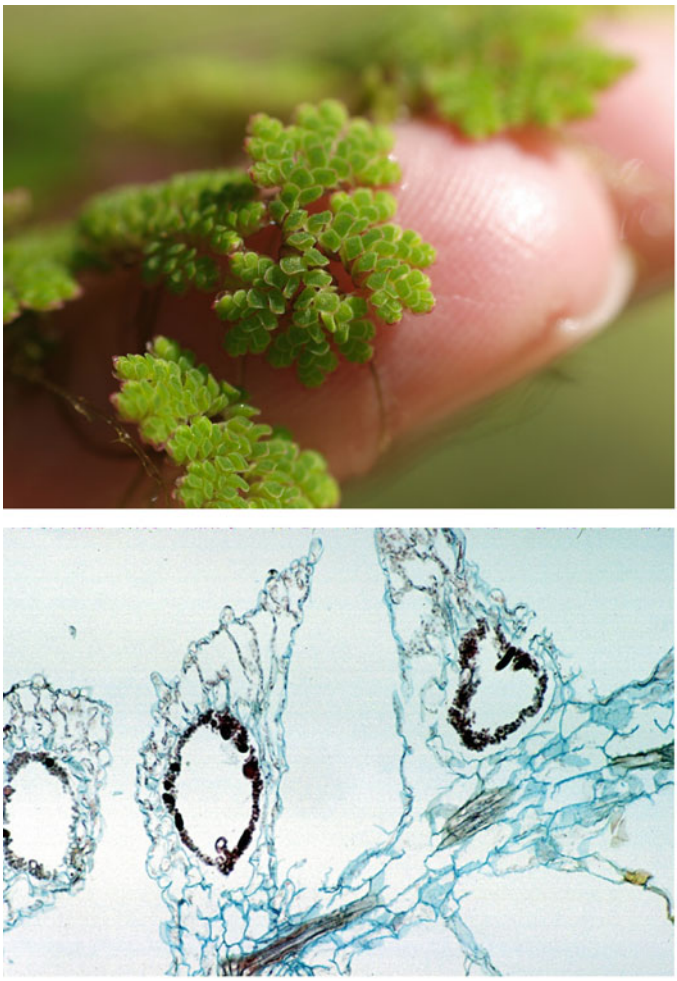


Fig. 26.21 The water fern *Azolla* and its symbiont *Anabeana.* Water ferns of the genus *Azolla*, also called mosquito ferns, contain leaf pockets in which are housed symbiotic cyanobacteria of the genus *Anabeana*. The *Anabeana* provide fixed nitrogen to the plant. The upper image is titled "Azolla caroliniana.jpg" by author Ingrid Taylar from San Francisco Bay Area—California, USA, and it is being used under a Creative Commons Attribution 2.0 Generic license. The lower image is titled "Azolla lvs LS with Anabaena.jpg" with the provided description "Light micrograph of a longitudinal section of Azolla, showing the cyanobacterium Anabaena in leaf pockets" by author Curtis Clark and is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license. In this lower image, the *Anabaena* are visible as a band of dark cells lining the interior of the leaf pockets

endosymbiotic cyanobacteria which are found in the leaf pockets of *Azolla* and the coralloid roots of cycads. What follows is the information that I received from him regarding these cyanobacterial endosymbionts:

Fig. 26.22 The difference between coralloid roots and more typical plant roots of cycad. This montage shows the difference between coralloid roots, which are shown as a cluster in the upper left of the cycad root zone, and the cycads more typical plant roots. These images are of *Zamia integrifolia* and used with permission of their author, Jennifer Possley of the Fairchild Tropical Botanic Garden, Coral Gables, Florida

The morphology of filaments have both been reported to be '*Nostoc*' like and '*Anabaena*' like in the corraloid roots of *Cycas*. Molecular evidence has given indications of proximity to *Nostoc* but these have not been very exhaustive studies.

What I feel personally (from my experiences as a taxonomist and a Professor who regularly conducts practical sessions on both Corraloid Root and *Azolla* fronds) is as follows:

Fig. 26.23 Coralloid roots associated with cyanobacteria on cycad. Coralloid roots can be visible on the soil surface and they can extend to approximately 30 cm beneath the surface. The title of this image is "Cycas revoluta coralloid roots. JPG" by author Esculapio and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

1. There could be more cyanobacterial genera inside both these symbiotic systems. Its just that some people need to do a proper global sampling collaboration to discover and describe them. Example: Last year another famous symbiont *Trichormus azollae* was reclassified to the new genus *Desikacharya*.
2. There could also be a complex of cyanobacterial symbionts and they may differ from place to place and with climatic conditions too at the same place.
3. Lastly, the filaments inside the root usually look very short (at least in case of the samples we have seen here in India). So, they could resemble *Anabaena* appearing straight and being less curved.

26.6.2 Proteobacteria as Symbionts that Provide Fixed Nitrogen for Their Host Plants

Proteobacteria also have developed a root-associated symbiosis in which they provide fixed nitrogen to their plant host, presumably in exchange for compounds provided by the plant. This section of the essay is about those proteobacteria known as *Rhizobium*, and I have included other members of the order Rhizobiales.

Hermann Hellriegel and Hermann Wilfarth were agricultural chemists who performed studies at the Anhalt Research Station in Bernburg, Germany.

Hellriegel and Wilfarth were studying legumes, which are plants of the family Fabaceae. Legumes are capable of forming very obvious root nodules. We have since learned that these nodules represent an association between the plants and symbiotic proteobacteria. There, in Bernburg, these two researchers examined the role of legume root nodules as a source of nitrogen for supporting plant growth (Hellriegel and Wilfarth 1888). Hermann Hellriegel was born October 21, 1831 in Mausitz, Germany and died September 24, 1895 in Bernburg, Germany. Hermann Wilfarth was born May 21, 1853 in Hamburg, Germany and died November 27, 1904 in Bernburg, Germany. Figure 26.25 is a photograph of Hermann Hellriegel. Unfortunately, I could not find a photograph of Hermann Wilfarth.

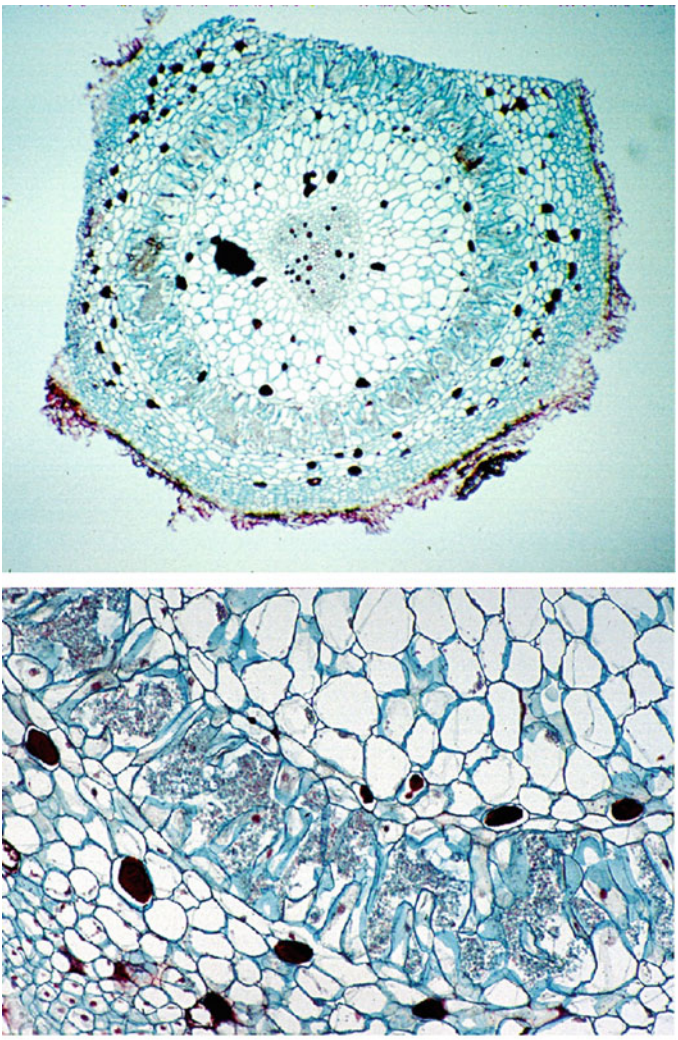


Fig. 26.24 Cycad coralloid root sections showing the location of endosymbiont cyanobacteria. The figure shows the ring shaped band of cyanobacteria located inside of a cycad coralloid root. In the upper image, the cyanobacteria are located wthin a circular band that begins slightly more than half the distance outward from the center of the root. The lower image shows a higher magnification of the root cross section, in which the cyanobacteria are visible as tightly clustered small blue dots within a wide arc that extends from the upper left downward to the lower right. The upper image is titled "Cycas coralloid root XS low.jpg". The lower image is titled "Cycas coralloid root XS high. jpg". Both of these images are by author Curtis Clark and are being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

Fig. 26.25 A Photograph of Hermann Hellriegel

Hermann Hellriegel and Hermann Wilfarth also were studying barley and oats, which belong to the family Poaceae and do not form root nodules.

Legumes include the genus *Lotus* whose members commonly are called trefoils, the genus *Ornithopus* which contains some species commonly called bird's-foot and other species commonly called serradellas, and the genus *Vigna* many of whose members are called beans or peas. Some additional legume genera are: *Aeschynomene*, *Discolobium*, *Neptunia*, and *Sesbania*.

The formation of rhizobial root nodules on legumes is a complicated process, involving interactions that include the bacteria suppressing the plants innate immune system. The plant limits the supply of nutrients to the nodules and seems to limit the number of nodules (Cao et al. 2017). The symbiosis which creates root nodules of legumes is in fact only one aspect of a complicated community of microorganisms that reside in and around plant roots (Zgadzaj et al. 2016). Rhizobial bacteria also can produce stem nodules, and those nodules occur at the site of dormant root primordia on some aquatic legumes and on legumes whose roots grow in flooded soil.

Figure 26.26 is a photograph which shows results from Hellriegel and Wilfarth's 1887 comparative cultivation of the plant species serradella, whose taxonomic name is *Ornithopus sativus*. The goal of this experiment was to determine if a microbe was responsible for the formation of root nodules which suppy nitrogen for use by these plants. Hellriegel and Wilfarth were particularly concerned about the possibility of their plant cultivation experiments being contaminated by fungal spores, because they believed that a fungus was responsible for inducing root nodule formation. The conclusion of Hellriegel and Wilfarth was that a microbe naturally present in soil did induce formation of those root nodules. We have come to learn that the root nodules are caused by *Rhizobium*, which is a name that now represents a genus and also a more general group of bacteria. The dominant rhizobial species found in the root nodules of lupines and serradella growing in europe are *Bradyrhizobium canariense* and *Bradyrhizobium japonicum*. Figure 26.27 shows *Ornithopus sativus* in bloom and with seed pods. Figure 26.28 shows examples of rhizobially induced root nodules on two different species of legumes. Figure 26.29 shows the appearance

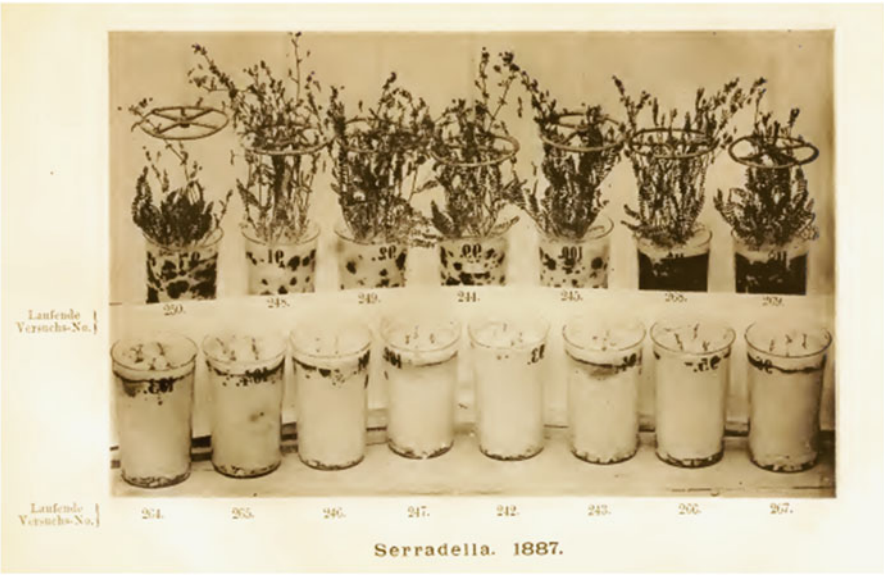


Fig. 26.26 Hellriegel and Wilfarth's results from a comparative cultivation of Serradella. This photograph shows some of Hellriegel and Wilfarth's (1888) results from a comparative cultivation of serradella (*Ornithopus sativus*), which is an important annual legume grown in pastures. Those plants in the back row were permitted access to a vital microbial symbiont that lives in soil. That symbiont microbe induces root nodule formation and fixes atmospheric nitrogen within the root nodules. Those plants in the front row were denied access to this microbial symbiont. The specific details of this experiment were that Hellriegel and Wilfarth surface sterilized their serradella seeds with a solution of mercuric chloride, and afterwards the seeds were rinsed with absolute alcohol and boiled water. The sterilized serradella seeds were planted in containers of quartz sand after the sand had been treated by a chemical washing and by dry heat sterilizaion. Next, a nitrogen-free nutrient mixture was added to the sand. The surface of the sand was then covered with a thick layer of sterilized cotton. That cotton was intended to prevent microbes in the air from landing in the soil. The container numbers are: in the back row from left to right 250, 248, 249, 244, 245, 268, 269; in the front row from left to right 264, 265, 246, 247, 242, 243, 266, 267. Irrigation of the planted seeds was done with distilled water. Containers 242 and 243 received nothing further except for distilled water. Containers 244, 245, 248, 249 and 250 additionally received an infusion prepared from light sandy soil from a lupine field, and that infusion provided a natural microbial inoculum. Containers 246 and 247 received the same infusion after it had been heated to boiling temperature to destroy its microorganisms. Containers 266 and 267 were supplemented with carbonated lime. Containers 268 and 269 received carbonated lime and unboiled soil infusion. Containers 264 and 265 received boiled soil infusion plus 0.041 g calcium nitrate = 0.007 g nitrogen. This photograph was made on August 1st, 1887 and published in "Untersuchungen über die Stickstoffnahrung der Gramineen und Leguminosen" by Hermann Hellriegel and Hermann Wilfarth (1888)

of bacteroids in a root nodule on *Glycine max*. These bacteroids are symbiosomes in which the nitrogen-fixing rhizobial microbes are surrounded by a host-derived peribacteroid membrane.

George T. Moore (Moore 1905) noted that some strains of rhizobial bacteria produced nodules but did not benefit nitrogen assimilation by leguminous crops and,

Fig. 26.27 *Ornithopus sativus* in bloom and with seed pods. These photographs are of *Ornithopus sativus*, which has the common name serradella and was the plant species grown by Hellriegel and Wilfarth as shown in Fig. 26.26. The upper part of this image is titled "Ornithopus sativus flower, serradelle bloem.jpg". The lower part of this image is titled "Ornithopus sativus flowers and seedpods, serradelle bloemen en peulen.jpg". Both images have been modified by cropping and auto-corrected. Both images are by author Rasbak and being used under a Creative Commons Attribution-Share Alike 1.0 Generic license

interestingly, some other bacterial strains seemed to fix nitrogen without the formation of root nodules. Crook et al. (2012) eventually discovered that a naturally existing plasmid can result in rhizobial strains that have enhanced competitiveness for nodule occupancy but impair nitrogen fixation, resulting in reduced benefit to the host plant.

Coba de la Peña et al. (2018) have presented a question of whether the symbiosis of rhizobia and legumes could result in coevolution that produces a nitrogen-fixing organelle.

Legumes can form a tripartite symbiosis which includes both *Rhizobium* bacteria which serve by fixing nitrogen, and arbuscular mycorrhizal fungi which help the plant to acquire phosphorus (Scheublin et al. 2004). I will mention arbuscular mycorrhizal associations later in this essay.

Rhizobium have an association with duckweeds that occurs without forming nodules (Kittiwongwattana and Thawai 2013). Duckweeds, as mentioned above, also have a nitrogen fixing association with cyanobacteria.

Fig. 26.28 Root nodules induced by rhizobia on legumes. This image shows the appearance of nodules which bacteria of the rhizobial group induce on the roots of legumes. The rhizobial nodules can differ in appearance depending upon the host plant species. The upper image shows roots of *Vigna unguiculata*, which commonly is called cowpea. I have grown *Vigna unguiculata* subsp. *sesquipedalis* in my yard. The common names of that subspecies include asparagus bean and Chinese long bean. The beans of *Vigna unguiculata* subsp. *sesquipedalis* taste very good when cooked Chinese style! The title of this upper image is "Rhizobia nodules on Vigna unguiculata.jpg" by author Stdout and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license. The lower image shows roots of *Lotus pedunculatus* containing nodules that similarly were induced by rhizobial bacteria. Members of the genus *Lotus* are called trefoils. The common names for *Lotus pedunculatus* include big trefoil and greater bird's-foot-trefoil. The title of this lower image is "Lotus pedunculatus11 ies.jpg" by author Frank Vincentz and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

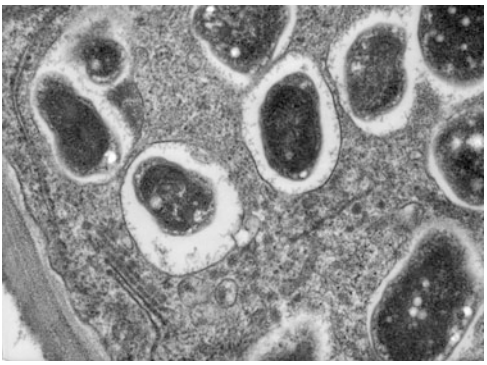


Fig. 26.29 Bacteroids inside a root nodule on *Glycine max.* This figure shows bacteroids containing *Bradyrhizobium japonicum* within a root nodule on soybean (*Glycine max*). The figure also shows endoplasmic reticulum, a dictysome, and the plant cell wall. *Bradyrhizobium japonicum* is one of the rhizobial bacterial species that establish nitrogen fixing symbioses with legumes. This figure is a public domain image titled "Root-nodule01.jpg" by author Louisa Howard—Dartmouth Electron Microscope Facility

It is important to understand that although I am mentioning in this essay a few very specific symbiotic associations, each host simultaneously will have symbiotic associations with a great many other groups of microorganisms. An example of this would be the leaf microbiome of duckweed plants (Acosta et al. 2020).

26.6.3 Actinobacteria as Symbionts that Provide Fixed Nitrogen for Their Host Plants

Frankia alni is a bacterial species that induces the formation of root nodules on black alder (*Alnus glutinosa*) trees, also called the common alder. In this regard the symbiosis of *Frankia alni* with alder trees is similar to the symbioses that rhizobial bacteria form with legumes (family Fabaceae). The mechanisms of *Frankia* symbiosis including signaling which occurs between the host and its candidate symbiont (Pawlowski and Demchenko 2012).

Frankia strains tend to be host specific with respect to the plant species in which the bacteria establish their symbiotic relationship. *Frankia* use their bacterial nitrogenase enzyme to fix nitrogen within those root nodules by converting atmospheric nitrogen into ammonia. These nodules can become visible above ground if the trees roots become exposed. Figure 26.30 shows root nodules that were induced by *Frankia* on alder.

Fig. 26.30 Root nodules induced by *Frankia alni* on black alder. *Frankia alni* is a actinobacterial species that induces the formation of root nodules on black alder (*Alnus glutinosa*) trees, also called the common alder. These nodules can become visible above ground if the trees roots become exposed. The genus *Frankia* is named in honor of Albert Bernhard Frank. The upper image is titled "Alder nodules2.JPG" by author Cwmhiraeth and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license. The lower image is titled "Frankia alni.jpg" by author Lebrac and it is being used under a Creative Commons Attribution-Share Alike 1.0 generic license. The authors description which accompanied the lower image is "Frankia alni, Aktinorrhiza, Wurzelknöllchen an Schwarzerle" which translates into English as [Frankia alni, actinorrhiza, root nodules on black alder]

26.7 Fungal Symbioses with Plant Roots

Fungi form many types symbioses with plants. These also would fit into the concept of cheating to gain evolutionary benefit against ones competitors.

Bonfante and Genre (2010) very nicely summarized the mechanisms and benefits of mycorrhizal symbioses including an explanation of the differences between ectomycorrhiza and arbuscular endomycorrhiza. The word 'mycorrhiza' means 'mushroom root' in its original interpretation, and that term was created by Bernhard Frank (Frank 1885).

26.7.1 Ectomycorrhizae

Ectomycorrhizal fungi produce an externally visible symbiosis with the roots of their host plants. Figure 26.31 {Ectomycorrhiza of *Amanita*} shows the appearance of plant root tips that are covered with a white layer of hyphae. That external layer is termed a fungal sheath or mantle. That fungal growth typically, although not always, extends inside of the root with fungal hyphae invading the intercellular spaces of the epidermis and cortex to produce what is described as a Hartig net. This net is an interface which facilitates exchange between the two symbionts. Hyphae also extend outward from the mantle into the surrounding soil. Presumably, the fungus helps the plant to take up nutrients and minerals from the surrounding soil and that action could include the fungus helping to increase availability of nitrogenous compounds. In return, the fungus may receive carbohydrate products from the plant. An individual plant may have ectomycorrhizal symbioses with more than a single species of fungus. Some of the ectomycorrhizal fungi are generalists that can form symbiotic associations with many different host plant species. Other fungi seem to enter ectomycorrhizal associations with only a single species of host plant.

The fruiting bodies of some ectomycorrhizal fungi, such as the *Amanita* shown in Fig. 26.31, emerge above the ground. Other ectomycorrhizal fungi produce fruiting bodies that must be excavated from the soil and then ingested by symbiotic animals in order to achieve dispersal of the fungal spores. Examples of the latter category are the fungal fruiting bodies know as truffles.

Bernhard Frank (Frank 1885) was the first person to describe ectomycorrhiza. He was, at that time, trying to develop practical methods for truffle cultivation. Frank believed that the organic connection between the root and the fungal mycelium formed a morphologically independent organ, with their being an interdependence of the growth of both parts which revealed a close relationship of physiological functions existing between the plant and fungus. By my translation, I would quote Frank as stating "The whole body is therefore neither tree root nor fungus alone, but similar to the lichen thallus, a union of two different beings into a single morphological organ, which can perhaps be aptly referred to as a mushroom root, a 'mycorhiza'".

Fig. 26.31 Ectomycorrhizal Growth of *Amanita* on Host Plants. This figure shows the externally visible part of ectomycorrhizal symbioses. The outside of these plant root tips are covered with a white layer of hyphae which is termed a fungal sheath or mantle. That fungal growth typically, although not always, extends inside of the root with fungal hyphae invading the intercellular spaces of the epidermis and cortex to produce what is described as a Hartig net. The title of the upper image is "Mycorrhizal root tips (amanita).jpg" by author Ellen Larsson and it is being used under a Creative Commons Attribution 2.5 Generic license. The host plant shown in the upper image was not identified by its author. The title of the lower image is "Ectomycorrhizae 001.jpg" by author Randy Molina, it is a public domain image showing an ectomycorrhiza of *Amanita muscaria* and Radiata pine (*Pinus radiata*)

Frank's opinion was that, in so far as the mycelium are concerned, the fungus must undoubtedly be regarded as a parasite to the living root because of the manner in which the fungus attaches and penetrates into the growing root. He believed that the underlying nutritional needs of the fungus, as it applied to all parasitic fungi,

mainly would relate to assimilating carbon-containing nutrients which the tree prepares through its chlorophyll-containing organs. On the other hand, the fungus would take its own mineral nutrients from the soil.

I easily can understand Frank's opinion that the fungus was acting parasitically. When I was a Boy Scout, long ago, during a camping trip I once noticed with a sense of horror that the roots of a fallen tree were absolutely covered with white fungal hyphae. My presumption was that the fungus had killed the tree. I finally realized, more than 50 years later while writing my essays for this book, that the fungus which surrounded those tree roots was the visible part of an ectomycorrhiza.

26.7.2 Endomycorrhizae

A second category of symbiosis between plant roots and fungi has been identified and given the name endomycorrhiza. As suggested by that name, hyphae of the involved fungi are most obviously internal to the plant roots.

The term arbuscular mycorrhiza describes a category of endomycorrhizal associations in which the hyphae penetrate root cells, grow in the space between the plant cells, and connect the internal compartments of neighboring plant cells. Nutrient exchange between the fungus and plant then occurs through an interface consisting of branching fungal masses called arbuscles that develop inside of the root. The development of an arbuscular mycorrhiza can include formation of arbuscules and vesicles. Arbuscular mycorrhiza may help plants adapt to abiotic stresses including drought, heat and sailinity (Begum et al. 2019). They also help plants to capture necessary elements such as nitogen, phosphorus, and sulfur from the soil.

Arbuscular mycorrhiza are formed by members of the phylum Mucoromycota, subphylum Glomeromycotina. Figure 26.32 shows arbuscular mycorrhiza in root cortical cells of flax (*Linum usitatissimum*) and the arbuscules shown are described as being paired.

Legume roots develop both rhizobial symbioses and arbuscular mycorrhizal symbioses (Scheublin et al. 2004). The rhizobial symbiosis involves a bacterial partner, as mentioned above, and that association characteristically but not necessarily results in the formation of root nodules. Rhizobial symbioses quite obviously serve by providing fixed nitrogen for the plant.

Plants have developed endophytic interactions with many types of symbiotic microbes (Strobel 2018; Zhang et al. 2019). Arbuscular mycorrhiza fungi can interact either synergistically or antagonistically with the plants other endosymbiont microbes, including those symbionts that are growing in aerial parts of the plant.

It has been suggested that arbuscular mycorrhizal associations appeared when plants began colonizing the land, perhaps 400–460 million years ago. Rimington et al. (2018) have written an article about the possible early establishment of arbuscular mycorrhizae. Arbuscular mycorrhizal symbioses thus may have represented an important step in the development of terrestrial plants. By now, arbuscular mycorrhizal symbioses have become established in perhaps 85% of the

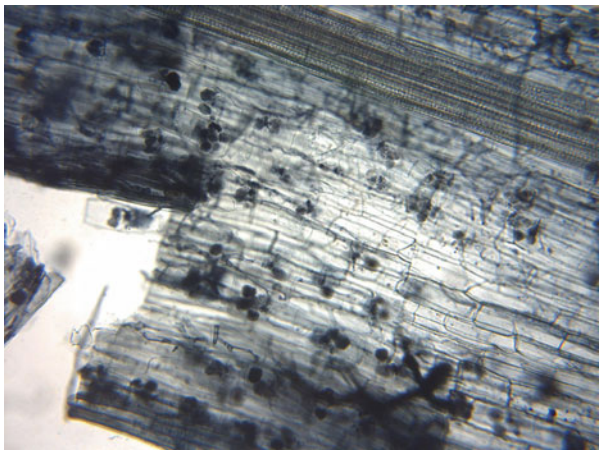


Fig. 26.32 Arbuscular mycorrhizae in flax root. This image shows arbuscular mycorrhizae in root cortical cells of flax (*Linum usitatissimum*) and the arbuscules shown are paired. The singular form of this term is arbuscular mycorrhiza, the plural form is arbuscular mycorrhizae. Arbuscular mycorrhizae are a type of endomycorrhizal symbiosis that is formed by members of the phylum Mucoromycota, subphylum Glomeromycotina. The title of this image is "Arbuscular mycorrhiza microscope.jpg" by author Msturmel, it is a public domain image, and hyphal growth can be seen connecting the internal compartments of neighboring plant cells. Arbuscular mycorrhizae help plants to capture necessary elements such as nitogen, phosphorus, and sulfur from the soil. The fungi that form an arbuscular mycorrhiza can simultaneously interact either synergistically or antagonistically with other endosymbiont microbes, including symbionts that are growing in aerial parts of the plant. Arbuscular mycorrhizal symbioses may have represented an important step in the development of terrestrial plants

terrestial plant families. Thus, it would seem that this type of symbiosis which once represented evolutionary cheating has become tremendously successful. In contrast to the popularity of arbuscular mycorrhizae, ectomycorrhizae may form with only a small percentage of plants.

26.8 Gastrointestinal Symbiont Microbes

Animals have gastrointestinal tracts where all of us maintain symbiont microbes. A few of those microbes are endosymbionts housed intracellularly within specialized tissues and organs. Most of the gastrointestinal symbiont microbes reside in the lumen, and often those symbionts are part of a biofilm.

26.8.1 Endosymbiotic Sulfur-Oxidizing Bacteria

There are interesting examples of host animals having developed endosymbiotic associations with chemoautrophic microorganisms. One of these is *Riftia pachyptila*, which is a species of vestimentiferan tubeworm that lives on the sea floor near hydrothermal vents. The *Riftia* depend upon receiving fixed carbon that is supplied by endosymbiotic sulfur-oxidizing bacteria housed in an organ called a trophosome. Juvenile *Riftia* accumulate these bacteria from the water by ingestion. An ensuing process similar to infection results in the bacteria becoming housed intracellularly within bacteriosomes. The trophosome arises from the animals midgut and the remainder of the worms intestinal system is absent in adults. Several species of endosymbiont bacteria have this type of association with *Riftia*. These symbionts all are members of the phylum proteobacteria, some belonging to the class gammaproteobacteria and others belonging to the class epsilonproteobacteria.

Figure 26.33 shows a colony of *Riftia*. The *Riftia* tentacles, which are visible protruding from the top of the animals tube, appear red because they contain hemoglobins which serve to bind O_2 and H_2S. Those gasses are transferred via capillaries to the chemosynthetic bacteria. Vesicomyidae clams (*Calyptogena magnifica*) and mytilid mussels (*Bathymodiolus thermophilus*) that live in communal association with the *Riftia* do similarly house endosymbiotic sulfur-oxidizing bacteria.

Fig. 26.33 *Riftia* tube worms. The title of this image is "Riftia tube worm colony Galapagos 2011.jpg" by author NOAA Okeanos Explorer Program, Galapagos Rift Expedition 2011, and it exists in the public domain. The description provided with this image is "Photo of one of the largest concentrations of Riftia pachyptila observed, with anemones and mussels colonizing in close proximity. From the 2011 NOAA Galapagos Rift Expedition." *Riftia pachyptila* lives on the sea floor near hydrothermal vents and depends upon fixed carbon that is supplied by endosymbiotic sulfur-oxidizing bacteria residing in the animals trophosome. The *Riftia* tentacles, visible protruding from the top of the animals tube, appear red because they contain hemoglobins which serve to bind O_2 and H_2S. Those gasses are transferred via capillaries to the chemosynthetic bacteria

26.8.2 *The Rest of us Also Use Gastrointestinal Symbiont Microbes*

Symbiotic interactions with intestinal microbes are a critical health component for all animals. Some flatworms have trophosomes as do the *Riftia*. Perhaps more than 10% of insects contain bacteriocytes within their midgut epithelium that house endosymbiont microorganisms, including bacteria and fungi which help with digestion and provide essential compounds such as amino acids. The rest of us animals depend upon mutualistic symbiotic microorganisms that reside within the lumen of our intestines and some of those microbes are in biofilms associated with mucosa. People tend to think of mucosal biofilms solely in association with pathogenesis, but the gastrointestinal microbes that contribute to our health generally also represent mucosal biofilms. Extending from that last thought, I would suggest the publication by Hillman et al. (2017) in which those authors presented a comparative study on the gastrointestinal microbial ecology of mammals.

26.9 Luminescent Bacterial Symbionts

There are many groups of animals, including teleosts (infraclass Teleostei) and squid (subclass Coleoidea), that house luminescent bacterial symbionts within specialized organs. Some squid may use their light organs as a form of camouflage against predators. There are angler fish which house luminescent bacteria in their esca, termed a lure, and the illumination from that organ serves to attract prey.

Fish belonging to the family Anomalopidae house luminescent bacteria in light organs located below their eyes (Hendry et al. 2016; Hellinger et al. 2017). These fish belong to the genera *Anomalops*, *Photoblepharon*, and *Phthanophaneron*. Figure 26.34 is a photograph of *Anomalops katoptron*, which is called the splitfin or two-fin flashlight fish. The Anomalopidae are capable of either revealing or concealing the illumination, in effect blinking their light, and that activity has been likened to the appearance of using a signal lamp or flashlight for sending Morse code messages. These nocturnal fish have no equivalent to Morse code, but the intervals at which they blink their light do vary in a way that has been associated with locating zooplankton prey. The blinking of their light also may help these fish to group as a school at night (Gruber et al. 2019).

26.10 Endosymbiotic Viruses

Organisms have mechanisms for acquiring and genomically incorporating DNA from environmental sources (Bininda-Emonds et al. 2016).

Fig. 26.34 *Anomalops katoptron*, Here's blinking at you, kid! *Anomalops katoptron* is called the splitfin or two-fin flashlight fish. Luminescent bacterial symbionts are housed within a special organ that is located beneath the fish's eye. Light constantly is emitted from that organ, although the fish can conceal this light by a blinking mechanism. The intervals at which this nocturnal fish blinks its light vary and have been associated with locating zooplankton prey. The light also may help these fish to group at night. This image was taken at the Steinhart Aquarium, is credited to "California Academy of Sciences, San Francisco", and is being used with the kind permission of John E. McCosker

Viruses are parasitic symbionts that aid the process of genomic evolution by acquiring genes from their hosts and then transferring those genes between hosts. It has been suggested that the coevolving interactions between virus and host may be one of the factors that drive evolutionary processes. The captured and transferred genes can evolutionarily benefit both virus and host, which suggests a mutualistic association.

A viral genome can become endogenous, which means the viral genome is inherited as a DNA copy when its host replicates. Endogenous viruses can become grounded, a term that signifies the viruses have lost their ability to form an infectious viral particle known as a viroid. But then, having become endogenous frees the virus from needing to be transmitted as an infectious viral particle. Even though an endogenous virus may have become grounded, genes of that endogenous virus can actively be expressed within the host cells.

Viral genes that have been captured by a host may become repurposed by the host. An example of such repurposing has been the usage of endogenous retrovirus envelope genes, which cause cell fusion, to change fetal trophoblast cells into placental syncytiotrophoblasts (Soygur and Sati (2016). Most, although perhaps not all, mammals use these gene products which are called syncytins to assist in producing a placenta. The process of placental development and the corresponding choice of retroviral genes differs between mammalian groups (Malik 2012). These choices have represented evolutionary changes and helped to mark a divide between monotreme mammals which lay eggs (clade prototheria, order monotremata) and placental mammals (clade eutheria).

The placenta is a transient organ cooperatively developed by the mother and the fetus. Our mammalian line, termed the placental mammals, has developed a placenta that allows the fetus to safely be transported internally by its mother rather than risking the parents needing to guard eggs. Placental mammals have claimed mammalian dominance, leaving the monotremes and even the marsupials marginalized. It certainly seems as though we, the placental mammals, thusly have gained an evolutionary advantage by successful cheating!

The viral envelope gene which humans and other primates use when initiating formation of a placenta comes from Human endogenous retrovirus W. The involved gene product is called Syncytin-1. Unfortunately, the envelope genes of endogenous retroviruses may contribute to the pathogenic inflammatory process associated with multiple sclerosis.

The upper image of Fig. 26.35 shows the monotreme *Tachyglossus aculeatus*, commonly known as either the Australian echidna or the Short-beaked echidna, and it lays eggs. The lower image shows the placental *Rangifer tarandus*, commonly known as reindeer. When I was a young child, I could not understand why this animal species seemed to have been named for the form of precipitation known as 'rain'. My understanding has improved and I now know that the term reindeer means the deer can be harnessed. Placental mammals have in one sense harnessed our endogenous retroviruses, although we lack full control of the reins.

26.11 Summary

The competition for evolutionary success has included discovering ways to do things differently. Competitive advances sometimes can result from changes which seemingly are minor but somehow prove to be powerful, such as genomic rearrangements which improve regulatory functions and changes in the efficiency of enzyme kinetics.

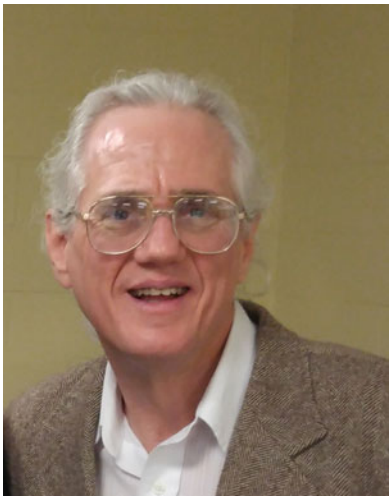
Development of effective symbiotic interactions has been one of the factors that drive evolutionary competition. Symbiotic interactions seem to have shaken up the entire order of life on several occasions, including the development of mitochondria, chloroplasts, and a placenta. All of these might be perceived as having represented ways of cheating to produce biased outcomes.

Success is defined by the winners, history is written by the victorious, and we the placental mammals . . . have won! We have either out competed or just simply eaten everything that presented a siginificant obstacle in our pathway to evolutionary success.

That declaration of victory by placental mammals will stand until microbes decide to reclaim the title. We could not exist without cooperative agreements that have been forged with the microbes. The microbes survived for a long time before we evolved, and the microbes largely don't need us.

Fig. 26.35 Optimistic. The evolutionary changes between monotreme mammals which lay eggs (clade Prototheria, order Monotremata) and placental mammals (clade Eutheria) have included repurposing endogenous retroviral genes. The upper image shows the monotreme *Tachyglossus aculeatus*, commonly known as either the Australian echidna or the Short-beaked echidna. The upper image is titled "Wild shortbeak echidna" and is being used with the kind permission of Henry Firus. The lower image shows the placental *Rangifer tarandus*, commonly known as reindeer. The lower image is titled "Reinbukken på frisk grønt beite.—panoramio.jpg" [Reindeer on fresh green pasture] by author Are G Nilsen, the image has been cropped, and it is being used under a Creative Commons Attribution-Share Alike 3.0 Unported license

Acknowledgements I wish to thank Henry Firus, Hwan Su Yoon, Rex Lowe, John McCosker, and Jennifer Possley for generously sharing their photographs. Prashant Singh helped me to understand the state of knowledge and questions that still exist regarding taxonomy of the endosymbiotic cyanobacteria associated with *Azolla* and cycads. Robert Thacker very kindly helped me with understanding the relationships that exist between marine sponges, cyanobacteria and diatoms.



Christon J. Hurst celebrating the publication of Advances in Environmental Microbiology

Dedication I dedicate my efforts on this essay to Alex Stewart Fraser. He was the most intelligent person whom I have known during this lifetime and his enthusiastic encourgement did in turn fuel the enthusiasm of his students. Alex could absolutely thunder with excitement when he was lecturing. My most memorable moment with Alex occurred when I was a senior and Alex asked if I would feel brave enough to face an oral examination on evolution to be presented by a group of my fellow students instead of sitting a written examination for my final grade of the quarter. None of his other students ever had agreed to that idea. I bravely said "Yes". The students who presented that examination were given a few weeks to prepare the questions which they wanted to ask of me. During the examination my inquisitors may have been more nervous than was I. After the examination, Alex declared that I received the grade of "A" and revealed that he already had chilled a bottle of champagne in honor of the occasion. That was the first celebratory bottle of champagne I have opened during my science career. Alex sadly left us nearly two full decades ago, when he passed away on Bastille Day July 14, 2002, but he certainly never will be forgotten. I thank you, Alex, for the encouragment which you so generously gave to myself and to all of your other students.

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Chapter 27
The Importance of Being Symbiont and the Role of Symbiosis as a Driving Force in Evolution

Francisco Carrapiço

Abstract We live in a symbiotic world where many living forms, from the simplest to the most complex ones, have associations with microorganisms. Thus, symbiosis plays a very important role in the origin, organization, and evolution of life. Given their role in the association, symbionts are among the key actors in this interplay. Traditionally, the symbiont is seen as a supporting actor, with a secondary role in the play, whereas the host is understood as having the leading role. However, bacteria and, more recently, viruses have gradually come to be seen as important elements of these symbiotic relationships or consortia, and as one of the key factors in the evolution and organization of the web of life, as well as its health and disease. The gut microbiome, namely in humans, is another important case study to understand the importance of symbionts. Misunderstood and diabolized for a long time, the human gastrointestinal microbiome represents a challenge and an opportunity to understand in a holistic way how the human body works and the effects that it has on our health. This could be the beginning of a new paradigm in science, namely in biology and medicine, that remains almost unexplored and that challenges the traditional concept of organism.

27.1 Introduction

Evolution is traditionally considered a gradual process essentially consisting of natural selection, conducted on minimal phenotypical variations that are the result of mutations and genetic recombinations to form new species (Darwin 1859; Dawkins 1976; Huxley 1942; Mayr 1942, 1982, 2001; Wilson 1975). This is the case even in models of evolutionary change, such as the punctuated equilibria, which can be considered as a rupture with the neo-Darwinian tenets (Eldredge and Gould

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_27

1972). Nowadays, however, it is no longer possible to ignore that in a ubiquous way, the biological world presents and involves symbiotic associations between different organisms to form consortia, a new structural life dimension and a symbiont-induced speciation. The acknowledgment of this reality implies a new understanding of the natural world, in which symbiogenesis plays an important role as an evolutive mechanism (Carrapiço 2015a; Gontier 2016; Margulis and Fester 1991). Within this understanding, symbiosis is the key to the acquisition of new genomes and new metabolic capacities, driving not only the evolution of living forms but also the establishment of biodiversity and evolutionary complexity of living systems (Archibald 2014; Carrapiço 2006, 2010a, 2012, 2015a, b; Corning 1983, 2005, 2018, 2020; Margulis and Fester 1991; Margulis and Sagan 2002; Reid 2007; Sapp 2003, 2009; Selosse 2017; Villarreal and Ryan 2011). Despite the existence of open-minded ideas on co-operative and synergistic approaches to the evolutive process, symbiosis and symbiogenesis have traditionally been considered by the majority of the scientific community as the "stepdaughters or stepsons" of the evolutionary theory (Carrapiço 2010a, 2012, 2015a, b; Corning 2005, 2018; Pereira et al. 2012; Sapp 1994, 2003; Suárez 2018), or in the case of symbioses, as biological anecdotes (Selosse 2000). These ideas reveal a limited understanding of evolutive process and do not correspond to the reality nor to the structure of the web of life in our planet and to the understanding of the role of symbionts as key actors and not only as supporting ones.

27.2 The symbiont's Long Road in Time

In 1867, the Swiss botanist Simon Schwendener proposed at the Swiss Natural History Society annual meeting, held in Rheinfelden, the dual hypothesis, relating to the structure and organization of lichens (Carrapiço 2015a). This hypothesis indicated that lichens are an association of two or three organisms, a fungus and an alga or a cyanobacterium, behaving as "master and slave" (Honegger 2000; Sapp 1994, 2003; Selosse 2000, 2017) (Fig. 27.1). The idea that an organism could be formed by two or more genetically separate organisms living together and working as one unit was regarded as so unusual at the time that it was largely rejected by the scientific community. Although the dual hypothesis was an innovative concept for the biology of the nineteenth century, as well as an epistemological rupture in the traditional concept of organism, it was considered by several experts of the lichenological field of the time as a "pure fantasy" (Carrapiço 2015a; Nylander 1896).

The symbiosis concept introduced in 1878 by the German biologist Heinrich Anton de Bary (1831–1888) and defined as the "the living together of unlike named organism" (Carrapiço 2015a; De Bary 1878, 1879) (Fig. 27.2) was another important step in recognizing the role of symbionts. This author used lichens and *Azolla* (aquatic fern) as biological examples to build this important concept in science (Carrapiço 2010b). The definition of symbiosis includes actually parasitism, commensalism, and mutualism (Villarreal and Ryan 2011), and it can be considered as a continuous

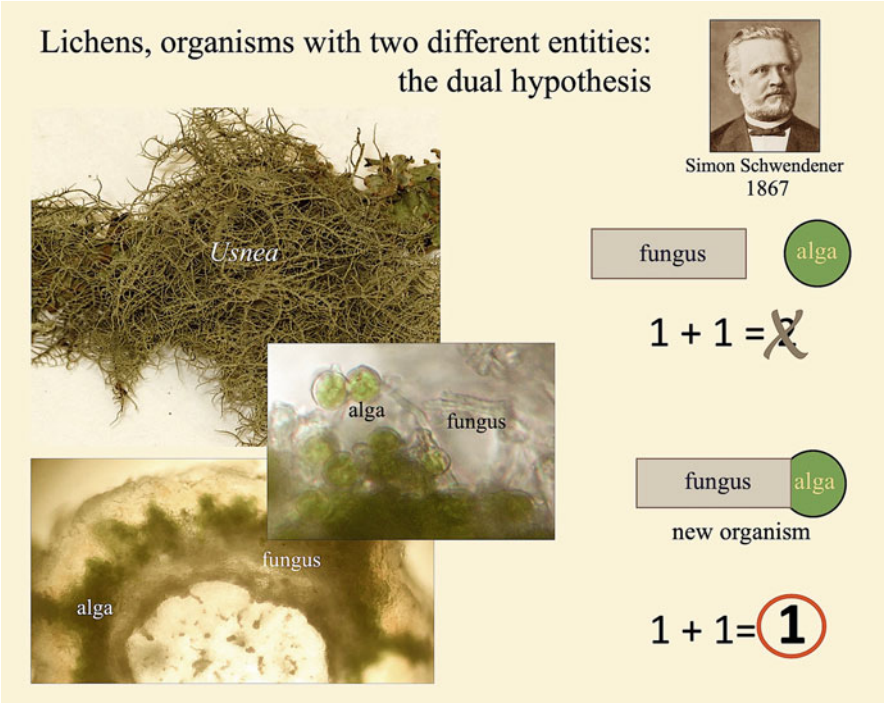


Fig. 27.1 The dual hypothesis applied to the structure and organization of lichens, using *Usnea* as a case study. Simon Schwendener's photo credits: Meise Botanic Garden, Belgium and Europeana. Lichen photos from the author

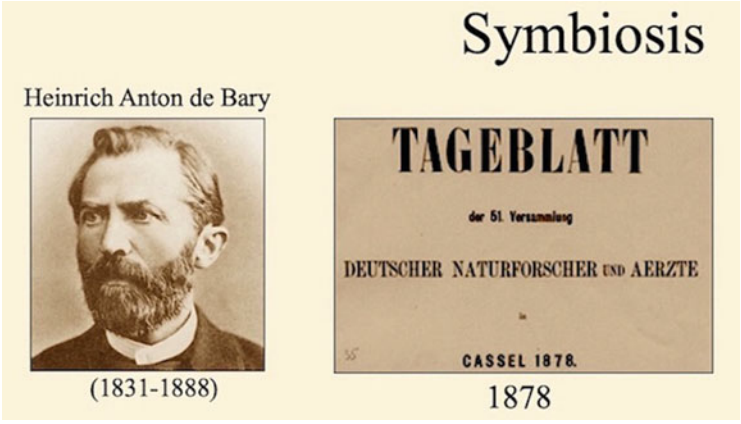


Fig. 27.2 Anton de Bary and the introduction of the symbiosis concept in 1878 (De Bary 1878). Heinrich Anton de Bary's photo credits: https://www.archi-wiki.org/Personne:Anton_de_Bary#/media/File:39176.jpg

integrative phase of interactions among organisms with physiological expression and genetic novelty (Apprill 2020; Carrapiço 2012; Munzi et al. 2019; Zook 2015). Three years before the symbiosis concept, in 1875, the concept of mutualism was introduced by the Belgian zoologist Pierre-Joseph Van Bénéden (1809–1894) and based on Pierre-Joseph Proudhon's (1809–1865) sociological ideas applied to the animal kingdom (Boucher 1985; Van Bénéden 1875). It was a significant step to understand the importance of the relations between organisms (Boucher1985). This concept can be defined as a beneficial relationship between different species of organisms present in the association (Boucher 1985). Another important concept was symbiotismus, which was introduced in 1877 by the German naturalist Albert Bernhard Frank (1839–1900) in a work on lichens (Frank 1877). Symbiotismus and symbiosis are very similar concepts in terms of biological definition, and although symbiosis was introduced one year after symbiotismus, it is symbiosis the most well-known and used in biology (Carrapiço 2015a).

To understand the importance of symbiosis and symbionts in biological science, we must also go back to the introduction of the symbiogenesis concept in 1909 by the Russian biologist Constantin Merezhkowsky (1855–1921). Working on symbiotic associations, namely lichens, Merezhkowsky developed the concept of symbiogenesis as "the origin of organisms by the combination or by the association of two or several beings which enter into symbiosis" (Carrapiço 2015a; Merezhkowsky 1909; Sapp et al. 2002). He also presented in 1905 coherent scientific arguments showing that plastids arose from free-living cyanobacteria, showing the importance of symbionts in evolution and in the complexification of organisms and also in the origin of life under a symbiogenic perspective (Carrapiço 2015a; Merezhkowsky 1905, 1909; Pereira et al. 2012). This work was further developed in 1920 in the article "La plante considerée comme un complexe symbiotique" (The plant considered as a symbiotic complex) about the symbiotic origin of chloroplasts and nucleus (Carrapiço 2015a; Merezhkowsky 1920). In opposition to contemporary views of the time, Merezhkowsky defended that chloroplasts did not evolve from mitochondria or protoplasm, but from free-living cyanobacteria, as he had presented in 1905 (Carrapiço 2015a; Merezhkowsky 1920). These ideas point out, in some way, the importance of the symbionts in the symbiotic process and their role in biological evolution.

Another Russian botanist, Andrey Famintsyn (1835–1918), contemporary of Merezhkowsky and also working on the symbiotic field, published in 1907 "On the role of symbiosis in the evolution of organisms,", where the author developed the idea that symbiosis has an important evolutionary, or even adaptive, meaning. Merezhkowsky, however, introduced a new classification of the living world using for the first time associations between organisms to understand their biological complexification and reinforce the importance of symbiosis, in particular the symbionts' role as leading actors in the biological world structure and in the evolutive process (Carrapiço 2015a; Merezhkowsky 1909; Sapp et al. 2002).

At the beginning of the twentieth century, Hermann Reinheimer, an almost unknown English author born in Germany, published several books in the field of symbiontology. Among them, three can be considered good examples of the author's

work and a coherent contribution to the field. In 1913, he published *Evolution by co-operation. A study in bio-economics*, followed by the 1915 *Symbiogenesis: the universal law of progressive evolution* and the 1920 book *Symbiosis: A socio-physiological study of evolution*. In these books, Reinheimer points out the importance of specific interrelations in the development of organisms as a whole, giving a holistic perspective on organismal evolution and the importance of cooperation in the evolutive process (Carrapiço 2015a).

Several other authors published key studies that were related to the development of symbiogenic ideas in biology during the first decades of the twentieth century. Among them, we must refer to the French biologist Paul Jules Portier (1866–1962) who published *Les symbiotes* in 1918 (Carrapiço 2015a; Portier 1918) (Fig. 27.3). In his work, Portier developed the idea that all organisms are constituted by an association of different beings. In the particular case of mitochondria, he argues that those cell organelles were symbiotic bacteria, which the author calls "symbiotes" (Carrapiço 2015a; Portier 1918; Sapp 1994). He also refers to the positive role of these prokaryote organisms as symbionts in the human body. These new ideas were rejected by the French scientific community, at a time when germ theory was the mandatory rule in medicine and biology (Carrapiço 2015a). A year later, the French academic Auguste Lumière (1862–1954) published a critical response to these ideas in the book *Le mythe des symbiotes* (Carrapiço 2015a; Lumière 1919).

Fig. 27.3 Paul Jules Portier and Ivan Emanuel Wallin and their key books referred in the text (Portier 1918; Wallin 1927). Paul Portier's photo credits: Institut océanographique, Monaco and Ivan Wallin's photo credits: U.S. National Library of Medicine http://resource.nlm.nih.gov/101431424

Related to the field of viral symbiosis, we must refer to the pioneering work of the French-Canadian biologist Félix d'Hérelle (1873–1949) associated with the Institut Pasteur in Paris, who published in 1917 the article "Sur un microbe invisible antagoniste des bacilles dysentériques" (On an invisible microbe antagonistic to dysentery bacilli) describing the phenomenon of the bacteriophage and its biological nature. He developed this hypothesis, based on previous experimental studies carried out by him, of the existence of viruses associated with bacteria, which he calls bacteriophages, seeing this biological phenomenon in a symbiotic perspective (d'Hérelle 1917; Sapp 1994; Summers 1999).

In Russia, Boris Kozo-Polyansky (1890–1957) published in 1924 the book *A new principle of biology: an essay on the theory of symbiogenesis*, where symbiosis has a determinant role in evolution, building the bridge between symbiogenesis and the Darwinian theory and introducing the idea of the organism as a consortium (Kozo-Polyansky 2010). This idea of organism as a consortium was initially presented in 1873 by the German botanist Johannes Reinke (1849–1931), to refer to the relationship between the fungus and alga in lichens (Carrapiço 2015a; Reinke 1873).

In 1927, Hermann Joseph Muller (1890–1967), an American geneticist working at University of Texas, publishes in the journal *Science* an important article entitled "Artificial transmutation of the gene," showing that X-rays could dramatically increase the frequency of gene mutations in *Drosophila melanogaster* (Carrapiço 2015a; Muller 1927). In this paper, the author refers that "Most modern geneticists will agree that gene mutations form the chief basis of organic evolution, and therefore of most of the complexities of living things" (Muller 1927). In 1946, he was awarded the Nobel Prize in Physiology or Medicine for the discovery of the production of mutations by means of X-ray irradiation. More important, he provided the foundational "tool" for neo-Darwinists to explain biological evolution and the complexity of life (Carrapiço 2015a). However, the same year, Ivan Emanuel Wallin (1883–1969), a Professor at the University of Colorado published *Symbionticism and the origin of species*, where the author defends the importance of symbiotic mechanisms in evolution, with emphasis on the symbiotic origin of mitochondria. Wallin also emphasized the importance of microsymbiosis in this process, pointing out "that bacteria, which are popularly associated with disease, may represent the fundamental causative factor in the origin of species" (Carrapiço 2015a; Wallin 1927). He considers symbioticism as a mechanism of speciation, suggesting that the primary source of genetic novelty for speciation was the periodic repeated fusion of bacterial endosymbionts with host cells, reinforcing the importance of these symbionts as leading actors in the biological evolutive process (Carrapiço 2015a, b; Wallin 1927) (Fig. 27.3).

All the ideas discussed above were represented in the innovative work of the American biologist Lynn Margulis (born Lynn Petra Alexander, 1938–2011) published in 1967 with the title "On the origin of mitosing cells" (Carrapiço 2015a; Sagan 1967). In this article, Margulis presented the theory on the origin of eukaryotic cells, explaining the transition between the prokaryotic and the eukaryotic levels of biological organization. It considers that several cellular eukaryotic organelles, such as mitochondria and chloroplasts, have derived from free-living

prokaryotes and views eukaryotic cells as the result of ancient symbioses (Carrapiço 2015a; Margulis 1970). This work formed the basis of the Serial Endosymbiotic Theory (SET), and it constituted the beginning of the rehabilitation and development of symbiogenic ideas applied not only to the cellular world but also to the construction of a new Biology for the current century (Carrapiço 2015a; Margulis and Fester 1991; Margulis and Sagan 2002). The works of Ris and Plaut (1962) on the detection of DNA fibers in chloroplasts, and the similar research by Nass and Nass (1963a, b) in mitochondria, contributed decisively to the acceptance of these new ideas. Furthermore, the pioneering work of Lynn Margulis and co-workers represents an important contribution to the development of the post neo-Darwinian approach to understand biological evolution in an adequate way, as well as to the establishment of symbiogenesis as an important evolutionary biological mechanism on Earth (Archibald 2014; Carrapiço 2010a, 2012, 2015a, b).

27.3 Cases of Symbioses and the Role of Symbionts

27.3.1 Azolla *as a Polygenomic Entity*

A good example of the importance of symbionts to the organism as a whole or biological unity can be found in the symbiotic system *Azolla-Anabaena*-bacteria.

Azolla is a small-leafed floating or semi-aquatic heterosporic fern with overlapping leaves that float or have submersed lobes. The chlorophyllous dorsal lobe of the leaves contains a prokaryote community (symbionts) in an ovoidal cavity with filamentous nitrogen fixing cyanobacteria, traditionally assigned to *Anabaena azollae*, as well a variety of bacteria strains, mainly assigned to the genera *Arthrobacter*, *Corynebacterium*, and *Agrobacterium*. However, the cyanobiont's identification is still controversial and involves beyond the genus *Anabaena*, the genera *Nostoc* and *Trichormus* (Carrapiço 2015a, 2017). Also present in the leaf cavity are three types of trichomes, which show an ultrastructure of transfer cells, about 20–25 simple hairs, one primary and one secondary branched hair in number of two (one primary and one secondary) (Carrapiço 2010b, 2015a). Since the *Azolla* leaf cavity has no direct connection with the vascular system of the pteridophyte, the trichomes are involved in the transfer and uptake of metabolites from the fern to the prokaryote colony and from this one to the plant (Carrapiço 2010b, 2015a).

The leaf cavity behaves as both the physiological and dynamic interface unit of this symbiotic association, where the main metabolic and energetic flows occur (Carrapiço 2010b, 2015a; Grilli-Caiola and Forni 1999). In this sense, it can be considered a natural microcosm, a microecosystem that reveals a self-organization and an ecological defined structure, where natural selection acts to evolve different cyanobacteria ecotypes (Carrapiço 2002, 2010b, 2015a; Papaefthimiou et al. 2008). It can also be considered as a natural photobioreactor (Carrapiço 2010b, 2015a; Shi and Hall 1988), with millions of years of evolution, where the prokaryote community (cyanobacteria and bacteria) is immobilized and immersed in a fibrillar network

present in the leaf cavity and driven by the fern to modify some of its own physiological and metabolic activities, which in turn change the fern's own metabolic activity (Carrapiço 2010b, 2015a). One good example can be found in the nitrogen metabolism associated to this symbiotic system shared by the host and partners. The filaments of *Anabaena* present special cells (heterocysts) where nitrogenase converts atmospheric N2 into ammonia which is released into the leaf cavity (Carrapiço 2010b, 2015a). It has been shown that intracellular ammonia pools of symbiotically associated *Azolla* are five times greater than those of *Azolla* free of cyanobacteria (Braun-Howland and Nierzwicki-Bauer 1990; Carrapiço 2010b, 2015a). The level of activity of ammonia-assimilating enzymes in the isolated trichomes of the dorsal leaf cavity are much higher than those in *Azolla* leaves, while some other activities in the *Anabaena* filaments are repressed to very low levels. These results indicate that *Azolla* trichomes cells play an important role in the nitrogen assimilation, which the cyanobiont fixes and releases into the cavity, transferring it to the fern (Carrapiço 2010b, 2015a; Uheda, 1986).

This symbiosis is sustained throughout the fern's life cycle, where the prokaryote community (cyanobiont and bacteria) is always present and transferred from one generation to the next, either in the dorsal leaf cavities or in the megasporocarps, indicating the obligatory nature of the symbiotic association (Carrapiço 2010b, 2015a). It also suggests a phylogenetic coevolution of the symbionts and the host, representing a good example of hereditary symbiosis and indicating that the complexity of the relationship between the host and the symbionts can be recognized as a new level of biological organization in evolution, which can be included in the concept of symbiogenic superorganism in biological and ecological terms (Carrapiço 2010b, 2015a, b). This implies and involves the development and acquisition of new metabolic and organic capabilities and also genetic sharing by the partners in synchrony with the host, extending beyond the capability of each individual forming the association or consortium (Carrapiço 2010b, 2015a, 2017, 2018).

With this concept, we have reinforced the idea that this symbiotic system works in a synchronized way and can be considered a polygenomic entity (Fig. 27.4), within which the different genomes operate together in a complementary and synergistic way that benefits the whole (*Azolla*, cyanobateria and bacteria). It is as if collectively they were a single living unity (a big one) acting as a symbiogenic superorganism (Carrapiço 2012, 2015a, b). We might describe them as being either a symbiome or holobiont (Carrapiço 2006, 2010b, 2015a, b, 2018; Guerrero et al. 2013; Sapp 2003; Suárez and Triviño 2019). This concept reinforces the principle that eukaryote organisms are not genetically unique entities, and individual must instead be seen as a complex biological ecosystem, formed by multiple interdependent parts coexisting symbiotically within the whole (Carrapiço 2010b, 2015a). As previous referred, natural selection acts at the symbiome level, which is composed of an integrated multigenomic genetic pool where all the genomes work together for the whole (Carrapiço 2015a). Thus, we believe that the new metabolic and organic capabilities acquired and developed by the partners establish a new level of organization that goes beyond the individual capabilities of any individual

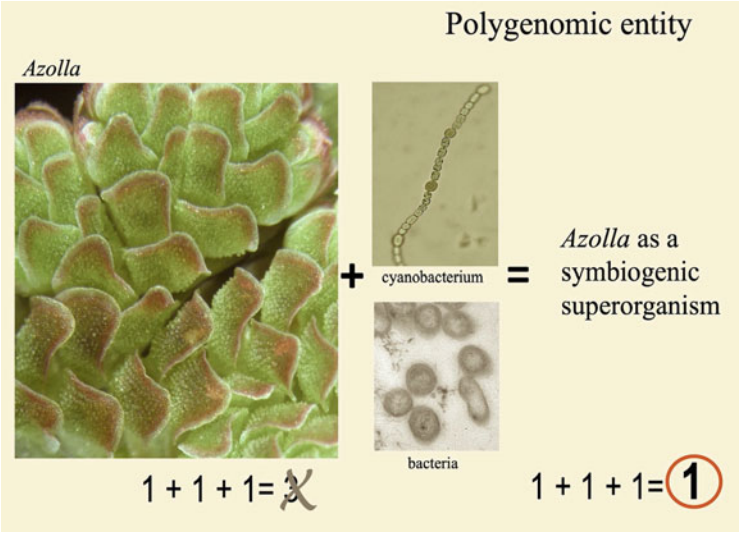


Fig. 27.4 The *Azolla-Anabaena*-bacteria symbiotic system as a polygenomic entity. (Photos from the author)

partner. It involves synergies associated to symbiosis and forms the bases of the symbiogenic superorganism (Carrapiço 2010b, 2015a, b; Corning 1983, 2005, 2018).

27.3.2 Koalas, the Perfect Symbiotic Adaptation

Koalas (*Phascolarctos cinereus*) are arboreal herbivorous marsupials native to Australia and they are also a good example of the importance of symbionts to the organism as a whole. Juveniles after birth spend 6 months in the marsupial pouch before becoming autonomous, while in their mothers pouch, the juveniles feed on breast milk and also ingest a fecal substance called *pap* produced by the mother's colon. This latter substance is very rich in bacteria, allowing these juvenile animals to acquire gastrointestinal microbial symbionts which will facilitate the koalas ability to digest eucalyptus tree leaves that are rich in tannins and contain toxic chemical compounds. Those leaves will serve as the only food of these mammalian herbivores as adults. This is how, during the period in the marsupial pouch, the juveniles microbiome is altered, which will allow the animals survival under those specific environmental conditions that the juvenile will face during the remainder of its lifetime. Evolutionary synchrony of the host and its microbes thus has been providing the ability of these herbivores to extract nutrients and detoxify their potentially toxic diet with the active involvement of the symbiont community living in the marsupial gut (Brice et al. 2019; Osawa et al. 1993).

27.3.3 Viral Symbioses

Another good example of the importance of symbionts in the evolutive process can be found in studying viruses and infectious subviral genetic parasites, which have been drivers of speciation since the origin of life. These genetic elements are as well key actors in RNA interactions and have been involved in the origin of group identity (Villarreal 2004, 2009; Villarreal and Witzany 2019). They are also important drivers in host evolution and concomitant evolution of biodiversity as has become clear from the content and regulation of chromosomes of host cells. There are indications that viral symbioses may have contributed to the origins of nuclei and might also be involved in chromosomal replication (Villarreal and Ryan 2011).

The discovery that part of the human genome and genomes of other mammals are of viral origin leads us to consider the importance of horizontal gene transfer in evolution and in the shaping of life on Earth. Since viruses can adapt rapidly to new environments, viruses might allow for the host to adapt faster and efficiently to new environmental conditions through the introduction of new genomic elements (Villarreal 2004, 2009; Villarreal and Witzany 2019). We suggest that an important evolutive interphase between new viruses and the human body as a host can be found at our gastrointestinal system, where a permanent and well-adapted community of microbial organisms coexist, allowing for the sharing and incorporation of new genetic information and adaptation by the viruses when they attempt to communicate and to establish a stable symbiotic relationship with human populations via symbiogenic mechanisms. Probably this is the case of the new coronavirus responsible for the disease Covid-19 and we are experiencing in this first phase of the outbreak the establishment of a genetic relationship which is, for now, temporarily more lethal for the host.

27.3.4 The Human Gut Microbiome

To understand the global importance of symbionts in relation to their host organism, we must refer to and discuss the gastrointestinal microbiome in humans.

The human microbiome consists of all microbial organisms and their genetic content in and on the human body and can be considered an intricate and complex ecosystem in which symbiotic bacteria, archaea, fungi, protists, and viruses coexist in the gastrointestinal system, on the skin and within others parts of the body. The gut microbiota represents the largest number and concentration of microorganisms in the human body, with the colonization of the intestinal tract starting at birth (Haahtela 2019; Salazar et al. 2017; Sender et al. 2016).

In 1886, Theodor Escherich (1857–1911), a German born physician, published a postdoctoral thesis as a monograph entitled *The intestinal bacteria of the infant and their relation to the physiology of digestion*, where he describes his findings on *Bacillus coli commune* (later named *Escherichia coli*) present in healthy infants and

in those with diarrheal disease (Shulman et al. 2007). A preliminary communication on this subject was made in 1885 at the Society for Morphology and Physiology in Munich (Shulman et al. 2007). Although this was the first scientific evidence that bacteria are part of the intestinal system, this situation was misunderstood and even diabolized for a long time.

The number of bacteria in the gastrointestinal tract reaches 39 trillion with a total mass of 200 g for a healthy young person. If we consider that human cells amount to 30 trillion, this finding refutes the traditional data of 10:1 (bacteria/human cells), showing that bacteria are the same order as the number of human cells (Haahtela 2019; Salazar et al. 2017; Sender et al. 2016). Metagenomic studies have indicated that the number of bacterial species in the gastrointestinal tract of a human body varies between 1000 and 1500, with each human being harboring around 150 different species, possibly amounting to their being more genes in our symbionts than in our own genome (Salazar et al. 2017).

The diversity and stability of these symbiotic microbiota are fundamental for human health and for our adequate physiological development. Reduced contact with the natural environment possibly is a factor that would result in a poor symbiotic microbiota, which in turn might lead to immune imbalance (Haahtela 2019). This might contribute to the allergy epidemic that exists in modern societies associated with global warming which prolongs and intensifies pollen seasons and increases allergy crises (Haahtela 2019).

A good example of the importance of gut symbionts can be found in the serotonin synthesis and role. Serotonin is a neurotransmitter of the central nervous system, namely involved in immune cell activation, in intestinal inflammation process and even in psychiatric disorders (Banskota et al. 2019). This molecule is synthesized in two different areas of human body: 5% in serotonergic neurons of the central nervous system and 95% in enterochromaffin cells of the gut (Banskota et al. 2019). We know that the precursor of this neurotransmitter is tryptophan, which is involved in metabolic pathways associated with bacteria of the gastrointestinal tract, and those microbes constitute a key factor of this molecular synthesis. However, it is not clear why a neurotransmitter involved in the function of the central nervous system is in great part synthesized in the gastrointestinal tract and has an important evolutive influence on the gut–brain axis (GBA). Other microbial metabolites produced in the gastrointestinal tract can also influence the GBA. Such is the case of short chain fatty acids such as butyrate synthetized by gut microbiota that can stimulate memory and synaptic plasticity and also influence the release of serotonin from the intestinal enterochromaffin cells (Kaur et al. 2019). Furthermore, several metabolites of bacterial tryptophan metabolism are important to modulate the health of the host, namely in cases of mental depression. Various studies have also shown the ability of certain bacteria such as *Enterococcus* and *Pseudomonas* to produce serotonin, when those microbes are in tryptophan-rich media. Thusly, these other microbes might also affect serotonergic neurotransmission, thereby in turn affecting the functioning of human central and enteric nervous systems (Kaur et al. 2019).

27.3.5 Termites: A Complex Microbial Community

Termites are detritivore insects with a complex social cast structure that originated in the early Cretaceous Period. Termites show phylogenetic relationships with cockroaches and are present a complex endosymbiotic microbial community in the gut (Fig. 27.5), which are involved in the degradation of organic matter (Duarte et al. 2017; Radek 1999). In fact, the two termites groups (lower and higher) have different feeding habits and present a variety of gut symbionts that are responsible for digesting lignocellulose and converting it into glucose and acetate which are used by the host. In lower termites, this digestion of cellulose is made by the symbiotic association of flagellate protists that live inside the termites' guts. These protists have bacteria associated with them that produce hydrolases, such as cellulases. Some of the bacteria associated with this symbiotic system also have the capacity to fixate nitrogen, serving as a significant source of nitrogen for the termites (Benemann 1973). Higher termites, on the other hand, do not have symbiotic protozoa present in their gut and have limited bacteria species (Evangelista et al. 2019; Duarte et al. 2017, 2018; Radek 1999). Some species of higher termites have the capacity to cultivate fungi of the genus *Termitomyces*, which have high nutritional value and

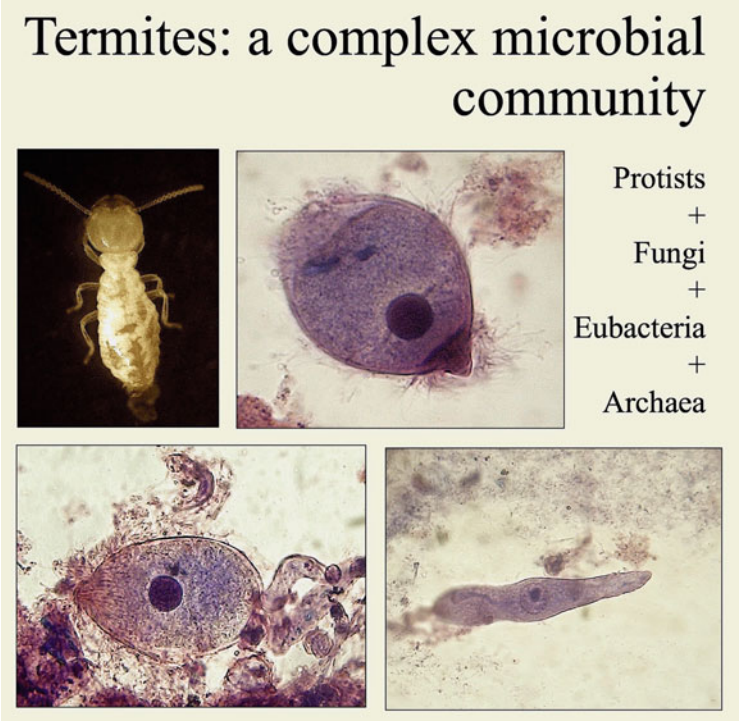


Fig. 27.5 Termites, an example of the importance of symbionts in the metazoan host. (Photos from the author and also adapted from Rita 2006)

also contain hydrolitic enzymes that catalyze the breakdown of cellulose materials into simpler monosaccharides (Radek 1999).

In 1923, the same year that Ivan Wallin publishes his work on *The mitochondria problem* emphasizing the bacterial symbiotic origin of these organelles (Wallin 1923), Lamuel Roscoe Cleveland (1892–1969) an American protozoologist published in the Proceedings of the National Academy of Sciences the article "Symbiosis between termites and their intestinal protozoa,", referring for the first time to the symbiotic nature of the intestinal flagellates of termites and demonstrating that a mutualistic relationship between endosymbiotic microorganisms and their metazoan host was present (Cleveland 1923).

The presence of these symbionts in the termites' complex symbiotic system helps us to understand the evolutionary history of these eusocial insects and the reason why they can be considered one of the most successful groups of insects on Earth (Evangelista et al. 2019).

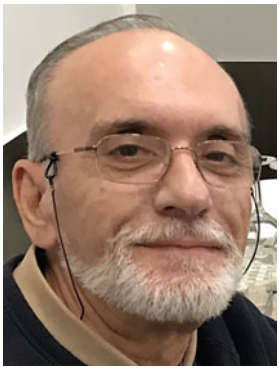
27.4 Concluding Remarks

Our planet is the stage of a multi-dimensional symbiotic world, where biological systems are related to non-biological ones establishing associations and connections to form a global and holistic web of life. It is in this dynamic scenario that antagonistic forces of nature coexist allowing the development and evolution of life. In this context, the dynamics of biological processes are mainly characterized, not by the isolation of characteristics from other life forms but by the integration of those biological capacities into the organism's evolution. We must believe and understand that one of the main characteristics of biological systems is that they establish associations or connections and biotic communication with other organisms. This manifestation is one of the main evolutionary drivers which creates and sustains the diversity of life. In some ways, life has not established or developed to exist alone but only exists together with its symbionts, and this is the main rule for success in the web of life. It is in this evolutive scenario that symbiotic associations develop, and the symbionts' role is effective.

Associated with this symbiotic approach to understanding the biological world, there needs to be a new concept of what represents an organism. This concept is important in the sense that eukaryote organisms are not genetically unique entities, and the concept of individual must be seen as a complex biological ecosystem, composed of multiple interdependent parts living symbiotically. It is also a challenge for the species concept in the traditional genetic point of view, which must adapt to this new reality. This biological complex forms a superorganism, a symbiome or holobiont entity, which shares information at multilevels and creates the ability for the organism, as a whole, to evolve and adapt to new conditions (Carrapiço 2006, 2012; Guerrero et al. 2013; Sapp 2003; Suárez and Triviño 2019). This idea reinforces the principle that all individuals of a species contain associated bacterial and virus populations that collectively act in determining the phenotype. In some

ways, evolution is a dynamic process that evolves and responds not in the sense of perfection or progress but in the sense of adaptation to new conditions and life is at the symbiome or holobiont level composed by of an integrated multigenomic genetic pool, upon which natural selection acts. In this sense, the presence of symbionts can help organisms to achieve a more efficient and faster adaptation, which is the key of evolutive success.

Acknowledgments The author is grateful and expresses his sincere thanks to Maria Helena Costa for the comments and suggestions on the text and to Helena Farrand-Carrapiço for the English revision of the manuscript. Last but not the least, particular thanks to Christon J. Hurst for the comments and suggestions.



Francisco Carrapiço

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Chapter 28
Viruses, Underestimated Drivers of Ecology and Evolution of Life

Antje Lauer

Abstract Viruses are the most understudied microbial agents on our planet; their polyphyletic origin is still shrouded in mysteries. At least some RNA viruses appear to have evolved from self-replicating molecules in the so-called RNA-world that researchers believe to have existed before DNA evolved billions of years ago. Many theories of viral origin and evolution are debated and, even though the fossil record of viruses is basically non-existent in the traditional sense, modern phylogenetic sequencing and electron microscopy have revealed astonishing secrets of viral diversity regarding genome, shape, and size and elucidated many sophisticated mechanisms behind their ability to thrive and replicate as obligate parasites, enslaving their host's replication machinery for their own purposes. Furthermore, evidence exists that many proviruses have evolved with their host, some being part of the so-called junk DNA that we find in prokaryotes and eukaryotes alike, whereas others shaped the evolution of multicellular species becoming even necessary for the survival of the host. With the detection of giant viruses with large genomes including genes coding for replication, one might consider a fourth domain of life for these agents. Ignored and despised, these representatives of the microbial world have been significantly involved in shaping the evolution and ecology of all living species on Earth, in the past, currently, and will continue so in the future. Therefore, viruses, as well as viroids, deserve to be considered as being part of the **foundation stone of Earth's biosphere**.

28.1 Introduction

It is undebated that microbial species on Earth are the true rulers of the biosphere; without them and their involvement in biogeochemical cycles, life, as we know it, would not be sustainable, not even exist. Thanks to the dedication and diligence of uncounted researchers, we are now aware of the microbial world around us. It has

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_28

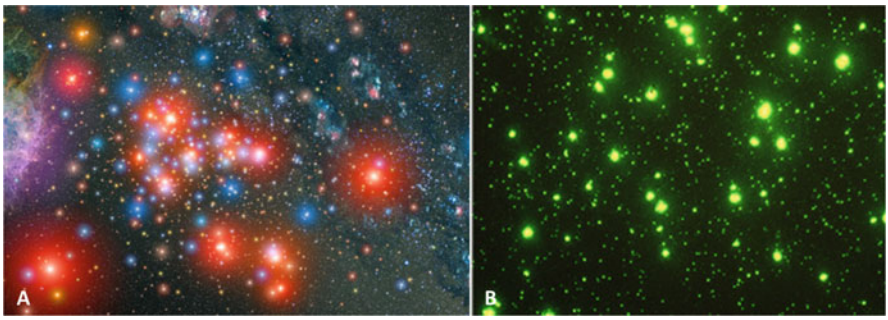

Fig. 28.1 Striking similarities between the macro and the micro cosmos. (**a**). A glimpse into the universe showing endless numbers of stars and planets in between clouds of star dust that some researchers believe contain the seeds of Life (photo credit: NASA/ESA/A. Schaller (for STScI) https://www.discovermagazine.com/the-sciences/the-biocentric-universe-theory-life-creates-time-space-and-the-cosmos-itself). (**b**). Fluorescent staining revealing the ubiquity of microeukaryotes and bacteria (large green dots), as well as viruses (small green dots) in a drop of sea water, as observed by fluorescence microscopy. The fluorescent stain SYBR green binds to DNA and RNA (photo credit: UiB https://www.uib.no/en/rg/mm/57832/viruses-and-bacteria)

been estimated that there are about 10^{33} viruses on our planet. They have been detected in all living species investigated, eukaryotes, and prokaryotes alike and are a major cause of mortality, drive global geochemical cycles, and comprise the greatest reservoir of genetic diversity on our planet (Suttle 2005) (Fig. 28.1).

We have learned that only a minority of prokaryotes and microeukaryotes are in fact dangerous pathogens to humans, animals, and plants and have recognized that also prokaryotes can become hosts of viruses known as phages. Nevertheless, we have just started to understand that we depend on microorganisms in many ways, have started to document microbiomes and viromes of different environments, and thus came to appreciate, respect, and fear their abilities to outsmart us in many ways. However, we must dive even deeper into the microsphere to understand how all living cells are influenced by so-called selfish genes or elements with various levels of autonomy such as plasmids, transposons, and viruses which can drive evolution (Hurst and Werren 2001; Szathmáry 2015; Koonin 2016).

Many species within the prokaryotic domains, Bacteria and Archaea, have been investigated and described regarding physiology, ecology, and genetic makeup, searching for their Last Universal Common Ancestor (LUCA), a progenote or a more defined cell comparable to known prokaryotes (Di Giulio 2019). However, one group of microorganisms or microbial agents has been understudied due to methodological limitations and its crucial underestimation of importance, until Scanning Electron Microscopy became available in the 1930s (McMullan 1995) and molecular biological tools became more widely used among scientists after 1980 (Mullis 1990): these agents are called viroids, phages, and viruses.

The initial interest of the scientific community in studying viruses has its origin in medical microbiology. Back in the nineteenth and early twentieth century, physicians failed to apply Koch's postulates determining the causative agent of some

infectious diseases that had haunted humanity for hundreds of years if not more (Rivers 1937; King 1952). The origin and transmission of diseases such as smallpox, influenza, yellow fever, Ebola, and other mostly zoonotic diseases were mysteries to physicians, nurses, and the general public. Only the development of molecular methods in the late 1980s allowed us to determine their presence in infected patients and determine their natural reservoirs in the living and non-living environment (Lipkin and Firth 2013).

Regarding structure and genetic makeup, viruses are the most primitive agents on our planet that contain genetic information and can reproduce, not considering viroids and other sub-viral agents that lack structure, such as a protective protein coat or envelope. Nevertheless, the more we learn about them, their diversity and their ability to evolve and adapt to different environments, viruses appear to be very sophisticated and not primitive regarding survival in different environments by not depending on metabolism that might limit their distribution. These agents and their ancestors have been the most successful entities that ever evolved and thrived on our planet for ~4 billions of years and should therefore be considered **as part of the foundation stone of the biosphere** which have survived while many highly evolved eukaryotic species are long extinct (Darroch et al. 2018; Dasgupta et al. 2019). Even though it is debated if viruses are alive (Moreira and López-García 2009; Boyer et al. 2010; Hegde et al. 2009; Forterre 2010), their impact on prokaryotic and eukaryotic diversity and survival has been recognized (Sullivan et al. 2017). Should environmental disaster wipe out life on our planet completely, it can rise again by using information stored in prokaryotes and viruses, just giving them time . . . .. but "time is an illusion" (quote: Albert Einstein).

Let's have a closer look at what is currently believed about the origin, evolution, structure, diversity, and impact of viruses on other organisms and how that helps us to understand the impact viruses have on Life on Earth, meaning why they should be considered as a **foundation stone of the biosphere**?

28.2 Origin of Viruses

The origin of viruses is a mystery, but several theories exist that try to explain how viruses came into existence.

Panspermia Hypothesis, also Referred to as the Hoyle–Wickramasinghe Model We know that Earth has been bombarded with comets, ateroids, and meteors bringing most of the water that filled our oceans for a long time (Chyba 1987), and Earth still receives material from outer space supported by strong solar winds that reach our planet (Hawkins 1964; Parkin and Tilles 1968; Brook et al. 2000). The origins of this theory date back to the ancient Greeks; the Greek philosopher Anaxagoras (500–428 BCE) asserted that seeds of life might be present in the entire universe (Temple 2007). Most modern researches are reluctant to accept this hypothesis, which was also proposed by William Thomson, known as Lord

Kelvin (1824–1907), due to the strong believe that viruses and other microorganisms would be deactivated or destroyed by cosmic radiation (Davies 1988). However, some sceptics still support the theory that a molecular cloud generated by meteorite force from the soil microbiome of a planet that sustains life can become a life-bearing ejectum transferred to other planets within a planetary system or even to the universe as a whole (Napier 2004; Wickramasinghe et al. 2010; Wickramasinghe and Smith, 2014). These ideas are supported by the facts that seeds of life can be protected from cosmic rays in asteroids, comets, and meteors (Lithopanspermia) and that viruses which lack a fragile phospholipid envelope might be able to survive unprotected in interplanetary space (Radiopanspermia). The carbon isotope ratio ($^{13}C/^{12}C$) of ancient rocks revealed that life on Earth developed between 3.82 and 4 billion years ago during active meteorite bombardment of our planet (Mojzsis et al. 1996), indicating that life on Earth developed early in a hostile environment, referred to as the Hadean epoch, where life as we know it today, would not be able to survive with the exception of some extremophilic prokaryotes and their viral parasites. Furthermore, amino acids were discovered in the Murchison meteorite that landed on Earth in 1969 (Schmitt-Kopplin et al. 2010). Due to radioactive decay, comets might also carry liquid water deep within protected from the −800 °C degrees of outer space to support fragile molecules or primitive life (Wickramasinghe et al. 2010). In fact, it has been shown that bacterial endospores, endolithic cyanobacteria, as well as lichens and dormant forms of primitive microeukaryotic species, such as Tardigrada, are able to survive outer space environments which include the full spectrum of solar extraterrestrial electromagnetic radiation. However, damage to outer layers of microbial colonies was observed, and these microorganisms did not survive the re-entry process to Earth in these so-called Lithopanspermia experiments (De La Torre et al. 2010; Persson et al. 2011; Horneck et al. 2012; Kawaguchi et al. 2016). If protected from harmful radiation that could damage the microbe's genetic information, re-germination is possible even after millions of years, as it was shown to be true for some spore forming bacterial species retrieved from ancient soils, sediments, glacial ice, or from within amber, and with them their viral parasites (Cano and Borucki, 1995; Ma et al. 1999; Willerslev et al. 2004; Nicholson 2009). The ability of many viruses to incorporate themselves into the genome of their host in form of a provirus allows them to survive adverse conditions together with their host. Recently, a spin-off of the Panspermia theory received new attention, the Nebula-Relay theory, that tries to explain life on Earth seeded by primitive life forms that may exist in the nebulae and in the celestial bodies of solar systems and which could be carried to our planet via meteorites that came in contact with them (Feng 2019). The lithopanspermia hypothesis is difficult or even impossible to prove, because some factors that were proposed in an equation to test the probability of this theory cannot be determined, and consequently different researchers who applied this equation came to opposite results (Nicholson 2009).

Nevertheless, scientists agree that viruses comprise the most abundant entities on the planet by far and have discovered that uncountable numbers of viruses that have been swept upward into Earth's atmosphere and higher by sea spray. Also dust storms can travel large distances, raining down onto Earth in high numbers each day,

inoculating and maybe conquering new hosts and territories (estimated 800 million/day/m^2), with numbers that are significantly higher compared to deposition of bacteria (Reche et al. 2018). Their small size allows viruses to be swept up higher than it can occur for larger organisms, leading to a longer residence time in the atmosphere, and thus viruses potentially can be transported longer distances, which possibly explains why genetically closely related viruses have been detected in very distant and different ecosystems (Short and Suttle 2005).

Different Origin Hypothesis First, the fact that viruses can infect species in all domains of life suggests that they have to be ancient (Cornish-Bowden and Cárdenas 2017; Gaia et al. 2018). Viral genome sequencing and comparisons revealed the vast diversity of viral genomes with five classes of viral genes currently recognized. These included dsDNA viruses (Baltimore classification group I, e.g., Herpesviruses), ssDNA viruses (Baltimore group II, e.g., Parvoviruses), dsRNA viruses (Baltimore group III, e.g., Reoviruses), +ssRNA viruses (Baltimore group IV, e.g., Coronaviruses), -ssRNA viruses (Baltimore group V, e.g., Orthomyxoviurses). Two additional classes included viruses that possess the enzyme reverse transcriptase (ssRNA-RT, Baltimore group VI, e.g., Retroviruses, and dsDNA-RT viruses, e.g., Hepadnaviruses, Baltimore group VII) (Baltimore 1971; Simmonds et al. 2017). No single phylogenetic marker has been detected that could serve as a marker for all viruses. Recent research also confirmed that lateral gene transfer occurred among unrelated viruses complicating taxonomical grouping (Diemer and Stedman 2012). This observation led to the hypothesis that viruses evolved from a variety of different ancestors and did not originate from one LUCA (Forterre 2017). This is also supported by the diversity of different structures of capsids, shapes in general, and mode of entry to the host cell, especially between phages and animal viruses, as well as the existence of RNA and DNA genomes. Structural analyses of capsid proteins have led to the current hypothesis that viruses formed at least during 20 independent events (Krupovic and Koonin 2017). It is currently assumed that with a few exceptions, viral proteins have no homologues in modern eukaryotic cells which contradict the idea that viruses have pickpocketed cellular genes. Therefore, it is now believed that viral genes originated in a so-called virosphere during viral gene replication influenced by genetic shift, with many genes recruited from cell-lines long extinct, or both (Iranzo et al. 2016; Zhang et al. 2018).

Unfortunately, the fossil record for agents smaller than microorganisms, such as bacteria and fungi is non-existent, but the presence of viral agents can be determined indirectly based on cytopathic effects on their host. For example, we know the virus that causes smallpox, a now eradicated viral illness which haunted humanity for hundreds of years and more, has left its marks on mummies such as Ramses V who ruled from 1149–1145 BC as discovered in a tomb in Egypt in 1898 (Strouhal 1996). It is being discussed if virions could be re-activated from mummified victims, then spreading into the environment upon meeting a suitable host, thus allowing the recurrence of epidemics with re-emerging and yet unknown viral entities. For example, due to climate change, the wooden vault full of nineteenth-century smallpox victims mummified in the permafrost of the Arctic Circle near Koltsova, near

Yakutsk in Russia has been investigated (Stone 2002), a nightmarish scenario indeed. One only needs to be reminded that smallpox killed more than three million people in a single epidemic when Hernando Cortéz and his conquistadores introduced smallpox to the New World (Patterson and Runge 2002).

28.3 Evolution of Viruses

Researchers generally agree that viruses evolved long before eukaryotes arose on our planet as outlined by the "virus-first theory" (Forterre 2006). However, evidence exists that viruses have multiple evolutionary origins (Durzyńska and Goździcka-Józefiak 2015; Koonin and Yutin 2018). Were viruses able to live an independent life in the past, meaning, were they once alive? This debate received new attention with the recent discovery of giant viruses or giruses (Colson et al. 2017). Some of these recently detected giant DNA viruses (e.g., Mimivirus, Mamavirus, Pandoraviruses, and Molliviruses among others), which replicate in the cytoplasm of an infected host, are considered to be ancestors of modern eukaryotes, explaining the formation of the nucleus which have striking analogies to viral factories in size and structure (Abergel et al. 2015; Forterre and Gaïa 2016). The authors also stated that the comparable large genome of giruses might have provided new genes to proto-eukaryotes and archaea via transduction or when virus became dormant in the host. Interestingly, DNA of giruses encodes for proteins previously only known from living cells, particularly those supporting the translation process which opens the door of introducing a fourth domain of life (Hurst 2000; Schulz et al. 2017).

Researchers also considered the "reduction theory," investigating the possibility that RNA viruses originated from RNA containing cells by regressive evolution (a new version of the reduction theory [Forterre 2006]). This concept is not new. We know that some members of the domain bacteria have reduced their genome and became obligate parasites, such as *Rickettsia* or *Mycoplasma* spp. (Meseguer et al. 2003; Blanc et al. 2007). Chloroplasts and mitochondria were once independent prokaryotic cells which led to the endosymbiotic theory (Zimorski et al. 2014). But what about ancestors of viruses? No fossil record exists, but we know that the most primitive self-replicating structures relate to ribozymes, quasi-species RNA, that can act as catalysts. They don't encode proteins, lacking the genetic code, but exhibit structural information in form of hairpin loops, able to interact with other molecules forming hydrogen bonds with amino acids and other complex molecules (Marek et al. 2011; Hayden et al. 2018). Reverse transcription as we know from certain RNA viruses can be performed by ribozymes performing the same function that we know from the enzyme reverse transcriptase, which led to the idea that RNA viruses derived from complex ribozymes in the RNA world, being the oldest form of viruses on Earth. Newest virological studies based on the gene coding for the viral RNA-dependent RNA polymerase (RdRP), the only gene that all known RNA viruses share, revealed that dsRNA viruses evolved from +ssRNA viruses on at least two independent occasions, whereas -ssRNA viruses evolved from dsRNA

viruses (Wolf et al. 2018). Viruses with a +ssRNA genome have the advantage that their genome functions as a coding strand equivalent to mRNA which can be translated directly by the host's ribosomes. DNA viruses however, particularly the circular, replication protein (Rep)-encoding ssDNA (CRESS-DNA) viruses, could have evolved in a very different way, namely from bacterial and archaeal plasmids using cDNA copies of capsid genes of eukaryotic +RNA viruses on three independent occasions as proposed by Kazlauskas et al. (2019) suggesting polyphyletic origins. In addition, viroids, which are basically pieces of RNA, can self-replicate and at least fulfill some of the criteria of life (Ding 2010). All this information led to the proposal of an RNA world to have existed before the DNA-protein world evolved (Leslie 2004; Robertson and Joyce 2012) (Fig. 28.2).

We know and fear viruses as obligate parasites dependent on host cells and able to cause devastating diseases affecting all branches of the tree of life. Interestingly, scientists have discovered that some viruses have significantly contributed to the evolution of some of their host species and were even named "editors of the biological code" (Villarreal 1999, 2012). Amazingly, the development of the mammalian placenta is encoded partially by viral genes that these eukaryotic species obtained from prior viral infections, established as so-called non-coding DNA (nDNA) but important for regulatory functions (Villarreal 2016). A lot is still unknown about the genetic information humans and mammals in general obtained from viruses, which makes up a substantial amount of our chromosomal information. In this sense, even though viruses are responsible for infections, some of them deadly, they should be considered as a driver of evolution for eukaryotes and prokaryotes alike, in the past, now, and will certainly also shape evolution of future life forms on our planet.

28.4 Diversity of Viruses

The development of modern next-generation sequencing allows us to detect viral genes in the environment, as well as in hosts, without the need for cultivation of those viruses. These sequences are then used to build databases that can help us to identify novel viruses and compare the diversity of viruses between environments. However, compared to nucleotide databases on the ribosomal gene that are used to identify bacterial, archaeal, and microeukaryotic species, the viral databases are very modest, comprising a fraction of the data deposited for prokaryotic species which limits the ability of alignment-based classification of viral agents. With time, however, these databases will grow and become indispensable for viral researchers in the medical field and environmental field alike. Unfortunately, viruses are so diverse that they lack a universally conserved gene analogous to the ribosomal gene that we use to compare prokaryotic and eukaryotic diversity. In addition, known members of some viral families have segmented genomes, such as members of the Polydnaviridae (10–11 segments), Orthomyxoviridae (6–8 segments), and Reoviridae (8–12 segments) (Lucía-Sanz and Manrubia 2017), which provide a

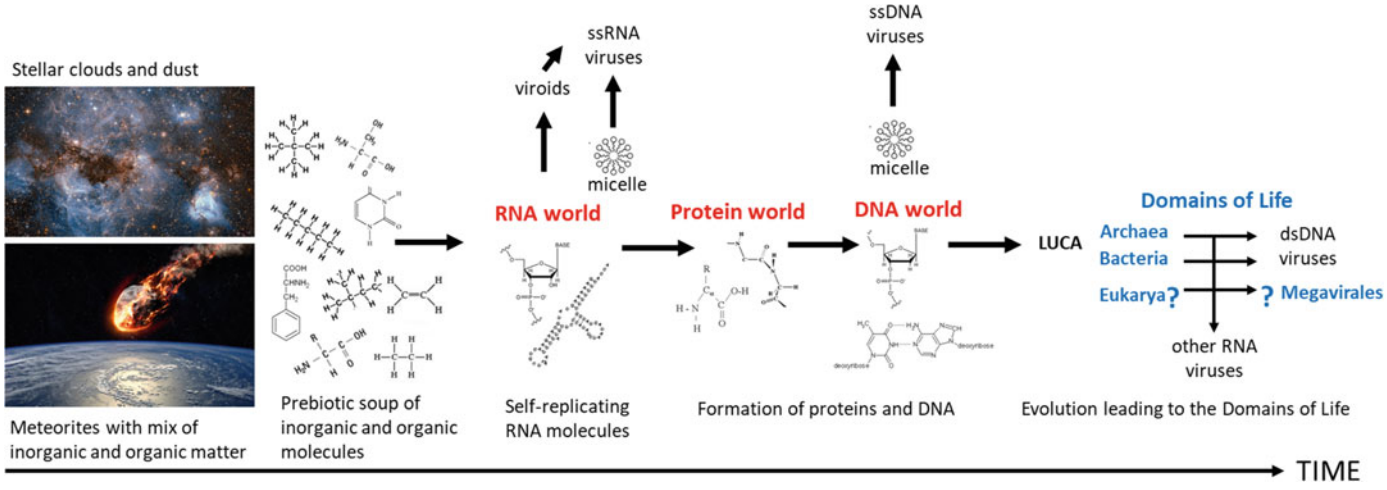

Fig. 28.2 Origin of RNA and DNA viruses as proposed by different authors (see text). A fourth domain of life for giruses is included in this simplified diagram that displays the multiphyletic origin of viruses (photo credits for the stellar clouds and dust: ESA/Hubble and NASA; asteroid falling to Earth: Vadim Sadovski/Shutterstock)

challenge to viral discovery and identification, because only portions of a novel virus might yet have been identified. Furthermore, viral sequences detected in metagenomic sequencing studies often have no related reference entry in established viral databases and are referred to as **viral dark matter** (Waldron 2015; Krishnamurthy and Wang 2017). It should be noted that the viral sequence database in GenBank (Benson et al. 2000) for example is biased toward mammalian, plant, and bacteria that either cause disease in humans or impact animal farms and crop production. Sequences for viruses that use archaea or protists as hosts comprise only a fraction of the database (currently less than 10%) and more research has to be accomplished to reveal the true extent of viral diversity (Krishnamurthy and Wang 2017; Greninger 2018). In addition to sequencing and aligning viral genomes with known database entries, other methods are available to identify and characterize virions, exploring their diversity and also revealing modes of entry and replication; the gold standard among them is viral culturing that allows the researcher to determine if an unknown sequence is indeed of viral origin and to observe potential cytopathic effects of viral infection. Classical viral isolation relies upon membrane filtration using 0.2 μm filters which allow the separation of viral agents from prokaryotes and eukaryotic cells, followed by RNAse and DNAse treatment that has no effect on virions protected by protein coats but will destroy any genetic material from lysed cells or viroids. In addition, viral tagging with fluorescent dyes can be useful to investigate viruses if they are permeable to the dye being used. For additional methods used by virologists to target and detect viruses please refer to (Boonham et al. 2014; Krishnamurthy and Wang 2017) and other references mentioned within this chapter. For the future, we will rely on improvements of viral culture methods to distinguish the so-called genetic **dark matter** from sequences of known viral origin and perhaps develop improved molecular methods that allow more sensitive and reliable cluster and differentiation analyses leading to the identification of as yet unknown viral lineages.

So, what do we know about the viral diversity in the environment? Studying the virome of different environments has continued to attract substantial U.S. and European government funding in recent years, as a result of major threats to humanity, to mention only the Ebola epidemic in West Africa in 2014–2016 (Matz et al. 2019) and the Coronavirus epidemic that started in Wuhan, China, in 2019 (Ralph et al. 2020). Research is needed because of difficulties in identifying the natural reservoirs of the pathogens and strategies to identify and successfully treat the patient zero of an outbreak. Furthermore, funds to develop new vaccines against these beforementioned emerging pathogens and others, such as viruses that cause livestock abortion storms (Dun 2019), and encephalitis in humans and animals (Centers for Disease Control and Prevention 2020a, b) are desperately needed. Recently, the Global Virome Project was launched with the goal of identifying yet unknown viral agents and their natural reservoir over a 10-year schedule, aiming to reduce global vulnerability to known and yet unknown potentially emerging viral zoonotic agents and to provide timely data for epidemiologists and other healthcare providers to prevent future pandemics. First results revealed hundreds of new viruses and their animal reservoirs (http://www.globalviromeproject.org/overview, see also

Afrough et al. 2019; Goldstein et al. 2019). Ongoing unrests, civil wars, violent attacks on ethnicities with different beliefs, uncontrolled population growth, poverty, and human greed collectively have resulted in extensive encroachment of humans and their livestock into highly biodiverse tropical forests and other pristine environments. Having encroached upon those environments, the humans and their livestock encounter native populations of animals that are natural reservoirs of viruses and other pathogens that have the potential to infect and cause disease in humans and livestock. As a result of increased contact with wild animals, a so-called spillover might occur where a virus is given the opportunity to infect a new host successfully with often fatal consequences for the host. So far, the global virome project has targeted primarily tropical areas in biodiversity hot-spots in Asia, Africa, and South America, in countries that traditionally struggle when dealing with public health crises. The project has identified a plethora of new viruses, some related to known viral pathogens that can infect humans. Furthermore, members of many unknown viral lineages were discovered, by investigating the blood of wild animals that could present as a natural reservoir of a virus and by screening for unknown viruses in the blood of villagers. This multimillion dollar effort is important for disease mitigation in humans and animals and will add substantial data to the GenBank viral database but does not focus on the diversity of phages or viruses that affect fungi, microeukaryotes, or plants that are not being used as food and which don't pose a threat to be able to infect humans directly (for details see https://www.ecohealthalliance.org/program/predict).

If we want to understand where viruses come from, we might want to focus on some of the oldest prokaryotes that exist, extremophilic Archaea, that survived in niches resembling conditions of early Earth and which harbor a unique and enigmatic diversity of viruses that might have evolved from non-viral mobile genetic elements able to self-replicate (Krupovic et al. 2018) originating at a time we call the dawn of Life.

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Chapter 29
Coevolution of Bryophytes and their Associated Microorganisms

Guillermo Reboledo and Inés Ponce de León

Abstract Plants colonized terrestrial habitats approximately 450 million years ago. This was one of the most important steps in the evolution of life on earth and the foundation of ecosystems. Fungal beneficial associations with plants have probably facilitated the conquest of land. Bryophytes (no-vascular plants: mosses, liverworts, and hornworts), and traqueophytes (vascular plants), are descendants of these early plants that evolved on land. Extant bryophytes, together with comparative analysis with traqueophytes, represent therefore excellent model organisms to reveal ancient mechanisms related to plant–microorganism interactions. In this chapter, we will present some of the current knowledge on bryophyte–microorganism interactions, including adaptation mechanisms that developed during coevolution of mosses and liverworts with microorganisms.

29.1 Introduction

Plants interact constantly with different microorganisms present in their environment, which influences plant development, health, and nutrient acquisition. For the host plant, these interactions can range from beneficial (mutualism) to pathogenic. Beneficial associations provide the plant a better access and uptake of nutrients and water from the soil, in exchange for providing the microbe with photosynthetic carbon sources. In contrast, colonization of plants by pathogenic microorganisms causes damage and often impairs plant growth and reproduction. These biotic interactions have coevolved during time through natural selection of genetic traits that increased fitness and helped to avoid disease in the plant, and we have been able to identify key traits associated with symbiotic and pathogenic microbial lifestyles. But our curiosity also results in asking several questions, three of those being: When were land plant–microorganism associations established during evolution? How did these different types of associations evolve? and, What adaptation mechanisms did

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_29

the host plants and the microorganisms develop to be successful? All these types of questions are starting to be answered by using fossil records and extant land plants, including bryophytes.

29.2 In the Beginning

Descendants of freshwater algae colonized terrestrial habitats long time ago in the Late Ordovician period, approximately 450 million years ago (Mya). At that time, the world was a barren place covered by rocks. The conquest of land by plants was one of the most important steps in the evolution of life on earth and the foundation of ecosystems. It changed the planet completely by reshaping the global environment, including land, oceans, and the atmosphere. However, the transition from an aquatic to a terrestrial habitat was not easy since it presented numerous challenges related to nutrient and water acquisition. In addition, environmental conditions changed dramatically, leading to desiccation, extreme temperatures, and ultraviolet radiation exposition. To survive these environmental stresses, the first land plants (embryophytes) developed different adaptation mechanisms. Cuticles and cell walls supported dehydration stress, and metabolites such as phenylpropanoids acted as shields against UV irradiance. Furthermore, things are always easier when you have a partner, and highly preserved fossils from this period have shown that embryophytes did not colonize the land alone. The presence of fungal structures in these fossils suggests that beneficial associations facilitated land colonization (Remy et al. 1994). The improvement of nutrient capture by plants was probably the main reason. In exchange for inorganic nutrients obtained from soil and water, early land plants provided photosynthesis-derived carbohydrates to the fungi. These interactions evolved into symbioses, and nowadays most land plants establish mutualistic symbiosis with fungi.

However, pathogenic microorganisms present in soil and air also composed the new terrestrial habitat. Fossil records suggest the existence of pathogenic fungi interacting with plants 400 Mya (Krings et al. 2007). These microorganisms might have imposed a selective pressure on early land plants leading to the development of defensive strategies to counteract invasion. During time, both plants and pathogens have evolved mechanism to avoid each other's defense strategies. This evolutionary arms race between plants and pathogens has resulted in the development of specialized and effective plant defense mechanisms and in adaptation strategies of the pathogen to changes occurring in host plants.

29.3 Bryophytes and Microorganisms

Bryophytes (non-vascular plants), including mosses, liverworts, and hornworts, and traqueophytes (vascular plants; lycophytes, ferns, gymnosperms and angiosperms), evolved from early land plants. Extant bryophytes represent therefore excellent model organisms to reveal ancient mechanisms related to plant–microorganism interactions. In addition, comparative analysis with tracheophyte lineages contributes to shed light on the origin and evolution of key land plant innovations during biotic interactions. As fascinating windows to the past, several fossil records from Ordovician sediments have shown the presence of fungal structures associated with early bryophytes, demonstrating the existence of ancient interactions that could persist nowadays. Arbuscular mycorrhizal (AM) fungi belonging to the groups Glomeromycotina and Mucoromycotina have been reported in bryophytes fossils (Strullu-Derrien et al. 2014). Both types of fungi are proposed to be implicated in land colonization by plants. Extant liverworts and hornworts also establish symbiotic associations with both types of fungi, sometimes simultaneously. The interaction with these AM fungi, as well as association with endophytic members of the ascomycota and basidiomycota groups, improves bryophyte growth and fitness through enhanced nutrient uptake. The AM fungi colonize the tissues of liverworts forming structures that are analogous to those observed in AM colonized roots of vascular plants, including intracellular hyphae, arbuscule-like structures, and vesicles. At present, plant symbiosis with both Glomeromycotina and Mucoromycotina are common in agricultural and natural ecosystems. The association of these fungal groups with different plant lineages has persisted during 450 million years of coevolution demonstrating a clear functional advantage. Thus, why are there no evidences that suggest the existence of a functional symbiosis with mosses and AM fungi? This is an intriguing issue and a plausible explanation is the loss of some symbiotic signaling genes in mosses.

Diverse microbial pathogens colonize bryophytes, including fungi, bacteria, oomycetes, and viruses. Several fungi and bacteria have been isolated from wild population of mosses and liverworts. In addition, wide host range pathogens that infect crops also colonize and cause disease in some of these bryophytes (Fig. 29.1), allowing comparative studies of defense activation and strategies developed by the pathogen to interfere with plant immunity.

29.4 Coevolution of Plants and Microorganisms

In the last 20 years, progress in understanding molecular aspects of coevolution of traqueophytes with their associated microorganisms has revealed complex responses on both sites. Plants have developed mechanisms to detect the presence of a microorganism, distinguish if the microbe is beneficial or pathogenic, and mount a response accordingly. Receptors at the plasma membrane act as sentinels and

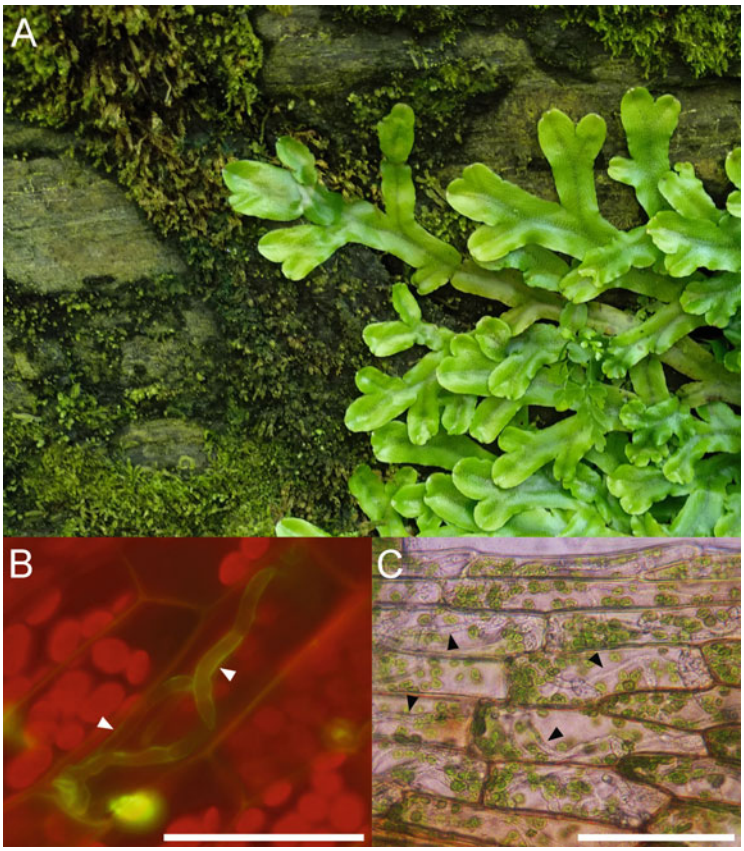

Fig. 29.1 (**a**) Moss and liverwort (the species shown in close-up is a liverwort), (**b**) the moss species *Physcomitrella patens* infected with a filamentous fungus, (**c**) *Physcomitrella patens* infected with an oomycete. Black-and-white arrowheads indicate the localization of hyphae. Scale bars: 50 μm

perceive molecules of the microorganisms, for example flagellin or chitin. After perception of the microorganism, a plant response is rapidly activated leading to a massive transcriptional reprogramming and change in hormone levels. However, microorganisms adapted to their host plants have evolved strategies to interfere and inhibit plant defense by the action of pathogen-secreted virulence factors known as effectors. For example, the bacterial pathogen, *Pseudomonas syringae*, can deliver over 30 effectors into plant cells through the type III secretion system. These effectors suppress the plant immune system at various levels, including receptor signaling, cell wall reinforcement, and hormonal signaling, among others. But plants have a second layer of immune system and recognize microbial effectors directly or indirectly by intracellular receptors encoded by resistance (R) genes.

Beneficial microorganisms have also evolved to avoid the plant immune system by producing less damage or by producing molecules to manipulate the host

signaling pathways for their own benefit. In addition to effectors, microbial pathogens interfere with plant defense responses by producing plant hormones.

29.5 Adaptation Mechanisms in Bryophytes and Microorganisms

Information regarding the cellular and molecular mechanisms operating in interactions of bryophytes and their associated microorganisms is quite limited. However, in recent years, our reference species of bryophytes have allowed us to make important advances, mainly with understanding interactions between plants and pathogenic microorganisms. The moss *Physcomitrium patens (previously Physcomitrella patens; P. patens)* and the liverwort *Marchantia polymorpha* (*M. polymorpha*) are currently used by several research groups to reveal ancient mechanisms in plant biology. They have several additional advantages compared to other bryophytes, including available genomic resources, targeted mutagenesis, and standardized protocols for genetic transformation. This allows the identification of crucial components in different types of bryophyte–microorganism interactions, including microbial colonization strategies and plant defense mechanisms to cope with them.

What we have known until present is that as traqueophytes, *P. patens* and *M. polymorpha* activate a defense response after pathogen invasion, with that response leading to cell wall associated defenses, increased levels of hormones, and expression of genes that encode proteins with different roles in plant resistance (Ponce de León and Montesano 2017; Carella et al. 2019). Some of these key defense components are conserved between traqueophytes and bryophytes, suggesting that they were probably present in the common ancestor of land plants. For example, traqueophytes have developed a system for sensing pathogens by monitoring the cell wall integrity, upon which they activate reinforcement of the cell wall, which importantly is required to prevent disease. Receptors at the plasma membrane can sense oligogalacturonides derived from the degradation of the cell wall by the action of microbial cell wall degrading enzymes (CWDEs). In *P. patens,* treatment with CWDEs and effectors of the bacterial pathogen *Pectobacterium caratovorum* activate a signaling cascade, suggesting a functional sensing mechanism. This leads to plant cell wall reinforcement by incorporation of phenolic compounds and callose to stop further damage. Similarly, *Phytopthora palmivora* infection of *M. polymorpha* involves the induction of a large set of genes encoding CWDEs, and consequently callose deposition occurs to prevent colonization. Fossil records have shown the presence of thickened cell walls in tissues of vascular plants infected with fungal structures 400 Mya, demonstrating that cell wall fortification is an ancient defense response.

Several hormones involved in traqueophyte defense against microbial pathogens are also present in bryophytes where they play a role in immunity, including the hormones salicylic acid (SA) and auxin. Both *P. patens* and *M. polymorpha* increase

the synthesis of SA after pathogen infection, leading to the activation of defense genes. The SA pathways could have been present in the common ancestor of land plants and played a role in defense since all genes of this pathway are present in bryophytes but not in algae. A remarkable discovery was the difference of the jasmonic acid (JA) pathway between bryophytes and traqueophytes, starting from the fact that the active hormone (JA conjugated to isoleucine; JA-Ile) is not produced in bryophytes. In traqueophytes, JA-Ile binds to the receptor COI1 (coronatine-insensitive 1) and activates a signaling pathway that reprogrammed the expression of hundreds of genes, while in *M. polymorpha* COI1 recognizes to its precursor, dinor oxophytodienoic acid (dinor-OPDA) (Monte et al. 2018). The reason for the evolutionary change in ligand specificity is a single amino acid substitution in COI1. Interestingly, dinor-OPDA play a role in bryophyte defense like JA-Ile in traqueophytes, and both hormones have an antagonistic effect with the SA pathway. Thus, ancient features of the jasmonate pathway were already present in early land plants.

Several evidences demonstrate that pathogen effectors influence bryophyte defense responses. For example, *P. palmivora* forms digit-like protruding structures within *M. polymorpha* living cells which invaginate, thereby forming haustoria-like structures that as in traqueophytes release effectors, mainly RXLRs (the family of phytopathogen effector proteins termed RXLR is defined by a secretion signal peptide followed by a conserved N-terminal domain which contains the sequence Arg-Xaa-Leu-Arg) (Carella et al. 2018). The expression of RXLR is associated with successful colonization events in the liverwort, demonstrating the importance of these molecules for pathogenic lifestyle. *Pseudomonas syringae* effectors target *M. polymorpha* defenses to provoke disease by interacting with receptors that sense its presence, by interfering with the SA response and by repressing host defense gene expression (Gimenez-Ibanez et al. 2019).

The recently gained knowledge on bryophyte defense mechanisms and pathogen infection strategies is just starting to reveal for us a very small piece of the complete picture of these interactions from the perspective of both organisms. Further research on these descendants of the first land plants and their associated microorganisms will certainly advance and improve our understanding on how bryophytes–microorganism interactions coevolved.

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Part VII
The Adventure of Microbiology Research

Chapter 30
BUBBLES in the MUD: A Reminiscence and Perspective

Ronald S. Oremland

Abstract I still feel a surge of excitement when, wading along some shore, my feet stir up an effusion of bubbles from the underlying muck. My thoughts ensue: Are they methane-rich, or perhaps composed of mixtures of nitrous oxide, hydrogen, carbon dioxide, and hydrogen sulfide? Do they contain exotics like ethane, dimethyl sulfide, trimethylamine, or even a methylated arsine? Suppressing an urge to grab a collection funnel, I resume my slog, leaving the unanswered questions for the next generation.

So, how did I first acquire this curiosity concerning gaseous emanations, especially ones of biotic origin? It is a long story, but it is in essence my career path allowing me to state unabashedly:

In small scientific circles, I am remembered for my gas.

30.1 A Reminiscence

My favorite high school subject was chemistry. Our teacher at Abraham Lincoln H.S. in Brooklyn was Mr. Hal Rosenthal, a caring, soft-spoken man who nonetheless had a clarity of presentation that allowed students to grasp difficult, novel concepts. He also performed numerous classroom demonstrations that wound up making gas bubbles, be they by reaction of metallic sodium with water or hydrochloric acid with "mossy" zinc. The latter used a glass reaction vessel consisting of a bottle stoppered with a small glass funnel inserted that allowed for the acid to be dribbled into the reaction bottle, and a glass exit tube through the stopper which connected to rubber tubing whereby the generated H_2 was collected by displacement of water from another small inverted bottle. Inserting a lit wooden taper into the opened collection bottle generated a small explosive "pop" thereby verifying that the product of the reaction was H_2. Similar demonstrations were of dilute acid with chalk to form CO_2,

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_30

which extinguished the wicker flame to the mild disappointment of the assembled class. Spirits were revived somewhat when hydrogen peroxide was reacted to form O_2, which notably enhanced the wicker's flame when inserted into the collection bottle. Oxygen supports combustion but is not by itself combustible was the take home message. Other gas bubble demo-spectacles included that of nitric acid with copper to form colorless NO, which when the test tube was opened to the air, formed the brown NO_2 gas. Wow! It was very heady stuff, especially for me and I was hooked.

At some point in the course, which occupied my entire junior year, I became a veritable chemistry nerd. I adored the exotic chemical glassware, the colored liquid reagents, and especially my acquired lab-poseur form of correctly holding a reagent bottle's ground glass stopper between the middle and fourth digitals of my right hand when meting out fluid. This became my version of being "cool" and added just a bit of swagger to my step. OK, it was not exactly an acquired James Dean sexy disillusioned look and persona meant to quicken the heartbeats of our high school's female student population, but it was good enough for me at the time.

Mr. Rosenthal also endowed us with a simple mnemonic device to understand chemical redox reactions that I employ to this day:

LEO the lion goes GER (LEO: Loss of Electrons = Oxidation; GER: Gain of Electrons = Reduction).

Next came Rensselaer Polytechnic Institute (RPI). At RPI, the curriculum was demanding and rigorous. There were, nonetheless, a few memorable instructors, one of whom was the late Professor K. Jack Bauer a naval historian. Dr. Bauer had a knack for telling a story coupled with a charismatic lecturing ability that could make virtually any topic interesting. That would include furniture. No kidding! This included a table, now owned by RPI that was once employed to end a war. Specifically, by the parties to the 1905 Treaty of Portsmouth that ended the Russo-Japanese War. Incidentally, it also gained President Teddy Roosevelt the Nobel Peace Prize. A second memorable instructor, recently deceased, was Professor Henry L. Ehrlich who taught general microbiology, along with his specialty, geomicrobiology. Professor Ehrlich was not a charismatic lecturer, but his talks were clear and interesting, especially because geomicrobiology had sealed into its core the basic chemistry that I first took a shining to back in high school. The Sixth Edition of Ehrlich's *Geomicrobiology* was published just a few years ago (Ehrlich et al. 2015).

30.2 Got Methane (and a Bathing Suit)?

In grad school, I pursued investigations on methane biogeochemistry (Oremland 2019), where much to my chagrin, the first gases I collected with a funnel while hovering over a seagrass bed were mostly composed of O_2 and N_2 with only a trace of CH_4 (Oremland and Taylor 1977). Yet when I incubated anoxic seagrass slurries,

they produced methane that ran concurrently with sulfate-reduction (Oremland and Taylor 1978). A few years later, observations I first made with Big Soda Lake sediments amended with methanol (Oremland et al. 1982a) led to the concept of non-competitive substrates. Hence, sulfate-reducers would not compete with methanogens for methanol, methylated amines (Oremland et al. 1982b; Oremland and Polcin 1982; King 1984), or methylated sulfur compounds (Kiene et al. 1986), while they would generally outcompete methanogens for the more plentiful H_2 and acetate.

Yet I digress back to graduate school, circa 1974. It would be remiss of me not to mention an event that coincided with my collection of gases bubbling out of the seagrass beds in Bimini Harbor. Tied up at the pier next to our research vessel (R/V Calanus; Univ. Miami) was a yacht whose occupants were engaged in making a dubious achievement: the first underwater pornographic movie. Also tied up at that pier was a sailboat with license plates from Boulder, Colorado (!!) whose occupants were two parapsychologists engaged in a search for the lost continent of Atlantis ostensibly hidden somewhere among the underwater carbonate geology of the Grand Bahama Bank. Local TV coverage came by and focused exclusively on these two groups. There was absolutely no interest whatsoever on their part concerning our efforts to quantify seagrass bed nitrogen fixation rates that my good friend, Doug Capone, was working on, let alone be impressed by the variable diurnal composition of bubble ebullition from the mats of *Thalassia testudium*, a species of marine seagrass also called turtle grass. The TV folks did recognize our presence, sort of, in that they asked us to turn off our generator so that their interviews could proceed more smoothly without background noise. Our chief scientist, Prof. Barrie Taylor (academic mentor to Doug and I) just refused their request. We had research work to do, and our instruments needed to be running.

30.3 Horror-Show, Tovarisch?!!?!

Back at RPI, I had declared a scientific curriculum rather than that of engineering. Hence, in my freshman year, I had to take a "scientific" language in lieu of engineering drawing. These were limited to either the study of German or Russian, and I chose the latter. Now I should make it clear that at this point in my life, although I liked science, I had little to make me think I would actually become a scientist, let alone make any possible use, whatsoever, of the two semesters of Russian I was required to pass. Indeed, I failed the second semester of the 3 credit course, and made up for it by taking a 5 credit course given by NYU over the summer which I did manage to pass. I had punched my course-requirement ticket in this regard. I simply moved ahead giving little thought, except sheer relief, to the Cryllic alphabet, the weird vowels I suffered pronouncing in the long language lab sessions and any latent ability to converse in this most unusual and challenging language.

In the spring of 1977, toward the end of my NASA postdoc, I found myself attending the Symposium on Environmental Biogeochemistry conference in Wolfenbüttel, Germany. It was my first international conference and my first time overseas. The meeting was chaired by Prof. Wolfgang Krumbein and had a number of intense, interesting sessions on ancient geobiology, extreme environments, and geologic structures having biogenic components (i.e., stromatolites). I had little prior exposure to these topics previously, and my appetite was whetted. Also, because its location was proximate to the border that once separated West and East Germany (this was at the height of the Cold War) there was a large scientific contingent from the Soviet Union (aka: Russia) also present. In walking around the conference during a coffee break, I espied a man of my age from that contingent, whose name I recognized from the marine methane literature: Dr. Sergey S. Bellayev. I walked up to him, and reaching deep into my brain, managed to retrieve a few words of standard greeting and self-introduction in Russian that had not altogether gone down the drain.

His eyes grew as wide as saucers because he assumed I was fluent in Russian, a rarity among the American contingent. In actuality, because I have an "ear" for sounds, all those tortuous hours spent in language labs endowed me with a refined/educated pronunciation of the Great Russian Language (*Balshaya Russkaya Yazike*) even though my vocabulary consisted of less than 100 words that I could barely string together in a coherent sentence. Yet to his ears I sounded like the Russian equivalent of trained English-speakers from foreign countries around the globe whose refined accents could easily put them in from of a BBC microphone.

Sergey called out to his Russian colleagues and I was within moments surrounded by their entire delegation, all speaking at once, and all trying to discern where this American oddity gained this capacity. Soon their most senior person, Professor Michael (Misha) Ivanov, came by and queried me, all while smoking a classic paparossi. After establishing my credentials which I used all the RPI/NYU Russian I had retained, he pronounced to his colleagues "Nash!" (literally . . .he is one of us). I made a number of wonderful new friends and colleagues that day, which was not at all bad for a kid who 12 years before had flunked Russian 2 and ruined his summer by repeating the course.

30.4 A Real Job and a Bit of an Uphill Struggle

My first few years at the United States Geological Survey (USGS, 1977–1980) were trying, especially since I had no lab. Moreover, I felt out of place, in that there was little interest by the hierarchy in allowing me to pursue work on anaerobic processes, especially methanogenesis. Yet somehow, I bootlegged and eventually published research dealing with methane. These included the marine plankton/fish fart hypothesis (Oremland 1979), detection of methanogenic activity in Deep Sea Drilling Project muds (Oremland et al. 1982c), and a refutation of Thomas Gold's deep methane hypothesis based on serendipitously collected field data (Oremland 1983).

In the summer of 1978, I was invited to give a talk on methanogenesis at the Gordon Research Conference on Organic Geochemistry which was very well received. Especially so, since most of the scientists came from a chemical analytical background, viewing petroleum, kerogen, coal, and hydrocarbon gases as static things to be collected, structures within identified and origins inferred. Treating sediments as live, dynamic experimental systems was at the time a novel concept to these folks. The positive feedback I received had a salutary effect upon me: It convinced me of the merits of continuing this research. Another invitation to make a methane biogeochemistry presentation to an International Symposia for Environmental Biogeochemistry further reinforced the positive feedback. Yet I also somehow had to make the entrenched hierarchy grasp the merits of studying anaerobiosis in regions where such things were not visually evident, such as pristine freshwater streams. Our discovery of active denitrification in stream periphyton communities got the ball rolling in this dimension (Triska and Oremland 1981; Duff et al. 1984) and helped me politically. Our use of dissolved gases (CH_4 and N_2O) to follow the propagation and rapid dissipation of a large municipal sewage spill in South San Francisco Bay reinforced the utility of understanding these processes (Cloern and Oremland 1983). By this time I had gained a good degree of scientific independence, and the "entrenched" folks who first doubted me just wanted more in the form of peer-reviewed research papers, which I was delighted to provide.

30.5 Ethane and Propane and Butane (Oh My)!

One take-away bit of information I acquired from interacting with organic geochemists was their method for classification of natural gases. Much of this was empirical, inspired by the search for petroleum reserves. The thinking was that accumulations of biogenic gases consisted almost entirely of methane, with perhaps only traces of the higher alkanes (ethane and propane) evident. Hence, a ratio of methane to ethane plus propane of greater than 100 (and usually much greater, say 1000 or higher) indicated a gas of biogenic origin. In contrast, when the ratio was less than 100 (and usually significantly less, where ethane and propane rise to percentage levels), it was indicative of a gas of thermogenic origin, which was often co-associated with petroleum deposits (Bernard et al. 1978; Hunt 1979). Yet the experimental literature was sparse in this regard to say the least (e.g., Davis and Squires 1954) and little was known about biogenic mechanisms for higher gaseous alkane formation.

My USGS colleagues Tim Vogel and Keith Kvenvolden, both marine organic geochemists, collaborated with me by modifying their sediment hydrocarbon extraction methodology. They routinely employed 1-gallon metal paint cans into which they placed their recovered, near-surface sediments and partitioned the associated gases into a small, sealed headspace after the can underwent prolonged violent shaking on a paint mixer. We modified this by placing anoxic, San Francisco Bay mud into the cans in order to form a time course of gas production. By using so much

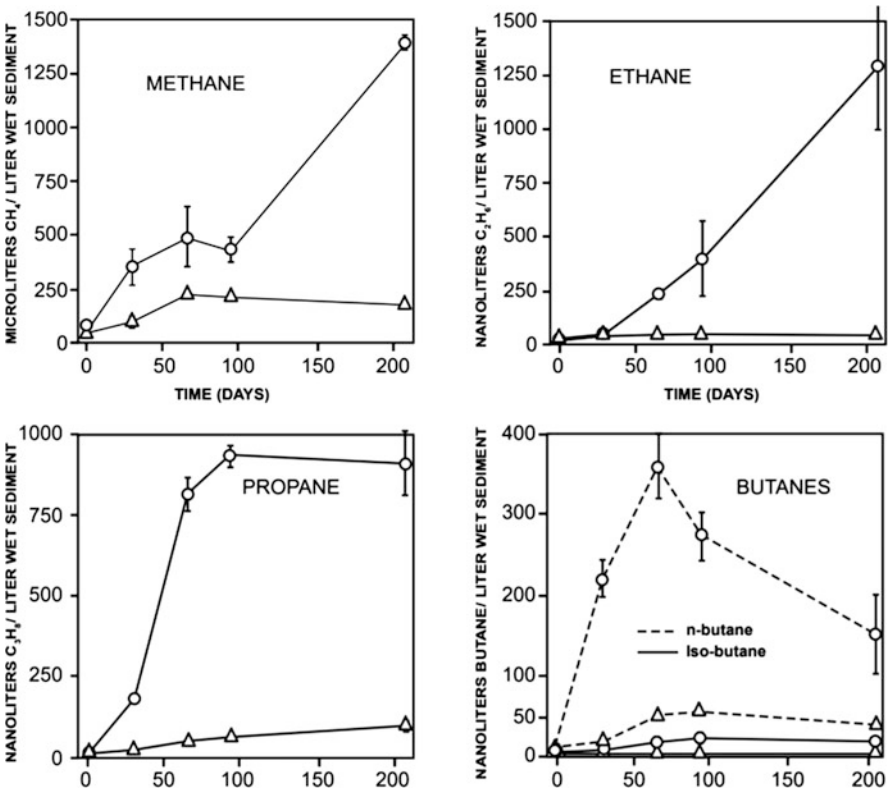

Fig. 30.1 Formation of methane, ethane, propane, and butanes by anoxic, San Francisco Bay sediments incubated in nearly completely filled gallon paint cans at 27 °C (open circles) and at 4 °C (open triangles). Error bars indicate ±1 standard deviation of 3 replicates. Reproduced from Vogel et al. (1982) with permission

material per sample, the idea was that we could detect robust production not only of methane but of ethane, propane, and butanes as well. The experimental group was incubated at 27 °C, while the control group was incubated at 4 °C in an effort to retard microbial activity (owing to the density of the sediment material, autoclaving or use of specific inhibitors were impractical). The results showed robust production of methane, ethane, propane, and butanes over a 7-month incubation period and that production of these gases was severely constrained at 4 °C (Fig. 30.1) implying biogenic sources for all C_1–C_4 alkanes. The final methane/ethane + propane ratio achieved was ~13,000, once again strongly indicative that a microbial production mechanism(s) was involved (Vogel et al. 1982). There was a suggestion that the unsaturated alkenes ethane and propene may have been precursors of the observed respective alkane formation, as the unsaturates accumulated at the end of the incubation, after ethene and propane formation ceased (Fig. 30.2).

The anaerobic nature of C_2+ light hydrocarbon production and its coincidence with methanogenesis provided circumstantial evidence that the two processes were

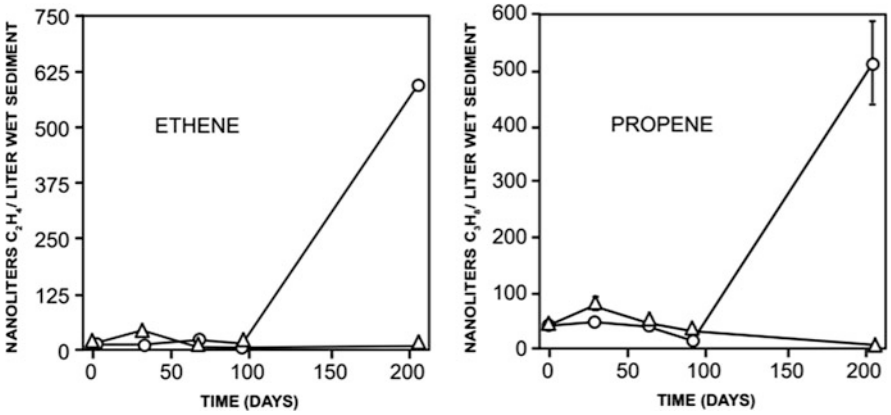

Fig. 30.2 Formation of ethene and propene during incubation of San Francisco Bay anoxic sediments. Conditions are the same as given in Fig. 30.1

linked in terms of sharing a common mechanism of formation. My colleague Rob Gunsalus (Gunsalus and Wolfe 1980) published dissertation work that provided a possible mechanism involving the methyl coenzyme M reductase (MCR) purified from *Methanobacterium thermoautotrophicum* (renamed as *Methanothermobacter thermautotrophicus;* Wasserfallen et al. 2000). It turns out that when coenzyme M reductase was provided in vitro with methylated CoM (methyl-S-CoM), methane was formed as would be expected. However, when the ethylated analogue was employed (ethyl-S-CoM), ethane was produced. However, this could not be extended to higher alkanes, as propane formation did not occur when the enzyme was fed propyl-S-CoM (Gunsalus et al. 1978). Nonetheless, a mechanism for ethanogenesis in sediment systems was suggested and it could clearly involve methanogens.

To pursue this question, I worked with dilute San Francisco Bay sediment slurries, which lent themselves readily to experimental manipulations (Oremland 1981). Incubation of the slurries under H_2 (an electron donor for many methanogens) stimulated methane production when compared against incubation under N_2. The observed methanogenesis was inhibited by 2-bromoethane sulfonic acid (BES). Addition of ethyl-S-CoM (kindly provided by Prof. Ralph Wolfe) to the slurries resulted in the formation of a small amount of ethane, which occurred simultaneously with methane production but at roughly 20,000-fold lower amounts than methane. Significantly, endogenous ethane formation was also noted (~4 nmoles) without the added ethyl-S-CoM but at lower levels than the slurries amended with ~2 mM ethyl-S-CoM (~24 nmoles). The BES inhibited ethane production by both amended and un-amended ethane slurries (Fig. 30.3). A highly purified enrichment culture of a coccoid methanogen, routinely cultivated in the presence of ethyl-S-CoM, was obtained from these slurries. It proved capable of ethane formation when provided with ethyl-S-CoM and after 4 days of growth yielded a final CH_4/C_2H_6 ratio of ~12,000. It was significant that neither tested sulfate-reducers (e.g.,

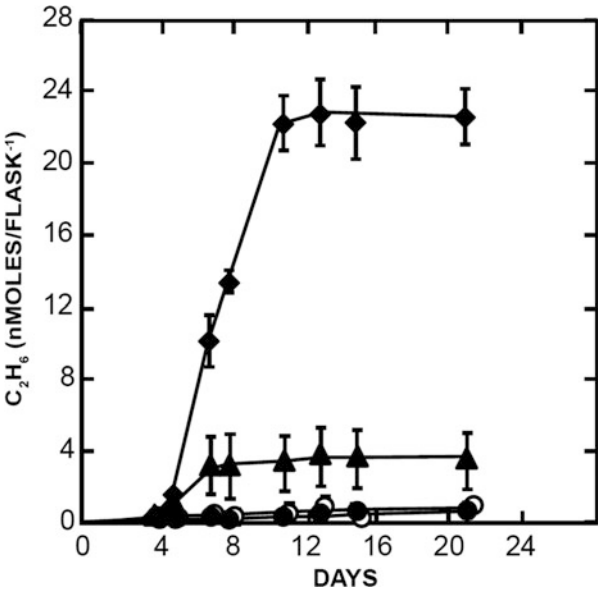

Fig. 30.3 Formation of ethane by anaerobic San Francisco Bay sediment slurries incubated under H_2. Symbols: amended with 2 mM ethyl-S-CoM (◆), without amendments (▲), inhibited by BES (●) and amended with ethyl-S-CoM and inhibited by BES (○). Results represent the mean of 3 slurries and bars indicated +1 standard deviation. Reproduced from Oremland (1981) with permission

Desulfovibrio desulfuricans) nor the HS-CoM impermeable *Methanobacterium bryantii* were able to produce ethane when provided with ethyl-S-CoM.

These results showed that a small amount of "natural" ethanogenesis occurs in anoxic sediments, achieved by a subset of the methanogenic flora. Ethyl-S-CoM served as a possible candidate for the type of molecules involved, but they were probably ethylated precursor molecules that link to CoM as a terminal step. Ethanogenesis in this instance appeared to be a non-energy generating co-metabolism, whereby if a molecule like ethyl-S-CoM gets inside the cell, it could make ethane via a biochemical pathway in vivo involving MCR.

Ralph Wolfe made a comment in the context of my request for some ethyl-S-CoM. He noted that his students first isolated *M. thermoautotrophicum* from mud, working out over years sophisticated new techniques for its mass culture, for breaking the cells open, then resolving and purifying the various sub-components by sophisticated biochemical techniques adapted successfully for low redox potential metabolites. These included the discovery of unique enzymes, co-factors, and substrates, including MCR, and its reactivity in vitro with molecular analogues like ethyl-S-CoM. After this monologue while shaking his head, Ralph was reputed to say: *And after all that time, progress, and effort, this guy Oremland just wants to throw it back into the mud!!!*

30.6 A Tale of Hydrocarbon-Rich Soda Lakes

I was first drawn to the study meromictic soda lakes because their anoxic water columns and high productivity which resulted in the accumulation of organic matter in their sediments, the first step in the process of petroleum formation. Fortunately, there were two lakes located relatively nearby, in the Great Basin Desert of the western USA: Big Soda Lake, Nevada, and Mono Lake, California. These seemed good field locations to pursue the biogenic side of light hydrocarbon production. Both also had sources of geothermal heat flowing into their basins. Big Soda Lake occupied an entire volcanic crater that erupted in the Pleistocene, while the much larger Mono Lake is surrounded (and penetrated) by volcanoes, volcanic features, and heat flow (e.g., hot springs).

The lakes did not disappoint, as each sported not only dissolved methane but also appreciable levels of ethane, propane, and butanes in their highly sulfidic anoxic bottom waters and sediments. Big Soda Lake sediments yielded strong signals for biogenic sources, for at a sediment depth of 1.75 m, methane was detected at 345 μmoles/kg. The methane had a "light" (e.g., ^{12}C-enriched relative to ^{13}C) stable carbon isotopic composition value ["δ^{13}C-CH_4"] of −74 per mil, along with a clear presence of ethane (0.18 μmoles/kg), propane (0.066 μmoles/kg), and butanes (collectively 0.02 μmoles/kg). The $CH_4/(C_2H_6 + C_3H_8)$ ratio at this depth was 1390, another strong biogenic indicator. All four alkanes were present in the anoxic monimolimnion and $CH_4/(C_2H_6 + C_3H_8)$ ranged between 161–333, depending on depth (Oremland and Des Marais 1983) likely reflecting biological activity which included methanogenesis and methane-oxidation in both the sediments and water column (Oremland et al. 1982a; Iversen et al. 1987). Similar results were obtained in Mono Lake, with extracted pelagic sediment CH_4 concentrations reaching saturation (~3 mM) by 0.6 m depth, and sediment $CH_4/(C_2H_6 + C_3H_8)$ ratios running between 600 and 750 over the length of the 1.1 m core (Oremland et al., 1987). Sediment and anoxic water column stable carbon isotopic values ("δ^{13}C-CH_4") mostly ranged between −70 and –85 per mil, again strongly enriched in ^{12}C relative to ^{13}C and clearly indicative of biological sources.

Yet because these lakes were located in regions of active heat flow, a thermogenic component to the source of C_2+ gases was also a possibility. It was time to investigate what possible metabolic precursors of alkylated-S-CoM compounds could serve to promote C_2+ hydrocarbon production in sediments from these and other locations.

30.7 Experiments with Smelly (Feh!) Alkylated Sulfur Compounds

We first conducted a series of experiments to expand the earlier work of Zinder and Brock (1978). We examined the role of methylated sulfur compounds including dimethylsulfide (DMS), methane thiol (MeSH), and dimethyldisulfide (DMDS) as methanogenic substrates in a variety of sediments types taken from different locales. These included soda lakes (Mono and Big Soda), as well as freshwater (Searsville Lake), saltmarsh (Flax Pond), and estuarine (San Francisco Bay) sites (Kiene et al. 1986). All responded positively to amendments with these compounds, showing a clear stimulation of methane production, although the quantity of added substance was critical as some concentrations of these noxious materials proved inhibitory, and this varied with the particular compound and with the various sediment types. A pure culture of an obligately methylotrophic methanogen was isolated from San Francisco Bay enrichment cultures which showed DMS-dependent growth and displayed MeSH as a transient intermediate. The culture was formally named *Methanolobus taylorii* (Oremland and Boone 1994).

In a follow-up series of experiments conducted along similar lines, we added a number of ethylated and propylated reduced sulfur, amines, and alcohols to sediments from five different sites to determine if they had a capacity to elicit ethane and/or propane formation (Oremland et al. 1988). The sites included Big Soda Lake (littoral and pelagic regions), Mono Lake, Searsville Lake, San Francisco Bay, and Hot Creek (a thermal spring site located near Mammoth, CA). All the sediment types responded positively in terms of enhanced ethane production when amended with either diethylsulfide (DES) or ethane thiol (ESH). As was previously noted with the methylated sulfur analogues, a range of applied concentrations was tested because some proved inhibitory. Figure 30.4 shows ethane formation by Big Soda Lake sediment slurries incubated under N_2 (rather than H_2), where there was clear stimulation by 1 mM ESH and 10 mM DES. There was also a notable endogenous ethanogenesis. Final levels of methane formation were about 30 μmoles, which yielded CH_4/C_2H_6 ratios of ~1000 for the ESH and DES amendments. It is notable that we saw only small amounts of stimulated ethane production when sediments were provided with ethylamine or ethanol, and we did not observe propane formation from propane thiol additions.

The methylotrophic methanogen *Methanolobus taylorii* was unable to grow on DES in lieu of DMS. Washed cells suspensions, however, showed a clear formation of methane and ethane when provided with DMS and DES, reaching a CH_4/C_2H_6 ratio of ~2000 by 11 days incubation. There was also a notable amount of ethene production, which was also observed in some of the earlier experiments with ethyl-S-CoM (Oremland 1981). Collectively, these results implied that there are naturally occurring ethylated reduced sulfur compounds that could serve as a basis for ethane production by methylotrophic methanogens. However, as was concluded earlier, these ethylated analogues were co-metabolized and did not support the growth of the methanogens.

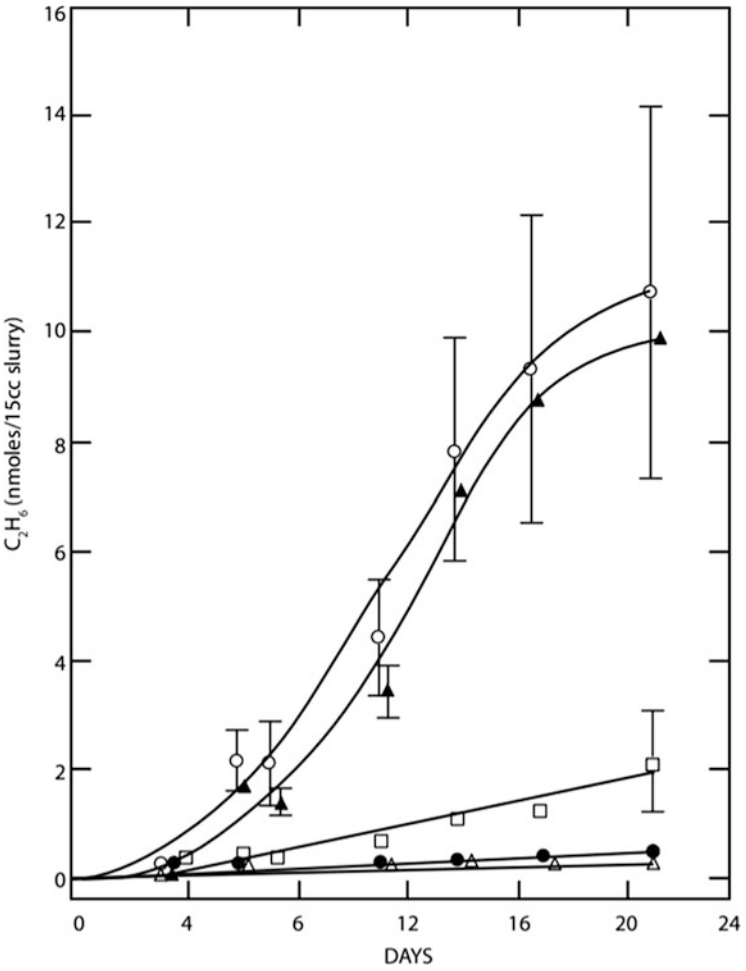

Fig. 30.4 Ethane production by anoxic sediment slurries from Big Soda Lake incubated under N_2. Symbols: amended with 10 mM DES (○), 1 mM ESH (▲), without amendments (□), with DES and BES (●), and autoclaved with DES (Δ). Results represent the mean of 3 slurries and bars indicate ±1 standard deviation. Reproduced from Oremland et al. (1988) with permission

At this point, I had no more desire to work with these compounds, as they each had a particularly and unique, penetratingly horrible odor. This would include DMS, MeSH, DES, ESH, PSH, and ethanolamine. The only compounds given a pass in this category were ethanol and propanol. I had had enough, and simply moved on to other areas of research, assuming that whatever merit these investigations had its publication would perhaps stimulate other researchers.

It took a while for some modest follow-up studies by other workers to accrue. Koene-Cottaar and Schraa (1998) reported on an enrichment culture composed of methanogens and an acetogen that with H_2 as an electron donor could carry out the

complete reduction of ethene to ethane, apparently as a co-metabolism. Hinrichs et al. (2006) concluded that biogenesis was responsible for the observed formation of ethane and propane detected in deep sea sediments primarily based on their relatively ^{12}C-enriched stable C isotopic compositions of recovered ethane and propane ("$\delta^{13}C$-C_2H_6" and "$\delta^{13}C$-C_3H_8") and theoretical reaction kinetics with availability of acetate and H_2. The experimental incubation approach and broad substrate testing was taken up by Xie et al. (2013) who examined ethane and propane formation by sediments amended with ethene, ethane thiol, or propane thiol. Again, some H_2 was required for the reactions to proceed, and a mixed enrichment culture composed of methanogens and acetogens was involved in what appeared to be a co-metabolism, but nonetheless could convert what is a well-recognized and naturally occurring gaseous alkene of biogenic origin (ethene) to ethane.

30.8 Working Backward to Move Forward?

The phenomenon of "AOM" (Anaerobic Oxidation of Methane) has now been well-established and extensively studied (e.g., Wegener et al. 2015; 2016). It involves a backward-running MCR in archaea similar to methanogens that converts ambient CH_4 outside the cell to CH_3-S-CoM inside the cell. The back reaction continues in reverse along the methanogenic pathway until the eventual product of CO_2 is formed, usually in syntrophy with other anaerobes. More recently, evidence has accumulated that in thermogenic natural gas seeps that contain appreciable higher alkanes besides CH_4 (e.g., C_2H_6, C_3H_8, C_4H_{10}) analogous archaea are also present that are specifically adapted to the anaerobic oxidation of these higher hydrocarbons, which would include ethane (Chen et al. 2019; and commentary by Ragsdale 2019) and butanes (Laso-Perez et al. 2016). These reactions are initiated by homologues of MCR, namely specific alkyl-CoM reductases (e.g., ECR, BCR) that function specifically for the given alkane, be it ethane, propane, or butane.

Aside from the excitement of new discoveries herein, there is also a practical interest in "reverse engineering" these reverse-running microorganisms. The thinking is that they could be coaxed to produce ethane, propane, or butane from CO_2, thereby undercutting the need to mine these gases via petroleum drilling or hydrofracking. It is relevant that ethane, for example, can be easily converted catalytically to ethene, thereby making it a valuable industrial feedstock.

In nature, there is usually a yin to answer to a yang. Hence, perhaps there are naturally occurring archaea that can potentially make a living by reducing CO_2 all the way to ethane or propane, using specific ACRs as their final step, just as methanogens use MCR. Hence, instead of being viewed as a co-metabolism carried out by methanogens converting ESH, DES, or ethene to ethyl-S-CoM, but requiring a simultaneous operative methanogenic pathway to yield energy, there could exist genuine ethanotrophs, propanotrophs, and butanotrophs. Maybe the reason they haven't been found yet is that no one has bothered to look for them, or that their activities and bioenergetics are so poor that they cannot compete with the more hale

methanogens or acetogens for available reducing power (e.g., H_2) to convert CO_2 to C_2H_6.

30.9 Other Gases I have Known

Somewhere in the mid-1990s, I sensed that I had pretty much gone as far as I could with methane, methanogens, and the ecophysiology of the process were concerned. The field was moving toward understanding AOM or of better characterizing the microbes in sediments using the new and evolving methods of molecular markers (i.e., culture-independent techniques). Although I had plenty of other research to keep me busy, I did regret falling out of the methanogenesis pack. The late Ralph Cicerone made a suggestion that I take up the biogeochemistry of methyl halides, specifically the fumigant methyl bromide, and I took his advice to heart. My associates and I had a lot of fun working in this realm because much of the microbiology was unknown. Some of the salient discoveries we made, along with our collaborating colleagues, was the isolation of an aerobic, methylotrophic bacterium, strain IMB-1 that grew on methyl bromide and other methyl halides (Connell et al. 1997; Miller et al. 1997; Connell Hancock et al. 1998). We named the isolate *Aminobacterium ciceronii* to honor Ralph (McDonald et al. 2005). We also conducted experiments on stable C isotope fractionation of methyl halides (Miller et al. 2001, 2004) and an attempt at using strain IMB-1 to remove methyl bromide from containerized fumigations (Miller et al. 2003).

Finally, I will make brief mention of a gas that has been with me through my entire career, namely acetylene. It was the subject of one of my very first papers (Oremland and Taylor 1975) and persisted over the years as we stumbled upon anaerobes that can grow fermentatively on acetylene (Culbertson et al. 1981, 1988). The effort encompassed touching on early (primordial) microbial food webs and exobiology (Oremland and Voytek 2008), practical efforts to discern how widespread "acetylenotrophy" is in nature (Miller et al. 2013), and a life detection system based on stable carbon isotope fractionation (Miller et al. 2015). Other efforts were along the lines of bioremediation of chlorinated ethenes (Mao et al. 2017), other facets including diazotrophy (Akob et al. 2017) and its possible widespread nature based on modern molecular databases (Akob et al. 2018).

In summary, if my opening statement about being remembered for my gas will fade with the passage of time, I sure do remember having a "gas" actually doing the work, making the discoveries, and working with the wonderful people with whom I collaborated over my career.

Ronald S. Oremland

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Chapter 31
Salty, Alkali-Laced Tales (Mostly True) from the Great Basin Desert, California and Nevada

Ronald S. Oremland

Abstract My career has managed to blend scientific inquiry with four decades of field work in the soda lakes of the Great Basin Desert. It has been quite an adventure. While many of our reported discoveries were serendipitous, they also involved curious interpersonal interactions that upon reflection were quite humorous. This writing, therefore, is meant as a tongue-in-cheek personal retrospective of scientific humor. Especially the "mostly true" caveat in the title. It stands in contrast to the usual gravitas of scientific works; after all no one would include such a statement accompanying a research paper, but given that these stories are 30–40 years old, one's memory cannot be entirely accurate and some embellishment occurs to make the episodes entertaining and (hopefully) tie together. So, in these Covid-19 days of sheltering-in-place, let me take you outdoors on a road trip of my adventures back into my past field trips (*cue the orchestra*: John William's theme music from *Raiders of the Lost Ark* or, if there are any aging followers of the late, great raconteur, Jean P. Shepherd and his N.Y. City radio shows broadcast on WOR/AM out there, *Frei Bahn*).

31.1 Part 1

31.1.1 The Mono Basin and a Trip to SNARL

It was during the summer of 1978 when I caught my first glimpse of Mono Lake as I descended from Tioga Pass (elevation: 9943 ft. or 3031 m) located at the eastern entrance of Yosemite National Park. I was tasked with driving a field vehicle up to the Sierra Nevada Aquatic Research Laboratory (SNARL) located about 25 miles south of Lee Vining and Mono Lake, near Mammoth Lakes, CA. The vehicle itself was an old, surplus U.S. Postal Service jeep, with a clunky manual transmission and a very disconcerting tendency to alternately sway left and right as it meandered along

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C. J. Hurst (ed.), *Microbes: The Foundation Stone of the Biosphere*, Advances in Environmental Microbiology 8, https://doi.org/10.1007/978-3-030-63512-1_31

the highway. Viewed from overhead, the jeep would display an evident sine wave function as I drove it east across California's Central Valley. By the time I reached Manteca, I had learned to drape my chest and arms over the steering wheel as a necessary expedient safety adaptation to dampen the amplitude of that sine wave so as not to veer into oncoming traffic or drive off that narrow 2-lane highway. A further test of the efficacy of that tactic came as I ascended the "Old Priest Grade" a series of switchbacks that eventually brought me up into the foothills of the Sierra Nevada's gentle western slope and the town of Groveland. I continued on east along highway 120 as it slowly rose toward the western entrance to Yosemite, after which I stayed on 120, rather than descend into Yosemite's fabled valley. The road gains altitude and emerges out of the forested region and continues along the exposed granite batholith with superlative views of the Valley below as well as Half Dome, exposed mountain peaks and many stop-and-see vistas of exfoliating granite, rushing streams, and other resplendent natural wonders (e.g., Tenaya Lake; Tuolmne Meadows). Compared to the very tame Catskill Mountains of my New York youth and 5 years spent in graduate school in very, very flat Florida (Miami), this was quite an eye-opening treat.

Highway 1 20 descending from Tioga Pass. Note the precipitous cliffs on the right side of the winding 2 lane road.

Highway 120 descending from Tioga Pass. Note the precipitous cliffs on the right side of the winding 2 lane road

Upon descending from Tioga Pass, I once again experienced driver's anxiety as Nature's vista opened before me: snow-covered, nearly vertically ascending mountain peaks on either side of the road with a precipitous chasm-like cliff in between (also known as a glacier-carved U-shaped valley). I sweated nervously as I struggled to keep the jeep on the road as it switched back and forth over its long descent. About half-way down the gradient eased and the vista opened up to encompass the Mono Basin, with its surrounding volcanic craters evident, Nevada's White Mountains afar in the east and Mono Lake itself, resplendent in the foreground as what appeared to be a vast inland sea. I clearly remember thinking: "*What the ---- is that*!?!!?" as I drove down to where route 120 intersects with Highway 395 and continued south toward SNARL. This route took me over Dead Man Summit (elevation: 8047 ft.; 2453 m) and by Convict Lake, all named after a posse caught up with a group of escaped convicts from Carson City in 1871 and commemorated the ensuing gunfight

and outlaw re-capture. This was the "Wild West" in every sense of those words. Yet as far as Mono Lake was concerned, it was to be another 6 years before I began working there. I first had yet to cut my teeth on a smaller version located further east (Big Soda Lake, NV), as well as to experience my first genuine California earthquake while wading through SNARL's experimental streams later in that fall of 1978 (see Oremland 1983a).

Mono Lake as it appears from Mount Dana, overlooking eastwards from 13,000 feet the Mono Basin.

Mono Lake as it appears from Mount Dana, overlooking eastward from 13,000 feet at the Mono Basin

In case you were worried, I arrived safely at SNARL and lived to tell the tale to thee.

In case you were worried, I arrived safely at SNARL and lived to tell the tale to thee

31.1.2 The Carson Sink and Big Soda Lake, Nevada

A couple of years later and then came another road trip, but this time in a reliable vehicle (a Jeep Cherokee) and accompanied by my two colleagues Yousif Kharaka and Steve Robinson who enticed me to visit Big Soda Lake (BSL), Nevada. As we headed east along I-80, I didn't realize it at the time that this would be a seminal moment in my career. The route is a far easier drive than going through Yosemite and one that I was quite familiar with owing to my skiing excursions onto the slopes around Lake Tahoe. It takes you up through Donner Pass (7506 ft.; 2151 m) named to commemorate the ill-fated Donner Party of 1846–1847, and thence downhill

toward and past Reno, Sparks, and Mustang (home of the Mustang Ranch brothel). The route is accompanied by the Truckee River which drains Lake Tahoe and runs east, eventually turning north at Fernley (then but a tiny, one flashing stoplight town) to empty into Pyramid Lake, while we turned south on 95A to drive another 25 miles to Fallon. Pyramid Lake is a site sacred to the Paiute Indian Tribe, and lies entirely within the bounds of their reservation. Indeed, it was what happened to the Truckee River, namely a man-made diversion (Newlands Reclamation Act of 1903) of some of its flow southward to fill the Lahontan Reservoir, thereby allowing for irrigation to sustain farming and cattle ranching in Churchill County that forms the basis of our story. The importation of surface water to the area recharged the groundwater and raised the water table so much so that Big Soda Lake filled up with an 18 m rise by 1920. It became meromictic (permanently stratified), its lower salinity surface water, the mixolimnion (27‰) overlying the higher salinity bottom water (89‰), the monimolimnion by a sharp pycnocline located at 35 m and extending to a depth of 62 m (Zehr et al. 1987). Hence, the monimolimnion had been anoxic for nearly 60 years, and that definitely piqued my interest. The lake level rise completely submerged the pumping station which in the early 1900s was still used to pump out lake-water for evaporative harvesting of its abundant "trona" (carbonate/sulfate salts). It also led to the rapid growth of submerged tufa towers, some ~5–10 m high, along the shore of its northern rim (Rosen et al. 2004).

First we drove to our lodgings: The Lariat Motel in Fallon owned and operated by an older couple (Al and Julia), immigrants from Lithuania after the war, and with whom I would come to know and cherish over the next several years. Inexpensive and well within our per diem financial limits, it certainly wasn't plush but it was adequate. Moreover, as I spent more and more time there over the years doing fieldwork, I brought my colleagues along as well. Al and Julia became very accommodating of our needs. They even allowed us to set up a makeshift lab in the spacious garage out back, where they had installed bench tops along the wall that we used for setting up instrumentation like gyratory incubators, pH meters, and gas chromatographs that we had schlepped along for the ride.

31.1.3 The Lake and the Lariat

Panoramic photo shot of BSL, courtesy of Michael Rosen.

Panoramic photo shot of BSL, courtesy of Michael Rosen

But I am getting ahead of myself. The next morning we drove out to the lake, located a few miles north of town to await a colleague (Patrick Glancy) who would provide us with a boat. The lake occupies a generally irregular circle/slightly oblong-shaped volcanic crater, about 1 km width × 1.5 l km length (39 °31′04″W; 118 °51′40″N; elev. = 1223 m) that explosively erupted back in the Pleistocene (i.e., ~15,000 years ago), when the entire region was covered by an inland sea (Lake Lahontan). The region still harbors geothermal activity. Standing on its shoreline, I got my first glimpse of how the denizens of Fallon viewed its utility: as a final resting place for their broken down cars and pick-up trucks, some of which were evidently pushed off the elevated rim of its eastern side. Moreover, the lake's "aroma" was stifling: whiffs of hydrogen sulfide and ammonia piquantly mixed with discernable traces of methylated sulfur compounds and methylated amines that tickled my nasal passages. The very shoreline was black, owing to the myriads of brine flies that were gorging themselves on washed up rotting effluvia. This mostly consisted of decaying *Ruppia* macrophytes uprooted and floated by big wads of the colonizing cyanobacterium *Anabaena*, whose emitted photosynthetic oxygen gas bubbles enmeshed in their matrix provided the necessary buoyancy (Oremland, 1983a).

An example of the colorful local flora that once commonly adorned theBig Soda Lake's basin. It is now a Churchill County Park and these eyesores have been removed.

An example of the colorful local flora that once commonly adorned the Big Soda Lake's basin. It is now a Churchill County Park and these eyesores have been removed

An odd phenomenon was to watch the brine flies depart your presence as you drew to the water's edge, a cloud arose and settled down a couple of meters away on either side of you, which in turn displaced another cloud, which continued on but eventually dampened out about a quarter of the way around the lake's circumference for a total distance of nearly 1.0 km. It did give one an underserved feeling of power and importance (a "Lord of the Flies" syndrome), until you realized the flies were only interested in detritus feeding and not at all in preying upon humans. Those came in the form of giant-sized mosquitoes which emerged from the shallow swamp back area around sunset and would even chase after the tires or windshield wipers of your field vehicles in search of sustenance if they couldn't grab hold of and devour you first before fleeing from their onslaught.

At around 9 AM, we noticed a field vehicle come over the eastern rim of the crater and head down the dirt road toward us. The vehicle was not from the USGS, but was a slick, late model of some sort outfitted with two blue flashing lights upon its roof, and it was towing a new rubber Zodiac fitted with big twin Evinrude outboard engines on its stern. It was a Navy EOD (Explosive Ordnance Disposal) team doing their monthly required practice SCUBA dives, this time to visit the submerged pumping station at about 20 m depth. As a former navy salvage diver, this exercise seemed somewhat recognizable to me. Fallon also hosts a Naval Air Station, and the presence of the EOD team led me to fret that sometimes maybe live ordnance accidentally falls off aircraft and into the lake, an unsettling thought for us at the time. About an hour later over that same rim came something more familiar, a beat-up USGS field vehicle with a small aluminum boat attached by rope atop its roof. It was Pat Glancy coming to lend us a hand. So, after the navy divers egressed, we launched our pathetic little boat and, with its 5 h.p. engine put-putted out to the lake's center and proceeded to hand-draw up water samples from depth with a van Dorn sampler, as well as to haul up some of the bottom sediments. Upon return to shore we processed the samples, thanked Patrick, and returned to the Lariat, planning to head back home the next morning. When I got back to my room to shower, upon disrobing I noted my body had acquired a distinct clinging ammoniacal odor, owing to the efflux/volatilization of NH_3 from the alkaline surface waters (pH = 9.8). Moreover, the shower's waters were "soft" in the sense that once you soaped up and showered off, it still felt as if the soap was always clinging to you, an odd oily sensation. The shower stall itself had an odd quirk, in that the fixtures seemed to have been designed for someone well under 5 feet tall, with the shower head aimed for below one's chest and the hot and cold valves situated below one's knees, requiring one to bend and contort in order to get clean. It was just one more uniquely weird, memorable aspect of sojourning at the Lariat Motel.

Early operations at BSL during summer, 1 981 . Note the small aluminum boat we (the author and Chuck Culbertson) used for all our initial sampling, including of the water column and taking gravity cores and sediment grabs of the deep sediments at 62 m.

Early operations at BSL during summer, 1981. Note the small aluminum boat we (the author and Chuck Culbertson) used for all our initial sampling, including of the water column and taking gravity cores and sediment grabs of the deep sediments at 62 m

Early field work at BSL, probably in the winter of 1981. The author is running Winkler titrations of dissolved O_2 using a trunk as a lab bench. The small boat is visible as is the high crater rim of the lake's eastern side.

Early field work at BSL, probably in the winter of 1981. The author is running Winkler titrations of dissolved O_2 using a trunk as a lab bench. The small boat is visible as is the high crater rim of the lake's eastern side

31.1.4 Ginfight at the O.K. Corral (in Back of the Lariat)

Once back home in my lab I was glad to find that the extracted gases contained abundant methane and other light hydrocarbons (Oremland and Des Marais 1983), and that there was evident methanogenic activity (Oremland et al. 1982) in the incubated sediment samples we collected. My interest was piqued—and I needed to go back for more. This became an evolving process which started snowballing as my interest and enthusiasm gained and I tried to recruit others, which included my friend and colleague Jim Cloern who I convinced to examine seasonal primary production in the lake's water column (Cloern et al. 1983a, b). Over the years, the investigations took on new colleagues and new perspectives, with something like a dozen or so papers directly focused on Big Soda Lake (BSL) published, along with several others that used BSL material (sediments) to delineate novel anaerobic process, plus summary reviews (Oremland et al. 1985, 1988). The final "coup" was a series of four papers published sequentially in a single issue of Limnology & Oceanography (Zehr et al. 1987; Smith and Oremland 1987; Iversen et al. 1987; Cloern et al. 1987).

By 1984 or so, we were feeling pretty cocky and started to think we should try for something bigger to tackle, like Mono Lake. It too had become meromictic owing to the large influx of freshwater runoff associated with two very wet El Nino winters. For a couple of years in the mid-1980s, we would sometimes combine our field trips to BSL with a follow-up to Mono. By this time we were driving newer vehicles, including a laboratory truck, and were towing well-equipped Boston Whalers for use on the lakes. I will write about some of our Mono Lake experiences in later, but I wish to recall an incident that occurred at the back of the Lariat's garage in about August, 1986.

By the mid-1980s our operational effort was aided by having access to better field vehicles and more suitable boats, in this case a 14' Boston Whaler.

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There were a bunch of us along on this trip, maybe seven or eight, who went out on the lake early that day with the hope of getting back and processing our samples before it got too hot (we were working in the Great Basin Desert and noontime temperatures daily rose above 100 °F). Among us were Doug Capone from University of Southern California, with whom I overlapped in grad school at University of Miami, and together we were set to write a chapter on the use of specific inhibitors in biogeochemistry (Oremland and Capone 1988). By now Doug had acquired a strong taste for all things tropical (including his casual attire) which extended to his preferred liquid alcohol refreshment (Barcardi white rum) mixed with lime and tonic water. My other good buddy (Jim Cloern) had a strong preference for Bombay gin and tonic, and when coming off 8 straight hours of sweltering work under the oppressive desert summer sun over an alkaline, smelly soda lake, one acquires a powerful thirst along with a primal need to cool off in the shade. We usually preceded this by a quick dip in the Lariat's pool to wash off the alkali salts and to initiate the cool-down process. We would follow this by meandering to the garage out back to prepare the sample processing and imbibe at least one good drink for the final chill out. Doug and I sat on our director's chairs, cocktails in one hand pad and pen in the other, and began to outline our inhibitors chapter. As the rum and tonics went down, we even accompanied our planning by singing songs of our shared grad school experience. We were greatly relishing and reveling in our reunion. At about this time Jim Cloern began to think about dealing with his own acquired thirst in his usual manner: his mind was set on a gin and tonic.

The other dramatis personae of this morality play were Larry Miller and Chuck Culbertson. Larry and Chuck had just pulled a 2 m long gravity core from the depths of BSL and were proceeding to process it out on the concrete slab in back of the garage. He and Chuck were all set, with the long core tied to a steady wall and a step ladder placed beside it so as to access the top. The idea was to extrude the core by placing a plunger at its bottom and pushing upward, slicing off 2–4 cm sections as they emerged and placing the hockey-puck sized sections into squeezers so as to access their interstitial fluids (pore waters) for later chemical analyses. Central to the

plot of this story is that Larry had neglected to pack the core extruder, a primitive cylindrical apparatus with a diameter matching the inner diameter of the plastic core tube. Ah, but necessity is the mother of invention. After futzing around the garage for several moments, Larry had a revelation. The circular diameter of Doug's half-full Barcardi bottle was such that it fit precisely into the core liner, able to step into the breach and be used as a make-shift push-up extruder. All that needed doing was to pour the white rum into the empty (and square-shaped) Bombay gin bottle. Eager to get the core processing work going, Larry neglected to label the Bombay bottle as Bacardi rum.

So there the entire team was happily engaged in their work: Larry and Chuck processing the core, Doug and myself outlining our chapter amidst a songfest, Dick Smith setting up his sulfate-reduction incubations, Nisan Steinberg looking to assay collected sediments for ^{75}Se-selenate reduction, and I think even Niels Iversen may have been there labeling the bottom water with ^{14}C-CH_4. At that moment out of the depths of the garage came a loud stream of shouted, unrepeatable expletives with regard to just what comprised the clear liquid occupying the Bombay bottle and who was responsible for this dastardly deed of gin mutilation? Jim had taken his first deep pull on his much needed, thirst quenching presumed gin & tonic and had received a most unwelcome surprise. After the cause of the mix-up was revealed to Jim, he was directed to the other bottle of Bombay, while Doug generously offered to consume his rum and tonic rather than let it go to waste. But Jim was in a touchy mood for the rest of the day and kept a sharp eye upon his prized possession. Larry and Chuck finished processing the core, and despite the smelly mud that they were covered with, gratefully partook in the G&Ts that Jim was able to dispense. Life resumed its previous course and another successful BSL field trip was concluded.

The Lariat Motel marque as viewed by night (left panel: from the south) and day (tight panel: from the north).

The Lariat Motel marque as viewed by night (left panel: from the south) and day (right panel: from the north)

By the 1990s our field trips became more involved, but we were better equipped with boats, vehicles, and a mobile lab truck.

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31.1.5 Epilogue

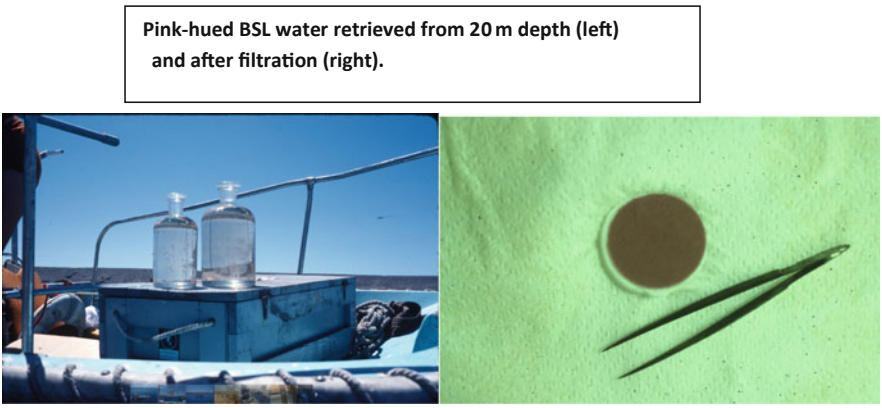
As the 1980s gave way to the 1990s and beyond, our interest in BSL waned as our attention shifted to the much larger and formidable Mono Lake. I had also initiated research on arsenic, which displaced to a large degree that of selenium. In 2006, I received an inquiry from Jolyon Jenkins, a journalist with the BBC who ran a radio show entitled "Name Your Poison" and he wanted to come out to California to interview me and see our field site, hopefully at Mono Lake, so he could do a program devoted to arsenic. It was April, however, and access to the Mono Basin was constrained because route 120 over Yosemite and through Tioga Pass was closed due to the still abundant snow depth covering the road. Yet we offered an alternative, a visit to BSL (also rich in arsenic) and an interview both lakeside and in the hotel room as well. Yet we were unable to get in touch with either Al or Julia at the Lariat, so we made alternative arrangements at an Econo Lodge or some such chain. When the three of us arrived (Jolyon, Larry, and myself) in town the first thing we did was to venture out to the site where the Lariat once stood and alas, there was nothing to see. Driving around the property, we could only make out the base of the former brick fence that ran its perimeter. There was nothing there, the Lariat had disappeared, certainly no sign of either Al or Julia, or even a listing for them in the local phonebook. Presumably they had both passed on. They were in their 70s when we first met them, a couple who did all the work at the Lariat, everything from making the beds, laundry, and restocking the rooms. They had only one son, living in San Francisco, who had no interest in taking on such a difficult business by himself, so we assumed the motel was razed to allow for easier sale of the property. Yet, although inevitable, Larry and I were deeply saddened. After all, Al and Julia had once invited the three of us (including Chuck) to come into their living area and

share a home cooked meal behind the curtains that divided the registration room from their home. It was my birthday and was meant to be a surprise (which it was) and included a home-baked cake. We became, in essence, their adopted sons. As we drove home, I noted how the area had changed: Hazen, barely a dot on a map as a former railroad stop was now being leveled for single family homes, and the two lane highway 95A was being widened to four lanes. Fernley was now a bedroom community of dozens of more single family homes for folks working in Reno.

And that was the last time I saw Fallon. I would have liked to have said farewell to Al and Julia too, and I did in a way by whispering those words under my breath as we drove north to I-80 as my eyes welled up with tears.

31.1.6 Afterthought

We did observe what is referred to as a "bacterial plate" located in the BSL water column in proximity to the thermocline/oxycline at about 20 m depth (Cloern et al. 1983a). The transmissometer analog readout (just a light source and a photocell separated by 1 meter "yardstick") would peg at 0 after recording at nearly 100% in the waters above that depth. When we drew up water from 20 m, it had a definite pink hue which became pronounced upon filtration. I even scuba dove to that depth where the plate had the appearance of a grey fog, the red end of the light spectrum being attenuated within the upper few meters depth. The plate was composed of photosynthetic bacteria, mostly Ectothiorhodospira, that situated themselves at the interface where there were reducing compounds present (e.g., sulfide and arsenite) which these microbes could use as an electron source to carry out anoxygenic photosynthesis. Our experience with this bacterial plate was to cloud our interpretation of what ensued when we began working at Mono Lake. The work at BSL, nonetheless, continued without my presence. Chad Saltikov and his students were interested in isolating phototrophs from the plate along with Shelley Hoeft McCann who continued to characterize the isolates (Hernandez Maldonado et al. 2016; Hoeft McCann et al. 2017). Larry Miller facilitated the operations and Mike Rosen was often along to futz with his tufa samples.



Pink-hued BSL water retrieved from 20 m depth (left) and after filtration (right)

31.2 Part 2

31.2.1 Mono Lake

A very strong El Nino winter (1982–1983) resulted in a large influx of freshwater into Mono Lake in the ensuing spring and summer, causing its surface waters to experience about an 18% decrease in salinity. It thereby became meromictic, and our interest was definitely piqued. We thought we could apply what we had learned with the much smaller BSL to Mono, although we needed to scale up in terms of our capabilities to tackle a much larger system. Our eventual boat of choice was a 21 foot Boston Whaler Revenge with twin 75 hp. Johnson outboards mounted off its stern and a stowage area beneath its bow. It also had a removable canopy that extended from its middle up to its windshield that could offer us some protection from the elements when conditions grew inclement. Yet our early trips to Mono Lake still made use of the 14 foot whaler, as the Revenge laid in our future plans once we fully grasped what was entailed by the scope of Mono operations. Mono Lake is roughly circular, with a diameter of ~15 miles (24 km), about an order of magnitude greater scale than BSL. Hence, when the wind comes up off the Sierras in the afternoon, as it often does, the lake's greater fetch can make for rough seas and hazardous operating conditions. Mark Twain noted this in his book "Roughing It" where he devoted an entire chapter to Mono Lake that centered on his exploration of Paoha Island in search of a rumored freshwater spring (it exists, but he never found it) and his hilarious but nearly disastrous attempts to row back to shore when the wind came up that afternoon.

So it was in the summer of 1984 that we (myself, Larry, and Chuck) decided to link our BSL trip with a preliminary excursion to Mono Lake to scout out the lake's possibilities as a research site and to grasp some of the logistical/operational factors

this would entail. We drove south on US Hwy 95 until it intersected with Nevada state highway 359 and ran by Walker Lake. We stopped for dinner in the town of Hawthorne, which is a major Army (formerly Navy) weapons depot. When viewed from the comfort of an airliner flying at 30,000 feet above, the area outside of Hawthorne consists of innumerable oddly shaped geometric patterns, which are weapon storage bunkers. After dinner, we continued east on Hwy 359 which wound through the hills of the Toiyabe National Forest. It was pitch dark and we were the only vehicles on the road for the entire 2 h trip, a weirdly odd and somewhat unnerving experience. Highway 359 descends into the eastern Mono Basin and becomes California State Hwy 167 which traverses the northern rim of the basin. Coming down from the eastern hills and entering the basin, we could discern from afar, some 15 miles ahead in the distance, what appeared to be a faintly yellow flashing. Since we were unfamiliar with our surroundings and the broad scale of geographic distance entailed, and were still enveloped in total darkness, we eased our way forward carefully until we eventually came to the yellow flashing light source and saw that it was a traffic light at the intersection of the terminus of Hwy 167 and the north–south running Hwy 395. Thence we made a left turn and traveled a few miles more along the western side of Mono Lake until the road began to rise a few hundred feet, bringing us into the town of Lee Vining, CA. The rise in elevation was the result of Lee Vining being situated atop a terminal moraine deposited by considerate glaciers way back in the Pleistocene.

The map shows Mono Lake and the Mono Basin, with Route 167 skirting its northern side. Arrow shows the approximate location of Lee Vining, CA. The arrow's length is about 10 map scale miles.

The map shows Mono Lake and the Mono Basin, with Route 167 skirting its northern side. Arrow shows the approximate location of Lee Vining, CA. The arrow's length is about 10 map scale miles

We checked into a motel, I believe it was the Yosemite Gateway Motel, and I immediately went to my room to chill out and sleep. Chuck and Larry, however, marched to a different drummer than I and elected to stroll down to the lake's shore blissfully unaware that it was several miles away in habitat patrolled nightly by mountain lions and black bears. They didn't get far before they realized the folly of their idea, but it does illustrate just how unfamiliar we were with the region.

Although we were separated in age only by a few years, I had long since given up trying to tamp down their exuberant foibles. For example, they were both devoted aficionados of the rock group "The Grateful Dead." I had never objected to them playing their group's music when we worked in our make-shift labs, or even when they applied one of the group's decals (5 gaily colored, lei festooned dancing teddy bears) to the back of our boat. Indeed, more than once that sticker proved helpful to us, as gas station mechanics were also into the Dead and Larry and Chuck immediately spun into what concerts they had attended in common. This may sound trite, but whenever we found ourselves in need of repairs or a spare part, the "Dead-Head" mechanics would do everything in their power to fix things for us in record time. It was like having a super-special consideration AAA card (American Automobile Association) and it saved our butts more than once. While I prefer classical music and enjoy the likes of Beethoven, Mozart, and Bach, a field trip just did not feel right unless I heard their tape player blasting out "*Goin' Down the Road Feelin' Bad"* several times a day.

The next day we linked up with Bob Jellison (U.C. Santa Barbara) who was headed out to the lake to do some routine sampling associated with UCSB's contract ecosystem monitoring work with Los Angelis Department of Water and Power (LADWP). This greatly facilitated our subsequent efforts later on, so that we didn't have to waste time searching for a suitable launch site and getting lost in the maze of tufa-lined backroads that circle around the western shoreline of Mono Lake. We likely did a few hydro-casts of our own, noticing a turbid layer in association with the thermocline at a depth of 16–18 m. It whetted our appetites and convinced us to make a return visit in the fall.

So that October we caravanned east towards Mono Lake but had to take the long route (I-80; Hwy 395) because route 120 over Yosemite was closed. This entailed driving east on I-80 and making a right turn south on U.S. route 395 at Reno. This way adds about 3 h to the trip compared with going via Yosemite, and somewhat longer owing to the slowness of our aged vehicles (we were towing a boat), and the need to huddle together in case of mechanical difficulties. Owing to a late start, we stopped overnight at a motel south of Carson City and continued on the next morning, reaching Conway Summit around noontime and its spectacular view of the Mono Basin.

Panoramic view taken from Conway Summit vista (~ 8,1 00 feet/2,482 m) above the Mono Basin.

Panoramic view taken from Conway Summit vista (~8100 feet/2482 m) above the Mono Basin

We continued south on Hwy 395 passing through Lee Vining and reaching SNARL an hour or so later in the early afternoon. We offloaded our vehicles of our lab equipment and made a quick run into Mammoth Lakes for food supplies to keep us going for a few days. The next morning we awoke, eager to tackle the rigors of Mono Lake and its limnological mysteries and headed north. Unfortunately, although it was only mid-October, a major winter storm was coming in from the north with sufficient force to take it over the Sierras and into the Mono Basin. By the time we reached Lee Vining, the sky had darkened and snow was gently coming down as big downy flakes. Although there was no appreciable wind yet we wisely decided to cancel the day's excursion. One look at the vastness of the lake and the puniness of our boat (the 14 foot Boston Whaler; pre-Revenge days), we decided to call it off as too risky and head back to SNARL. Good thing that we did because the wind soon picked up, and the snow came down harder, becoming a zero visibility white-out. We had a full scale blizzard on our hands that persisted over the next few days, piling up 3 feet of snow on the roadways, the wind being so intense that it blew one of our vehicles clear across the icy tarmac parking area around SNARL. Luckily, because we made a previous food/supply shopping trip into Mammoth when we first arrived, we had enough grub to see us through the storm, which abated a few days later giving the opportunity for the roads to be cleared and us having another shot at a Mono Lake excursion under more clement conditions. This we were successful in achieving, taking several vertical profiles of salinity, dissolved oxygen, methane, sulfide, and turbidity. We returned to SNARL processed the samples and packed off the next day for Fallon and a shot at the much tamer and gentler Big Soda Lake. We had amassed our first data gathering on Mono, the first of many to come, and had gained a very healthy respect for weather conditions in the region and how they can shift quickly from mild to life threatening. It was a factor closely monitored when we were engaged in the region and was nothing to be cavalier about.

We were eager for a return visit and made plans for an exclusive Mono trip the following summer (1985), but we needed a base of operations closer to the lake, hopefully in Lee Vining. We required to be housed of course, but just as important a site where we could set up our analytical equipment much as we had done in the Lariat garage. The Lakeview Lodge seemed amenable to our needs, having not only a typical multi-level motel building out front but several old cabins in the rear as well as storage rooms and a garage. It also had an area out back where we could safely park our boat and make use of freshwater hoses to wash it (and the engines) free of the corrosive Mono salts after each outing. The hotel was ran by the multi-generation Banta family starting with great-grand-dad Bill, his son the patriarch and grandfather Don, in turn his son Bill who ran the place, and Don's three toddler grandsons Tim, Matt, and Jeff. Over the years, these kids would watch us with wide eyes as we towed the Revenge into the back reaches of their property, and their curiosity was piqued further when they would peek into the temporary makeshift laboratory we made out of their garage, or even to come into one of the back cabins we rented, a darkened room in which we housed our epifluoresence microscope. The kids were "wowed" by the unusual and on-site nature of our business, something they had never witnessed before. The years flew by and we saw the kids grow through

adolescence and beyond. Tim married, started a family and took over the running of the motel from his dad Bill; Matt set his goals on a different path. He attended college and became a consulting hydrologist and traveled the world professionally. He confided in me many years later that it was the excitement of watching our activities that pushed his mind to consider new, scientific possibilities to shape his life. Tim and his wife reworked the Lakeview, making it into more hospitable and upscale place, and eliminating the possibility of our ever again molding it into our temporary rustic needs as a laboratory. By then our work had wound down as well, but I am getting ahead of myself here in telling our Mono Lake story.

31.2.2 Mono in '85: The Summer of Mickey and Methane

On this trip we came through Yosemite and headed directly for the Lakeview Lodge where we spent the first day setting up our makeshift lab and then getting our analytical equipment (gas chromatograph; epifluorescence microscope) up and running. Along with us was Jon Zehr, my postdoc at the time. The second day we headed off to the lake, launching our boat from Danburg Beach on its northern shore. Launching was easy, but recovery was always challenging because of westerly afternoon winds would throw the boat off just when it needed to align properly with the half-submerged trailer. Nonetheless, we persisted in this folly for several years, including difficult winter excursions, because we knew of no other sites that proved better. Eventually, we were able to access a better launch/recovery site on the western shore, much aided by Tom Crowe who ran a kayak rental and zodiac interpretive lake tour from that location. Tom was to prove an invaluable asset and friend for us over the years, but once again, I am getting ahead of myself.

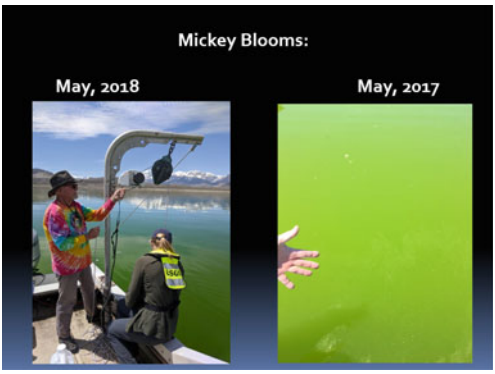
The first day on the lake we ran water column profiles and established that there was a thermocline at about a depth of 16 m, which roughly coincided with the pycnocline and oxycline (the depths below 16 m were anoxic), as well as a clear redox-cline below which the water had appreciable dissolved methane, ammonia, and sulfide. There was also a pronounced turbidity maximum at around this depth, just as we had seen previously in Big Soda Lake. Yet water samples pulled from this depth were not pink-hued but instead had a pale green color. When we examined the water under the microscope, we did not see bacteria, but instead some oddly trilobite particles, about 2–3 um, that fluoresced red, indicating the presence of chlorophyll *a*. We were stumped because this did not conform to the paradigm we had co-opted from BSL that we naively assumed would be extended to Mono Lake. Factual hubris had struck home against our over-confident preconceptions! Chuck and Jon first hypothesized that the particles were pollen grains blown into the lake from the surrounding vegetation that accumulated at the density gradient. So they spent a couple of days collecting pollen from sage, tumbleweed, mesquite, and other plants that abound locally but could find no obvious matches. It wasn't until Chuck returned to our Menlo Park labs with some of the water samples, set up enrichment cultures on the window shelf of his office that he found he could actually cultivate

these trilobite particles (nicknamed "Mickey" for obvious reasons) that we determined it was really a eukaryotic algae. Chuck went on to carry out in situ seasonal studies on Mickey's inorganic carbon fixation rates and found it contributed ~50% of annual primary productivity, along with some lab-based studies on its unusually adaptive physiology, including its ability to grow at very low light levels (Roesler et al. 2002; Oremland et al. 2017). Much later, Stamps et al. (2018) detected mRNA sequences of photosynthetic machinery that implied Mickey was still carrying out "cryptic" oxygenic photosynthesis at depths below the lake's pycnocline where there was no detectable light. Mickey was also capable of forming a variety of organo-arsenic compounds, including an unusual 2-O-methyl riboside lipid which was transmitted via "trophic transfer" (i.e., eating) to the lake's brine shrimp (Glabonjet et al. 2020).

Once upon a time, maybe 30 years ago, I could snorkel around the tufa towers in Mono Lake and the water was crystal clear. The winter bloom of Mickey in the surface water had been consumed by the first emerging generation of brine shrimp (Artemia), and by the summer, they had pretty much cleared out the lake's green color. Other than the brilliant white of the submerged tufa and the hordes of brine shrimp, there wasn't much to see. Mono Lake is a simple ecosystem and is too chemically extreme to host fish or other invertebrates besides brine flies. The only interesting facet are the many migrating waterfowl that sojourn on the lake to feast on brine shrimp and flies to fuel their long seasonal voyages north or south, and they can be viewed comfortably from the boat. Indeed, snorkeling in Mono Lake has its hazards, as you definitely do not want to get lake water in your eyes or in your throat, as it has a burning sensation on delicate tissues. So I had to put fresh water in my mask (to guard it from fogging) and in my snorkel as well and remember never to dunk my head below the surface, thereby replacing the snorkel's freshwater with Mono water. SCUBA diving is even worse, as one must use extra weights to compensate for the salinity buoyancy factor, and submerged freshwater springs can represent a real hazard as an over-weighted diver would soon encounter low density freshwater and be forced to quickly drop one's weights or be drawn to one's death into the maw of the spring itself.

Yet over recent years, we noticed that Mono Lake's surface water is always green and turbid, hosting a perpetual bloom of Picocystis. The reason(s) for this are not clear, but because productivity in the epilimnion is usually nitrogen-limited (there is an abundance of phosphate ~1 mM) it could be due to: (1) a decrease in Artemia filter-feeding or (2) perhaps an input of key nutrients (N) to the surface waters that were previously constrained by thermal or salinity density stratification. The latter could arise from a linked biological source (N_2-fixation) or from wet/dry atmospheric deposition (e.g., ash fall from California's recent mega-fires) or decade-long drought-driven dust inputs or again from spring stream runoff from the Sierra snowmelt.

Testable hypotheses for the next generation of interested young scientists to tackle.



In recent years the lake's surface waters appear to have a continuous bloom of Picocystis, turning them green where the once were clear.

In recent years, the lake's surface waters appear to have a continuous bloom of Picocystis, turning them green where the once were clear

The second incident that trip came about later when we did a bit of exploring, which took us to the southeast corner of Paoha Island to view its hot springs. Paoha Island sits atop a magma chamber, which pushed bottom sediments out of the lake about 500 years ago and is also manifested by a large cinder cone on its northern side. On drawing close, we could see the steam rising and smell the hydrogen sulfide present in its Hades-like volcanic emanations. In the water itself, we saw hundreds of gas bubble seeps with flows ranging from about 50 mL/minute to some in excess of 4 liters/minute. At this point Larry, Chuck, and Jon started cajoling me to collect some of the gases to determine their methane content. I countered that because the lake had so much sulfate (~130 mM) bubble ebullition from biogenic sources was unlikely. Moreover, I pointed out that the gases were most likely composed of volcanic CO_2. After a while, I relented and collected some of the gas to inject into our gas chromatograph back at the makeshift garage-laboratory. I did this as a sop to placate my heckling colleagues, but when I injected the first sample into the GC, I was shocked. The gas was composed mainly of methane, with traces of higher alkanes present as well. This was a serendipitous discovery that, ironically, I would have missed entirely, if not finally relenting to the pleas (and catcalls) of my colleagues.

The view from Hot Spring Cove on the SE corner of Paoha Island. Steam and malodorous sulfur gases abound there.

The view from Hot Spring Cove on the SE corner of Paoha Island. Steam and malodorous sulfur gases abound there

Over the next year Larry and I, with the collaboration of Mike Whiticar, were able to establish that there were four types of methane present in Mono Lake (Oremland et al. 1987). First, there was currently formed biological methane present in its bottom sediments that diffused out to the water column and was oxidized at the pycnocline/oxycline by methanotrophs (Joye et al. 1999), resulting in the residual, un-oxidized methane in the surface water being enriched in ^{13}C. There was still a sufficient abundance of dissolved methane in the surface water to achieve a measurable outward flux to the atmosphere (Miller and Oremland 1988). The vents and seeps, however, came in two categories: ancient biogenic methane and thermogenic methane. The former were enriched in ^{12}C and had little co-associated ethane and propane while the latter were enriched in ^{13}C and had appreciable ethane and propane. The thermogenic gases were only found in proximity to the hot spring, while the ancient biogenic seeps occurred all around the lake. The clincher was determining that all of the seeps and vents were radiocarbon (^{14}C) dead, indicating they were older than ~20,000 years, while the methane dissolved in the anoxic monimolimnion was radiocarbon active, actually somewhat "future dated" stemming from ^{14}C-fallout from open air atomic testing conducted decades earlier. We concluded that Mono Lake was underlain by a natural gas deposit formed much earlier in its existence, a fact that was later confirmed by our colleague, Dr. Angela Jayko. Angela conducted extensive bottom sound surveys of the lake and determined about half of its underlying deep benthos were gas-charged (Jayko et al. 2013). In addition, the major gas vents we found on the lake's surface appeared to originate from cracks and fault lines detected in the geologic structure beneath the bottom sediments.

The collection of sufficient vent gases to conduct their radiocarbon analysis did not present a problem as there was a surfeit of supply and it was just a matter of waiting for collection bottles to be filled (~700 mL) with enough methane. We did this in the days before radio mass-spectrometry was widely available, so we had to collect a considerable amount of sample in order to achieve enough material be "counted" using older, slower methodology. The dissolved methane in the bottom water presented more of a challenge, but Larry came up with an ingenious solution. He continuously pumped water up from beneath the pycnocline into a floating cylindrical plastic chamber, where it was equilibrated with its helium headspace. The helium itself was pump recirculated from a reservoir contained in a floating air mattress and served to bubble strip the methane out of the bottom water. We kept the mattress tethered to the Revenge, but kept a close eye on it lest the wind would pick up and carry it aloft into the distant reaches of the Great Basin Desert. Indeed, we also had to keep it filled to slightly below full capacity, as our return trip home would carry us up and over Tioga Pass, where the volume would increase due to the drop in pressure from driving from Lee Vining (altitude = 6781 feet) up to Tioga pass (9943 feet) and bursting open the air mattress. You got to think of this kind of stuff when you're running the show, and it's not a topic covered in the literature.

You also get to worry a lot, as in the time Larry and Mike were operating out on the lake when a powerful thunderstorm rolled in suddenly, as can occur when summer monsoonal moisture comes up from the Gulf of California. For about 2 h,

I was at my wits end, as they did not respond to my frantic calls on the CB radio, which was inoperative as they knew enough to lower the metal whip antenna. They had the presence of mind to put the boat into the nearest shore (Paoha Island) and hunker down, thereby lessening their target profile to lightning until the storm passed. Yet during this time, I fretted with every lightning bolt (they are quite fearsome at this altitude) and thunder roll, checking the lake from time to time with my binoculars. When the storm eventually receded eastwards, I could finally see their white Revenge pulling around the island against the dark background of storm clouds, and I only then could I let out a big sigh of relief.

31.2.3 Toro! Toro! Toro!

As the 1980s transitioned to the 1990s, we continued our investigations of Mono Lake on a seasonal basis, where Chuck performed ^{14}C-labelling measures of primary productivity, Larry collated biogeochemical data on sulfide, methane, and ammonia as responses to and breakdown of meromixis-caused stratification (Miller et al. 1993), and I focused on N_2-fixation (Oremland 1990). Unlike BSL, Mono Lake lacks rooted macrophytes like Ruppia that become colonized by big globs of nitrogen-fixing Anabaena (Oremland 1983b). What it has instead are small, marble-sized aggregates of the alga Ctenocladus, which in turn host internal populations of light-driven (cyanobacterial) and heterotrophic-bacterially driven anaerobic diazotrophs. I also deployed chambers along the shoreline of the north-eastern quadrant of the lake and measured N_2-fixation via the acetylene reduction assay. Not surprisingly, rates were highest in the light and during summer but fell off to zero during winter, when nighttime temperatures routinely plummeted to −20 °F (−28 °C). In total, this activity was minimal compared to the nitrogen requirements needed to sustain primary productivity (Oremland 1990), but the work did have its compensations and hazards.

Accessing the site, located in the lake's NE quadrant playa, required driving out on route 167, turning off at the 10 mile signpost onto a dirt road and proceeding about a mile down through brush and bramble toward the lake until it dead-ends into a set of barriers. After that it is a 1 mile hike down to the water's edge, which I would do for retrieving samples twice daily (morning and early evening). The morning sampling was best because it was relatively cool, but by the summer afternoons, the temperature would rise above 100 °F (~38 °C). During summer, the playa hosts big nasty biting/clawed flying insects like horseflies that, if given the opportunity, will alit on your exposed flesh and proceed to dig out a chunk of your personage to feast upon. I warded them off by emulating a horsetail, swinging a motel's hand-towel over my head as I hiked to and fro from the water's edge.

During summer, the lake can actually be observed shrinking in size because of evaporation. The NE quadrant is shallow, and there would be a moist long ribbon of wetted, green–grey exposed sand (about a meter wide) at the water's edge that looked as if there was an ebbing tide. Pieter Visscher was quite enthusiastic about

this proto-biofilm/biomat, as he determined the in situ porewater concentration of dimethylsulfide to be about 1 mM. It reeked.

On one morning, after clearing the fence, I noticed off to my left about a quarter mile away a large bull walking briskly in my direction, following the lake's fenced boundary perimeter. How he got there and to where he was headed were questions I wished answered but did not dare to await to interview the subject. Great horned male bovines like these are reputed to be quite irritable, readily insulted, and easily aroused to violence. I did not wish to inflame the wandering ruminant by my towel's rotary motions, thereby becoming the Mono Basin's first (and last) amateur matador-scientist, in an American parody of what occurs in Pamplona. If gored, I would suffer my wounds in the great isolation of the region, likely bleeding out well before anyone back at the motel would notice my absence (this was the pre-cell phone era). Fortunately, by the time I finished sampling the chambers, the bull had disappeared westward in search of its grazing herd from which he became separated, thereby leaving only the aforementioned horseflies as the only serious hazard I encountered upon hiking back up to my vehicle.

The author (below, left panel) in his old Coney Island lifeguard attire presiding over the vast emptiness of Mono Lake's NE quadrant (facing SW). Below right panel, Pieter Visscher samples the headspaces of the incubation chambers at the water's edge. The photo faces east. Note the green-grey wet edge (bio-mat) caused by the retreating lake boundary caused by water loss attributable to a typical summer day's evaporation and not any non-existent tidal forces.

The author (below, left panel) in his old Coney Island lifeguard attire presiding over the vast emptiness of Mono Lake's NE quadrant (facing SW). Below right panel, Pieter Visscher samples the headspaces of the incubation chambers at the water's edge. The photo faces east. Note the green–grey wet edge (bio-mat) caused by the retreating lake boundary caused by water loss attributable to a typical summer day's evaporation and not any non-existent tidal forces

The next few years while we were doing seasonal trips, we saw something of a parade of colleagues coming out to Mono Lake and participating in the field work. These included, among folks already attached to my Project, Dick Smith, Niels Iversen, Nisan Steinberg, Gary King, Mandy Joye, Pieter Visscher, Tim Hollibaugh, and Derek Lovley. Many were listed as co-authors on a grab-bag chapter on diverse aspects of the methane biogeochemistry of Mono Lake and its surrounding basin that was published as part of an International Symposium on Environmental Biogeochemistry volume that (ahem) I edited (Oremland et al. 1993). We even delved into

the metabolism of methyl bromide by the lake's planktonic bacteria (Connell et al. 1997). We were floundering to some extent, casting about for a new Mono discovery to sink our teeth into, when we came to the realization it was staring us right in our faces all along: Arsenic!

31.2.4 Tufa and Tephra and Tuff! Oh My!!!!

Some brief mention of basic pertinent geological information is required here. Mono Lake, although it is circular and lies at the bottom of a closed basin (water flows in but not out), is not in itself the remnant of a giant volcanic caldera. It looks like one, but it isn't. It is a subsided region that sank about one million years ago, as the mountains to its west were still rising. Back in the Pleistocene (10,000–20,000 years ago) the lake occupied five-times the area and contained eighteen-times the water than it does in modern times. It even over-filled its basin, spilling out from its SE corner and establishing fluvial transport south, extending even to Searles Lake in the Panamint Valley. One of the advantages of the Mono Basin is, owing to the lack of trees, to easily discern past geological features, be they the glacier-carved U-shaped valleys of the neighboring Sierras or the former lake levels, presented as obvious striations on the hillsides circling the basin. Indeed, while driving along Hwy 167, looking down at the lake gives one the impression of a draining bathtub, with its prominent islands as exposed rubber duckies and loofa sponges in the residual water. The surrounding striations are reminiscent of the need to scrub the tub clean of its accumulated soap rings. It must have been quite spectacular back in its heyday, not only was the lake greater in size (Lake Russell) but it was infringed upon by giant glaciers (1000 meters high) descending from the Sierras. One only needed to await the coming of the volcanoes for some further excitement.

Yet because of the grinding and subduction of the Pacific plate's peripatetic boundaries, the region itself is seismically active and has its own past history of eruptive volcanoes, the most prominent that of the mega-sized Long Valley Caldera. This pressure cooker blew open about 700,000 years ago with a force estimated to have been 300-fold greater than that of the Mount Saint Helens 1980 showstopper. The Long Valley eruption spread pyroclastic flow throughout the region that solidified into a long series of tan-colored block-like rocks that abound and have served well as backdrop for many a Hollywood Western movie or TV series. The subsequent active volcanism moved northwards toward the lake, and a number of rhyolite-type volcanoes (e.g., the Mono-Inyo Craters) line either side of US-395 when one drives north from Mammoth Lakes to Lee Vining, including the most recent eruption of Panum Crater on the lake's south shore (erupted about 600–700 years ago; you can hike into this one and see all the obsidian piles too), and extended through the lake (Paoha and Negit Islands, the latter a black cinder cone) and onto the northern shore (Black Point).

Trivia question: what two movies had sequences filmed at Mono Lake?

1. *Fair Wind to Java* (starring Fred MacMurray and Vera Johnston; 1953; Republic) had its volcanic eruption sequences filmed on the lake, enhanced by erecting a scaffold with smudge pots behind it on a small islet near Paoha Island so to look more eruptive and volcanically menacing as a Krakatoa stand in. The islet and its scaffolding are still there.
2. *High Plains Drifter* (starring Clint Eastwood and Verna Bloom; 1971; Universal) shot mostly on the lake's southern shore near a forested region that once provided lumber to the mines and the present ghost town of Bodie, CA.
3. *Close but no cigar*: *Out of the Past* (Robert Mitchum and Jane Greer; co-starring Kirk Douglas and Rhonda Fleming; 1947; Warner Bros.) had sequences filmed outside the county courthouse in Bridgeport, CA, some 30 miles north of Mono Lake but no lake scenes. Sean Penn served prison time for reckless driving in the Bridgeport hoosegow back when he was married to Madonna (1987).
4. *Again close, but now 30 miles further south*: *The Secret of Convict Lake* (starring Glen Ford; Gene Tierny; Ethel Barrymore; 1951; 20th Century Fox) dramatizes the escaped convicts escapade from Carson City in 1871 as mentioned earlier in this chapter.

Mono Lake is of course famous for its exposed tufa mounds formed when calcium-laden freshwater springs encounter the alkaline/carbonate-rich brine and precipitate $CaCO_3$. Tephra is a light-weight rock spat out of an eruptive volcano, and many blocks of tephra abound by the lake's northern margin surrounding Black Point. And then there is pumice, a very light, gas-filled volcanic rock that abounds along the shoreline in the NE quadrant playa. The smooth, pebble-sized stuff can actually float atop Mono Lake water and get blown all around the lake. CBS news recently televised a short film clip of the lake's tufa preserve which is well-worth watching: https://www.cbsnews.com/video/nature-californias-mono-lake/?fbclid=IwAR3qnHSvgrZC7KifVelFsha6YthYhXaSsKBs8uB67jWUFmmcPDt_gf0iVPA

The region also has a history of precious metal mining that got started in earnest during the second half of the nineteenth century, as the now ghost towns of Bodie, CA, and Virginia City, NV, can attest. In simple geochemical terms, hot magma essentially volatilizes metals such as gold, silver, and platinum so that they become upwardly mobile within the bedrock, eventually aggregating into concentrated ore veins where they cool and solidify. Along with the precious metals comes the dense, mobilized arsenic bearing minerals, which are leached out of the rock by hot geothermal waters, and subsequently drain into the closed-basin lake. Mono Lake's unique alkaline chemistry keeps the arsenic in solution, so that in addition to the oxyanions arsenate and arsenite, the anoxic waters also contain soluble arsenic–sulfide complexes, compounds that would normally precipitate into the sediments of a freshwater lake. Add to this, the evapo-concentrative effects of an arid climate of the past few thousand years, and voilà: Mono Lake's current concentration of dissolved inorganic arsenic is ~200 μM.

31.2.5 Got Arsenic?

We first isolated two new species of halo-alkaliphilic bacilli from the lake's bottom sediments that proved able to grow via dissimilatory reduction of arsenate (Switzer Blum et al. 1998). This whetted our appetites, so that by the following year, we successfully designed and deployed a radiotracer experiment to measure in situ arsenate respiration in Mono Lake's anoxic bottom waters. We measured rates ranging from 1–5 μmoles/L/d, which when integrated and calculated as a sink for electrons wound up mineralizing 8–14% of annual primary production, with another 40% contributed by sulfate-reduction. This was a major revelation for us, because it illustrated that respiratory arsenate reduction has quantitative significance as an electron acceptor for carbon mineralization in an actual ecosystem and not just as a curious microbiological sidebar (Oremland et al. 2000). Our experimentally determined rates were borne out when independently calculated via lake-wide mass balance considerations (Hollibaugh et al. 2005), thereby solidifying the importance of arsenic biogeochemistry in this peculiar ecosystem.

Since arsenate respiration was occurring in the lake's anoxic water column, we set out to try to cultivate some of the arsenotrophs from that location (Hoeft et al. 2002, 2004) including arsenite-oxidizers (Oremland et al. 2002). The result of these efforts, largely accomplished by Shelley Hoeft McCaan, was the isolation of the facultatively chemoautotrophic, facultatively anaerobic arsenite-oxidizer, *Alkalilimnicola ehrlichii* strain MLHE-1 (Hoeft et al. 2007), a very flexible γ-Proteobacterium. Strain MLHE-1 is a bacterium that proved crucial to our uncovering further mysteries of arsenic metabolism based on the discovery of its "reverse" functioning arsenate reductase (ArrAB) homolog, an anaerobic arsenite oxidase we designated ArxAB in further collaborations with John Stolz at Duquesne University and Chad Saltikov at University of California Santa Cruz (e.g., Richey et al. 2009; Zargar et al. 2010, 2012). Shelley also isolated the difficult-to-grow δ-Proteobacterium strain MLMS-1which, we eventually realized, disdained organic substrates and used sulfide as its electron donor to achieve growth via arsenate reduction. Strain MLMS-1 showed little inclination in doing much of anything else of interest with its time, except a perhaps a disproportionation of thioarsenate (Hoeft et al. 2004; Planar-Friederich et al. 2015).

Curiosity drove Larry and me to once again explore the hot springs on Paoha Island, but this time actually stepping ashore to see if there was anything of interest growing within them. There clearly was, as these springs (~45 °C) hosted red-colored biofilms as coatings on the rocks and pebbles over which the spring water flowed. The biofilms were thin (~1 mm) and could easily be removed by gently scraping the surfaces with a soft toothbrush. We were able to obtain a lot of sample in this manner, which we stored within anaerobic serum bottles held in a refrigerator. We later discovered that they remained blood-red in color and still proved viable even after several months of such dark storage. The hot spring waters were anoxic and contained dissolved methane, sulfide, and arsenite in abundance, all of which were possible electron donors that could support anoxygenic

photosynthesis in the biofilm. So we set out on a field trip specifically devoted to determining whether or not arsenite could function in this manner. We brought along an HPLC in our lab truck, thereby allowing us to run arsenic speciation incubation results from our makeshift field locale, headquartered in the back of the Lakeview Lodge.

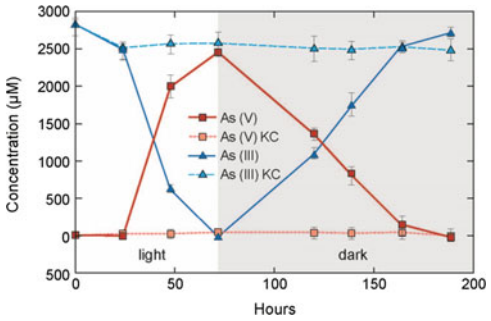
Paoha Island hot spring (left) viewed from the boat, and sampling of its abundant red biofilm waters (right).

Paoha Island hot spring (left) viewed from the boat and sampling of its abundant red biofilm waters (right)

Unfortunately, our first efforts were unsuccessful, largely because Tom Kulp employed a powerful outdoor high-wattage light for sample illumination that "fried" the sensitive photosynthetic machinery of the phototrophic bacteria. I came to this realization after consulting the literature once we got back to Menlo Park. So I implored Tom and Shelley to turn around, drive back to Mono, link up with Tom Crowe and his Zodiac and head straight back out to Paoha Island to obtain fresh samples, and then head home *tout de suite*. This they did, and upon setting up their incubations under low light intensity, obtained after an overnight incubation, a positive indication that the experiment was working. It was early October, on the Friday of the long Columbus Day weekend, when I looked at Tom and told him that if he and his wife had made plans for a getaway, he needed to cancel them. It was necessary that he babysit the experiment over the weekend and obtain a 4 day long incubation time course that was also a publication-quality result. This he achieved, with the caveat that I allowed him a 3-day weekend makeup and first authorship on the paper (Kulp et al. 2008). In that paper, we also reported on a "green" mat, presumably cyanobacterial that we found in a higher temperature regimen (~60 °C) which also proved capable of light-driven arsenite oxidation. Upon returning the following season, we were eager to tackle the green mats, but they were nowhere to be found. Over the years, we have found these biofilms and their hot spring water taps to be somewhat ephemeral, appearing or disappearing upon whims we did not perceive. Complicating factors included lake level drops or rises due to drought or high winter precipitation. The rule of thumb now being that although the red biofilms

are always present, the rare green ones will only make an appearance when one has given up all hope of ever seeing their re-occurrence and is thus entirely unprepared to tackle them experimentally. We found one during a 2019 visit with the International Geobiology Course, but alas were unprepared to conduct any additional research on this subject.

Shelley went on to demonstrate an alteration of processes occurring over diurnal light/dark cycles within the biofilms, with a net anaerobic arsenite oxidation occurring in the light, and anaerobic arsenate respiration dominating in the dark, primarily carried out by chemoautotrophs (Hoeft et al. 2010). Shelley also reported on the isolation and characterization of 3 strains of anaerobic photosynthetic bacteria (Ectothiorhodospira) recovered from Paoha Island and Big Soda Lake that were capable of using arsenite as their electron donor, while Hernadez-Moldonado et al. (2016, 2017) established that these strains all had functional *arxAB* genes as their mechanism for exploiting arsenite. Moreover, his in situ transcriptional data showed that *arxAB* genes were expressed by the hot spring biofilms indicating that arsenite serves as an electron donor for ongoing anoxygenic photosynthesis even in the presence of the more abundant sulfide.



Cyclical light-driven oxidation of arsenite to arsenate, and dark-driven reduction of arsenate to arsenite in red biofilm samples from the Paoha island hot spring. (from Hoeft et al., 2010, with permission). KC = killed controls.

Cyclical light-driven oxidation of arsenite to arsenate, and dark-driven reduction of arsenate to arsenite in red biofilm samples from the Paoha island hot spring (from Hoeft et al. 2010, with permission). KC = killed controls

Our discovery of ArxAB anaerobic arsenite oxidation genes linked to bacterial photosynthesis led us to speculate about its origins in deep time, going back to the Archean era when Earth was anoxic, and crustal geothermal activity was more common. We hypothesized that owing to the sequence similarity of *arxA* vs. *arrA* genes that they were clearly closely related neighbors. Based on biogeochemical observations, it made "sense" that *arxA* preceded that of *arrA* with the latter arising from the former to take advantage of arsenate-rich niches created by the ArxA's activity. Hence, one did not need to invoke the presence of strong oxidants such as oxygen or nitrate in the Archean to generate arsenate. All that was needed was light (Oremland et al. 2009). Indeed, Sforna et al. (2014) presented geochemical evidence from 2.7 Gya stromatolites that indicated such an active biological arsenic cycle

preceded the Great Oxidation Event of 2.4 Gya, although they suggested an environment more akin to the salt-saturated, arsenic-rich extreme of Searles Lake than Mono Lake (Oremland et al. 2005; Kulp et al. 2006, 2007).

Whenever one posits a chicken vs. egg precedence argument in biology, there are always countering positions taken by those whose research is more invested with chickens than with eggs. Such was the argument posited by van Lis et al. (2013) who, although they agreed with the antiquity of a biological arsenic cycle, they countered that aerobic arsenite oxidation arose first, achieved by oxygen-linkage of the *aioBA* arsenite oxidases, which then in turn created niches for anaerobic arsenate respiration via *arrAB* genes from which anaerobic arsenite oxidation via *arxAB* later evolved. They used largely bioinformatic approaches which showed that while neither *arxA* nor *arrA* occur in the domain Archaea, those of *aioA* do and in addition are present in the domain Bacteria, thereby implying a deep-time origin in the Last Universal Common Ancestor (LUCA).

A recent study by Wells et al. (2020) demolished this counter argument. Making much greater use of the broad sequence databases now available (~1568 species) and employing more sophisticated current bioinformatics techniques, they investigated the deep origins of several CISM-genes (Complex Iron Sulfur Molybdoenzymes) which include the arsenic reductases and oxidases. They showed clearly that *arxA* preceded the origin of *arrA*, and that *aioA* arose later in a different CISM realm, likely from a sub-group of assimilatory nitrate reductases. While there was a deep time origin for the *arrA* and *arxA* genes it came after LUCA diverged into Bacteria and Archaea, occurring in the former but not the latter. Although *aioA* genes are found in the Archaea, they occur only in extreme halophiles and not in extreme thermophiles, the latter thought to be models of the LUCA physiology. Hence, their acquisition occurred much later in time via lateral gene transfer events and did not arise independently in LUCA, and thence to Archaea and Bacteria, but first arose in aerobic Bacteria and from thence to some Archaea. Curiously, some extremely thermophilic archaea can respire arsenate; they achieve this via a respiratory tetrathionate reductase TtrA, the gene for which aligns closely with the later-emerging bacterial *arxA* and *arrA* (Wells et al. 2020).

31.2.6 Arsenic in DNA?

I would be remiss if I did not offer in this retrospective piece a final, brief commentary upon our claim of arsenic substituting for phosphorus in the DNA of *Halomonas* strain GFAJ-1 (Wolfe-Simon et al. 2011). Although almost 10 years have elapsed since the work first appeared in the journal *Science*, aspects of it are still too painful for me to recount in detail.

Wolfe-Simon proposed a highly original and very bold idea, namely that arsenate could act as a substitute for phosphate in the 3′ and 5′ di-ester linkages of the deoxyribose molecule in the outer helix that binds the nucleotides together. I liked the idea and offered that she use Mono Lake sediment as an inoculum source, its

high arsenic content perhaps making the success of this risky venture more likely. Aerobic enrichment cultures were setup using a mineral salts medium, glucose and arsenate in lieu of phosphate. These were taken through several transfers and decimal dilutions and plated on agar from which a colony was isolated. A growth curve was followed by inoculating the isolate, strain GFAJ-1, into liquid medium lacking either arsenate or phosphate. No growth was noted in these controls, but addition of 40 mM arsenate or 1.5 mM phosphate resulted in obvious enhanced growth. It was clear from a number of experimental protocols that arsenic had entered the cells, especially in the arsenate replete condition (+As/-P). NanoSIMS analysis of extracted DNA indicated it was denser and had a higher As/P ratio (13.4) than that of the –As/+P condition (6.9). Finally, X-ray spectroscopy (EXAFS) of DNA in whole cells (+As/-P condition) as modeled on As substituting for P closely aligned with the observed experimental spectra. Moreover, these data further indicated that the arsenic atom was bonded to oxygen (i.e., arsenate), which in turn was bonded to the carbon of the deoxyribose sugar. This ruled out the possibility of direct arsenic to carbon bonds as occurs in such molecules as arseno-betaine. The paper underwent internal review and then was submitted to *Science*. It was favorably reviewed and published online on December 2, 2010.

For reasons I will not delve into, high praise soon turned into severe criticism. The biggest viable criticism we faced was that arsenate-carbon ester bonds are inherently unstable and break apart quickly in aqueous medium.

Laboratories from around the world requested strain GFAJ-1 for testing. We plated out the cultures and shipped them to certain bona fide laboratories, but instead of using arsenate, for safety reasons we replaced with phosphate. I was aware of the possibility that removal of the arsenate selective factor could work against us. We sent the microbe to Rosie Redfield at the University of Vancouver, as well as Julia Vorholt, at the ETH in Zurich. Alas, neither of these labs detected appreciable arsenic in the DNA they extracted from grown cultures of GFAJ-1 (Reaves et al. 2012; Erb et al. 2012), although Elias et al. (2012) reported the bacterium's interesting ability to discriminate in favor of phosphate uptake against a high background of arsenic, while Wu et al. (2018) reported on the exceptional arsenic-resistance capacity of strain GFAJ-1. Scavenging of phosphate from ribosome breakdown was suggested as the source of GFAJ's phosphate as evidenced by experimental work with *E. coli* (Basturea et al. 2012).

Our refutation of these papers is that the efforts by Reaves et al. and Erb et al. employed additional treatments to the extracted DNA before analyses. After an initial phenol chloroform extraction, Reaves et al. purified the DNA with RNase A and Proteinase K followed by phenol chloroform extraction and ethanol precipitation. Erb et al. did not use RNase A but repeated 70% ethanol rinses. The DNA was then resuspended in water before it was analyzed. Hence any susceptible arsenate-esters would have been broken down by the extraction process itself. We did not employ such methods, relying on NanoSIMS and X-ray spectroscopy for our results. Indeed, in our paper we made note of the instability of the molecule in water and suggested that it could have been stabilized internally by such molecules as poly-β-hydroxybutyrate. Moreover, Wolfe-Simon pointed out that DNA within

cells is not the linearly arranged molecule illustrated in textbooks but is "super-coiled" in such a fashion that would defy the best attempts of human knot experts recruited either from the seafaring community or the Eagle Scouts to unravel. In such microenvironments, the activity of water molecules is likely reduced, and thereby the longevity of arsenate-esters would likely increase.

In the nearly 10 years since it appeared in *Science,* it has been cited 547 times. Most of these were inconsequential citations in papers that make reference to arsenic and phosphate. Some were experimental refutations like those mentioned above, some were theoretical criticisms (Singh and Giri 2018) that were roughly balanced by quantum chemical calculations saying it was possible (Denning and Mackerell 2011). A good number were ministrations of what to avoid in the realm of scientific reporting and intense media attention, cautions against flying too close to the sun like Daedalus, and the oft stated Carl Sagan mantra *exceptional claims require exceptional proof*. I still have confidence in our report and stand by our results and interpretation. I believe it is a subject of viable inquiry for the future.

The "Three Amigos" on our last field sampling reunion at Mono Lake, circa 2013. Left to right: myself, Larry Miller, and Chuck Culbertson, aboard the venerable Revenge Boston Whaler.

The "Three Amigos" on our last field sampling reunion at Mono Lake, circa 2013. Left to right: myself, Larry Miller, and Chuck Culbertson, aboard the venerable Revenge Boston Whaler

31.2.7 Some Final Thoughts

Forty plus years of field research in the Great Basin Desert has some Biblical connotations if one is seeking to link one's semi-obscure career to something far bigger, say that of the Hebrews wandering in the wilderness of Sinai. Yet even though I have gained much, it is a big stretch to infer anything more here than sheer coincidence. Alas, time passes and some things must come to an end. The "Three Amigos" pictured above have all retired, although Larry still makes field excursions

to Mono Lake with colleagues (e.g., Alex Sessions, Jerad Leadbetter, Victoria Orphan) from Caltech. The International Geobiology Course instituted a leg of its summer field instruction at Mono Lake, which Larry, Mike Rosen, and myself eagerly took part in when it was first under the auspices of University of Southern California and Colorado School of Mines, then shifting to Caltech. We were able to make use of the fine auditorium of the interpretive Visitor Center in Lee Vining to facilitate our lectures before spending a day sampling the lake. The enthusiasm of the students (and faculty) for this unique location is gratifying, because it assures me that research will likely continue into the foreseeable future.

Lee Vining itself is a curious, small town (2010 population = 222). Yet on a summer day you can sit on the benches outside its only market and see people wander by that are not just locals, native American Indians, and Bay Area tourists, but often visitors from overseas (Europe, Asia, Middle East) speaking their diverse languages and obviously enchanted by the wide open spaces of the region and the public's easy access to them. Sometimes I would fantasize about having a research facility erected on Mono's shores, maybe proximate to the Mono Inn, a restaurant owned by the Adams Family, descendants of the photographer the late Ansel Adams, whose collected works glorified Yosemite.

Well, it is time now to thank those with whom I have worked over the years and who all helped out in the writing of this essay by keeping me on track, correcting my mistakes, and providing photos of our exploits: Chuck Culbertson, Larry Miller, Mike Rosen, and John Stolz. Along with Tim Hollibaugh, Chad Saltikov, Jodi Switzer Blum, Shelley Hoeft McCann, and Shaun Baesman, I thank them from the bottom of my heart along with many other folks too numerous to mention.

Ronald S. Oremland near the Mono Basin Interpretive Center in California

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