Cellular Origin, Life in Extreme Habitats and Astrobiology 17

Joseph Seckbach
Martin Grube
Editors

Symbioses and Stress

Joint Ventures in Biology

Springer

SYMBIOSES AND STRESS

Cellular Origin, Life in Extreme Habitats and Astrobiology

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Cover Illustration: *Trapezia* crab between the branches of *Stylophora pistillata* (Photo by I. Brickner). See Chapter by Barneah & Brickner in this volume.

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FOREWORD

When one picks up a multiauthored book in a series like this, one wonders what will be distinctive about its contents. One wonders about the "Concept of *Symbiosis*." Does it have the same meaning for all authors and all potential readers? One is further tempted to question the concept of *stress*. What is the meaning of the concept of stress? Some change in the biotic or abiotic aspects of the environment or habitat of the symbiotic partners? Many might support the more general definition of symbiosis credited to de Bary (1879), that symbiosis is the living together of separately named organisms. Something like Smith's (1992) more restricted POLLNPIA (Permanent Or Long-Lived Intimate Associations between different organisms, usually of different sizes, in which the larger organism, the host, exploits the capabilities of one or more smaller organisms) seems to be a better fit for a book centered on the effects of stress on symbiosis. POLLNPIA implies an integrated holobiont system that has adapted itself to living successfully in a particular environment that could be construed as harsh for nonsymbiotic systems. Often, when queried for examples, one thinks of lichens, of corals living in oligotrophic tropical waters, of Pompeii worms living in association with chemolithotrophic bacteria, and of all sorts of herbivorous animals living in associations with microorganisms. Presumably, the hosts could not survive, or thrive, in their habitats without their smaller partners doing their trophic work for their holobiotic systems.

As could be expected from his previous writings, Sapp (1994, 2004) in his chapter goes through a very detailed history and discussion of the various shades of meaning and the connotations associated with concepts of symbiosis. If there is any nuance of meaning used by someone in the past and missed by Sapp, the reader is challenged by the author of this foreword to report it.

Fortunately, the authors of each chapter have come to grips with their concepts of *symbiosis* and *stress* and leave the reader with little doubt as to their ideas of the boundaries of the meaning of the phenomena they have worked with. What happens, or could happen if the external or internal conditions of the partners change? That is the essential theme of this book.

Particularly fitting the current concern for global warming, as it relates to coral reef ecosystems, is the focus of the chapter by Barneah and Brickner. Their interpretation of *symbiosis* is a broad one. They begin their chapter by comparing coral reefs to rain forests. In this, they include numerous other organisms that grow and interact with each other and the coral holobiont in a complex array of symbiotic associations. Their chapter focuses on multibiont symbioses involving corals, zooxanthellae, cyanobacteria, and endolithic algae (microsymbionts), as well as associations that involve the coral, and macroorganisms such as mussels,

barnacles, and fish (macrosymbionts). Following this multibiont theme, they discuss aspects of metabolic contribution, symbiont effectiveness, host gain/loss, and the establishment of the multibiont symbiotic ecosystem.

They describe the coral–algal adaptive bleaching hypothesis that grew out of molecular systematic studies of two dominant Caribbean Sea corals. Three distinct taxa of algal symbionts were associated with *Montastraea annularis* and *M. faveolata*; at shallow-to-intermediate depths, they host Clade A or B *Symbiodinium* in the well-illuminated parts of the colony and in their shaded surfaces, they host clade C. When such intracolony irradiance gradients were manipulated, the symbionts reestablished patterns of zonation. The authors discuss later research that showed that different strains of *Symbiodinium* vary in their thermal tolerance and photosynthetic response to irradiance. Barneah and Brickner feel that this variation underlies adaptations to high temperatures and irradiance and are the primary causes of coral bleaching (the breakdown of the symbiotic relationship between the coral and its zooxanthellae). Thus the adaptive bleaching hypothesis (ABH) predicts that when environmental circumstances change, the loss of one or more types of zooxanthellae followed by the formation of a new symbiotic consortium with different zooxanthellae are more suited to the new conditions in the host's habitat. In support of this idea, the authors cite Baker's experiment that showed that when stony corals were taken from deep water and transplanted into shallow water they bleached (the loss of their suboptimal low-light symbionts). The newly vacant hosts were subsequently repopulated with "high-light algae." They argue that Baker's experiment did not show whether the new combination of symbionts in the host is due to new acquisition of symbionts or whether the phenotypic change was caused by the increase in the proportion of rare genotypes of symbionts that were always there.

Noga Stambler's chapter on "coral symbiosis under stress" reviews much of the same literature as did the chapter by Barneah and Brickner, but it does so in such a different perspective that her chapter and theirs fit together like hand and glove. Systematically she reviews potential sources of stress (physical, chemical, and/or biological) on coral colonies and their varied responses to them. Each stressor can affect one of the symbiosis partners: the animal, the zooxanthellae, both partners, or the relationship between them. Stress can affect the growth rate, reproduction, and/or survival of the holosymbiont. Respiration is usually the most sensitive, and as such, is a good indicator of stress. Photosynthesis is one of the first processes affected by stress on the zooxanthellae. In most cases, there is damage to the photosystem, the thylakoid membrane, or proteins such as D1 or on the coral host due to reactive oxygen species (ROS) damage. Two defense mechanisms exist against high-temperature bleaching: (1) change in the amount of heat shock proteins (HSPs); and (2) oxidative enzymes, including copper/zinc superoxide dismutase (SOD), manganese SOD, iron SOD, ascorbate peroxidase, and catalase. These prevent subsequent cellular damage from active species of oxygen. The major bleaching mechanism involves expulsion of zooxanthellae in response to environmental stress in in situ degradation followed by exocytosis.

Other options are pinching off, programmed cell death (apoptosis), host cell death resulting in loss of zooxanthellae (necrosis), and host cell detachment.

She ends her chapter with a consideration of eutrophication as a stress on coral reefs. The zooxanthella density in *Pocillopora damicornis* and *Stylophora pistilllata* increases in response to eutrophication. At the same time, chlorophyll per area increases, photosynthetic efficiencies are reduced, and the coral growth rate decreases. In *Acropora pulchra*, nutrient enrichment causes unbalanced growth between organic tissue and the carbonate skeleton. High nitrate concentrations enhance zooxanthella volume and chlorophyll contents per *P. damicornis and Porites lobata* cells. Variations exist between species: 30% of *P. damicornis* colonies remain healthy in contrast to 90% of *Porites lobata.* The branching of *P. damicornis* is significantly affected by the addition of nitrate, whereas *P. lobata* is significantly influenced by water temperature.

What a world we live in! It challenges the imagination to try and understand how life can exist near the intense super-heated seawater boiling up from the ocean depths at plate boundaries, subduction zones, and volcanoes. Naganuma delights the reader with his account of animal–bacterial endosymbioses of gutless tube-dwelling worms in marine sediments. Tubeworms in seeps and muddy sediments have greater longevity (~200 years or longer) than individuals at hydrothermal vents (~100 years) where focused fluxes of heat and chemicals sustain much larger masses of tubeworms. These worms lack a digestive tract and share the same unique strategy for life dependence on endosymbionts for energy capture and nutrition. These pogonophoran and vestimentiferan worms have endosymbiotic bacteria that are thiotrophs (oxidize sulfur for autotrophic production) or methanotrophs (oxidize and assimilate methane) to capture energy and use the Calvin Benson Cycle to fix carbon. Although most of the pogonophoran and vestimentiferan tubeworms possess single thiotrophic 16S rRNA genes (16S rDNA) related to Gammaproteobacteria, some pogonohorans have a single methanotroph species or even dual symbionts of thio- and methanotrophs. Among different specimens of the vestimentiferan *Lamellibrachia* sp. L1, 16S rDNA sequences suggested that the worms collectively hosted alpha-, beta-, gamma-, and Epsilonproteobacteria. They all had RuBisCO form II gene (*cbbM*) sequences related to Betaproteobacteria.

The endosymbionts in the vestimentiferan trophosomes are transferred horizontally. Trophosomes have been regarded as a modified gut or endodermal tissue. Originally, it was thought that vestimentiferan larvae take up ambient microorganisms through the mouth to the gut cavity when it is present in the larva, and subsequently select some for endosymbioses when converting gut to trophosome. Newest evidence suggests that trophosome is a mesodermal tissue formed de novo and that endosymbionts migrate across the epithelium into the mesodermal trophosome.

How do animal/bacteria symbioses in sulfide-rich habitats not only survive high sulfide concentrations, but also metabolize sulfide efficiently? This ability has been attributed to a sulfide detoxification system. Uptake of oxygen and hydrogen

sulfide is facilitated through the anterior plume of the animals. This body region was found to be a very efficient gas exchange organ. Abundant extracellular hemoglobin in the blood and coelomic fluid binds with high affinity, simultaneously and reversibly, to oxygen and sulfide. However, no sulfide was measured at plume level in the seep vestimentiferan *Lamellibrachia luymesi.* Records taken at plume level of the "long and thin" morphotype of *Ridgeia piscesae* showed very low concentrations of hydrogen sulfide. In both species, the posterior part of the body is very long ("roots"). This area is permeable to sulfide uptake. The take-home lesson is that in the sea, chemoautotrophy in sulfidic, otherwise hostile environments is a major factor driving symbioses coordinated by mutually beneficial associations with bacteria.

One would have thought that the subject of the origins of mitochondria and plastids in eukaryotes might be out of place in this book; however, it is not. Curtis and Archibald, in their contribution, discuss the "ox-tox hypothesis." This hypothesis emphasizes that the new partnership between the alphaproteobacterial mitochondrial progenitor and anaerobic protoeukaryotes was an adaption to the stress caused at the time that oxygenic photosynthesis was changing the Earth's atmosphere. Aerobic respiration and ATP synthesis served to detoxify the increasingly aerobic environment. The Alphaproteobacterium entered into an endosymbiotic relationship with such an organism, consuming oxygen within its cytoplasm, and the relationship between the two cells was eventually cemented by the evolution of host-derived ATP/ADP translocases.

Vesteg and Krajčovič discuss the emergence of the eukaryotic cell as a response of a two-membrane-bounded sexual pre-karyote to an aggressive alphaproteobacterial infection. They speculate that the pre-karyote (the host for alphaproteobacterial ancestors ofmitochondria) possessed two membranes (outer and inner) and was sexual. Alphaproteobacterial ancestors of mitochondria are assumed to have been parasites of prekaryote periplasm (intermembrane space), whose infection might have features similar to the infection of negibacterial periplasm by *Bdellovibrio* sp. It is proposed that eukaryotic-plasma membrane descended from prekaryote outer membrane, while prekaryote inner (plasma) membrane is proposed to have been ancestral to eukaryotic nuclear/ER membrane. They speculate that the best evidence in favor of nuclear/ER membrane originating from prekaryote plasma (inner) membrane is the fact that eukaryotic cotranslational import of proteins into ER is homologous to cotranslational secretion of proteins through prokaryotic plasma membrane. (Eukaryotes do not secrete proteins cotranslationally through their plasma membrane). Eukaryotic nucleoplasm is thought to be derived from prekaryote cytoplasm, while pre-karyote periplasm is thought to be ancestral to eukaryotic cytoplasm. Their view that the eukaryotic nucleoplasm was ancestrally pre-karyote cytoplasm is supported by the following: (1) eukaryotic nucleolus with 109 unique eukaryotic protein domains is still the place of assembly of ribosomal subunits; (2) some components of signal recognition particle (SRP) are still present in the nucleolus; and (3) there is some evidence

that translation is still occurring in the eukaryotic nucleus. The transitions of periplasm to cytoplasm and cytoplasm to nucleoplasm, as well as the evolution of nuclear/ER membrane topology and nuclear pore complex are suggested to have been driven by the aggressive alphaproteobacterial parasite and the responses of the host. The result of this intriguing essay is that the reader is reminded once again how much of life has evolved as a result of symbiotic phenomena.

Kawano and Kadono provide yet another different twist on the theme of stress on symbiotic systems. For them, *Paramecium bursaria* is a model organism they can use for testing the ecological impacts of various chemical pollutants. Their main theme centers on reactive oxygen species (ROS). The endosymbiotic algae, *Chlorella* spp, are rich in antioxidants and the ROS-detoxifying enzymes that protect their hosts from ROS stress.

How does a propagule survive? One rarely thinks about the stress caused to symbionts released into the free-living environment. If the environment is inhospitable, how do the propagules survive under such great stress? Hirsh in her chapter discusses how rhizobia survive in the absence of their symbiotic partner. The issue is relevant to the theme of the book because these bacteria are nonspore formers and do not have a resting stage. How do they do it?

"Three in a Boat" is the catchy title of the chapter by Applebaum, Ichelczik, and Humber. It starts with a description of how the development and physiology of a fungal entomopathogen is affected by stress either directly (depletion of insect metabolites needed by the fungus) or indirectly (interactions between the environment, the host plant, and the insect). Various factors affect the degree of adhesion of the fungus to the insect epicuticular surface, decreased germination, and penetration of the cuticle, and even compromise the innate immune response of the insect. They go on to describe how the interaction between fungal entomopathogens and their hosts can be exploited (integrated pest management) to control pests important to food production. The application of fungal metabolites as potential bioinsecticides is also examined.

The chapter on stress on symbiotic foraminifera by Altenbach, Böhmer, Gitter, Läuchli, and Wieczorek begins with a general introduction and guide to previous reviews of the subject. Starting with the affects of global warming, sea-level changes, and the details of bleaching that have been observed in populations of larger foraminifera, they review the most recent additions to our knowledge. High light was considered the triggering stressor for the intracellular anomalies observed by Talge and Hallock, because heat stress alone (32°C or more) was observed to induce symbiont loss, but not the degradation of the host endoplasm. As is well known, micropaleontologists and paleoclimatologists often use the tests of planktonic foraminifera as tools to interpret past oceanographic conditions. During photosynthesis, the algal symbionts preferably remove the lighter 12C leading to enriched incorporation of 13C during test calcification. The fractionation gradient steepens with enhanced photosynthesis by the algal partners and diminishes with symbiont loss. The authors suggest that other factors in the sea

might also affect host–symbiont interactions and cause subtle test compositional changes that might be used to detect them.

New research by Bernhard and her colleagues has disclosed still another facet of symbiotic phenomena in granuloreticulopods. A newly described "allogromiid" with high numbers of sulfur-oxidizing bacterial endosymbionts thrives under long-term sulfidic conditions in the deep sea. Rod-shaped bacterial endosymbionts, tentatively identified as sulfide oxidizers, were also observed in V*irgulinella fragilis* from sulfidic environments. In both cases, the metabolism of the symbiotic prokaryont might provide a source of sulfide detoxication for the eukaryotic host that still needs oxygen as a terminal proton acceptor. Chloroplasts from ingested algae are also sequestered in the cytoplasm of *V. fragilis.* As chloroplasts produce hydroxyl radicals, they may offer a source of oxygen for the host. Bernhard and Bowser have shown that kleptoplasty is not just restricted to *V. fragilis.* Many foraminiferal species that thrive near redox boundary conditions shelter kleptoplasts. They are often sequestered at water depths where photosynthetic activities are excluded.

After a first glance at the title ("Physiological responses to stress in the vibrionaceae"), a reader of this book might begin to wonder what is this chapter doing in a book on symbiosis? Soto, Lostroh, and Nishiguchi have a wonderful perspective on a topic familiar to most scientists with broad knowledge of symbiotic phenomena. Most recognize the importance of photoluminescent symbioses between bacteria and squids or with various groups of marine fishes. It is also common knowledge that the bacteria are transferred laterally; each generation must acquire fresh symbionts from the sea. Microbiologists also are aware of diseases transferred by marine vectors and spoilage of refrigerated fish.

Members of the vibrionaceae are abundant in the sea (some estimates 1×104/ml). The thrust of this review is to discuss the physiological responses of noncholera vibrios to stress, especially to stressors likely encountered during symbiosis or during transitions from one host or lifestyle to another. They also draw connections to studies that tackle vibrios from evolutionary, ecological, and molecular physiological points of view. For example, chemotaxis important for virulence of the fish pathogen *V. anguillarum*, is strongly affected by temperature. It is very chemotactic at 25°C, and the response diminishes in both cooler (5°C) and warmer (37°C) conditions. Temperature also controls production of a capsular polysaccharide, an important virulence factor in *V. vulnificus.* With respect to the familiar Vibrionaceae involved in luminescent symbioses, *Photobacterium profundum*, *V. logei*, *V. wodanis*, and *V. salmoncida*, *Photobacterium* spp. have been more frequently observed Vibrionaceae in the cold deep sea, whereas the genus *Vibrio* is more common in ocean surfaces. Ecologically and evolutionarily, pH stress is significant because symbiotic vibrios must somehow survive the acidic challenge encountered by entering the digestive tracts of their hosts. Nutritional stress is another factor to be overcome by laterally transmitted symbionts. Except for coastal waters, most of the open ocean is oligotrophic. Host organisms, however, are nutrient rich. As vibrios experience transient free-living and host-associ-

ated life cycles, these microbes thus encounter feast or famine conditions. They must therefore undergo long intervals with little or no growth and metabolic dormancy in their free-living state, followed by brief periods of rapid growth during symbiosis. Oxidative stress is another important factor. The authors cite evidence to suggest that bioluminescence may have evolved as a response to oxidative stress. In sum the authors' perspective on stress factors in these bacteria bring a deeper mechanistic understanding to familiar symbiotic phenomena.

The interesting chapter by Little uses the fungus-growing ant–microbe symbiosis as a model system to study ways in which parasites shape the ecological and evolutionary dynamics of mutualistic organisms. The effect that parasites, microbes in particular, have on mutualists is an area of interest in symbiosis biology that has not been well addressed by others. Parasitism can affect mutualists at many levels. The ant–plant mutualism is a classic example of protective mutualism. Plants provide nutrient rewards to ants that protect them from herbivores. In the fungus-growing ant symbiosis, there are similar patterns. Because the mutualism is tripartite, there are two defensive mutualisms. First, there are ants that defend their fungus gardens from fungivores. In the absence of ant mutualitsts the fungus garden would need an alternative strategy to survive fungivory. However, bacterial mutualists defend the fungus garden from microfungal parasites through antibiosis. Without antibiotic protection, the fungus garden would suffer significant mortality and, consequently, so would their ant mutualists. In each case, forming mutualistic associations with phylogenetically distant partners has been an effective solution to the threats posed by natural enemies. Step by step, Little develops the argument that mutualism may be a viable solution to ameliorate the effects of parasitism and, in turn, discusses the possibility that parasitism may in fact contribute to evolutionary stability of persistent mutualisms.

Torres and White Jr. discuss alkaloids and their functions in grass endophyte-mediated plant stress tolerance. They present evidence for the physiological roles of secondary fungal metabolites like the ergot alkaloids in enhancing their symbiotic relationships with plants as well as to function primarily to deter animal and insect herbivores from consuming the fungus or its host plant. The consistent and widespread production of ergot alkaloids by clavicipitaceous plant biotrophs is evidence that they may have adaptive value for the fungi. The broad diversity of their forms further suggests that they have multiple cellular targets and perhaps multiple functions.

The adaptation and survival of plants in high stress habitats due to stress tolerance conferred by fungal endophytes written by Rodriguez, Woodward, and Redman is extremely interesting. They theorize that the intergenomic epigenetic communication responsible for endophyte-conferred stress tolerance gave some plants the quantum evolutionary leaps necessary for their establishment and survival in high stress habitats. Collectively, fungal symbionts have been shown to confer several fitness benefits to plants including increased root and shoot biomass, increased yield, and tolerance to abiotic stresses such as heat, salt, metal, and drought.

One of their most interesting examples is the tolerance to heat in geothermal habitats conferred on *Dichanthelium lanuginosum* (panic grass) by the fungal endophyte *Curvularia protuberata.* All plants are known to commence complex biosynthetic responses to elevated temperatures; these include the synthesis of heat shock proteins and antioxidant systems, and adjustments in osmotic potential and membrane lipids. However, few plants are capable of thriving in geothermal soils that impose temperature and drought stress. There are only nine plant species that thrive in, and appear restricted to, geothermal soils in Yellowstone National Park. The geothermal soil that panic grass grows in reaches root zone temperatures as high as 57°C in the dry summer!! By removing seed coats and briefly surface-sterilizing seeds, it was possible to generate nonsymbiotic plants that were free of the endophyte. Comparative studies with symbiotic and nonsymbiotic plants indicated that *C. protuberata* confers thermotolerance to panic grass and that this plant/fungal symbiosis is responsible for survival of both species in geothermal soils.

They relate details of research on a similar type of symbiotic relationship that involves salt tolerance in dunegrass (*Leymus mollis*) and its fungal partner (*Fusarium culmorum*). In addition, they discuss reports describing drought tolerance conferred to plants via fungal symbionts and the details of some of the mechanisms by which this is accomplished. They end with a section focusing on evolutionary outlook mentioned above. They opine that the ability of some plants to establish and survive in high stress habitats was driven by dependence on fungal endophytes.

Another twist on the same theme is the chapter by Koltai and Kapulnik on arbuscular mycorrhizal symbiosis under stress conditions. They view stress as either a shortage or excess in the availability of a given resource or a factor damaging biological structures of the organism (membranes, proteins, or nucleic acids). The latter damage may have a negative effect on metabolism, provoking a response that is intended to limit or reduce the stress effect. They discuss how arbuscular mycorrhizae affect plant resilience to drought conditions via several different mechanisms. Arbuscular mycorrhizae affect the rate of water movement into, within, and out of the plant in order to maintain water content, cell turgor, and associated cellular processes. Mycorrhizae enhance the plant cell's ability to accumulate organic compounds, such as sugars and amino acids, and to increase levels of ions (of magnesium [Mg2+], potassium [K+], and calcium [Ca2+]) and sugars, including soluble sugars and soluble starch. Drought conditions lead to increased oxidative stress in plants. One of the routes to mitigating oxidative stress in plants is by producing or activating reactive oxygen species, such as superoxide dismutases (SOD). In droughts, plants with mycorrhizae have elevated levels of SOD activity relative to those, which do not have mycorrhizae. Increased concentrations of antioxidant enzymes and nonenzymatic antioxidants were found in citrus plants with mycorrhizae. These antioxidant compounds protect the plants against oxidative damage and enhance drought tolerance. The affects of salinity and mineral depletion are discussed more broadly in this chapter than

in the chapter of Rodriguez, Woodward, and Redman. The latter took the tack of using more detailed examples of models. Although the two chapters overlap in terms of content, we have good examples of how different approaches can both successfully lead to deeper understanding of the subject matter under review.

The rhizosphere of plants is also the focus of the contribution of Berg, Egamberdieva, Lugtenberg, and Hagemann. *Stenotrophomonas maltophilia* is reported to be associated with a long list of plant species, which includes all branches of plant phylogeny: potato (*Solanum tuberosum*); oilseed rape (*Brassica napus*); rice (*Oryza sativa*); sweet flag (*Acorus calmus*); tropical orchids (*Paphiopedilum appletonianum* and *Pholidota articulate*); coffee (*Coffea arabica*); grape vine (*Vitis vinifera*); poplar (*Populus* spp.), and marram grass (*Ammophila arenaria*). They are also associated with bryophytes and some lichen symbioses (where they fix nitrogen). Strains of *Stenotrophomonas* induce systemic resistance in plants and act as antagonistic bacteria for pathogens (Whipps, 2001). As antagonists they give plants traits enabling them to interfere with the growth of pathogens and the ability to survive infection. The mechanisms responsible for antagonistic activity include inhibition of pathogens by antibiotics, toxins, and biosurfactants; competition for colonization sites and nutrients; competition for minerals; and degradation of the pathogens' toxins. *Stenotrophomonas* promote the growth of wheat, tomatoes, lettuce, sweet peppers, melons, celery, and carrots in the highly salinated soils of Uzbekistan. The authors believe that *Stenotrophomonas strains* have potential as biocontrol agents.

We have learned much in the more than 4 decades since Buchner's (1965) book first drew attention to associations involving aphids and their bacterial symbionts. Jurkevitch delights the reader with a narrowly focused and detailed perspective that the symbiosis has been so successful and has evolved as a result of the partnership giving the host abilities to feed on diets that would otherwise be suboptimal. Specific nutrients lacking in an insect's diet are complemented by its microbial symbionts. He details examples where temperature stress to an insect was directly linked to the nutritional output of its bacterial symbionts. To be more specific, a point mutation in the *Buchnera aphidicola* that became fixed in laboratory lines of the aphid *Acyrthosiphon pisum* diminished the reproductive capabilities of the host under high temperatures but improved reproduction under cool conditions. Buchner (1965) observed that endosymbionts in insects are associated with deficient diets. Details and deeper understanding of the phenomenon have followed in the decades that followed. Insects that feed on plant sap experience shortages in essential amino acids. Phloem sap (the nutritive resource of aphids, psyllids, whiteflies, mealybugs, and stinkbugs) is relatively rich in sugars, but deficient in essential amino acids. Therefore, the utilization of such deficient resources is only feasible if the missing nutrients are supplied by microbial symbionts. Experimental data back this idea. Some species of aphids can grow on synthetic diets without added essential amino acids, but that growth is strongly impaired by the addition of antibiotics to the medium, clearly suggesting that the bacterial symbionts provide the missing nutrients. The author gives the

details of many experiments in different partnerships and ends with a discussion of how genome analysis is in accordance with physiological studies. Genes for biosynthesis of the amino acids essential for the aphid host were present, whereas those for the nonessential amino acids were almost completely missing from the bacteria.

Most microbes living in symbioses with plants have cycles of harmony and stress if you agree with the views of Crilli, Caiola, and Canini. They suggest that associations of plants with bacteria are beneficial mainly for plants, the microbes being eliminated at the end of symbiosis. They examine cycad–cyanobacteria, *Azolla–Anabaena*, legume–rhizobia, and tomato–*Azospirillum* symbioses and point out that the associations commonly end by eliminating most microbes. In fact, only a few individual symbionts may survive in the soil or inside the resting form of the host. The life of the symbiont appears to be more comfortable inside the host than in the free-living stage outside, especially during the first steps of associations. Afterward however, symptoms of stress occur in the microbe such as a decrease in growth and division, appearance of trehalose, PHB, melanin, SOD, resting cells like akinetes, and spore-like cysts.

Taken as a whole, this is a book that will have a broad appeal for enthusiasts of symbiosis. The authors of each of the various chapters differ widely in their concept of symbiotic phenomena and in their interpretation of stress on the relationships they focus upon. Nonetheless, all the authors have a message to send to their readers. The result is a compilation of valuable essays to entice readers; it certainly deserves a coveted place on every symbiologist's bookshelf.

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Dr. John J. Lee is a distinguished professor of Biology at City College of City University of New York and a research associate at the American Museum of Natural History in New York and at the IOLR National Center for Mariculture in Eilat Israel. He earned his Ph.D. at New York University (1960). Dr. Lee and his students have published more than 125 papers and book chapters on symbiosis in "Living Sands" (larger foraminifera). He was one of the founders of the International Society of Symbiosis and served as its first President. He also has served as president of the Society of Protozoologists and was the editor-in-chief of the first and second editions of *The Illustrated Guide to the protozoa.* He was named an Honorary member of the Society of Protozoologists in 2001 and was given the Joseph A. Cushman Award by the Society for Foraminiferal Research in 1999.

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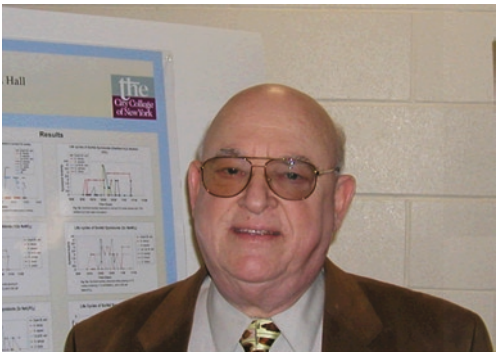

PREFACE

"Two are better than one: because they have a good reward for their labor … and a threefold cord is not quickly broken."
Ecclesiastes 4:9, 12

Everything flows and nothing stands still, Sokrates says in the Kratylos dialogues ("Πάντα χωρεῖ καὶ οὐδὲν μένει"). This insight holds particularly true today, more than ever before in the history of mankind. We face a profound change in our environment, due perhaps to changing natural habitats, overall pollution, or the induced climatic change. Our chances to escape this situation are very limited. Because we are able to create our own habitats such as cities, shopping malls, offices, etc., we are often distracted from the fact that – being highly organised metazoans – we are obligately associated and dependent on the stability of the living biosphere around us. As a matter of fact, game theoreticists describe preservation of the global climate as a huge public goods game. They also claim that it can only be solved by cooperative behavior, which is favored after we are better informed about the serious risks of planetary gambling (Dreber and Nowak, 2008). Under these premises, cooperation is in our own interest to maintain a symbiotic relationship with life on the Planet, and to persist as a species among so many others. The estimation of our impact and role will, hopefully, grow if we know more about the web of biological relationships, and the fragility or robustness of biotic networks under changing conditions. Symbiosis as a key concept in Biology is coupled with aspects of stress to reflect on this theme from diverse angles.

Symbiosis (derived from greek "sym": together, and "biosis": living) describes a situation in which two or more dissimilar organisms live together for an extended period of time. This wide-sweeping definition encompasses a continuum of organismal interactions, and is widely maintained in the international scientific community, although the connotation of mutual benefits prevails in public discussions. The term "Symbiotismus" was introduced by Frank (1877) in his study of crustose lichens and taken up as "Symbiosis" by de Bary (1879). In de Bary's original sense of a living -together of unequally named organisms ("Zusammenleben ungleichnamiger Organismen"), the outcome of the interactions for either organism does not play a role and symbiosis also encompassed pathogenic interactions. Interestingly, de Bary quoted the parasitologist Van Beneden as using the term "mutualism" in a similar sense (the term mutualism thus predates symbiosis; see Van Beneden, 1873). Also, in the scientific community, symbiosis was then more frequently used for mutualistic interactions in which the participants benefit from the association (especially in German speaking countries), while symbiotic interactions with negative effects for one of the

engaging organisms were usually called pathogenic or parasitic. Today, we are aware that such value-laden terms are sometimes difficult to apply when interactions are weak or when they may change owing to varying external or intrinsic parameters. Douglas (1994) points out an evolutionary aspect and characterizes symbioses as long-term interactions that lead to new structures and metabolic activities.

Irrespective of the direct effects on the symbiotic organisms, symbioses are indeed major drivers of evolution. Intracellular endosymbioses, in which cells of one organism may have internalized symbiont cells into a host cell, led to the perhaps most important evolutionary innovation, the emergence of Eukaryota. The founder of this theory of symbiogenesis was Constantin Mereshkovsky (1855–1921). In the early decades of the twentieth century, he postulated that chloroplasts were symbiotic cyanophytes (cyanobacteria) and that the prokaryotic precursors of some eukaryotic organelles had been free-living organisms. Other examples of "living together" also include an enormous range of exosymbioses, where symbionts maintain their cellular integrity. The involved species maintain a close – usually physical – association, which can result in unique symbiotic morphologies. One of the richest, in terms of species numbers, of such symbioses are the lichens, traditionally seen as mutualistic symbioses between fungi and algae. Ecologically and economically very important are the mycorrhizal mutualisms of fungi with plants. Some plant groups also maintain bacteria in distinct root nodules of their hosts, but it is now known that specific bacterial communities are a common fraction on plant roots. Recent research has shown that many symbioses often involve more species than previously thought, and rather diverse organisms are involved in these symbiotic associations with variable degrees of specificity and interaction strengths. There are many other examples of well-studied symbiotic systems, such as the paramecia and green algae, plants and endophytic fungi, rhizosphere bacteria, and others that will be covered in this book. The list is virtually endless and a closer look will reveal new cases of symbiosis. New exciting cases of symbioses can be discovered in tropical rainforests, with their density of life, but even a walk outside in a park may lead to unexpected discoveries. One such case, perhaps, is the enigmatic presence of unicellular green algae in cells of the widely known park tree *Ginkgo biloba* (Tremouillaux-Guiller et al., 2002).

Stress involves a range of factors with harmful effects to organisms, which can cope with such unfavorable conditions by acclimatization or adaptation. Species have evolved protection and repair systems to tolerate stress in their environment. Stress responses have been studied in a wide range of organisms and at various levels of investigation, but still much work is required to understand the role of stress in symbiotic systems, which are so widespread on our planet. May stress facilitate the evolution of cooperation among unlike organisms, is stress naturally involved in the interaction of dissimilar species, and how do symbiotic systems cope with our changing environment? These and many more questions are pending further research. With this book we are attempting to promote research in this direction, the fascinating area of symbiosis and stress.

This volume is number 15 in the ***Cellular Origins, Life in Extreme Habitats and Astrobiology*** (COLE) series. It is a continuation and complement to the series volume 4 ***Symbiosis: Mechanism and Model Systems*** edited by J. Seckbach (2002). The purpose of this current collection is to present functional and evolutionary aspects of stress and mutually beneficial symbioses, i.e., the manifestation of biological cooperation among unrelated organisms. Mutualisms are ubiquitous in nature and may contribute to stress tolerance, ecosystem stability, and major evolutionary radiations. Rather than focusing on the particular organism groups involved, the structure of the book concentrates on general aspects. This includes metabolic processes, the structure of genomes in symbioses, evolutionary processes, and the ecology of symbioses. The 31 chapters contributed to this volume, by 60 scholars from a dozen countries, cover a variety of topics in the symbiosis and stress fields. The editors thank the authors for their contributions and their cooperation during the compilation of this book. We also acknowledge the efforts of many individuals for their careful reviewing of its chapters. We hope that the readers (whether they are biologists, ecologists, geneticists, or general readers interested in science) will extract new knowledge from this collection of articles.

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Among his publications are books, scientific articles in the lines of phytoferritin, cellular evolution, acidothermophilic algae, and life in extreme environments. He has also edited and translated several popular books. Dr. Seckbach is the coauthor (with R. Ikan) of the *Chemistry Lexicon* (1991, 1999) and a coeditor of ***Proceeding of Endocytobiology VII Conference*** (Freiburg, Germany, 1998) and the ***Proceedings of Algae and Extreme Environments meeting*** (Trebon, Czech Republic, 2000). His new edited volume (with Richard Gordon) entitled ***Divine Action and Natural Selection: Science, Faith, and Evolution*** has been published by World Scientific Publishing Company. His recent interest is in the field of enigmatic microorganisms and life in extreme environments.

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PART I:
GENERAL INTRODUCTION

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Biodata of **Jan Sapp,** author of ***"On the Origin of Symbiosis"***

Professor Jan Sapp obtained his Ph.D. from the University of Montreal in 1984. He was professor at the University of Melbourne from 1984 to 1990, and Andrew Mellon Fellow at the Rockefeller University (1991–1992) before his appointment at York University. His scientific interest is in the areas of symbiosis and lateral gene transfer as modes of evolutionary change, microbial phylogeny, and the evolution of complex systems. He has authored many scholarly papers and numerous books, including *Beyond the Gene* 1987; *Evolution by Association: A History of Symbiosis* 1994; *What Is Natural? Coral Reef Crisis* 1999; *Genesis: The Evolution of Biology* 2003; and *The New Foundations of Evolution: On the Tree of Life* (2009).

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ON THE ORIGIN OF SYMBIOSIS

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> Parasitism, mutualism, lichenism etc., are each special cases of that one general association for which the term symbiosis is proposed as the collective name.
>
> Anton de Bary (1878, pp. 21–22)

> The double danger of research into this type of phenomenon lies, on the one hand, in bringing to them preconceived ideas of too subjective a nature, bordering on an illusory anthropomorphism, and on the other hand, trying to reduce complex facts to simple elementary reactions.
>
> Maurice Caullery (1952, p. 2)

1. The Genesis of the Word

The concept of symbiosis took root in evidence that lichens were "dual organisms," comprised of fungi and algae (Sapp, 1994). Simon Schwendener's (1868, 1994) "dual hypothesis" was confirmed by many botanists who isolated and identified the algae that enter into association with various kinds of fungi to make particular species of lichens. Some attempted to produce lichens synthetically by culturing the fungus component and the algal constituents separately and uniting them to study the formation of the lichen thallus, an emergent property resulting from the symbiosis (Reess, 1872; Bornet, 1873; Stahl, 1877; Bonnier, 1889). The algae could be cultured separately when supplied with the proper nutrients, but the fungi were thought to have become so reliant on the algae that they had lost the ability to propagate alone (see, however, Stocker-Wörgötter, 1995).

Schwendener perceived of the lichen relationship in terms of fungal master and algal slave, but others saw the relationship to be more a matter of cooperative living. The alga (autotrophic) synthesizes carbohydrates and borrows from the fungus (heterotrophic) the nitrogenous and albuminoid material that the latter builds up with the help of the carbohydrates furnished by the alga; besides this, the fungus draws up water and mineral substances. Johannes Reinke (1873) suggested the use of the term "consortium" to express the relationship. Albert Bernhard Frank at Leipzig agreed that the term "parasitism" with its connotations of disease and destruction was inapt for such relationships, and he proposed the neologism

"*Symbiotismus*" under which to gather such cases: "We must bring all the cases where two different species live on or in one another under a comprehensive concept which does not consider the role which the two individuals play but is based on the mere coexistence and for which the term Symbiosis [*Symbiotismus*] is to be recommended" (Frank, 1877, p. 195). Frank investigated lichens and other kinds of intimate associations between microbes and plants, especially mycorrhizal fungi associated with the roots of forest trees (Frank, 1892).

Still, the term "symbiosis" was not attributed to Frank, but rather to Anton de Bary at the University of Strasbourg. He first used it in an address on "The Phenomena of Symbiosis" delivered at a conference of the Association of German Naturalists and Physicians at Kassel in 1878. Like Frank, de Bary (1879) defined it as "the living together of unlike named organisms" to embrace both parasitic and mutualistic relationships." The term "mutualism" had been introduced into biology a few years earlier by the Belgian zoologist Pierre-Joseph van Beneden (1873, 1876) to describe relationships in the animal kingdom, which, he said, were as varied as those found in human societies. He classified them in terms of "parasitism," "commensalism" and "mutualism."

1.1. THE PARASITE

"Is he whose profession it is to live at the expense of his neighbour, and whose only employment consists in taking advantage of him, but prudently, so as not to endanger his life" (van Beneden, 1876, p. 85).

1.2. THE COMMENSAL OR "MESSMATE"

"Is he who is received at the table of his neighbour to partake with him of the produce of his day's fishing. … The messmate does not live at the expense of his host; all that he desires is a home or his friend's superfluities" (van Beneden, 1876, p. 1). Van Beneden was thinking, for example, of pilot fish swimming alongside sharks from whom they receive aid and protection.

1.3. THE MUTUALISTS

> Are animals which live on each other, without being either parasites or messmates; many of them are towed along by others; some render each other mutual services, others again take advantage of some assistance which their companions can give them; some afford each other an asylum, and some are found which have sympathetic bonds which always draw them together. (van Beneden, 1876, p. 68)

Many insects while sheltering in the fur of mammals for example, cared for the toilet of their host by feeding on epidermal debris and excretions. Some

mutualists, such as the Egyptian plover that "keeps the teeth of the crocodile clean," rendered services that van Beneden (1876, p. 107) compared to medical attendance.

De Bary argued that commensals in the sense of van Beneden could not be found in the plant kingdom, but relationships approaching mutualists could be found there. He described the intimate association of blue-green algae, Anabaena, and a genus of aquatic ferns, Azolla, that float on the surface of freshwater ponds and marshes. The algae live inside special sealed cavities of the leaves. He suspected that the host protected the algae, but he had "no idea of a reciprocal service they rendered in return" (de Bary, 1879, p. 21). He noted blue-green algae that produced nodules inside the cells of the roots of the palm-like cycads in which they lived. Finally, he pointed to lichens which he himself had long studied: there were thousands of species, and all were constituted by the association of fungi and algae. In his view, the relationship between fungi and algae could be one of parasitism or of mutualism depending on the species involved in the lichen in question.

The most significant aspect of symbiosis, as de Bary saw it, was that it could lead to morphological variations that were not pathological. So he came to his central thesis: that symbiosis was a means for generating evolutionary novelty that could be investigated experimentally. It was a mode of evolutionary innovation in addition to gradual evolution based on the accumulation of individual variations within populations of species. "Whatever importance one wants to attach to natural selection for the gradual transformation of species, it is desirable to see yet another field opening itself up to experimentation. This is why I wanted to call your attention to these here, though they can only shed light on a part of the phenomena" (de Bary, 1879, pp. 29–30).

2. Symbiosis as Division of Labour

During the 1880s, several other microbe–plant relationships were added to those deemed symbiotic (Sapp, 1994, 2004). Bacteria in the root nodules of legumes and fungi in roots of trees were among of the best studied examples. The bacteria were isolated and the nodules were produced experimentally by growing the plant in sterile soil and seeding the bacteria into the soil (Wilson and Fred, 1935). The bacteria fixed nitrogen and the plant benefited from it. The root tubercles of legumes were regarded as functionally analogous fungi associated with the roots of forest trees. The fungi had been supposed to be parasites until Frank (1885) suggested otherwise for the "mycorrhiza" and distinguished between "ectotrophic" mycorrhiza that remain external to roots, and "endotrophic" mycorrhiza that penetrate into the cells of the root. Far from being parasites, ectotrophic fungi functioned as root hairs: bringing minerals and nitrogenous food to the plant, which for its part, yielded carbohydrates to the fungus. The endotrophic fungus would be digested by the plant and thus providing it with nitrogen. It seemed to

him that endotrophic fungi benefited trees without receiving anything in return, and he suggested that plants somehow attracted the fungi.

Some of the most widely discussed theories emerged from studies of "animal chlorophyll" in sponges, protozoa to worms and sea-anemones, the so-called plant-animals: the phytozoa. The main interest in these chlorophyll-containing organisms lay in their bearing on the long-disputed taxonomic relations between plants and animals (Sapp, 2009). While the chlorophyll was typically considered to be an animal-specific product, many of those green animals could also be found in a colourless state, and they did not turn green when exposed to light. In some cases, "chlorophyll bodies" could be extracted, and still the animal survived. Based on these arguments, a number of biologists concluded that these chlorophyll bodies were symbionts (Brandt, 1881; Geddes, 1882). The alga and host relationship was understood as one of mutual dependence: The translucent animal cell provided CO_2 and nitrogen for the symbiont, which in turn provided its host with oxygen and starch.

Algae living in the animal cell came to be seen as a microcosm for a view of the relations of plant and animal worlds as a whole functioning as an integrated superorganism (Spencer, 1899; Reinheimer, 1915). Mutualistic symbiosis was perceived by some as manifesting another relationship borrowed from sociopolitical theory: the division of labour, complementary types mutually integrated into an organic whole. Just like each of the cells derived from a single fertilized egg of a complex organism lived for itself and for the benefit of the whole, so do different species of organisms functioned for the benefit of the whole (Sapp, 1994, 2003). In the nineteenth century, the concept of division of labour was applied to every level of organization, single cells, multicellular organisms, ecological communities, and human societies. As C. O. Whitman (1891, p. 19), first director of the Marine Biological Laboratory in Woods Hole, stated:

> On the same grounds that the sociologist affirms that a society is an organism, the biologist declares that an organism is a society.
>
> A society is an organized whole, the unity of which consists in, and is measured by, the mutual dependence of its members. The living body is an organization of individual cells with the same bond of unity. The principle of organization in both cases is the division of labor or function.

This concept of mutual dependence was applied to intracellular relations of nucleus, cytoplasm, centrioles, chloroplasts, and mitochondria, each of which were proposed to have once arisen as symbionts – concepts that could be systematically investigated only much later in the twentieth century (Sapp, 1994, 2009).

The analogy between symbiotic algae living in the cells of many translucent animals and the chloroplasts in plants was obvious to many (see Sapp, 2009). De Bary's former student Andreas Schimper (1883, pp. 112–113) first proposed that chloroplasts might be symbionts, while providing evidence that those cell "organs" reproduced by division:

> Should it be definitively proven that the plastids are not formed anew in the egg cells, then their relationship to the organism that contains them would more or less remind

> us of a symbiosis. It is possible that the green plants indeed owe their origin to the union of a colourless organism with one evenly stained with chlorophyll.

Between 1905 and 1918, Constantin Merezhkowsky (1905, 1910, 1920) wrote a series of papers arguing that chloroplasts (chromatophores) were symbiotic microorganisms, and that the nucleus and cytoplasm also emerged through a symbiosis of two phylogenetically distinct organisms. Merezchkowsky offered the term *symbiogenesis* for "the origin of organisms by the combination or by the association of two or several beings which enter into symbiosis" (Sapp et al., 2002).

Whitman's student, Shôsaburô Watasé (1893) proposed that nucleus and cytoplasm, as well as centrosomes originated from symbiosis. Watasé did not speculate on what the earliest living units that formed cells actually were. But, in assuming their existence, he was already on safe ground since leading biologists agreed that there existed microscopically invisible living units or hypothetical "elementary organisms" standing somewhere between the cell and the ultimate molecules of living matter. As Watasé saw it, his concept of symbiosis gave a more concrete meaning to the idea expressed by Darwin (1868, p. 404): "Each living being must be looked at as a microcosm – a little universe, formed of a host of self-propagating organisms inconceivably minute and numerous as the stars in the heavens."

That mitochondria arose as symbionts was postulated later. The concept is typically traced to Richard Altmann at Leipzig. But Altmann's theory was quite different. His model accounted for the origin of bacterial-like cells, as well as nucleated cells. The cytoplasmic granules that he wrote of had been reported by others who believed them to be inert. But Altmann (1890) named them "bioblasts": they were diverse "elementary organisms" and he suggested that the primordial cells without nuclei first emerged when bioblasts came together into a colony and a membrane formed around them. That mitochondria were symbionts that lived within a nucleated cell was developed at the *Institute Océanographique de Monaco* by Paul Portier. In his book *Les Symbiotes*, Portier (1918), constructed an all-encompassing theory of symbiotes in metabolism, nutrition, development, parthenogenesis, heredity, cancer, and the origin of species. Based on his theory that

> all animals from Amoeba to Man, all plants from Cryptograms to Dicotyledons are constituted by an association, the "*emboitement*" of two different beings.
>
> Each living cell contains in its protoplasm formations which histologists designate by the name of "mitochondria." These organelles are, for me, nothing other than symbiotic bacteria, which I call "symbiotes." (Portier, 1918, p. vii)

A similar idea was subsequently developed by Ivan Wallin (1927) at the University of Colorado, who also proposed that repeated acquisition of mitochondria would account for the origin of new genes, and for genomic complexity. He suggested that part or all of the genome of symbionts would be given up to the cell nucleus as symbiosis develops, leaving the remains of the symbiont in the cell cytoplasm. The idea that infectious microbes might be the source of new genes had been previously postulated – by the leader of the Mendelian genetics in England, William Bateson (1913). It was not until the rise of genomics some 80 years later that such

lateral gene transfer was recognized to be a major mechanism of evolutionary innovation (Sapp, 2009).

3. Ethics of Symbiosis

By the early twentieth century, two meanings of the term symbiosis had emerged, and they persist to the present. For those who studied intimate symbiotic associations symbiosis included parasitism as well as mutualism. They believed that they could detect as de Bary (1887, p. 369) put it, "'every conceivable gradation' … between the parasitism which quickly destroys its victim and that in which parasite and host mutually and permanently further and support one another." For others, symbiosis was restricted to mutualism. To understand why this restricted definition became so prominent we have to place the science in its larger social context and examine its anthropomorphic metaphors. Anthropomorphic metaphors. To the extent to which the phenomena of symbiosis implied something other than parasitism with its destructive connotations, they were understood to be in opposition to the germ theory of disease. Botanical studies of symbiosis, beginning with the lichen, paralleled, but had not interacted with research on the development of the germ theory of disease. A medical perspective on microbes as germs as disease-causing entities competed and overshadowed an ecological and evolutionary perspective of microbes at the foundations of life (Sapp, 2009). During the 1870s and 1880s, when botanists discussed cases of symbiosis, microbe hunters were celebrated for the identification of pathogens such as the anthrax bacillus: *Staphylococcus*, *Neisseria gonorrhoeae*, *Salmonella typhi*, *Streptococcus*, and *Mycobacterium* tuberculosis (Bulloch, 1938). These studies closely preceded Elie Metchnikoff's phagocytosis theory according to which natural immunity was reduced to the direct action of phagocytes that engulf and digest invaders (Tauber and Chernyak, 1991). The extent to which studies of symbiosis were separated from studies of disease and immunity was well exemplified in Frank's suggestion that forest trees had developed a mechanism for attracting and capturing the beneficial fungi so as to procure nitrogen.

Studies of symbiosis were also in conflict with the main focus of evolutionists in illustrating "the struggle for existence," and nature as "red in tooth and claw." Competition and progress through individual life struggle were dominant themes of both natural and social science. As Darwin (1859, p. 490) concluded in *The Origin*, "Thus from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows." As critics saw it then, as well as today, much of Darwinism seemed to be an application of laissez-faire socioeconomic theory (Lewontin, 1968; Young, 1985).

So too mutualism had roots in human social relations. In Britain, trade unions, Chartism, and the "friendly societies" were formed to help workers deal with catastrophes such as illness or funerals. The analogous organizations in France were the mutual aid associations: *Mutualité* societies were a hotbed of socialist ideas (Boucher, 1985). These socioeconomic aspects of mutualism converged with

another, originating in natural theology. Mutual interactions were favourite examples of Divine Providence in the natural theology of the seventeenth and eighteenth centuries. Van Beneden was a Catholic with deep religious convictions. His mutualisms were imbued with ideas from natural theology, and they were examples of perfect adaptations created by Divine wisdom:

> All these mutual adaptations are pre-arranged, and as far as we are concerned, we cannot divest ourselves of the idea that the earth has been prepared successively for plants, animals, and man. When God first elaborated matter, He had evidently that being in view who was intended at some future day to raise his thoughts to Him, and do Him homage.
>
> (van Beneden, 1876, p. xxvii)

Followers of natural theology opposed the views of Thomas Hobbes who, in *The Leviathan* (in 1651), represented the state of nature as a war of all against all, and that without a powerful government, humans would live without virtue and morality, agriculture and arts and letters. Mutualism was posited implicitly or explicitly in opposition to this conception, and of evolutionary and social progress resulting from a pitiless struggle for individual advantage. Russian anarchist Peter Kropotkin's best-selling book *Mutual Aid: A Factor of Evolution* argued that cooperative behaviour and altruistic feelings themselves were important progressive elements in evolution, that the extent of the struggle for existence in its narrowest sense had been exaggerated while the importance of sociability and community had been underrated. Those animals which practiced mutual aid were much more "fit," intelligent and highly developed than those which were constantly at war with each other. Nature's message was clear (Kropotkin, 1915, p. 62):

> "Don't compete! – competition is always injurious to the species, and you have plenty of resources to avoid it!" That is the *tendency* of nature, not always realised in full, but always present. That is the watchword which comes to us from the bush, the forest, the river, the ocean. Therefore combine – practice mutual aid! That is the surest means of giving to each and to all the greatest safety, the best guarantee of existence and progress, bodily, intellectual, and moral.

4. Entangled in Anthropomorphism

Kropotkin's *Mutual Aid* reflected well the ethical context in which reported cases of cooperation between microbe and host for mutual benefit was discussed and understood by biologists. Examples of such cooperation could be found in some areas of the animal kingdom. But it was absurd to believe that "unconscious mutual support" existed among "lower" organisms such as algae, fungi, sponges, and ciliated protozoa, or among plants generally. As Danish botanist Eugenius Warming (1909, p. 95) commented: "In the plant community egoism reigns supreme." The principles for understanding the

complex relations between species, he argued, had been laid down by Darwin: species were never "at peace with one another." Every species "endeavoured" to extend its area of distribution by any means of migration as it possessed (Warming, 1909, p. 349). Warming interpreted all intimate associations in terms of parasite–host interactions. The lichen symbiosis reflected a case of "slavery" or "helotism": "The algae is in a condition of slavery in relation to the fungus, which is a kind of parasite differing from ordinary parasites in incorporating the host and in providing a portion of the food consumed in the host maintenance" (Warming, 1909, p. 85).

Similar views were upheld by the American botanist Roscoe Pound (1893, p. 519):

> Ethically, there is nothing in the phenomena of symbiosis to justify the sentimentalism they have excited in certain writers. Practically, in some instances, symbiosis seems to result in mutual advantage. In all cases it results advantageously to one of the parties, and we can never be sure that the other would not have been nearly as well off, if left to itself.

Pound found it absurd to believe that the microbes benefited their hosts without gaining anything in return. Microbes, Pound (1893, p. 519) argued, were parasites, thieves that steal from the host its rightful inheritance:

> It is not necessary, as Frank seems to think, in order to establish mutualism to show that the organisms do no injury to each other. Mutualism of the kind we meet with in the vegetable kingdom involves sacrifice on the part of the host. The parasite is not there gratuitously. It is there to steal from its host the living it is hereditarily and constitutionally indisposed to make for itself. If the host gains any advantage from the relation, it can only do so by sacrificing – by giving the parasite the benefit of its labor that it may subsist.

Some botanists were reluctant to use the term symbiosis if it meant adding mutualism to parasitism. The impression of species harmonizing their functions for the good of the community, some argued, resulted from confusing cause and effect, and it would disappear, at least in part, if one examined the way in which associations originated in terms of "attack" and "defense" of the "antagonists." Studies of mycorrhiza in orchids by Noël Bernard (1902, 1909a, b) became the exemplar for this perspective. During the 1890s fungi had been identified in the cortical cells of the roots of about 500 species of orchids. Bernard isolated the fungi in pure culture and demonstrated that the penetration of the fungi made it possible for the seed of the orchid to germinate. By inoculating seeds of the same orchid with fungus from different species, he showed that the fungus penetrates the seed and one of three results may ensue: (1) The resistance of the seed may overcome the virulence of the parasite and the latter is destroyed, (2) the virulence of the fungus outweighs the resistance of the seed and the seed is destroyed, or (3) finally, the virulence of the one and the resistance of the other balance, and a symbiosis results which is in effect, a constrained parasitism. "An impartial examination of these facts," he concluded (Bernard, 1909b, p. 371),

"reveals clearly that symbiosis is an exceptional state, rarely accomplished and bound together by gradual transitions of an infectious disease under its diverse forms." Bernard scoffed at Franks' interpretation that the plant attracted the fungus. In his view, the distinction between the terms "parasitism" and "symbiosis" only reflected the extent to which studies of symbiosis had been divorced from studies of the pathological effects of microorganisms in "higher" animals. From "an experimental point of view," he declared (Bernard, 1909b, p. 372), symbiosis was "*à la frontier de la maladie*," and "the *terrain de choix* for understanding the laws of plant pathology."

The supposition that symbiotic relations arose initially from parasitic microbes certainly jibed better with germ theory and the struggle for existence. But the nature of relationships between microbe and host was no less speculative; they were, in effect, "just-so stories." Indeed, the evolution of the relationships themselves was difficult to demonstrate. The only crucial method available for determining the nature of a symbiosis was first to break the association, study each partner separately, determine what it can or cannot do when alone, then reestablish the partnership and study the united partner symbiosis.

Bernard's argument that began as parasitism was common. British microbiologist George Nuttall (1923) found it difficult to imagine that "symbiosis" originated in any other way than through a preliminary stage of parasitism, the conflict in the course of time, ending in mutual adaptation. "Symbiosis," he said was a balancing act between two extremes: complete immunity and deadly infective disease. He suspected that some of the supposed "symbionts" described in the literature would prove to be actually "parasites" on further investigation. Famous microbiologist, K. F. Meyer (1925, p. 95) agreed that "symbiotic" "communal life" was "merely an example of true parasitism or even of disease" (Meyer, 1925, p. 97). But he raised another issue when discussing his experiments on bacterial symbionts in snails. It was "easy to assume" that the mollusks derive some benefit from the intracellular bacteria as anabolists or catabolists of metabolic waste products. But Meyer found it difficult to fathom what benefit the bacteria derived. He searched the literature for other examples, but found only speculations: "The function of the microscopic 'symbiotes' and their benefit to the host are explained, but little or nothing is said regarding the possible advantage of the microorganisms" (Meyer, 1925, p. 95). Meyer found it difficult to believe that these bacteria should manage to live and propagate better in the cells of animals than outside. The microbial "symbiotes" would secure more benefit as true parasites or disease producers.

Not all biologists agreed that symbiosis was due to parasites gotten under control. There was no evidence to indicate that parasitism was the usual manner of development of symbiotic complexes. Wallin (1927, p. 66) insisted that in many instances the physicochemical properties of the symbionts entering into a relationship are of such a nature that the terms "parasitism," "infection" and "disease" could not be applied to the relationship. In his view, one had to understand the evolution of symbiosis in the same way as one understood

the development of any multicellular organism from a single fertilized cell. Symbiosis and parasitism were closely akin, not because the former was derived from the latter, but because, they were both "end responses in the expression of one and the same biological principle which accounts for the aggregation of cells in complex plants and animals as well as aggregations of organisms" (Wallin, 1927, p. 59).

Whether one should understand symbiotic associations in the same terms as one understood the relations among cells of an individual plant or animal remained central to discussions of symbiosis. Some took a genetic view, arguing that "the progressive evolution" of such intimate cooperative relations was primarily restricted to organisms of the same or similar germplasm. Others maintained that such cooperation was due to the physiological characteristics of organisms and their environmental context. "Commonsense" views of human social relations – of cooperation, exploitation, partners, enemies, masters and slaves, marital relations and international affairs – continued to be used to explain relations between microbes and their hosts, as well as to the tissues of our own individual bodies. The discussion of symbiosis by H. G. Wells, Julian Huxley and G. P. Wells in their popular work *The Science of Life* (1931) is exemplary.

In a chapter entitled "Some Special Aspects of Life," the authors described how "the "struggle for existence" among individuals could make and break cooperative relationships, and "how difficult it may be to distinguish service from slavery" (Wells et al., 1931, p. 992). At first glance, they argued, it looked as if there were many cases of mutual arrangements by which both partners gain: nitrogen-fixing bacteria in the root nodules of legumes; green algae within the tissue of many kinds of translucent animals; luminescent bacteria in special organs of cuttlefish, sea squirts and a few fishes. But Wells, Huxley and Wells argued that when an ecological context was added one could see that such mutualism was underlain with hostility.

They pointed to the common scotch heather and its partnership with fungi: the fungi receive carbohydrates from the heather, which in turn appropriates nitrogen made available by the fungi. However, they emphasized, the partnership worked this smoothly and with mutual benefit only in certain environmental circumstances; that is, in the presence of little or no nitrogen. If the seeds are grown in the presence of more nitrogen, mutual help gives way to exploitation: the fungus grows too vigorously, and becomes a parasite and kills the heather seedling:

> The phrase "hostile symbiosis" has been used to describe the state of our own tissues – all of the same parentage, all thriving best when working for the common good, and yet each ready to take advantage of the rest, should opportunity offer. There is a profound truth embodied in the phrase. Every symbiosis is, in its degree, underlain with hostility, and only by proper regulation and often elaborate adjustment can the state of mutual benefit be maintained
>
> (Wells et al., 1931, p. 932).

Wells et al. (1931, p. 935) were careful to point out that our "simple human categories of exploitation and mutual benefit, although useful, are artificial and break down when confronted with the complex and inhuman, or at least non-human, realities of other life." Yet their revelations of "hostile symbiosis" were so twisted and coiled around human social relations that it was difficult to untangle them. Their stories of symbiosis were also about international relations on the eve of National Socialism in Germany, about gender relations – about conflict, instability and dependence. As illustrated in the relations between lower organisms, nations, men and women had to be wary of exploitation. All relations of any intimacy were "supported, as it were, on a knife-edge. They may so readily overbalance and change into something different and even opposite" (Wells et al., 1931, p. 936).

5. Symbiosis as Functional Field

Mutualistic symbiosis received brief attention from ecologists and evolutionists of the 1950s and 1960s. There was little progress in studies of the evolution of symbiosis. In 1952, an English translation of Maurice Caullery's book *Parasitism and Symbiosis* appeared. Though the French version had been written 30 years earlier, it was hardly updated, and the arguments remained the same: "Commensalism, parasitism and symbiosis are man-made categories which in nature are not discontinuous but are really different aspects of the same general laws" (Caullery, 1952, p. xi). The significance of symbiosis, in Caullery's view was not in mutualism, but in the degree of integration, and interdependence of the associates into a biological unity. All symbiotic associations, even "hereditary symbiosis" could be understood in terms of conflict "terminated by domination of one of the organisms over the other and by a stable equilibrium corresponding to a novel function" (Caullery, 1952, p. 276). Still, he argued, the result was integration, a physiological division of labour.

Conceiving symbiosis in terms of integration, not of mutualism, was also the consensus of a group of British microbiologists who assembled in 1952 to discuss the meanings of the word. H. G. Thornton (1952, p. 171) framed the problem:

> The term symbiosis has been used with different meanings, and the question of its correct meaning and even of the desirability of its use at all has been debated. The term, indeed, raises the question as to how far it is possible to distinguish a definitely beneficial association between two or more organisms from certain states of parasitism on the one hand and from complex ecological associations on the other.

Though symbiosis could not be defined strictly in terms of mutualism, it was clear to participants that those stable intimate relationships between two or more species could not be understood in terms of strict parasitism either. Some equilibrium was necessary, and some exchange of function bonded symbiotic organisms. F. G. Gregory (Gregory et al.1952, p. 202) offered a solution similar to that intimated by Caullery

himself: the meaning of the term lay in functional integration and broadening the definition of the organism to include symbiotic complexes as such:

> The "struggle for existence" presupposes antagonism between organisms whether or not they belong to the same or diverse species. On the other hand, the question remains whether associated species tend to provide for each other a favourable environment. The analysis of the relations between organisms has been dominated by the notion of "competition" or "struggle," and the converse notion of "cooperation" has in consequence been disregarded. … The data of ecology serve as a challenge to this view of the predominant role of "struggle."

Whether it emerged from parasitism or not, Gregory asserted that the unity of symbiotic complexes was comparable to the division of labour among organs of any individual organism. The only significant difference between the "unitary organism" and symbiotic associations was that in compound organisms the unity was not dependent upon a single genetic mechanism, nor was the transmission of characters secured by the intervention of a single germ cell provided with a complete set of genes. Gregory argued that mutual benefit among the associates was not necessary for such unity. "Interlocking of function" and the establishment of an equilibrium could exist in gradations of relationships from "mutual advantage" to complete exploitation of one organism by the other.

> The value of the concept of symbiosis resides in the widening of the concept of organism to include heterogeneous systems overriding the limitations of genetic uniformity, and the supplementation of the concept of a structural unity by that of a "functional unity" or functional field.

Other participants at the meeting agreed that symbiosis, therefore, represented "a steady state of dynamic equilibrium." There were grave practical difficulties in assigning associations to one of the categories based on cost and benefit accountancy when several different kinds of symbionts were involved. "In view of these difficulties," F. Baker (Gregory et al. 1952, p. 204) remarked, "it may well be necessary, as Professor Gregory has recommended, to discontinue the use of the word symbiosis, substituting for it the more appropriate term 'functional field.' … If this were done, questions could profitably be raised regarding the degree of integration of symbiotic associations considered as a function of the intensity of the field established and of the internal and external resistances surmounted in its establishment."

Discussions of definitions of symbiosis opened virtually every paper and volume on symbiosis during the 1960s, and images from human social relations continued to be employed (Sapp, 1994). The idea that microbial symbiosis arises from parasitism persists, but the difficulties of cost–benefit analysis for complex associations is also emphasized as dynamic concepts of symbiosis are developed. While neo-Darwinian evolutionists continue to trivialize significance of symbiosis as a mode of evolutionary innovation, others argue that the concept of the organism needs to be enlarged to embrace the symbiotic complex, or "symbiome" (Sapp, 2003).

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SYMBIOSES AND STRESS

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1. Introduction

The "living together of unlike organisms" in symbiosis implies the confrontation of different physiological properties and ecological preferences. To be successful, organisms in association need to resolve these differences and to account for situations of stress experienced by the symbiotic partners. In the following, we will review aspects of symbiosis and stress from several perspectives and elaborate on some general patterns.

Symbioses are frequently classified by simple terms such as mutualism or parasitism when the interaction results in visible positive or negative, respectively, effects on one (usually the larger) partner. These effects may be obvious in many cases, but the lines between negative or positive outcomes for partners in symbioses are sometimes hard to draw, e.g., in the case of commensalism or amensalism. Also, many fungi known as virulent pathogens are able to reside in plants without expression of symptoms. Depending on the genotype of these hosts, fungi apparently switch their lifestyle to live as commensals or even mutualists (Redman et al., 2001). This phenomenon is known as symbiotic lifestyle switching (SLS; Rodriguez and Redman, 2008). Rather than maintaining the value-laden traditional categories, the concept of a symbiotic continuum has been established for relationships without a clear-cut separation of mutualistic and parasitic relationships (Starr, 1975; Schulz and Boyle, 2005; see Fig. 1). To avoid anthropocentric interpretations, symbioses can better be seen as a long-term intimate association of organisms that lead to new structures and metabolic activities (Douglas, 1994). The eukaryotic cell is the prime example showing that such novelties evolved to support and maintain the associations over extended periods of time. Mutualistic symbioses are sometimes also interpreted as biological markets, where products or services that are easily produced by one partner are

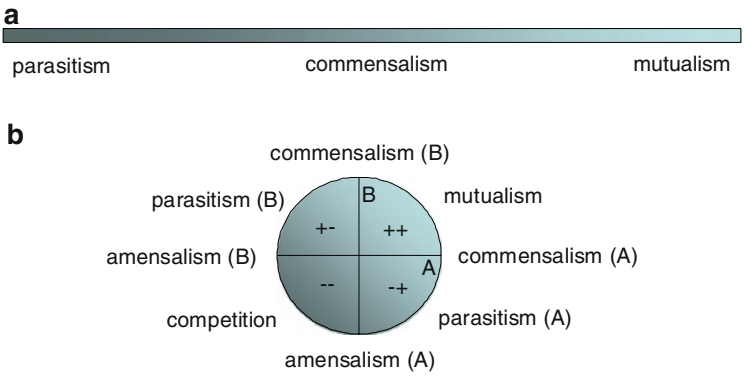

Figure 1. Continuum of interspecific interactions. (**a**) Single species perspective, (**b**) the theoretical two-species interaction continuum, e.g., parasitism (A) indicates that species A parasitizes species B.

distributed to the others in exchange for other products (Noë and Hammerstein, 1994). Because it took hundreds of millions to billions of years to evolve such capacities, symbionts often do not have the ability to quickly switch strategies in their associations, as it might possible in, e.g., human economy. Changing conditions may thus impose stressful situations for symbioses. In the following discussion, we will concentrate on the roles of stress in those symbioses, which are traditionally perceived as mutualistic cases.

Symbiotic interactions are often studied under the premises of rather constant environments. Yet, natural situations are clearly different because ecological parameters change over time with diverse dynamics: rapidly and both regularly (e.g., seasonal changes) and irregularly (e.g., rainfall), or slowly (e.g., global climatic shifts). Deviations from a presumably optimal stage impose stress on any of the partners in a symbiotic system, which will also influence interactions among the organisms. Apart from the stress imposed on the symbiotic systems, colonization with a symbiont itself can include induction of a latent or chronic stress response in the hosting organism. This leads to the beneficial priming effect (Pozo and Azcón-Aguilar, 2007). A priming effect is thought to be involved in many microbial interactions with plants, and mediates significant tolerance to further biotic and abiotic stress (see below).

Abiotic stresses such as drought, flooding, and temperature stress have similar effects in a broad range of organisms. Depending on their predisposition, organisms are nevertheless variably sensitive to stress. Biotic stress exerted by pathogens can be rather specific to the host organisms. Hosts have evolved recognition systems for elicitors excreted by pathogenic symbionts (Bar and Avni, this volume), which are further processed by common signaling pathways. Parts of the signaling cascades and systems are overlapping in mutualistic and pathogenic systems (e.g., Soto et al., 2006, 2009). Particularly the first steps of the signaling machinery are similar in both cases, while subsequent steps differentiate between

the beneficial and detrimental interactions. Both mutualistic and pathogenic colonization and establishment of bacteria in their hosts involve quorum-sensing signals and mechanisms to suppress the hosts defense responses.

2. Stress is Alleviated by Mutualistic Interactions

Dan Janzen (1985) first identified the phenomenon of defensive mutualism, which he called "protection mutualisms." These are symbioses where a primary outcome is that the host or one of the symbiotic partners is protected in some way. The example of a protection mutualism used by Janzen (1985) was "ant plants." These are plants that associate with ants for protection. The plants provide sugars and in some cases shelter to the ants; in turn, the ants attack insects or other herbivores that come to feed on the plants. Kieth Clay (1988) applied the concept to fungal endophytes and epibionts of plants in the ascomycete family Clavicipitaceae, where research demonstrated that the fungi increased resistance of plants to insect and mammalian herbivores, but also to viruses (Lehtonen et al., 2006).

Associations between fungal symbionts (mycobionts) and plants (or photobionts) are somewhat more complex than ant–plant systems. Further, they frequently impact on how hosts tolerate both biotic and abiotic stresses. A good example of this complexity can be seen in the lichen symbiosis. The lichen is resistant to desiccation, UV radiation, many pathogens, and herbivores, but individually, the photobiont (alga) and mycobiont (lichen fungus) are much more vulnerable to these stresses (Lawrey, 2009). The lichen symbiosis is a defensive mutualism because the symbiotic unit (lichen) shows protection from a range of abiotic and biotic stressors. In the lichen symbiosis, many stresses (dehydration, rehydration, UV radiation, etc.) are accompanied by the formation of reactive oxygen species (ROS; Kranner et al., 2005). Lichen mycobionts and photobionts are protected from ROS by antioxidants (see Kranner et al., 2005; Weissman et al., 2005). Oxidative stress is a factor in evolving symbioses with photoautotrophs. Therefore, selective pressures will promote the evolution of stress-tolerant host species with ROS-detoxification mechanisms, as suggested by Kawano and Kadono for the symbiosis of paramecia with endosymbiotic coccal algae (this volume). It is very likely that this also played an important role in the evolution of the lichen exosymbiosis. The lichen has also been suggested to be protected from herbivory of insects by lichen compounds (generally phenolics) produced by the mycobiont (Lawrey, 2009). The combination of antioxidants to protect against ROS and other secondary metabolites renders a lichen resistant to many oxidative stresses and feeding by many herbivores.

Plant endophytes are also complex defensive mutualisms that provide resistance from both biotic and abiotic stresses (Rodriguez and Redman 2008; Redman and Rodriguez, this volume). The clavicipitaceous endophytes produce alkaloids such as lolines, peramines, and ergot alkaloids that have insect-deterrent properties (Bacon et al., 1977; Schardl et al., 2004). However, they also result in enhanced levels of

phenolic antioxidants in plants. It has been proposed that many of the beneficial effects that come from endophyte infection may be the result of this enhanced antioxidant capacity (Malinowski et al., 2005). Since most stresses result in ROS production, an enhanced antioxidant content results in an increased capacity to manage ROS and increased stress tolerance. Specifically in the case of the clavicipitaceous endophytes, the presence of the endophytes results in enhanced drought tolerance, heavy metal tolerance, nematode resistance, and fungus disease resistance, all potentially associated by increased levels of phenolic antioxidants. In our view any symbiosis that is capable of ameliorating stress may also be considered to have some component that is defensive mutualism; and all defensive mutualisms will result in a stress reduction for at least the dominant partner.

Further interesting examples for stress amelioration are also found in symbioses of invertebrates. Both obligate and facultative endosymbionts of aphids have significant effects on the thermal tolerance of the insects (Dunbar et al., 2007). Some insects contain bacteria as protective endosymbionts to counteract biotic stress. For example, *Hamiltonella defensa* is a facultative endosymbiont of aphids and other sap-feeding insects. The phages in these bacteria produce toxins that kill parasitic wasp larvae and let the aphid hosts survive. Degnan and Moran (2008) investigated the evolutionary genetics of these defense bacteria, and identified four new toxins due to nonhomologous recombination in the virus, which also resulted in reassortment of the downstream lysozyme and holin genes. Association with phages facilitates ongoing gene exchange among heritable endosymbionts with effects on the symbiotic relationships at a higher level.

Recently, defense mutualism plausibly explained the puzzling presence of the fungus *Scopulariopsis brevicaulis* in American dog ticks (*Dermacentor variabilis*). Yoder et al. (2008) found out that this apparently harmless and maternally inherited fungal infection helps the ticks to become resistant against the common fatal fungal pathogen *Metarhizium anisopliae*.

3. Is There a Cost to Stress-Reducing Symbiosis?

There is increasing evidence that hosts cannot engage in stress-reducing symbioses or any comparable symbioses without paying a price. The cost may involve restrictions in the life cycles, range, or habitat of the host or both participants in the symbiosis; and often there is a nutritional price. Although considered a mutualism, in the lichen symbiosis the photobiont alga pays dearly with a restricted cell cycle where the alga becomes asexual, which is likely retarding evolution (cf. the 'Red King' hypothesis, Bergstrom and Lachmann, 2003). The mycobiont maintains its capacity to undergo sexual reproduction and is sometimes considered the "dominant partner" in the symbiosis (Lawrey and Diederich, 2003). Many symbionts suffer comparable restrictions in their reproductive cycles. Another example is seen in a fungal endophyte of a tropical palm. Alvarez et al. (2008) demonstrated that the fungal endophyte *Diplodia mutila* protected its host, palm *Iriartea*

deltoidea, from herbivory by stem borer insects in Peruvian forests. Without the fungal endophyte the stem borers frequently caused mortality in palm seedlings. The price for protection from insects involves a drastic restriction in the plant's habitat. The level of light determines whether endophytic *D. mutila* expresses a quiescent endosymbiotic phase or its pathogenic phase that may be mortal to seedlings. Seedlings that are exposed to bright light of the forest gaps succumb to fungal disease, while those in the shaded understory of the forest show few symptoms and benefit from protection from the stem borer. In the case of the *Diplodia*–palm symbiosis, the price is evident in the habitat restriction to shaded understory and the seedlings that succumb to disease due to light exposure.

There is some evidence that symbioses come with a nutritional cost. In situations where the host cannot afford to pay the nutritional cost, the symbiosis may be detrimental to the host. Cheplick (2007) compared endophyte-infected and endophyte-free grasses grown in soils where soil nutrients were limiting to those in soils with adequate nutrients. Under limiting nutrient conditions, plants with endophytic fungi performed significantly worse than those without endophytes in terms of shoot and root mass; where soil nutrients were adequate, endophyte-infected grasses outperformed those lacking the endophyte. In this experiment, the nutritional cost of hosting the endosymbiotic fungus is evident. If the host cannot support the added load of the endophyte with nutrients, the symbiont becomes a liability.

Observations from natural grass endophyte populations suggest that the symbiosis will not be maintained unless the symbiosis is beneficial under the conditions of the population. This is likely due to the nutritional cost of maintaining the symbiosis. This principle is illustrated by the association of an endophytic fungus (*Neotyphodium* sp.) in the grass *Bromus setifolius* growing naturally in the deserts of the Andes mountains in Argentina (White et al., 2001). In populations in the Andes high frequency of the endophyte in individuals from the grass population were seen only in grass populations in communities that contained leaf-cutting ants. The leaf-cutting ants constitute a primary herbivore in these desert communities. The presence of the endophyte in grass individuals resulted in avoidance of the infected plants. In the absence of the leaf-cutting ants in the plant community, the endophyte was a liability in the impoverished desert soils and as a consequence the infection levels in the grass populations decreased. In another example, the need for continuous selective pressure to maintain the symbiosis may explain the frequent observation of reduction of arbuscular mycorrhizal diversity and population size in soils where abundant nutrients are present (Duke et al., 1994; Egerton-Warburton et al., 2007).

4. Some Functional Aspects

Wilcox et al. (2003) used full-genome microarrays to evaluate transcriptional response to temperature stress in *Buchnera* symbionts of aphids. Only modest shifts occurred in the transcriptome, primarily of heat-shock genes. However, these

genes are constitutively elevated in the symbionts also under nonstress conditions, with GroEL representing 10% of total protein in *Buchnera* cells (Baumann et al., 1996). This investment in protein stabilization appears to compensate for accumulating mutations of the proteins. A single nucleotide deletion affects a homopolymeric run within the heat-shock transcriptional promoter for ibpA, encoding a small heat-shock protein, also negatively affects thermal tolerance in *Buchnera* symbionts of the aphid *Acyrthosiphon pisum* (Dunbar et al., 2007). Another involvement of heat-shock response in mediation of thermotolerance was shown in the interaction of a rhizosphere fungus with *Arabidopsis*. While Monocillin I produced by the fungus inhibits function of HSP90, this metabolite promotes heat tolerance of *Arabidopsis* seedlings after induction of HSP101 and HSP70 (McLellan et al., 2007). There seems to be a general function of chaperones and other function-stabilizing proteins in symbiosis-mediated stress tolerance.

On the other hand, marked differences among mutualists can occur at other metabolic interfaces. The mutualism conferred to plants by a mutant *Colletotrichum* strain (Path-1) involved that peroxidase and phenylalanine ammonia-lyase activity and lignin deposition increased within 24 h after exposure to a virulent pathogen (Redman et al., 2001). The priming effect is localized to tissues colonized by the mutualist and is not systemic. Contrarily, the root endophyte *Piriformospora indica* is apparently systemic and is thought to resist necrotrophs by the increase of glutathione-ascorbate antioxidant systems (Waller et al., 2005). Stein et al. (2008) showed that two jasmonate-signaling mutants were nonresponsive to *P. indica*. Jasmonic acid-responsive vegetative storage protein expression was otherwise primed and elevated in response to powdery mildew, which is indicative of induced systemic resistance (ISR). The fungal effector involved in this mechanism is not known yet, but thought to be a component of the fungal cell walls, also because autoclaved fungal material seemingly invoked a similar response in the plants (A. Molitor, pers. comm.), as it does promote growth (Vadassery et al., 2009). These results indicate that several biochemical mechanisms are involved in biotic stress tolerance of symbiotic associations of plants with endophytic fungi.

5. Evolutionary Consequences

Symbiotic interactions shape evolution of the involved partners to variable extent. Long-term relationships including obligate and heritable partners left traces in the genomes of the symbionts. On the other hand, facultative interactions and horizontal transmission of partners drive the evolutionary success of populations.

Heritable symbiotic associations have profound consequences on the genomic architecture of the involved partners. This has been revealed most profoundly with the insect symbionts, including the smallest and fastest evolving genomes known (Moran et al., 2008, 2009; Nakabachi et al., 2006). Small genome sizes are also known in other endocellular symbioses, e.g., '*Candidatus* Glomeribacter gigasporarum' in arbuscular mycorrhizal fungi is with 1.35–2.35 Mb (depending on the method to

assess genome size) the smallest in Betaproteobacteria (Jargeat et al., 2004). Genomic erosion of obligately endosymbiotic bacteria of insects involves many genes considered to be essential for metabolism, as well as regulatory sequences of the residual set of genes (Dale and Moran, 2006). Facultatively symbiotic bacteria with a more recent history of association with their hosts often have genomes in transitional stages, with intermediate sizes. In contrast to the obligate primary symbionts, these genomes harbor mobile elements that may confer horizontal genetic exchange, as well as secretion systems with partly reduced functionalities, e.g., lacking genes encoding effector proteins (Dale and Moran, 2006). The dynamics of genomic erosion in symbiotic bacteria has recently been studied by Moran et al. (2009). Mutational rate in *Buchnera* genomes is ten times higher than in other known bacteria, and involves a shift toward higher AT content. This increases the frequency of homopolymeric sequences and indel mutations by replication slippage. As purifying cannot eliminate all mutations, genomes are prone to a vortex of genomic and functional reduction.

The hyphae of the rice seedling blight fungus *Rhizopus microsporus* harbor *Burkholderia* sp. as endosymbionts (Partida-Martinez and Hertweck, 2005). These bacteria produce the polyketide macrolide rhizoxin, which blocks mitosis in most eukaryotes. The fungal host remains not only unaffected but rather requires the bacterial endobiont to complete its life cycle (Partida-Martinez et al., 2007a, b). Schmitt et al. (2008) found by rhizoxin sensitivity assays amino acids, which convey rhizoxin resistance. Sensitivity to rhizoxin likely represents the ancestral character state and evolution of resistance took place in the ancestor of extant-resistant Zygomycota. These findings suggest that endosymbiosis became possible through a parasitism – mutualism shift in tolerant fungi. Such a shift has also been observed experimentally after long-term (5 years) culture of bacteria-infected amoeba by Jeon (1972). Initially, the bacteria were harmful to the amoeba, but after a few years the amoeba became dependent on the internalized bacteria. Transition toward mutualism from pathogenic ancestors are also hypothesized for clavicipitaceous endophytes (Schardl and Leuchtmann, 2005), as well as for *Hamiltonella* symbionts of sap-feeding insects (Degnan et al., 2009). The shift from parasitism to mutualism is apparently not a one-way course. Analysis of fungal evolution shows that multiple losses of lichen symbioses likely took place in the evolution of the ascomycetes, with parasitic lineages stemming from lichen-forming ancestors, likely with lichen–parasitic intermediate stages (Lutzoni et al., 2001).

The ubiquitous facultative symbiotic partnerships may have significant roles in natural ecosystems. Endophytic fungi that enhance the tolerance of plants to abiotic stress factors and diverse community of helper bacteria in the rhizosphere strengthen the performance of the plant root system. It is now well established that plants have species-specific bacterial communities, but abundance and composition of the associated bacterial communities of plants varies among species and differ in slightly different ecological settings (Smalla et al., 2001; Berg and Smalla, 2009). The question is raised how these plant species-specific patterns evolve. Are they a consequence of plant evolution and adaptation to new niches,

or is niche colonization enabled by shifting associated bacterial communities? Perhaps both. In a study by Germida and Siciliano (2001), the evolutionary history of plant–microbe interactions was revealed with cultivated plants: old wheat cultivars were colonized by phylogenetically diverse rhizobacteria, whereas the rhizosphere of modern cultivars was dominated by fast-growing proteobacteria.

We hypothesize that symbiotic associations modulate the fitness of hosting individuals and populations, as it is also postulated by the hypothesis of habitat-adapted symbioses (Rodriguez et al., 2008). Positive fitness effects exerted by symbionts likely influence their genotype frequencies. Such impact of associated organisms is in our opinion an underestimated factor in the evolution of biodiversity. Survival of the fittest may be the result of selection of those who live in optimal symbiotic associations. A beneficial bacterial community could even compensate for fitness-lowering mutations in the symbiotic hosts. Symbiosis may thus bypass selective forces that would act on the hosting partner in solitude. This may be a first step toward evolution of obligate symbiotic associations. We generally envisage an evolutionary phase transition in the evolution of symbioses: from a phase of dynamic selection of habitat-specific facultative mutualists toward evolving specific and lasting partnerships with division of function among the partners. A fascinating example for the final stage of division of nutritional functionalities is the symbiosis of the sharpshooter *Homalodisca coagulata* with the bacteria *Baumannia cicadellinicola* and *Sulcia muelleri* (Wu et al., 2006). The insect feeds on xylem fluids of the host plants as a carbon source, but this diet lacks essential amino acids and vitamins that cannot be produced by the insects. This function is mutually complemented by the two bacterial symbionts: *B. cicadellinicola* synthesizes vitamins and cofactors, whereas *S. muelleri* produces most or all of the essential amino acids.

Direct effects of symbionts on the life cycle of a host can rapidly alter the genetic structure of populations. In the absence of the endosymbionts, the host failed in vegetative reproduction (Partida-Martinez et al., 2007b), implying the symbiont produces factors essential for completion of the fungal life cycle. In other symbioses there are more specific effects on the reproductive capabilities. The endosymbionts *Wolbachia*, *Cardinium*, and *Spiroplasma* can modulate the sex rate shifts toward female offspring of its arthropod hosts (Moran et al., 2008). Interspecific cytoplasmic incompatibility caused by reproductive manipulators can lead to hybrid inviability and reinforcement, and consequently to reproductive isolation and speciation (Jaenike et al., 2006).

6. Flexibility

The relative amounts of symbionts vary in some symbioses and may expand the ecological amplitude of the symbiotic organisms. Climatic alteration experienced by a lichen species over the latitudes of its distribution causes a shift in the relative abundance of algal producer versus fungal consumer. Due to higher respiration

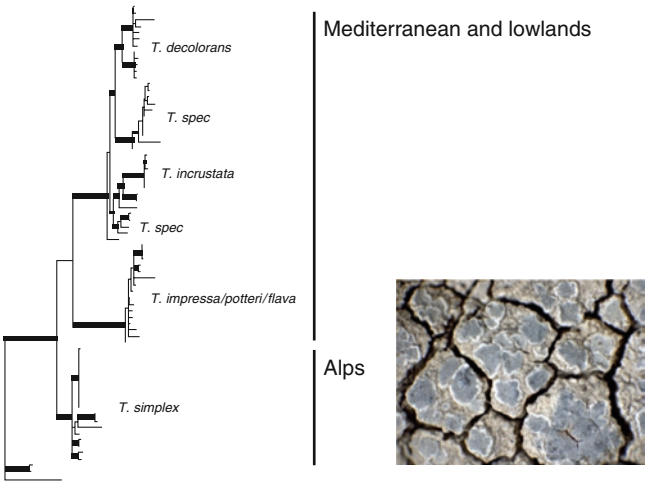

Figure 2. Symbiont switches in a widespread lichen species correlated with habitat ecology. Phylogeny of algal symbionts with strains found in *Lecanora rupicola*, with bars indicating photobionts selected in different habitats in Europe. *Right*: habit of *Lecanora rupicola* (see Blaha et al., 2006).

rates at warmer sites more algal mass is required to support fungal growth in the thallus. This phenomenon was used to prove the community adaptation hypothesis introduced by Sun and Friedman (2005). However, if the demands of the hosting (i.e., structure-providing) symbiont cannot be fulfilled by changing the ratios of hosted symbionts, switching to a more appropriate symbiont is observed in different types of symbioses, including coral symbioses with *Symbiodinium* or lichen symbioses with *Trebouxia* algae (Fig. 2). Capacity to switch symbionts according to ecological requirements contributes to robustness of symbioses (e.g., Baker et al., 2004). Nonetheless, it is not yet clear if this robustness is also strong enough to prevent irreparable damage to populations of many coral symbioses that are now under threat with raising temperatures of seawater.

7. Increasing Robustness by Multisymbioses?

Many cases of symbioses that have been traditionally considered as partnerships of two species turned out to be more complex and finely balanced systems. In addition to the previously recognized key partners, they include a considerable number of additional microbial partners (Fig. 3). Multibiont systems are clearly evident and species-rich if one considers the gut flora of animals, which are well studied in a wider range of organisms (e.g., Brune, 2005; Moran et al., 2005; Rajilic-Stojanovic et al., 2007). The human body is a rather massive symbiotic system on two feet, carrying ten times more bacterial than human cells, with a

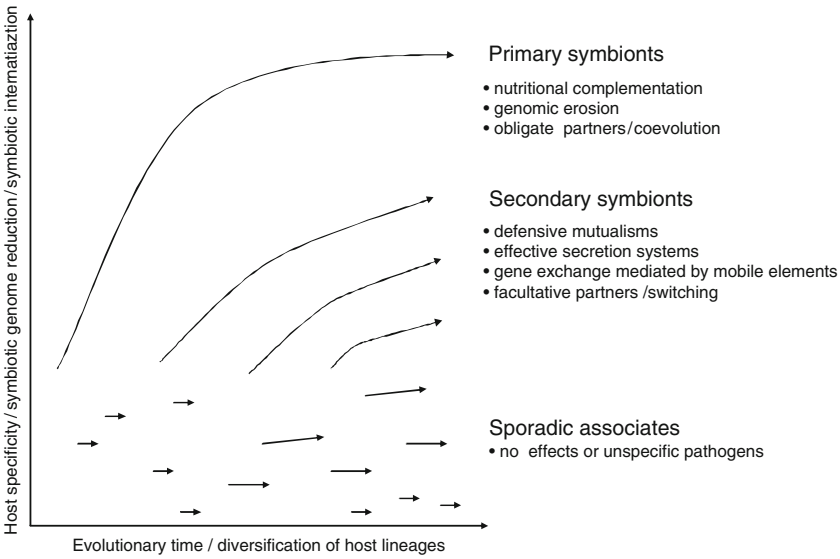

Figure 3. Scheme of symbiotic evolution with evolutionary trajectories, as seen with insect bacterial symbioses. Symbioses can comprise primary symbionts that show a high level of genomic erosion and are often involved in resolving nutritional stress. Secondary symbionts, which can switch among hosts, are more frequently involved in other functions, including defensive mutualism or abiotic stress tolerance. They are also less subject to genomic erosion and may maintain many other functions (see Dale and Moran 2006). Among secondary symbionts, mutualists may arise from pathogenic ancestors, while sporadic associates do not express relevant effects on the hosts.

great variety of microhabitats (such as skin, oral cavity, intestine, etc.). We are only at the beginning of developing an understanding of how deeply our symbionts influence our lives. Considering that "every eukaryote is a superorganism" and "all plants and animals involve complex ecological communities of microbes," Sapp (2003) coined the term "symbiomics" as a research field to study these symbiotic systems, or symbiomes (Zook 1998).

Modern research contributes in many ways to a better understanding of symbioses. First, we must reexplore traditional symbioses with new tools to discover additional partners. The leaf-cutter ant symbiosis was seen as an example of fungal agriculture by ants, to ferment plant leaf material with the help of a fungus (*Leucoagaricus* spp.). Meanwhile, additional symbiotic partners are being recognized: the fungal culture of the ants can be invaded by a fungal parasite (*Escovopsis*). To defend against this pathogen, the ants carry bacteria (*Pseudonocardia*, belonging in the order Actinomycetales) on their thoraces. These antagonists produce a substance that counteracts the *Escovopsis* contaminations. The actinobacterial effect compromises of a black fungus occurring in the ant colonies, with affinity to *Pseudonocardia* (Little

and Currie, 2008; Little, this volume). Latest research indicates that even nitrogen fixation by Alphaproteobacteria takes place in the ants nests (Pinto-Tomás et al., 2009). The multiple players with their beneficial and antagonistic roles in this symbiosis contribute to the stability in the ants' "homoeostatic fortresses" (Hughes et al., 2008).

A tripartite association between a virus, a fungus, and a plant confers thermal tolerance on both the fungus and the plant (Márquez et al., 2007). Márquez et al. found two viral RNA segments and subsequently purified infectious viral particles from *Curvularia protuberata* and named the virus *Curvularia* thermal tolerance virus (CThTV). This virus appears to be essential in abiotic stress tolerance, which clearly shows that functional interactions in a multisymbiont context may have been previously underestimated. Viruses can significantly interfere with the fungal host metabolism and are able to suppress the host's RNA silencing machinery (Hammond et al., 2008). Not surprisingly, recent research by Herrero et al. (2009) indicates that viruses are frequently found in endophytic fungi. The roles of viruses in symbiotic interactions are still little explored, but we expect exciting new insights from future research.

The fungus *Geosiphon pyriforme* (Glomeromycota) forms a unique phototropic association formed by club-like bladders exposed on soil surface. The huge vesiculate cells of *Geosiphon* contain large numbers of *Nostoc* chains internally (Schüßler and Kluge, 2001), and proteobacteria as well. They were originally termed bacteria-like organisms (BLOs; Schüßler et al., 1994), and later assigned to the *Oxalobacter* group of Betaproteobacteria (Volz, 2004). It is still unclear what role the proteobacteria have in the *Geosiphon* symbiosis.

Complex symbioses can also comprise endobacterial bacteria, as was shown for mealybugs, which feed on plant sap. Von Dohlen et al. (2001) showed that the cells of Betaproteobacteria in the mealybug bacteriomes host Gammaproteobacteria. The authors suggested that this endobacterial symbiosis could represent a kind of compensation of the primary betaproteobacterial symbionts through genetic exchange with their intracellular symbionts.

These are merely a few examples of the complexity of symbioses with many partners, and a list of further examples would fill yet another book. What can be extracted as a general pattern observed in these multisymbioses? Depending on the tightness of interaction, there are dominant partners that shape the morphology of the systems (we may call these the "macrosymbionts"). These symbionts also build the structures to host obligate microsymbionts (such as the primary or P-symbionts in bacteriomes of insect symbioses). In addition, further and often facultative partners can be more diverse and are more often horizontally transmitted; thus, switches between symbionts among macrobiont lineages are more likely (e.g., the secondary or S-symbionts of insect systems). The secondary symbionts can contribute to various complementary and partly redundant functions of the symbiomes, which may or may not be essential for the dominant partners. We argue that these partners could have profound beneficial effects when a symbiotic system suffers diverse stress conditions. In many symbioses, obligate

symbionts are not evident, but facultative symbionts are common and important regulators of natural host populations. Populations with facultative symbiotic partners that help ameliorate various stresses contribute to genetic propagation of the hosts and further support the evolution of symbiotic interactions. Redundant functionalities of multiple microbial symbionts, which co-occur or occur in different habitats may buffer stress factors, and thereby contribute to homoeostasis and adaptivity of the whole symbiotic system, especially if the symbionts have different ecological optima. We suggest that weakly linked facultative partners in symbioses may also promote evolution of highly diversified macrosymbiont groups, including higher plants, insects, lichenized fungi, and others. Future work should seek to explore the functional roles of symbionts and their importance in symbiotic systems under changing natural conditions.

8. Conclusions

Alleviation of stress is a common function in mutualistic symbioses. In contrast, in pathogenic interactions, an increase in biotic stress is the primary impact to the host organism. Both mutualistic and pathogenic interactions involve similar interaction mechanisms that need to be studied in greater detail in the future. To investigate the functional contributions in symbioses is not a simple task, especially if the obligate partners are hard to grow in vitro, and when genomic information is lacking. New tools are now available to analyze metabolic, enzymatic, and transcriptional activities, as well as epigenetic effects of symbiotic partners directly (meta-metabolomics, metaproteomics, metatranscriptomics, metamethylomics, etc.). Also, new microscopic approaches such as confocal laser scanning microscopy and fluorescence in situ hybridization lead to new insights into spatial patterns in symbiotic systems (e.g., Grube et al., 2009), while microspectroscopy or secondary ion mass spectrometry imaging can be used to study ecophysiology of single cells (Wagner, 2009). These methods pave new ways for exciting discoveries on symbiotic interactions in the next decade.

Stress amelioration in symbioses may contribute massively to evolutionary progress and diversification of life (Margulis and Fester, 1991). The effects of stress can drive symbioses to develop optimally functioning partnerships. This may occur by selection for optimal symbiont combinations (short-term strategy) and/or by genetic adaptation to an optimized symbiotic intercourse (long-term strategy) at the limits between facultative and obligate partnerships. Rodriguez et al. (2008) suggested that habitat-adapted symbiosis represents a common non-Darwinian adaptation in biology. Evidence is increasing for the fact that colonization of hosts by symbionts, which confer stress tolerance, is an important component of plant adaptation to environmental stresses and allows plants to conquer new habitats. Stress, in all of its aspects, is an important factor influencing health and vitality of organisms. Because this also affects our agricultural organisms, all of which live in tight symbiotic relationships with other organisms, it is

in our interest to further expand research on stress and symbioses in times of environmental change.

Thus, the practical impact of research on symbiosis and stress is clear since we are facing changing environments worldwide. For example, progressing aridity and salinity of soils is threatening future agriculture, especially in warmer regions. Various microbial inoculants are already available, which promote plant growth and health by antagonism against detrimental organisms and other stresses (Berg, 2009). The practical exploitation of beneficial symbiotic interactions will offer an environmentally compatible alternative to chemical pesticides and fertilizers for sustainable crop production.

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PART II:
SYMBIOTIC ORIGIN OF EUKARYOTES

Curtis
Archibald
Vesteg
Karjčovič
Löffelhardt

Biodata of **Bruce A. Curtis**, author with **John M. Archibald** of ***"Problems and Progress in Understanding the Origins of Mitochondria and Plastids"***

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PROBLEMS AND PROGRESS IN UNDERSTANDING THE ORIGINS OF MITOCHONDRIA AND PLASTIDS

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1. Introduction

Mitochondria and plastids (chloroplasts) are membrane-bound organelles of endosymbiotic origin, the energy-generating functions of which lie at the heart of eukaryotic cell biology. All known eukaryotes have, or are believed to have once had, mitochondria (Embley and Martin, 2006) and in the case of plastids, which evolved after mitochondria, even secondarily non-photosynthetic algae and plants retain the organelle as the site of important processes such as isoprenoid and heme biosynthesis (Gould et al., 2008). These observations underscore the extensive coevolution that must have occurred between endosymbiont and host during the initial establishment of these quintessential eukaryotic organelles.

While the idea that mitochondria and plastids descend from once free-living prokaryotes has now been proven beyond all reasonable doubt (Gray and Doolittle, 1982; Gray, 1992), the details surrounding the earliest phases of their evolution remain obscure. This is due in large part to the tremendous evolutionary gulf that exists between mitochondria and plastids and the modern-day relatives of their putative bacterial predecessors to which they can be compared. Decades of research has shown that plastids and mitochondria are both fundamentally prokaryotic in nature (Gray and Doolittle, 1982; Gray, 1992) but also that they are highly derived entities whose evolution was (and to a certain extent still is) greatly influenced by the hosts in which they evolved. The host cell in which plastids evolved was undoubtedly a nucleus- and cytoskeleton-containing eukaryote, given that "primary" plastid-bearing organisms clearly represent a distinct sub-branch on the eukaryotic evolutionary tree (Reyes-Prieto et al., 2007). In contrast, there is no consensus as to the nature of the host involved in the origin of mitochondria. Was it a eukaryote or prokaryote, or something in between? What metabolic capabilities defined the host and endosymbiont cells and what role did they play in shaping the

trajectory of this landmark event? To what extent did changing environmental parameters (e.g., oxygen concentration) play a role?

This chapter addresses contemporary views on the early events leading to the establishment of mitochondria and plastids. The similarities and differences in our perceived knowledge of the genesis of these organelles are discussed in the context of advances in the areas of comparative genomics, biochemistry, and cell biology.

2. What is an Organelle?

The term "organelle" is often applied loosely in reference to any discrete subcellular structure with a specialized function, such as the nucleus, endoplasmic reticulum, Golgi apparatus, basal bodies, and vacuoles. For the purposes of this chapter, we shall limit its use to include only membrane-bound, DNA-containing structures of exogenous (i.e., endosymbiotic) origin: mitochondria and plastids.

Opinions vary as to precisely what distinguishes an organelle from an endosymbiont, but most definitions center on the extent to which the endosymbiont and host are genetically and cell biologically integrated. Comparison of the coding capacity of present-day mitochondrial and plastid genomes to their prokaryotic counterparts clearly indicates that organelle genesis involves massive intracellular DNA transfer from the endosymbiont to the host cell nucleus (Martin and Herrmann, 1998; Martin et al., 2002), a process referred to as endosymbiotic gene transfer (EGT; Martin et al., 1993). Together with EGT, the evolution of a dedicated system for importing the protein products of transferred genes back to their compartment of origin is seen as the critical step in the transition from endosymbiont to full-fledged organelle (Theissen and Martin, 2006; Cavalier-Smith, 2007). Mechanisms for the exchange of metabolites between the endosymbiont and host must also have been important (Andersson and Kurland, 1999; Weber et al., 2006).

In the case of canonical mitochondria and plastids, the distinction between endosymbiont and organelle is clear-cut. However, as will be discussed below, the boundary becomes blurred when one considers organisms such as the testate amoeba *Paulinella*, an enigmatic protist with permanent photosynthetic "chromatophores" of "recent" cyanobacterial origin (Nowack et al., 2008). We shall deal first with advances in our understanding of the evolution of mitochondria and mitochondrion-derived organelles.

3. Mitochondria

Textbook accounts of the origin of mitochondria usually invoke an association between some sort of primordial host cell and an oxygen-respiring bacterial endosymbiont, the latter gradually surrendering most (but not all) of its genetic material to the host and evolving into the cellular "powerhouse" whose biochemistry is now so well understood. This vague scenario is appropriate given that beyond

this level of detail, there are few issues on which specialists in the field unanimously agree. Hypotheses for the origin of mitochondria are of two general sorts, those that posit the evolution of many or most eukaryote-specific cellular features prior to the origin of the mitochondrion, and those proposing that the organelle evolved prior to, or contemporaneously with, the eukaryotic cell. These ideas are continuously evolving in response to advances in our understanding of eukaryotic phylogeny and new data from evolutionarily diverse eukaryotic microbes.

3.1. MITOCHONDRIAL DNA

What do genome sequences tell us about the origin of mitochondria? It is perhaps fitting to begin with this question, as the first definitive DNA sequence-based evidence in favor of a bacterial ancestry for mitochondria was obtained in the 1970s at Dalhousie University in Nova Scotia, Canada (Bonen et al., ; Cunningham et al., 1977; Gray, 1992). While the coding capacity of mitochondrial genomes is extremely limited – ranging from a mere five genes in the apicomplexan parasite *Plasmodium* to 97 in the jakobid flagellate *Reclinomonas* (Gray, 1998; Gray et al., 1999) – they retain sufficient historical signal to roughly pinpoint their closest bacterial relatives, whose genomes are typically an order of magnitude greater in size.

Several decades' worth of phylogenetic analyses using ever-increasing amounts of genomic data point to the same bacterial lineage originally posited as the mitochondrial progenitor prior to the availability of DNA sequences, the purple non-sulfur bacteria, belonging to Alphaproteobacteria (John and Whatley, 1975). More specifically, the Rickettsiales, a group of obligate intracellular parasites, have emerged as strong candidates for the closest modern-day relatives of mitochondria. The complete genome of the typhus pathogen *Rickettsia prowazekii* was sequenced in 1998 (Andersson et al., 1998), providing an interesting point of comparison and at the same time highlighting the difficulties of inferring ancient evolutionary events from genomic data.

At ~1.1 million base-pairs in size, the *R. prowazekii* genome is obviously much larger than a mitochondrial genome, but it is nevertheless significantly smaller than most bacterial genomes and shows many of the hallmarks of reductive evolution, such as the presence of pseudogenes and the absence of genes involved in nucleotide and amino acid synthesis (Andersson et al., 1998). It is thus tempting to speculate that perhaps mitochondria evolved from a *Rickettsia*-like bacterium. However, on balance, the data suggest that the intense genome reduction seen in mitochondria and the modest reduction observed in rickettsial genomes is the result of independent adaptations to intracellular life (Gray, 1998). Indeed, while even the most sophisticated molecular analyses typically resolve mitochondria and Rickettsiales as sister lineages (e.g., Andersson et al., 1998; Barbier et al., 2005), there are still nagging concerns over whether this relationship is an artifact. This is because gene sequences from both mitochondria and Rickettsiales are characterized by accelerated rates of substitution and their

proteins exhibit amino acid composition biases, features that are known to cause problems in molecular phylogenies (Barbier et al., 2005). It is interesting to note that a recent comprehensive analysis of mitochondrial, bacterial, and nuclear genomic data (Esser et al., 2004) suggested that another alphaproteobacterium, *Rhodospirillum rubrum*, appears to be at least as closely related to mitochondria as are members of the Rickettsiales.

While there is general consensus as to the alphaproteobacterial (and perhaps rickettsial) origin of the mitochondrion, the same cannot be said for the mitochondrial proteome. Modern-day mitochondrial genomes encode at most ~100 proteins, but 1,000 or more proteins are needed to service the organelle. Many nucleus-encoded, mitochondrion-targeted proteins such as chaperonin 60 do exhibit an alphaproteobacterial affinity (reviewed by Roger, 1999), as one would predict, but many others do not (Esser et al., 2004). A recent large-scale mitochondrial proteomic analysis in the ciliate *Tetrahymena paramecium* revealed that <20% of functionally annotated proteins were in fact demonstrably alphaproteobacterial in origin (Smith et al., 2007), similar to percentages gleaned from the yeast *Saccharomyces cerevisiae* (Karlberg et al., 2000). The mitochondrion itself very likely has a unique origin, but its proteome appears to be a mosaic of proteins with diverse evolutionary histories.

3.2. EVOLVING VIEWS: ARCHEZOA, OXYGEN, AND PROTEIN IMPORT

Early models for the evolution of mitochondria can be summarized roughly as follows: (1) both prokaryotic (Sagan, 1967) and eukaryote-like (Whatley et al., 1979) cells were considered as possible hosts, (2) some sort of aerobic bacterium was typically cast in the role of endosymbiont (John and Whatley, 1975), and (3) assuming the host cell was an anaerobe, oxidative phosphorylation was usually invoked as the prime selective advantage driving endosymbiont integration (Whatley et al., 1979). Building upon previous suggestions that cytoskeleton-mediated phagocytosis should be a prerequisite for endosymbiosis (de Duve, 1969; Stanier, 1970), Cavalier-Smith (1987) proposed the Archezoa hypothesis as an attempt "to dissociate the symbiogenetic origin of mitochondria from the most fundamental changes in eukaryogenesis" (Cavalier-Smith, 2007). An important prediction of the hypothesis was that living descendents of a possible amitochondriate phase of eukaryotic evolution might still exist. Several protist lineages thought to lack mitochondria, including the diplomonads (e.g., *Giardia*), parabasalids (e.g., *Trichomonas*) and microsporidia (e.g., *Vairimorpha*), quickly became the focus of intense study by cell evolutionists.

The first molecular phylogenies that included small subunit ribosomal RNA (SSU rRNA) gene sequences from putatively mitochondrion-lacking organisms were consistent with the Archezoa hypothesis in that the amitochondriates branched from the main eukaryotic line prior to the divergence of mitochondrion-containing

groups such as animals, plants, and fungi (reviewed by Roger, 1999). These trees generated considerable excitement, but the "deep branching" position of diplomonads, parabasalids, and microsporidia in rRNA trees was eventually deemed to be a phylogenetic artifact (Philippe and Germot, 2000). More importantly, all of the original archezoan taxa are now believed to possess mitochondrion-derived organelles (see below).

While the existence of extant primitively amitochondriate eukaryotes no longer seems likely, Cavalier-Smith continues to argue strongly for the importance of phagotrophy and for the evolution of a cytoskeleton *prior* to the origin of the mitochondrion (Cavalier-Smith, 2007). Somewhat more controversially, he has also proposed that the alphaproteobacterial mitochondrial progenitor was a phototroph with "tubular chromatophores," as seen in *Rhodospirillum rubrum*, which ultimately gave rise to the cristae seen in today's mitochondria (Cavalier-Smith, 2007). (Recall that genome phylogenies do not exclude the possibility that *R. rubrum* and its relatives could be the closest extant ancestors of mitochondria; Esser et al., 2004). The idea is that the products of photosynthesis generated by such an endosymbiont would have been of great benefit to the proto-eukaryotic host, providing a very strong initial selective pressure toward permanent "enslavement." Access to photosynthate was made possible by the insertion of host-derived carrier proteins. Over time, the photosynthetic abilities of the endosymbiont were lost in conjunction with the evolution of additional carriers, including ATP/ADP translocases that allowed the host to take full advantage of the ATP produced by the endosymbiont (Cavalier-Smith, 2007).

The "ox-tox" hypothesis of Kurland, Andersson, and colleagues (Andersson and Kurland, 1999) is a variation on the classical endosymbiont hypothesis that emphasizes aerobic respiration and ATP synthesis as key features of the mitochondrial progenitor. In this scenario, the alphaproteobacterium was a facultative aerobe, oxygen consumption of which served to detoxify the increasingly aerobic environment in which anaerobic proto-eukaryotes found themselves. The alphaproteobacterium entered into an endosymbiotic relationship with such an organism, consuming oxygen within its cytoplasm, and the relationship between the two cells was eventually cemented by the evolution of the above-mentioned host-derived ATP/ADP translocases (Andersson and Kurland, 1999).

Although often overlooked, central to any scenario for the origin of mitochondria is the evolution of the protein import machinery. In modern-day eukaryotes the apparatus is comprised of several large, multi-protein complexes, including TOM (translocase of the outer membrane), TIM (translocase of the inner membrane), and SAM (sorting and assembly machinery; Dolezal et al., 2006). Together these complexes serve to translocate hundreds of nucleus-encoded mitochondrial proteins, most of which possess short amphipathic amino-terminal extensions ("pre-sequences") that are recognized by TOM receptors. How did this complex machinery evolve?

Cavalier-Smith believes that the mitochondrial protein import system is ultimately derived from the bacterial protein export apparatus, with some

"key novelties" having been provided by the host cell (Cavalier-Smith, 1983, 2007). Efforts to confirm or refute this notion have been hindered by the fact that most TOM–TIM complex proteins are highly divergent and thus not amenable to comparative sequence analysis. Nevertheless, a number of them are demonstrably bacterial in origin, such as the β-barrel protein Sam50, which is a clear homolog of the bacterial outer-membrane protein Omp85 (Gentle et al., 2004; Dolezal et al., 2006). Others (e.g., TIM22 and TIM23 complex proteins) have no obvious bacterial counterparts and may be host-derived (Dolezal et al., 2006). Cavalier-Smith has proposed that during the early stages of endosymbiont integration, extensive EGT would have resulted in the erroneous targeting of many bacterial surface membrane proteins to the host cell's endomembrane system. This potentially deleterious situation could be rectified if the amino-terminal signal sequences of such proteins were modified so that they were no longer recognizable by the host's signal recognition particle and instead conferred specificity for the precursors of today's TOM receptors. In this model, the evolution of pre-sequence-mediated mitochondrial protein import can be thought of as an example of "phenotypic suppression," whereby the system evolves simply as a way of coping with the inevitable flood of genes moving from the endosymbiont to the host nucleus (Cavalier-Smith, 2007). There is presently very little in the way of solid data to go on, other than to say that regardless of how it evolved, the mitochondrial protein import pathway was undoubtedly established in the common ancestor of all known eukaryotes with contributions from both endosymbiont and host (Dolezal et al., 2006).

3.3. MITOCHONDRION-DERIVED ORGANELLES

Mitochondria are generally thought of as aerobic organelles. Less well appreciated is the fact that they are also capable of generating ATP under anaerobic conditions, e.g., by using terminal electron acceptors other than oxygen (e.g., fumarate), as in some parasitic worms (Tielens et al., 2002). It is now clear that on multiple occasions and in distantly related eukaryotic lineages, mitochondria have been radically transformed into specialized anaerobic organelles, in some cases almost to the point of becoming unrecognizable.

The best-known mitochondrion-derived organelle is the hydrogenosome, first described in the parabasalid flagellate *Tritrichomonas foetus* by Müller and colleagues in the 1970s (Lindmark and Müller, 1973). Hydrogenosomes are double-membrane-bound anaerobic organelles that generate ATP by substrate-level phosphorylation, acetate, CO_2, and, as the name suggests, hydrogen. These organelles are remarkable in that they completely lack a genome, a fact that has made their evolutionary origin difficult to discern. In addition to parabasalids, "textbook" hydrogenosomes are found in anaerobic ciliates and chytrid fungi (Hackstein et al., 2007). Even more unusual is the "mitosome," a vaguely defined double-membrane organelle found in a diverse array of anaerobic/microaerophilic parasites, including diplomonads, entamoebids (e.g., *Entamoeba histolytica*), and microsporidia

(e.g., *Trachipleistophora*; reviewed by Tovar, 2007). Where studied, mitosomes appear to lack a genome. Remarkably, in the diplomonad *Giardia*, the organelle no longer participates in energy generation, with the only known function currently ascribed to it being the synthesis of iron–sulfur (Fe-S) clusters (Tovar et al., 2003).

On the basis of its unusual biochemistry, the parabasalid hydrogenosome was initially suggested to have evolved from an anaerobic *Clostridium*-like bacterium, distinct from the endosymbiotic event that gave rise to the mitochondrion (Whatley et al., 1979). This hypothesis received little in the way of empirical support and has been replaced by the idea that hydrogenosomes share a common origin with mitochondria (Embley et al., 2003). In hindsight, this seems obvious in the case of hydrogenosome-bearing ciliates and fungi, which are phylogenetically nested within aerobic, mitochondriate groups. However, the same logic cannot easily be applied to the hydrogenosomes of parabasalids or the mitosomes of diplomonads, neither of which have obvious mitochondriate sister lineages. In the absence of a genome, phylogenetic analysis of nucleus-encoded proteins targeted at the hydrogenosome and mitosome provided some of the strongest initial clues. Such analyses have occasionally produced conflicting results (e.g., Dyall et al., 2004; Hrdy et al., 2004), but on balance they support the idea that hydrogenosomes and mitosomes are derived from mitochondria (reviewed by Embley et al., 2003; Barberà et al., 2007).

Ultimately, the strongest link between hydrogenosomes and mitochondria has been the discovery of "transitional forms" exhibiting characteristics of both organelles. A hydrogenosome with a genome has been identified in the ciliate *Nyctotherus ovalis* (Boxma et al., 2005) and, more recently, the anaerobic organelle of the stramenopile (heterokont) *Blastocystis hominis* has been shown to possess a genome that is demonstrably mitochondrial in nature (Perez-Brocal and Clark, 2008; Stechmann et al. 2008). The *B. hominis* "mitochondrion" possesses some (but not all) components of the classical mitochondrial electron transport chain as well as a partial tricarboxylic acid cycle, but also contains pyruvate:ferredoxin oxidoreductase (PFO) and iron hydrogenase, as in hydrogenosomes (Stechmann et al., 2008). This suite of features is curiously similar to that seen in the unrelated ciliate *N. ovalis* (Boxma et al., 2005), and represents a remarkable example of convergent evolution in response to anaerobiosis (Stechmann et al., 2008).

In sum, when the full range of eukaryotic diversity is considered, mitochondria can be seen as a continuum ranging from "standard" aerobic mitochondria at one end to the highly reduced Fe–S-cluster-forming mitosomes at the other (Howe, 2008). Beyond Fe–S-cluster biogenesis, it remains to be seen what (if any) biochemical processes are truly a feature of all mitochondria and mitochondrion-derived organelles.

3.4. SYNTROPHY

We conclude this section with a brief discussion of two recent and provocative ideas for the origin of mitochondria, focusing on the hydrogen hypothesis

of Martin and Müller (1998). The hydrogen hypothesis emphasizes metabolic symbiosis (i.e., syntrophy) as the driving force behind a concomitant evolution of mitochondria and eukaryotes, resting on the assumption that eukaryogenesis occurred in an anaerobic environment. As in traditional schemes, Martin and Müller posit that the mitochondrial precursor was an alphaproteobacterium, but in this case it made a living by anaerobic fermentation and generated CO_2 and molecular hydrogen as waste products (Martin and Müller, 1998). This bacterium entered into an association with an autotrophic methane-producing archaeon (archaebacterium), the metabolism of which was dependent upon the CO_2 and H_2 generated by its partner. Over time, the metabolic link between the two cells became increasingly intimate, to the point that the alphaproteobacterium ended up inside the methanogen and completely dependent upon it for energy. This was made possible by the initial transfer of key genes from the symbiont to the host that allowed the methanogen to import organic molecules, carry out glycolysis, and provide its endosymbiont with ATP (Martin and Müller, 1998).

The syntrophy hypothesis of Moreira and Lopez-Garcia (1998) is conceptually related to the hydrogen hypothesis and will not be elaborated upon here. Briefly, the scenario begins with a metabolic association between two prokaryotic entities, an H_2- and CO_2-generating, sulfate-reducing Deltaproteobacterium and a methanogenic archaeon (Moreira and Lopez-Garcia, 1998). The main difference between the two models is that in the hydrogen hypothesis, the mitochondrial-hydrogenosomal progenitor evolved directly from the alphaproteobacterial (endo)symbiont, while the syntrophy hypothesis posits the involvement of both Delta- and alphaproteobacteria, the latter ultimately evolving into the proto-mitochondrion.

Critics of the hydrogen and syntrophy hypotheses (e.g., Poole and Penny, 2007) point to the mechanistic issues surrounding cellular engulfment: there are many examples of syntrophic associations between methanogens and hydrogen-producing organisms and organelles (e.g., hydrogenosomes; Fenchel and Finlay, 1995), but archaea are not known to take up bacterial endosymbionts, and without a cytoskeleton it is not clear how they would do so. An additional shortcoming of both hypotheses is that neither explains the selective pressures and processes that ultimately gave rise to the myriad of cellular features that characterize modern-day eukaryotes, such as a cytoskeleton, endomembrane system, and nucleus. Perhaps the most controversial aspect of the hydrogen hypothesis is that it demands that the proto-mitochondrion possesses all the enzymes necessary for the eventual evolution of both classic (i.e., aerobic) mitochondrial and hydrogenosomal biochemistry. At face value, the patchy distribution of hydrogenosomes and mitosomes across the eukaryotic tree (Barberà et al., 2007) is more in line with the notion that these organelles evolved from mitochondria multiple times independently, as an adaptation to life in an anaerobic environment. Thus far, an important prediction of the hydrogen hypothesis, that hydrogenosome-specific proteins such as PFO and iron hydrogenase from diverse eukaryotic lineages should be monophyletic, has yet to be definitively confirmed or refuted (Barberà et al., 2007; Hackstein et al., 2007).

4. Plastids

Plastids arose from the engulfment and retention of photosynthetic cyanobacteria by heterotrophic eukaryotes. The general outline for this concept was first put forward at the beginning of the twentieth century (Mereschkowsky, 1905) and was later popularized by Margulis (1970). Transformation of a heterotrophic eukaryotic cell into a primary producer was a key event in the evolution of life, leading to a vast array of photosynthetic organisms capable of harvesting the sun's energy and, in the process, further oxygenating the atmosphere. While this vital acquisition of a cyanobacterium is indisputable, the exact mechanisms and processes by which it was established are not, and continue to generate considerable debate.

4.1. OXYGENIC PHOTOSYNTHESIS AND PLASTID EVOLUTION

Key among the questions of plastid evolution is how many times the organelle evolved. The preponderance of evidence seems to suggest that the successful engulfment and, more importantly, retention of a photosynthetic bacterium occurred once (Palmer, 2003). All subsequent eukaryotic photosynthetic organisms, no matter how complex, ultimately derive their plastids from this landmark "primary" endosymbiotic event. It is generally agreed that there are three primary photosynthetic lineages: the glaucophytes, red algae, and green plants. Because of their apparent monophyly these groups have been placed in the supergroup Plantae or Archaeplastida (Adl et al., 2005). This placement rests upon molecular phylogenetics and comparative analysis of a variety of host- and plastid-associated features.

When considering the origin and diversification of plastids, it is important to distinguish between the phylogeny of the plastids themselves versus that of the host organisms in which they reside (Howe et al., 2008). Various characteristics, such as the similarity of gene content in plastids, as well as plastid gene phylogenies, were considered as early evidence in support of the monophyly of plastids (reviewed by Palmer, 2003). However, initial phylogenetic analysis of the nucleus-encoded largest subunit of RNA polymerase II (Stiller and Hall, 1997) demonstrated a separation between red and green algae. Only with the advent of large-scale multi-gene, multispecies data sets has it been possible to recover the monophyly of primary plastid-containing eukaryotes with strong statistical support (Rodriguez-Ezpeleta et al., 2005; Burki et al., 2008). Nevertheless, some researchers are still not convinced, and suggest that there may be as-yet poorly understood artifacts contributing to the results of such analyses (Stiller, 2007).

4.2. SPREAD OF PLASTIDS

Despite their obvious significance, primary plastid-containing algae represent a small fraction of the known diversity of photosynthetic eukaryotes. Major algal

groups, such as cryptophytes, haptophytes, heterokonts, dinoflagellates, and euglenids, acquired their plastids by "secondary" endosymbiosis. In this process, a non-photosynthetic eukaryotic organism engulfs a photosynthetic eukaryote and retains the primary plastid (Archibald and Keeling, 2002; Palmer, 2003).

There are a number of significant differences between organisms containing primary plastids and those containing secondary plastids. In addition to the double-membrane plastid envelope derived from the cyanobacterium, secondary plastids possess one or two additional membranes, i.e., three or four in total. The first (outermost) membrane appears to be derived from the phagosomal membrane of the host cell, while the second additional membrane is thought to represent the plasma membrane of the algal endosymbiont. In addition, unlike primary plastids, which are found in the cytosol, secondary plastids reside within the lumen of the host cell's endomembrane system. As elaborated upon below, these additional membranes further complicate the targeting of plastid proteins encoded in the nucleus.

The scarcity of glaucophyte algae in nature, and thus the reduced opportunities for engulfment, may explain why, of the three primary plastid lineages, only red and green algae have been involved in secondary endosymbioses. Plastids derived from green algae are found in euglenids, a common group of flagellated protists, and in the relatively obscure chlorarachniophytes, an amoeboflagellate group characterized by long pseudopodia (Hibberd and Norris, 1984). The presence of chlorophylls *a* and *b*, which is a characteristic of green algae and plants, as well as molecular data, unequivocally identifies the origin of their plastids. However, it is equally clear that these two lineages acquired their green algal plastids in separate events. The two lineages are morphologically dissimilar (Hibberd and Norris, 1984), and extensive phylogenetic analysis of both host and plastid genes does not support the hypothesis that they share a common ancestor (Rogers et al., 2007; Takahashi et al., 2007).

Secondarily acquired red algal plastids have a much wider distribution and account for a significant portion of the photosynthesis carried out by aquatic organisms. Among their hosts are haptophytes and heterokonts (or stramenopiles) with the latter group including both unicellular (e.g., diatoms) and multicellular (e.g., kelps) forms. Cryptophytes contain a plastid of red algal origin, as well as a nucleomorph, which is the vestigial nucleus of the engulfed alga (Archibald, 2007). Along with chlorarachniophytes, cryptophytes are the only lineages known to possess this relict of the endosymbiotic process. Some dinoflagellates contain a secondarily acquired red algal plastid, while others possess tertiary plastids (see below). Finally, in a rather surprising turn of events, it is now clear that some apicomplexan parasites contain a plastid most likely derived from red algae (Waller and McFadden, 2005). Because these parasites are non-photosynthetic, their plastid is highly reduced, which has made it difficult to undertake phylogenetic studies to determine the placement of these plastids amid others whose primary purpose is photosynthesis.

One of the most energetically debated and researched areas of plastid evolution of the last 2 decades is the number of times red algal plastids have been

acquired by secondary endosymbiosis. Cryptophytes, heterokonts, and haptophytes were linked early on based on similarities in ultrastructure, and the kingdom Chromista was erected (Cavalier-Smith, 1986). All three lineages possess a four-membrane-bound plastid residing in the lumen of the endoplasmic reticulum: given this unusual feature, it was thought likely that such a capture could have occurred only once (Cavalier-Smith, 1986). It has been argued that the tubular mastigonemes of the flagella of heterokonts and cryptophytes are homologous (Cavalier-Smith, 1986). Heterokonts and haptophytes both possess fucoxanthin and chrysolaminarin, and share similarities in mitochondrial structures such as tubular cristae and three thylakoids per stack in their plastids (reviewed by Archibald and Keeling, 2002).

However, phylogenetic results, at least initially, tended not to confirm the placement of cryptophytes, heterokonts, and haptophytes as each other's closest relatives. Analysis of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (Daugbjerg and Andersen, 1997) and plastid small subunit ribosomal RNA (SSU rRNA) (Medlin et al., 1995) pointed toward separate endosymbiotic events as did nuclear SSU rRNA trees (Bhattacharya and Melkonian, 1995; Müller et al., 2001). More recent studies (Hackett et al., 2007; Burki et al., 2008) using concatenated datasets have consistently linked cryptophytes and haptophytes, but their relationship to heterokonts is ambiguous.

Central to the debate over the spread of plastids has been the chromalveolate hypothesis (Cavalier-Smith, 1999), which posits that not only did chromists acquire their plastids in a single endosymbiotic event, but that plastids in the alveolate lineages – dinoflagellates and apicomplexans – also arose from the same event. The chromalveolate hypothesis thus links all secondarily acquired red algal plastid-containing organisms into a single super group (Keeling et al., 2005).

The paucity of molecular data from the plastids of dinoflagellates as well as the highly reduced non-photosynthetic nature of apicomplexan plastids has made the link between alveolates and chromists very difficult to prove. There are certainly structural similarities. Cryptophytes and dinoflagellates both produce starch, and dinoflagellates also possess the aforementioned mitochondrial characteristics of tubular cristae and three plastid thylakoids per stack. Perhaps the most convincing molecular data in support of the chromalveolate hypothesis comes from analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This enzyme, ubiquitous across plastid-containing lineages, is encoded twice in the nucleus with one copy targeted at the plastid and the other functioning in the host cytosol. In primary plastid lineages, the plastid-targeted isoform of GAPDH is clearly related to its bacterial homolog while the cytosolic copy is of eukaryotic origin. However, in dinoflagellates, apicomplexans, heterokonts, haptophytes, and cryptophytes, plastid-targeted GAPDH is derived from a duplication of the cytosolic version. This suggests that these groups share a common ancestor wherein the eukaryotic version of GAPDH was duplicated, acquired the necessary targeting signals to function in the plastid, and ultimately replaced the cyanobacterial-derived copy (Fast et al., 2001). While not definitive, the unusual

history of GAPDH is consistent with the chromalveolate hypothesis. Further evidence is provided by the gene history of fructose-1,6-bisphosphate aldolase (FBA) (Patron et al., 2004). Similar to the situation with GAPDH, FBA endosymbiotic gene replacements suggest a single acquisition of red algal plastids in chromalveolate lineages.

However, it has been difficult to reconcile molecular and nonmolecular pieces of the chromalveolate puzzle. A series of recent phylogenomic studies using nucleus-encoded genes have tended not to support the monophyly of the chromalveolates (Burki et al., 2007, 2008; Patron et al., 2007). Burki et al. (2008) resolved haptophytes and cryptophytes together with Plantae in a monophyletic group. Even more interestingly, the study provided further support for grouping Rhizaria with stramenopiles and aveolates in a monophyletic group dubbed SAR. Hackett et al. (2007) also found phylogenomic evidence for placing Rhizaria within the chromalveolates, although unlike Burki et al. (2007, 2008) haptophytes and cryptophytes did not group with Plantae.

At present it is too early to tell what the effect will be of placing Rhizaria firmly in the middle of the chromalveolates, in terms of revision of existing classification schemes. Researchers have begun to float potential scenarios involving various degrees of plastid gain, loss, and transfer to explain the results. Any hypothesis must of course reconcile the phylogenomic analyses with existing data such as the duplication of GAPDH and similarities in plastid protein import mechanisms (Archibald, 2009). It is hoped that the recent rash of genome projects for organisms from the chromalveolate lineage as well as the rhizarian *Bigelowiella natans* will help considerably to untangle the current uncertainty about the origin and spread of secondary and tertiary plastids.

As previously mentioned, the evolutionary history of plastids in some dinoflagellates is even more complicated than that exhibited by a "typical" chromalveolate. *Peridinium balticum* has a plastid derived from a heterokont (Chesnick et al., 1997), which would make this an example of tertiary endosymbiosis. *P. balticum* lost its original secondarily acquired red algal plastid and replaced it by engulfing a diatom and retaining its photosynthetic apparatus. It has been suggested that the mixotrophic lifestyle of dinoflagellates is conducive to this loss and gain of plastids from various lineages. Needless to say the plastid proteome of such organisms is encoded by a patchwork of genes transferred to the host nucleus from the various endosymbionts involved. While it is possible that nucleus-encoded proteins targeted at the original plastid can simply be recycled and used in the tertiary plastid, it is also possible that the nucleus experiences further gene transfer from the newly acquired endosymbiont followed by gene replacement of the originals. Indeed, such is the case with the phylogeny of oxygen-evolving enhancer 1 (PsbO) in the dinoflagellate *Karenia brevis* (Ishida and Green, 2002). Dinoflagellates are also not limited to replacing their original plastid with a chromalveolate one. *Lepidodinium viride* has a plastid most likely derived from a green alga (Watanabe et al., 1990). Such an acquisition is referred to as serial secondary endosymbiosis.

4.3. HOST AND ENDOSYMBIONT INTERACTIONS

The early stages of the endosymbiotic relationship between the engulfed free-living cyanobacterium and the host cell were characterized by massive gene transfer to the host nucleus and the subsequent loss of similar genes in the endosymbiont (Martin et al., 2002; Timmis et al., 2004). Typical cyanobacterial genomes have several thousand genes, while most plastids contain 60–200 open reading frames. Because this gene complement is insufficient to maintain the operation of the plastid, many of the proteins derived from genes transferred to the host nucleus are targeted back to the plastid. As elaborated upon below, proteins produced in the cytosol have amino-terminal pre-sequences, which permit passage across the plastid envelope.

4.3.1. Primary Endosymbiosis

As is the case with mitochondria, increasing genetic integration between host and endosymbiont is central to views on the initial establishment of the plastid. While recent genetic experiments indicate that plastid-to-nucleus gene transfer is a rampant and ongoing process in some lineages (Martin, 2003), most researchers believe that the bulk of this transfer occurred in the very early stages of the host–endosymbiont relationship. Overall, plastid genomes are similar in their structure and coding capacity, suggesting that rapid loss and integration had occurred prior to diversification of modern-day plastid-containing lineages.

Mere transfer of genetic material from the plastid to the host nucleus is insufficient to establish integration: transferred genes need to be expressed by the host; their protein products need to be "useful" (or at least not overly harmful) to the cell; and if they are to be targeted to their compartment of origin, they need to acquire the appropriate topogenic signals. Martin and Herrmann (1998) believe that establishing stable expression in the nucleus was the rate-limiting step for integration. If a transferred plastid gene was successfully expressed, it could become fixed in the nucleus independent of the evolution of proper targeting signals or import mechanisms, as long as it was of use to the host cell. Indeed, a substantial number of plastid-derived proteins are known to have non-plastid roles (Martin et al., 2002; Reyes-Prieto et al., 2006), either providing novel and useful cellular components or else replacing pre-existing eukaryotic counterparts. A comprehensive examination of the cyanobacterial contribution to *Arabidopsis* (Martin et al., 2002) estimated that ~18% or ~4,500 genes of the nucleus genome were derived from the endosymbiont. However, only about 1,300 of these encode proteins targeted to the plastid. However, given the size of the plastid genome and the complexity of the plastid proteome, it is clear that development of a targeting and import mechanism was also central to the eventual fate of the photosynthetic endosymbiont.

The principal method by which nucleus-encoded proteins are targeted to the plastid is amino-terminal transit peptide extensions. These extensions are typically between 20 and 150 amino acids in size and although not conserved in primary

sequence, they share a number of general characteristics, including hydrophobicity, enrichment of hydroxylated amino acids, and a net positive charge. The transit peptides interact with receptor components of the import complexes permitting the proteins to cross the plastid envelope. After protein import the transit peptides are cleaved, releasing the mature protein into the stroma for further distribution (Gould et al., 2008).

Translocation across the two plastid membranes is accomplished via the TOC (translocator of the outer chloroplast membrane) and TIC (translocator of the inner chloroplast membrane) complexes. These intricate suites of proteins, which mediate passage into plastids, appear to be conserved among all primary plastid organisms, although there is currently very little information available for glaucophytes (Gould et al., 2008). The similarity of such a vital apparatus is often cited as evidence for a single origin of all primary plastids (Palmer, 2003; McFadden and van Dooren, 2004) and as is the case for mitochondria, the TIC–TOC machinery appears to be a combination of both host- and endosymbiont-derived proteins.

Recent work has demonstrated the presence of numerous chlamydial genes in photosynthetic eukaryotes and led to speculation as to how they got there and their possible role in the evolution of plastids. Chlamydiae are obligate intracellular bacteria, and while they have never been reported in plastid containing lineages, comparative genomic studies show that a surprising number of chlamydial genes are most similar to plant genes (Huang and Gogarten, 2007).

A comparison of the only red algal genome currently sequenced, that of *Cyanidioschyzon merolae* (Matsuzaki et al., 2004), against chlamydial homologs (Huang and Gogarten, 2007) found at least 21 instances of transfer from chlamidiae to the algae. Moreover, most of the plant homologs contained plastid-targeting signals. Especially interesting from the standpoint of plastid development is the gene encoding ATP/ADP translocase. In *Chlamydia*, the translocase allows the parasitic bacterium to acquire ATP from its host while shuttling ADP back. In plants, ATP/ADP translocase is used by the plastid in a similar fashion.

It has been proposed that such an abundance of chlamydial genes in the nuclear genomes of photosynthetic eukaryotes could best be explained by an ancient relationship between a chlamydial endosymbiont and the ancestral photosynthetic eukaryote with subsequent degeneration and loss of the endosymbiont (Huang and Gogarten, 2007). Furthermore, it was speculated that the presence of this additional endosymbiont had a significant impact on plastid establishment. Without key genetic transfers from the chlamydiae to the eukaryote cell, transformation of the engulfed cyanobacterium to a plastid would not have been possible. Because of its parasitic lifestyle, the chlamydia-like bacterium possessed genes that were involved in the transfer of energy and metabolites between host and endosymbiont, unlike the original cyanobacterium, which because of its phototrophy was self-sufficient. Genetic transfer to the nucleus of the chlamydial genes for ATP/ADP translocase and other transporters permitted their co-option for use with the engulfed cyanobacterial cell. Once the endosymbiotic relationship between the cyanobacterium and the host was firmly

established, the argument goes, the chlamydial endosymbiont was no longer useful and over time degenerated, leaving behind a suite of genes vital for cooperation between the newly evolved plastid and the host.

Other researchers are less convinced of the need to invoke additional endosymbiotic events to account for the presence of chlamydia-like genes in Plantae. It has been proposed that an ancient evolutionary relationship between cyanobacteria and chlamydiae exists (Brinkman et al., 2002), which could explain the chlamydial phylogenetic signal seen in some plant and algal genes. Alternatively, the genome of the cyanobacterial progenitor of plastids could have been a mosaic of genes derived by lateral gene transfer (LGT) from a variety of different bacterial groups, included chlamydiae (Dagan et al., 2008), which seems reasonable given the pervasive role LGT has played in prokaryotic evolution (Doolittle, 1999). Indeed, Gross et al. (2008) have provided evidence for genome chimericism in the cyanobacterial ancestor of plastids. Huang and Gogarten (2007) consider these alternatives less likely since some of the genes in question have no cyanobacterial homologs and no clear-cut examples of LGT between chlamydiae and cyanobacteria have been identified thus far. Perhaps with the availability of additional genome sequences from a much broader range of photosynthetic lineages the full extent and origin of chlamydiae-like genes in plants will become apparent.

4.3.2. Secondary and Tertiary Endosymbiosis

As mentioned above, secondary plastids are characterized by the presence of three or four plastid membranes. Consequently, organisms with secondary or tertiary plastids have had to evolve an even more complex mechanism for protein import. While a transit peptide can be used to cross the two inner plastid membranes, as in primary plastids, proteins targeted to secondary plastids must first cross one or two additional barriers. In heterokonts, haptophytes and cryptophytes this is accomplished via a bipartite N-terminal extension comprised of a signal peptide and a transit peptide. The signal peptide allows the protein to cross the outermost membrane into the ER lumen. The transit peptide is then used to cross the remaining barriers after cleavage of the signal peptide. In cryptophytes, heterokonts and apicomplexans, crossing of the second membrane appears to involve endoplasmic reticulum-associated degradation (ERAD) components recognizing the transit peptide (Sommer et al., 2007). The co-option of the normal ER translocase for import into secondary plastids has been suggested to be an additional character in support of the chromalveolate hypothesis (Gould et al., 2008).

In some secondary plastid-containing organisms, the cytoplasm of the engulfed eukaryote persists between the second and three plastid membranes. This periplastidial compartment (PPC) is therefore also a potential destination for nucleus-encoded proteins. Targeting to this compartment appears to be achieved by a minor modification to the transit peptide. A phenylalanine (F) residue upstream of the transit peptide proper causes the protein to continue into the stroma of the plastid while its absence results in the protein remaining in the PPC (Gould et al., 2008).

4.3.3. A Second Primary Plastid?

Although it is generally agreed that primary plastids are the product of a single primary endosymbiotic event in a common ancestor shared by red, green, and glaucophyte algae, the protist *Paulinella chromatophora* and its subcellular structures known as chromatophores raise the intriguing possibility of an additional primary endosymbiotic event, one that occurred fairly recently. *P. chromatophora* is a testate amoeba containing two kidney-shaped structures, chromatophores, that are thought to be derived from cyanobacteria. Indeed, the chromatophore genome has recently been sequenced (Nowack et al., 2008), and it appears to be closely related to *Synechococcus*, although the gene complement is much reduced: 867 protein-coding genes versus ~3,300 in *Synechococcus*. As alluded to above, there is some debate about whether the chromatophores are still endosymbionts or whether they have been reduced and sufficiently integrated with the host to now be considered an organelle (Theissen and Martin, 2006; Bhattacharya et al., 2007). On one hand, chromatophores cannot be cultivated independently of the host, their division appears to be regulated by the host, and they provide photosynthate to the host. However, what has been retained and what has been lost or transferred from the chromatophore genome does not resemble "typical" plastid genetic integration with its host. Rather than having many of the essential cellular processes maintained by nucleus-encoded proteins, the chromatophore appears to have retained most of these essential genes.

Unfortunately, until the *P. chromatophora* host nuclear genome is sequenced, the true extent of the genetic integration between host and endosymbiont will not be known to the degree that it is in typical plastid-containing eukaryotes. If this organism is indeed an independent example of a primary acquisition of a photosynthetic body, perhaps it is too much to ask that it follow the "rules" and "definitions" of typical plastids, which, after all, are believed to have evolved only once. Given that its close relative *Paulinella ovalis* is non-photosynthetic but feeds on cyanobacteria (Johnson et al., 1988), the chromatophores clearly represent a recent acquisition, one in which the interplay between host and "guest" has yet to be fully worked out.

5. Conclusions and Prospectus

From the above discussion it is clear that there are both similarities and differences in the current models put forth to explain the origins of mitochondria and plastids, and in the degree of confidence ascribed to them. On balance, the evolution of plastids is much better understood, as it was clearly a more recent event and completely disentangled from eukaryogenesis. What are the prospects for further advances in our understanding of the origins of both organelles?

In the case of mitochondrial evolution, the most significant development in the last decade has been the increasing empirical evidence in support of the notion that all eukaryotes have a mitochondrion or mitochondrion-derived

organelle (Embley et al., 2003; Barberà et al., 2007). Nevertheless, ideas about the earliest events leading to the evolution of this organelle are likely to remain firmly in the realm of speculation. This is due to the complete lack of any sort of a modern-day representative for the putative host and endosymbiont cells involved (the precise nature of which can differ significantly from model to model), and very little in the way of consistent data on the environmental and biochemical conditions in which the mitochondrion evolved. The situation is somewhat better for plastids in that species such as *Paulinella chromatophora* (Nowack et al., 2008) at least provide an opportunity to explore "recent" associations between eukaryotic hosts and cyanobacterial endosymbionts. The extent to which these systems can provide meaningful insight into the evolutionary events giving rise to the singular origin of plastids remains to be seen, but at least we can be reasonably confident in the general features of the host (a heterotrophic eukaryote) and endosymbiont (a cyanobacterium-like prokaryote capable of oxygenic photosynthesis).

Perhaps the most promising area where advances can be made is in our understanding of the proteomes of mitochondria and plastids, and the targeting of nucleus-encoded proteins to these organelles in present-day organisms. Mitochondrial and plastid proteomes have turned out to be surprisingly heterogeneous and malleable, even over short evolutionary timescales, containing both endosymbiont- and host-derived proteins, as well as proteins from neither of these sources (e.g., Martin et al., 2002; Esser et al., 2004; Smith et al., 2007). It behooves us to more precisely pinpoint the evolutionary origins of these proteins in diverse eukaryotic lineages and to better understand the diversity of ways in which these proteins make their way to the organelle. While the predominantly cyanobacterium-derived TIC–TOC protein translocon system of plants and algae appears to be the dominant pathway for targeting proteins to plastids (Gould et al., 2008), recent research has shown that routing through the endomembrane system is also possible (e.g., Villarejo et al., 2005), raising questions about the nature of the initial mode of protein import (Bhattacharya et al., 2007). The same can be said of mitochondria, as bioinformatic predictions of mitochondrial proteomes do not capture the full spectrum of such proteins inferred from proteomic analyses (Millar et al., 2006). Finally, a better understanding of the biochemical components underlying the metabolic exchange between mitochondria and plastids and their hosts should make it possible to better speculate on the nature of the selective forces involved in forging the initial interactions between the two cells involved, and thus fine-tune our evolutionary scenarios.

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Biodata of **Matej Vesteg** and **Juraj Krajčovič**, authors of ***"The Origin of* Eukarya *as a Stress Response of Two-Membrane-Bounded Sexual Pre-Karyote to an Aggressive Alphaproteobacterial Periplasmic Infection"***

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THE ORIGIN OF *EUKARYA* AS A STRESS RESPONSE OF TWO-MEMBRANE-BOUNDED SEXUAL PRE-KARYOTE TO AN AGGRESSIVE ALPHAPROTEOBACTERIAL PERIPLASMIC INFECTION

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1. Introduction

The organisms were classified as either prokaryotes or eukaryotes in pre-genomic era. While eukaryotes possess the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, and are sexual, none of these features is present in prokaryotes. The view of prokaryote–eukaryote dichotomy has dramatically changed, when the rRNA sequence comparisons of Woese and Fox (1977) revealed that two kinds of prokaryotes exist: Eubacteria and Archaebacteria. Woese et al. (1990) later suggested that three domains of life exist: *Bacteria* (Eubacteria), *Archaea* (Archaebacteria), and *Eukarya* (eukaryotes). Moreover, it has been proposed that *Archaea* and *Eukarya* are more closely related to each other than both these domains are related to *Bacteria* (Woese et al., 1990). The historical view that eukaryotes evolved from bacteria now seems to be over-simplified. Each model for the origin eukaryotes should nowadays count with the existence of *Archaea*. Though some models even suggest that the root of the tree of life is within archaea (Wong et al., 2007), the logic of this thinking is not far from the historical imagination that the last universal common ancestor (LUCA) must have been a prokaryote. On the other hand, a challenging view has been also proposed suggesting that many eukaryotic-like feature might have been present in (at least some members of) the population of LUCA (Glansdorff et al., 2008). In our opinion, if one wants to explain the origin of *Eukarya*, one should explain the origin of *Archaea* and *Bacteria* as well. Perhaps the best way to evaluate scenarios for the origins of all three domains of life is to start to think about the nature of LUCA. We can be quite sure that it possessed everything what is universally distributed among all cells within all three domains, but nothing else. If one takes it like this, there is no direct comprehensive evidence that LUCA was either bacterium or archaeon, as well as there is no clear evidence that LUCA was a eukaryote.

2. The Last Universal Common Ancestor (LUCA)

2.1. RIBOSOMES AND LIPIDS

The LUCA of all organisms should have possessed all components that are universally present among all extant organisms. These include ribosomes with their key rRNA components (Woese, 1998, 2000, 2002), membrane lipids (Peretó et al., 2004; Jékely, 2006), and membrane secretory and protein insertion apparatus (Jékely, 2006).

Fifteen proteins associated with the small ribosomal subunit, 18 proteins associated with the large ribosomal subunit, nine class I aminoacyl tRNA synthetases (aaRS) and seven class II aaRS, seven GTPases associated with various aspects of translation, and at least two other translation factors are traceable to LUCA (Anantharaman et al., 2002). LUCA also certainly possessed the signal recognition particle GTPases linking translation and secretion, and a variety of RNA-modifying enzymes. In addition, rRNA genes, some parts of sequences of particular classes of tRNAs and of the nucleotide-binding domains of ABC transporters are the most conserved nucleotide sequences on Earth (Isenbarger et al., 2008).

The phylogenetic study of enzymes involved in lipid biosynthesis of Peretó et al. (2004) clearly indicates that LUCA had membrane lipids, but their synthesis was most likely non-stereospecific. This is consistent with the previous suggestion of Wächtershäuser (2003) that LUCA most likely possessed heterochiral membrane(s) composed of a mixture of bacterial (fatty acid esters linked to sn-glycerol-3-phosphate) and archaeal (isoprenoid ethers built on sn-glycerol-1-phosphate) lipids. The universal presence among all organisms of proteins and pathways translocating proteins through, and mediating insertion of proteins into, plasma membrane (or ER membrane in eukaryotes), in particular SecY, SRP54, FtsY/SRα, and signal peptidase, as well as universal conservation of F_0F_1-ATPase further confirms the view that LUCA was certainly bounded by membrane proteolipids (Jékely, 2006).

2.2. DNA, GENOME, AND REPLICATION

There exist two lines of compelling evidence that LUCA almost certainly possessed DNA: the universal distribution of two largest (the catalytic) subunits (β and β′ in bacterial nomenclature) of the DNA-dependent RNA polymerase (Woese, 2002) and the universal distribution of RecA/Rad51 homologs (Cavalier-Smith, 2002a), the proteins which are involved in DNA recombination.

However, several proteins with analogous functions in DNA replication (DNA polymerase, primase, and helicase) are not orthologous in *Bacteria* and *Archaea*, while some of them are orthologous in *Archaea* and *Eukarya* (Edgell and Doolittle, 1997; Forterre, 1999, 2006; Leipe et al., 1999), suggesting that modern type of DNA replication probably did not exist in LUCA. This might be

surprising. Nevertheless, it was shown that there have been multiple exchanges of genes whose products are involved in DNA metabolism between viruses and cells in the life history (Filée et al., 2003). Thus, it is possible that modern type of genome replication of extant cells evolved twice (Leipe et al., 1999; Forterre, 2002, 2005; Woese, 2002) or three times and viruses played an import role in its origin (Forterre, 2006).

Evolution of genome structures likely depends on mechanisms of genome replication (Woese, 1998). Thus, there is no reason to think that the genome of LUCA reminded modern genomes of extant prokaryotes or eukaryotes. Instead, LUCA might have possessed either mixed RNA–DNA genome (Leipe et al., 1999) or a genome composed of multicopy small plasmid-like either linear (Woese, 1998) or circular DNA molecules of operonal organization (Wächtershäuser, 2003, 2006). A cluster of about 40 genes (the products of which are involved mainly in translation, two genes in secretion, three are DNA-dependent-RNA-polymerase subunits, and one encoding NusG antitermination factor) is shared by *Bacteria* and *Archaea*, suggesting that this cluster was present in LUCA (Wächtershäuser, 1988, 2003, 2006). If such cluster was present on circular multicopy plasmids, it would be ideal for rolling circle transcription and/or replication (Wächtershäuser, 2003, 2006). Alternatively, reverse transcription (by reverse transcriptase obtained from, e.g., symbiotic RNA virus) of LUCA's polycistronic RNAs and subsequent circularization via recA/rad51-homolog-mediated mechanism might have represented another simple mechanism of replication of LUCA's genome. Nevertheless, taking into account that eukaryotic conventional telomeres are replicated by ribonucleoprotein complex (a potentional relict of RNA world), and that various types of nonconventional telomeres exist in extant organism (Nosek et al., 2006), genome of LUCA might have been represented by linear molecules as well. Another alternative is that various types of DNA replication mechanisms as well as topologies of DNA molecules might have coexisted within different subpopulations of LUCA, depending on viruses and other mobile elements that these subpopulations possessed and exchanged.

2.3. QUASI-SEXUAL TWO-MEMBRANE-BOUNDED LUCA EXCHANGING MUCH GENETIC INFORMATION

Under "Pre-cell Hypothesis" of Kandler (1994a, 1994b, 1998) adopted by Woese (1998, 2000, 2002), LUCA was an entity with most basal attributes of cells, which was, however, unable to limit frequent mutual exchanges of genetic information. This hypothesis seems to be quite consistent with LUCA as described above. Moreover, the theoretical models of Santos et al. (2003) seem to be fully consistent with the view of frequent horizontal gene transfer within LUCA population. Because horizontal gene transfer of metabolic operational genes is common (Kurland et al., 2003), different subpopulations of LUCA might have occupied different habitats (Kurland et al., 2006; Wächtershäuser, 2003). In addition, LUCA possessing unstable heterochiral membrane lipids might have undergone frequent quasi-sexual fusions and fissions

(Wächtershäuser, 1988, 1992, 2003, 2006). Fusions and fissions could have also contributed to mutual exchanges between subpopulations of LUCA.

We have recently suggested that LUCA was bounded by two membranes of heterochiral composition (Vesteg et al., 2006), because it would be more stable than if it were bounded by a single unstable heterochiral membrane. Another reason for assuming two-membrane-bounded LUCA is that it is mechanistically easier to imagine enclosure of molecular components necessary for free-living lifestyle by two membranes than enclosure by one membrane only. Four simple scenarios of the origins of two-membrane-bounded LUCA were suggested: the intravesicular fusion occurring at the orifice of a single gastruloid membrane vesicle (Blobel, 1980), the fusion of two cup-shaped membrane vesicles (Cavalier-Smith, 1987, 2001), vesicular budding analogous to endocytic invagination (Griffiths 2007), and the fusion of more membrane vesicles (Vesteg et al., 2006; Vesteg and Krajčovičč2008a). As there exist both two-membrane-bacteria (negibacteria including the majority of bacteria) as well as two-membrane-bounded archaea (though only minority; Rachel et al., 2002; Nather and Rachel, 2004), the idea of two-membrane-bounded LUCA seems to be plausible. Interestingly, the periplasm (intermembrane space) of two-membrane-bounded archaeal genus *Igniccocus* occupies up to three times more volume than the cytoplasm and is filled with membrane-bounded vesicles derived from cytoplasmic (inner) membrane (Rachel et al., 2002; Nather and Rachel, 2004). In addition, the theory of membrane heredity refusing de novo formation of genetic membranes from dissimilar membranes (Blobel, 1980; Cavalier-Smith, 1991a, 2000; Warren and Wickner, 1996) would fail to explain the origin of two-membrane-bounded archaea, if the last archaeal common ancestor (LACA) was bounded by single membrane only. On the other hand, if LUCA, LACA, as well as LBCA (last bacterial common ancestor) were bounded by two membranes, the origin of single-membrane-bounded bacteria (posibacteria) and single-membrane-bounded archaea (the majority of archaea discovered so far) could be easily explained by membrane losses.

3. The Origins of Domains *Bacteria* and *Archaea*

It is reasonable that evolution could have proceeded from the less-stable heterochiral membranes to homochiral ones with much higher probability than in opposite direction (as suggested, e.g., by most models for the origins of eukaryotes, or model suggesting that LUCA was a bacterium) (Wächtershäuser, 2003, 2006). Following the hypothesis of Wächtershäuser (1988, 1992, 2003, 2006), subpopulations of LUCA practicing highly promiscuous fusions and fissions might have segregated into subpopulations with bacterial (subpopulations of type B) or archaeal (subpopulations of type A) lipids dominating strictly by physical and chemical forces. This would of course not mean that a LUCA of type B could not have transformed into type A or vice verse. It would depend most likely on with which

types and how frequently it would fuse. The origin of *Bacteria* might be explained by the emergence of an enzyme for the stereospecific formation of glycerol-3-phosphate units (Koga et al., 1998) and the emergence of fusion-prohibiting cell wall (Woese, 1983; Kandler, 1994a, 1994b, 1998) in a subpopulation of LUCA B. In similar manner, the origin of *Archaea* might have been a consequence of the emergence of an enzyme for the stereospecific formation of glycerol-1-phosphate units (Koga et al., 1998) and the emergence of fusion-prohibiting cell wall (Woese, 1983; Kandler, 1994a, 1994b, 1998) in a subpopulation of LUCA A. The phylogenies of informational genes revealing that *Archaea* are more closely related to *Eukarya* than to *Bacteria* are consistent with the hypothesis that LBCA arose prior to LACA (Woese, 1998, 2000, 2002). In addition, similar r-selective pressures for fast and precise DNA replication and reproduction might have been responsible for the convergent parsimonious genomic organization of *Bacteria* and *Archaea* (Penny and Poole, 1999; Poole et al., 1999).

4. The Origin of the Domain *Eukarya*

4.1. THE PITFALLS OF MODELS SUGGESTING PROKARYOTIC ORIGIN OF THE DOMAIN *EUKARYA*

Several hypothesis for the origin of eukaryotes suggest that prokaryotes, their chimeras, and/or symbiotic associations were direct ancestors of *Eukarya* (Cavalier-Smith, 2002b, 2006, 2009; Cox et al., 2008; de Duve, 2007; Jékely, 2007a, 2007b; Horiike et al., 2001, 2004; López-García and Moreira, 2001, 2006; Margulis et al., 2000, 2006; Martin and Müller, 1998; Martin and Koonin, 2006; Moreira and López-García, 1998; Pisani et al., 2007; Rivera and Lake, 2004; Saruhashi et al., 2008; Shinozawa et al., 2001; Yutin et al., 2008). On the other hand many other authors (Baluška et al., 2004a, 2004b; Doolittle, 2000; Forterre, 2006; Glansdorff et al., 2008; Hartman and Fedorov, 2002; Hartman et al., 2006; Kurland et al., 2003, 2006; Poole and Penny, 2006; Poole et al., 1999; Penny and Poole 1999; Wächtershäuser, 1988, 1992, 2003; Woese, 1998, 2000, 2002; Vesteg and Krajčovič, 2007, 2008a, 2008b; Vesteg et al., 2006) have suggested that eukaryotes are direct descendants of neither prokaryotes nor their associations/chimeras. Prokaryote(s)-to-eukaryote transition models have several pitfalls. These models have to suggest that either LECA (last eukaryotic common ancestor) or LACA underwent selectively highly unlikely and costly stage with heterochiral membrane(s) (composed of mixture of archaeal and bacterial lipids), which is (are) less stable than homochiral archaeal or bacterial one(s) (Wächtershäuser, 2003, 2006). These models also generally do not define the nature of LUCA and do explain the origin of LBCA and LACA (Woese, 1998, 2002). As molecular phylogenies group mitochondria with Alphaproteobacteria (Andersson et al., 1998, 2003; Gray et al., 1999; Lang et al., 1999) and plastids with cyanobacteria (Cavalier-Smith, 2000, 2002c; Martin et al., 2002), there is no doubt about prokaryotic

origins of these eukaryotic organelles. If an archaeal cell was involved in the origin of eukaryotes, as suggested by many of prokaryote(s)-to-eukaryote transition models, the phylogenies of informational genes should place *Eukarya* within some of archaeal branches (Forterre, 2006; Poole and Penny, 2006). However, *Eukarya* are sisters of *Archaea* in phylogenies of informational genes (Forterre, 2006; Poole and Penny, 2006). The true chimera models have to propose almost certainly lethal interdomain hybridization. Prokaryote-to-eukaryote transition models also do not explain how exactly eukaryote-specific proteins (Hartman and Fedorov, 2002; Hartman et al., 2006) and features with no prokaryotic counterparts arose (Forterre, 2006).

Following the ideas of Cavalier-Smith (1991b) and Doolittle (1991), followers of prokaryotes-to-eukaryote transition models such as López-García and Moreira (2006), Martin and Koonin (2006), and Koonin (2006) suggested that the massive intron spread was the main driving force leading to the appearance of the nucleus to separate slow splicing from fast translation to avoid the synthesis of aberrant proteins. However, massive intron spread would most likely require sexual life cycle (Poole, 2006) and would probably need the duplication of many ancient eukaryotic paralogs (see Makarova et al., 2005). Ignoring to explain how eukaryote specific meiosis and sex arose is one of the major pitfalls of hypotheses suggesting prokaryotic origin of eukaryotes (Vesteg and Krajčovič, 2007).

The paradox of sex includes (for review see Otto and Lenormand, 2002; Rice, 2002), e.g., breakup of co-adopted gene combinations (Fisher, 1930), the risk of fusion, the risk of infection, the time and energy costs while looking for the partner, etc. Prokaryote(s)-to-eukaryote transition models have to suggest that eukaryote specific sexual reproduction with all its costs and risks evolved from the less costly and less risky prokaryotic asexual reproduction. Moreover, prokaryotes undergo gradualistic Darwinian evolution under which mutation either increases or decreases the fitness of their carriers, and either lets the prokaryotic interactor survive and make its clones or not. The hypotheses supposing prokaryotic origin of eukaryotes have to propose highly unlikely unparsimonious back-transition steps such as loss of prokaryotic cell wall(s), de novo evolution of fusion process, and switch from Darwinian evolution to that described by Dawkins (1976), because LUCA probably did not possess cell wall, it underwent fusions, and the evolution of "selfish genes" was probably dominating in the era of LUCA.

It has also been argued that alphaproteobacterial ancestors of mitochondria had to have been engulfed by phagocytosis (Cavalier-Smith, 2002c; Poole and Penny, 2006), while prokaryotes are unable to phagocytose. One of the arguments in favor of phagotrophic hosts is that there exist numbers of examples of prokaryotic and eukaryotic obligate endosymbionts that live within eukaryotic cells (Cavalier-Smith, 2002c; de Duve, 2007, Jékely, 2007b; Poole and Penny, 2006). On the other hand, some parasitic bacterial species are known which can get inside other cells via their own predatory apparatuses without any need to be phagocytosed (Davidov and Jurkevitch, 2007). Dagan and Martin (2007) also mention the example of bacterial symbionts in the cyanobacterium *Pleurocapsa minor*;

however, nearly nothing is known about this symbiotic association, though the report about it is 30 years old (Wujek, 1979). Another report of bacteria in bacteria includes mealybug betaproteobacterial endosymbionts containing gammaproteobacterial endosymbionts (von Dohlen et al., 2001). Nevertheless, this is the case of very specific endosymbiotic relationship of "bacteria" in "bacteria," which themselves live in eukaryotes. Another interesting putative example of "bacteria" in "bacteria" (apparently analogous to previous one) is the presence of prokaryotic endosymbionts in the chloroplast stroma (ancestrally cytoplasm of a cyanobacterium) of the dinoflagellate *Woloszynskia pascheri* (Wilcox, 1986). Taken together, although there is the lack of examples of mutualistic endosymbiots in prokaryotes in contrast to numbers of such examples in eukaryotes, it seems that phagocytosis is not absolutely necessary for getting inside the cells.

Here, we suggest that the prevalence of evidence for mutualistic endosymbionts in eukaryotes in comparison to prokaryotes might likely reflect the incapability of prokaryotes to practice sex. For an asexual prokaryotic species to maintain a permanent endosymbiont, the division and distribution of an endosymbiont would have to become almost spontaneously coupled with the division of host cell and distribution of its cytosol into daughter cells. In the beginning of endosymbiotic relationship, even if it was strongly selectively advantageous to possess an endosymbiont, it would be only randomly distributed to daughter cells and often lost. Moreover, an endosymbiont would not be distributed to other individuals of population of host species due to the incapability of asexuals to fuse with one another. In contrast, in sexual population in which entities can fuse and divide into four cells, the distribution and persistence of endosymbionts would be more probable (although in the very beginning of symbiotic association also random). Thus, it is possible that fusion and two-step division could have contributed to the evolution of permanent endosymbiotic relationships. Therefore, the establishment of alphaproteobacterial ancestors of mitochondria might have occurred in sexual population with higher probability than in asexual population.

4.2. THE ORIGIN OF SEXUAL TWO-MEMBRANE-BOUNDED PRE-KARYOTE AND ITS LIFE CYCLE

In our view, it is proposed that after the origins of domains *Bacteria* and *Archaea*, there might have survived some relict post-LUCA (pre-cellular) lineages that were unlucky to have evolved a fusion-prohibiting cell wall. All of them were soon outcompeted by fast-reproducing bacteria and archaea possessing rigid cell surface, except for the pre-karyote – the lineage of B-type (possessing predominantly bacterial lipids) sharing a common pre-cellular ancestor with *Archaea*. It is suggested that pre-karyote without rigid cell surface would hardly survive if it had a single membrane only. It is proposed that pre-karyote was probably bounded by two membranes inherited from LUCA (Vesteg et al., 2006). It is also suggested

that the pre-karyote outer membrane might have been the ancestor of eukaryotic plasma membrane and pre-karyote inner membrane might have been the ancestor of eukaryotic ER/nuclear membrane. It is likely that the pre-karyote might have possessed at least some ancestors of eukaryotic cytoskeletal proteins (Vesteg and Krajčovič, 2008b), e.g., the protein ancestral to actin might have been major component of pre-karyote periplasm. It has been recently proposed by Baluška et al. (2004a) that an entity with outer and inner membrane system with actin-based "cell periphery" and tubulin-based "cell body" (see Baluška et al., 2004b) ancestral to eukaryotes existed prior to the origin of mitochondria.

In competition with prokaryotes, the pre-karyote might have evolved different strategies how to limit risks of fusion and to resist the horizontal gene transfer. These might have included different regulation of cell cycle, and revolution in repair processes with evolution of true sex as an outcome (Vesteg and Krajčovič, 2007). True sex and two-step meiosis could have helped to uncouple and thereby emasculate acquired parasitic genomes (such as viruses, transposons, and other mobile elements; Sterrer, 2002).

One famous hypothesis proposes that sex evolved to repair errors such as double-strand DNA breaks (Bernstein et al., 1981; Maynard Smith and Szathmáry, 1995). The recent phylogenetic analysis of cell cycle kinases revealed Chk1 DNA damage checkpoint kinase as the earliest branch (Krylov et al., 2003). Meiotic kinase Ime2 branched prior to cyclin-dependent kinase, which represented the latest branch (Krylov et al., 2003). Krylov et al. (2003) have suggested a scenario under which Chk1 might have been a basal regulator of ancient eukaryotic cell cycle at the level of DNA damage checkpoint and only duplication and diversification of ancestral Chk1 gene might have led to diversification of mitosis and meiosis. However, the multiple duplications of kinases genes would most likely themselves require sexual cycles.

On the basis of the study of Krylov et al. (2003) we have recently suggested that DNA damage checkpoint might have been the only ancestral pre-karyotic checkpoint regulating both pre-karyote asexual and sexual reproduction cycles (Vesteg and Krajčovič, 2007). It is noteworthy that prokaryotes lack a mechanism to stop DNA replication when their DNA is damaged (they do not have DNA damage checkpoint nor any other checkpoints; Kuzminov, 1999), though DNA recombination is essential for restarting DNA synthesis when the replication fork is stalled or broken in prokaryotes as well as eukaryotes (Haber, 1999, 2000; Kuzminov, 1999). Under our hypothesis (Vesteg and Krajčovič, 2007), the key role for pre-karyotic DNA damage checkpoint was to allow the pre-karyote to divide only when its DNA was completely replicated without mistakes. The role for the DNA damage checkpoint might have been to monitor the DNA damage; if present, stop replication, and leave the repair system to fix it. As there were no checkpoints other than DNA damage checkpoint, the pre-karyote is supposed to have started the DNA replication immediately after cell division, and thus pre-karyote was probably in haploid stage only for a very short time period immediately after cell division and instead was partially diploid for the vast

majority of S-phase. If the DNA damage was huge and unlucky, partially diploid pre-karyote was not able to repair its DNA and to restart the DNA synthesis alone, it could fuse with the partner pre-karyote (most likely partially diploid, and perhaps also damaged) and it could repair the DNA and restart the DNA synthesis via recombination of its DNA with the DNA of the partner. After repairing and restarting the DNA synthesis, the replication of both haploid sets would be completed resulting in a tetraploid entity. Now the cell division would be allowed to produce two diploid pre-karyotes. These would, however, "feel" that they have DNA-replicated and would have no need to start the DNA synthesis. Instead, both diploid pre-karyotes would be allowed to divide to produce haploid pre-karyotes.

4.3. THE ORIGIN OF EUKARYOTIC COMPARTMENTALIZATION TRIGGERED BY ALPHAPROTEOBACTERIAL ANCESTORS OF MITOCHONDRIA

Recently we have suggested (Vesteg et al., 2006; Vesteg and Krajčovič, 2008a) that alphaproteobacterial ancestors of mitochondria might have been selfish parasites of the pre-karyote periplasm (intermembrane space). The alphaproteobacterial infection might have reminded the infection of negibacterial periplasm by *Bdellovibrio* sp. After the infection of negibacterial periplasm, *Bdellovibrio* secretes macromolecular degradative enzymes into the host cell cytoplasm causing the host cytoplasm to shrink (Saier, 1994). It deserves to be mentioned that *Bdellovibrio*-like alphaproteobacterial predators have been recently discovered, and it has been suggested that this might have implications for the origin of mitochondria (Davidov et al., 2006).

Although the nuclear membrane has outer and inner layer, these two layers are interconnected in nuclear pores, and the outer layer of nuclear membrane is further continuous with ER membrane, and thus nuclear/ER membrane are one continuum (Cavalier-Smith, 2002c; Martin, 2005). It has been suggested that fusion of phagocytic (endocytic) vesicles around the genetic material could have resulted in nuclear/ER membrane topology (Cavalier-Smith, 2002c; de Duve, 2007; Jékely, 2007b;). Nevertheless, exocytosis clearly predates endocytosis, as revealed by the phylogenetic analysis of Ras-family GTPases that are key regulators of cytoskeleton dynamics, vesicular trafficking, and nuclear function (Jékely, 2003). In our opinion, the phagocytosis (which is clearly just a modification of endocytic process) itself would most likely require many complex eukaryotic features such as endomembrane system (including ER, GA, and lysosomes), cytoskeletal network, and probably also mitochondria.

It has been suggested that the early alphaproteobacterial symbionts might have been using secreted and membrane proteases, such as metacaspases, paracaspases, and HtrA-like proteases, to kill their host cells and to move to another cell (Frade and Michaelidis, 1997). It has been also shown for example that a

pore-forming toxin produced by *Aeromonas hydrophila* causes vacuolation of ER membranes (Abrami et al., 1998). We have suggested that pre-karyote inner membrane (similar to inner membrane of negibacteria, mitochondria, and plastids) might have created various secondary (similar to mitochondrial cristae) and tertiary (thylakoids) structures (Vesteg et al., 2006). We have hypothesized that the alphaproteobacterial parasites (ancestors of mitochondria) might have had the capacity to disrupt the pre-karyote inner membrane (Vesteg and Krajčovič, 2008a), perhaps to uptake metabolites from host cytoplasm for own division and development after infection. The lipid layers arising from the disrupted pre-karyote inner membrane after infection could have likely been forced by hydrophobic interactions in hydrophilic cellular environment to form membrane vesicles and/or vacuoles. These vesicles and other vesicles derived from pre-karyote inner membrane prior to alphaproteobacterial infection might have fused in exactly the same manner as proposed by endocytic (phagocytic) model. The result of the fusions of the vesicles would be nuclear/ER membrane topology present in extant eukaryotes. This process might have also allowed the pre-karyote to restore essential compartmentalization, and might have arrested the progress of parasite infection. Our model is (as well as endocytic model) consistent with the finding that components of eukaryotic curved membrane structures such as coated vesicles and nuclear pore complexes share a common molecular architecture (Devos et al., 2004). In addition, as the eukaryotic nucleolus is the place of eukaryotic ribosomal subunits assembly, the nuclear pore complexes and protein export–import pathways might have (at least partially) evolved to avoid the formation of chimeric ribosomes composed of host and symbiont ribosomal proteins after the transfer of some ribosomal proteins from alphaproteobacterial to host genome (Jékely, 2008).

The nuclear pore complex of extant eukaryotes seems to have been tinkered together from a small set of largely bacterial, some ancestral archaeo-eukaryotic, and some unique domains (Mans et al., 2004). The alphaproteobacterial origin is proposed for several components of the nuclear pore complex, in particular, RanGDP import factor NTF2, the HEH domain of Src1-Man1, and probably also key domains of karyopherins and nucleoporins, the HEAT/ARM, and WD40 repeats (Mans et al., 2004). These ancestral alphaproteobacterial proteins and/or proteins bearing these ancestrally alphaproteobacterial domains might have been once used by alphaproteobacterial symbionts to interfere with the evolving membrane repair system of their hosts. The series of events that accompanied divergence of nuclear and ER-specific functions from an ancestral set of functions in the pre-karyote is thought to have been in part the formation of paralogous proteins through duplication and divergence of ancestral pre-karyotic proteins (Mans et al., 2004).

The insertion of ADP/ATP translocase into the alphaproteobacterial inner membrane might have represented another immunity response of the infected pre-karyote. The efflux of ATP could have attenuated the aggressiveness of alphaproteobacterial parasite. The ATP pool in the pre-karyote periplasm might have

been further reused by the infected pre-karyote. The export of ribosomal subunits and mRNAs and thus most of the pre-karyote energetically costly metabolism to the pre-karyote periplasm might have evolved to use this ATP pool. If we consider that RNAs are transported into eukaryotic cytoplasm in complexes with proteins (ribosomal or cap-binding proteins), the evolution of the export process into the pre-karyotic periplasm does not seem to be unlikely. The addition of a few RNA-binding motifs to some few pre-karyote periplasmic proteins and/or the addition of a transport sequence driving the proteins into pre-karyote periplasm to some few RNA-binding proteins might have resulted in evolution of an export pathway driving RNA-protein complexes into pre-karyote periplasm. The evolution of such export system might have not only been advantageous, because it allowed the use of ATP pool in the pre-karyote periplasm, but it might have also allowed the escape of most pre-karyotic enzymes (and thus most biochemical pathways) from alphaproteobacterial degradative enzymes and toxins secreted by the parasite into the pre-karyote cytoplasm. In addition, export of mRNAs and ribosomal subunits into pre-karyote periplasm might have allowed the pre-karyote to separate splicing (of ancestrally alphaproteobacterial group II introns that massively invaded the genome of sexual pre-karyote) from fast translation, and to avoid the synthesis of aberrant proteins. Nevertheless, the RNA-protein complex export system must have coevolved with changes of pre-karyote inner membrane topology, with formation of nuclear pore complex, and with eukaryotic innovations in RNA processing and translation. For example, it has been suggested that mRNA cap-binding complex and the translation initiation factor eIF4G evolved from a common precursor containing NIC domain (Aravind and Koonin, 2000).

5. Conclusions

We propose that the pre-karyote (the host for alphaproteobacterial ancestors of mitochondria) possessed two membranes (outer and inner), and was sexual. Alphaproteobacterial ancestors of mitochondria are assumed to have been selfish parasites of pre-karyote periplasm (intermembrane space), the infection of which might have reminded infection of negibacterial periplasm by *Bdellovibrio* sp. It is proposed that eukaryotic plasma membrane descended from pre-karyote outer membrane, while pre-karyote inner (plasma) membrane is proposed to have been ancestral to eukaryotic nuclear/ER membrane. The strong evidence in favor of nuclear/ER membrane originating from pre-karyote plasma (inner) membrane is the fact that eukaryotic co-translational import of proteins into ER is homologous to co-translational secretion of proteins through prokaryotic plasma membrane (e.g., negibacterial inner membrane), while eukaryotes do not secrete proteins co-translationally through their plasma membrane. Eukaryotic nucleoplasm is thought to be derived from pre-karyote cytoplasm, while pre-karyote periplasm is thought to be

ancestral to eukaryotic cytoplasm. The view that the eukaryotic nucleoplasm was ancestrally pre-karyote cytoplasm is supported by the following:

1. Eukaryotic nucleolus with 109 unique eukaryotic protein domains is still the place of assembly of ribosomal subunits (Staub et al., 2004).
2. Some components of signal recognition particle (SRP) are still present in the nucleolus (Politz et al., 2000).
3. There have been reports about translation still occurring in the eukaryotic nucleus (Iborra et al., 2001; Hentze, 2001; Pederson, 2001).

The transitions of periplasm to cytosol and cytosol to nucleoplasm, as well as the evolution of nuclear/ER membrane topology and nuclear pore complex are suggested to have been driven by selfish conflicts between the aggressive alphaproteobacterial parasite and the immunity respond of the host that resulted in obligatory cooperation. Phagocytosis might have evolved later, perhaps at the final stage of pre-karyote-to-eukaryote transition, and ancestrally served probably as another mechanism of protection against aggressive bacterial parasites.

6. Summary

The universally present components suggest that the LUCA was bounded by membrane lipids, the synthesis of which was non-stereospecific. LUCA further certainly possessed ribosomes and protein apparatus linking translation and secretion and probably also DNA, but otherwise the nature of LUCA is unclear. We propose that LUCA was a population of pre-cells bounded by two membranes of heterochiral composition (mixture of bacterial and archaeal lipids) that probably practiced frequent fusions and fissions. The LBCA and the LACA arose convergently via the emergence of enzymes for stereospecific lipid syntheses for bacterial and archaeal lipids, respectively, and via the emergence of fusion-prohibiting cell walls. Parsimoniously organized genomes of bacteria and archaea might be also a result of convergent evolution mediated by r-selection for fast and precise replication and reproduction. We further suggest that the pre-karyote (host entity for Alphaproteobacteria), though sharing common precellular ancestor with *Archaea*, was unlucky to have evolved fusion-prohibiting cell surface and thus could have evolved sex. DNA damage checkpoint might have been the only ancestral checkpoint regulating both pre-karyote asexual and sexual life cycle. Sex might have represented useful repair strategy, the strategy of limiting of horizontal gene transfer, the strategy of transmission of symbionts increasing the fitness of their carriers, as well as strategy of attenuation of aggressive viral and cellular parasites. All these strategies together might have allowed the pre-karyote to survive in competition with prokaryotes bearing rigid cell surface. We further suggest that pre-karyote was bounded by two membranes (the inheritance from LUCA) and alphaproteobacterial ancestors of mitochondria were parasiting in

the pre-karyote periplasm. This infection might have reminded the infection of gram-negative bacteria by *Bdellovibrio* sp. Parasitic Alphaproteobacteria might have had the ability to disrupt pre-karyote membranes and to secrete degradative enzymes into the pre-karyote cytoplasm. Under this selective pressure pre-karyote might have evolved various strategies to resist the aggressive infection. These might have included the repair of membranes via fusion of membrane vesicles arising after the disruption of inner pre-karyote membrane, what could in fact have resulted in nuclear/ER membrane topology. Further immunity responses might have been the insertion of ADP/ATP translocase into alphaproteobacterial inner membrane and the export of ribosomal subunits and mRNAs into pre-karyote periplasm. We propose that eukaryotic plasma membrane was ancestrally pre-karyote outer membrane, eukaryotic endomembrane system (i.e., ER/nuclear membrane) was ancestrally pre-karyote inner membrane, eukaryotic nucleoplasm was ancestrally pre-karyote cytosol, and eukaryotic cytosol was ancestrally pre-karyote periplasm.

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Biodata of **Wolfgang Löffelhardt**, author of "***Low CO_2 Stress: Glaucocysto-phytes May have Found a Unique Solution"***

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LOW CO_2 STRESS: GLAUCOCYSTOPHYTES MAY HAVE FOUND A UNIQUE SOLUTION

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1. Glaucocystophytes and Their Position in Plastid Evolution

Glaucocystophytes maintain a special position among the archaeplastida: their cyanelles constitute the "missing link" in plastid evolution. They are considered to be the most ancient phototrophic eukaryotes known to date and can be assigned the status of "living fossils" (Löffelhardt and Bohnert, 2002). The plastids of the archaeplastida, i.e., the cyanelles, the rhodoplasts of red algae, and the chloroplasts of green algae and higher plants, are surrounded by two membranes and are thought to result from a single primary endosymbiotic event between a heterotrophic protist and a cyanobacterium. This postulated monophyly of the kingdom "Plantae" is supported by concatenated phylogenetic analyses of plastid and nuclear genes (Martin et al., 2002; Rodríguez-Ezpeleta et al., 2005) and by the demonstration of homologous protein import apparatus in cyanelles, rhodoplasts, and chloroplasts (Steiner and Löffelhardt, 2005). The denomination "cyanelle," though incorrect, is kept for historical reasons. Cyanelles are not endosymbiotic cyanobacteria but primitive plastids. However, among plastids they are the closest relatives to their free-living ancestors: A peptidoglycan wall between the inner and outer envelope membranes was retained in cyanelles but lost in rhodoplasts, chloroplasts, and all other plastid types (Fig. 1). Thus, the name "muroplast" has also been proposed. A central body containing the bulk of cyanelle Rubisco might be the second unique cyanobacterial heritage, a eukaryotic carboxysome (Fig. 1). In this review, the pros and cons of this hypothesis will be discussed.

2. *The Need for a Carbon-Concentrating Mechanism (CCM) in Aquatic Microorganisms*

Rubisco, the key enzyme of the Calvin cycle, has to face several problems: (1) Its substrate, CO_2, amounts to only 0.038% of the atmospheric gases; (2) its "second substrate," oxygen, though disfavored by a specificity factor of 50 to 250 has an

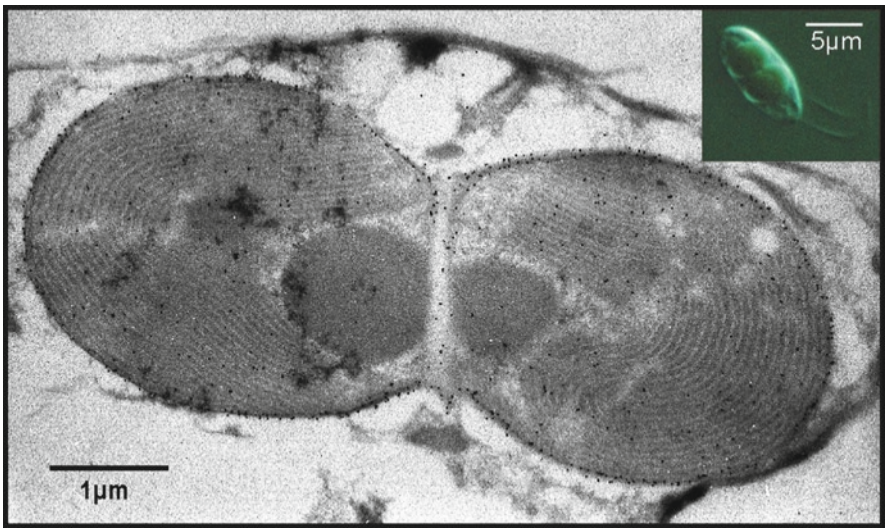

Figure 1. Immuno-EM of a dividing cyanelle: gold particles decorate the unique peptidoglycan wall. CS, (putative) carboxysome; PG, peptidoglycan septum. Insert: Interference contrast micrograph of the biflagellate.

air concentration of 21%; (3) prematurely bound substrate and substrate analogs such as the "misfire product," xylulose-1,5-bisphosphate (Pearce, 2006), or 2-carboxyarabinitol-1-phosphate (formed in the dark) act as inhibitors necessitating the chaperone action of Rubisco activase (Portis, 2003). The thermosensitivity of the latter, led to the development of the C_4 and CAM pathways among higher plants living in warm or hot climates, respectively.

In addition to all that, aquatic microorganisms have to cope with the low solubility of CO_2 in water, which is dependent upon temperature and pH. Therefore, all cyanobacteria and most algae invented a strategy for inorganic carbon concentration, the CCM (Badger et al., 2005; Giordano et al., 2005). Unlike higher plants, no pre-fixation of CO_2 via phosphoenolpyruvate (PEP) carboxylase is involved. Instead, the concentrations of both substrate and enzyme are substantially increased at the site of CO_2 fixation. A prerequisite for the CCM is the organization of the bulk of Rubisco in microcompartments where it is highly concentrated in a quasicrystalline form and nevertheless displays full enzyme activity: carboxysomes in cyanobacteria, and pyrenoids in algae. The increased substrate concentration is achieved via induction of transporters and/or pores for inorganic carbon. Since the gas CO_2 is membrane-permeant to some extent, bicarbonate is the storage form of choice in the cytosol of cyanobacteria and in the stroma of algal plastids. Therefore, carbonic anhydrase (CA) plays a crucial role in cyanobacterial and algal CCM in providing the substrate for Rubisco through conversion of accumulated bicarbonate.

Although the carboxysomal and the pyrenoidal CCM have some general features in common, they differ regarding certain details, as outlined below.

Furthermore, in-depth studies of the mechanisms involved have only been conducted using β-cyanobacteria such as *Synechococcus* sp. PCC 7942, *Synechococcus* sp. PCC 7002, and *Synechocystis* sp. PCC 6803 (Badger et al., 2005) and the green alga *Chlamydomonas reinhardtii* (Moroney and Ynalvez, 2005; Yamano and Fukuzawa, 2009).

The extent of bicarbonate enrichment relative to the environmental Ci concentration is quite different for the two types of CCM: cyanobacteria are able to concentrate Ci several thousandfold via their high affinity transport systems, whereas the accumulation factor of Ci is about 70 in the stroma of green algal chloroplasts.

Several small polyhedric carboxysomes (diameter about 50–100 nm) are located in the centroplasm of cyanobacteria (Kaneko et al., 2006). The cytosolic bicarbonate concentrated through the action of bicarbonate transporters and conversion from influxed CO_2 at the expense of photosynthetic energy enters the carboxysomes by diffusion. CA co-packaged with Rubisco catalyzes the rapid conversion into CO_2, which is then present in near-saturation conditions, allowing efficient fixation by the surrounding Rubisco.

In algal plastids, in general, one large rounded pyrenoid (diameter 200–500 nm) is present in the stroma, often, but not always traversed by unstacked thylakoid membranes (Osafune et al., 1990). The location of the crucial CA in *C. reinhardtii* chloroplasts is different from the situation in cyanobacteria: CAH3 resides in the lumen of the thylakoids penetrating the pyrenoid (Karlsson et al., 1998). In the light, passive transport of bicarbonate through channels into the thylakoid lumen occurs, which is acidified during photosynthetic electron transport. The equilibrium is shifted toward CO_2 (and rapidly attained by CAH3, leading to further influx of bicarbonate, etc.), which diffuses across the membrane into the surrounding pyrenoidal Rubisco and is fixed (Moroney and Ynalvez, 2005; Mitra et al., 2005).

Another difference between carboxysomes and pyrenoids is the presence in the former of an electron-dense "shell," a proteinaceous layer (consisting of the CcmKLMN proteins) surrounding the microcompartment. Recent structural studies on the CcmK proteins shed light on how such a polyhedral shell could be formed: carboxysomes are proposed to function not simply as a containment for Rubisco and CA, but they appear to display selective and controlled permeability for metabolites, perhaps including CO_2 and O_2 (Kerfeld et al., 2005; Tanaka et al., 2009). Recent data indicate a function of the shell as diffusion barrier for CO_2 (Dou et al., 2008) reducing the loss of substrate from the Rubisco microcompartment. Interestingly, CcmM was recently reported to form – together with the carbonic anhydrase CcaA – a multiprotein HCO_3^- dehydration complex in *Synechocystis* sp. PCC6803.

It is presently unknown how far these models can be generalized, e.g., for beta-cyanobacteria and other green or red algae, respectively. The beta-CA CcaA is co-packaged with Rubisco in the carboxysomes of several but not all cyanobacteria. There are indications that shell proteins with CA activity could compensate for the lack of CcaA (So et al., 2004). In some green algae, e.g., *Oocystis lacustris* (Stoyneva et al., 2009) the pyrenoid is not traversed

by thylakoid membranes, which also applies to diatoms (A. Schmidt, personal communication). In these cases a mechanism of the pyrenoidal CCM different from that in *C. reinhardtii* has to be envisaged.

3. The CCM in *Cyanophora paradoxa*

In the 1990s, the presence or absence of a CCM in *C. paradoxa* was discussed controversially: preliminary experiments of different groups seemed to favor either opinion but did not result in published papers. Gas-exchange measurements to quantify Ci uptake and the determination of photosynthetic affinity with cultures grown under high and low CO_2, respectively, clearly showed the operation of a CCM (Burey et al., 2007). The effects of the CA inhibitor ethoxyzolamide indicated a crucial role for CA in this process. This was not unexpected because the presence of a microcompartment is indicative for a CCM (Badger et al., 2005). An expressed sequence tag (EST) project allowed to set up microarrays for the search of CO_2-responsive genes that are upregulated or downregulated upon shift of the cultures from 5% CO_2 in air to air only (Burey et al., 2007) and to compare these data with results obtained *for Synechocystis* sp. PCC6803 (Wang et al., 2004) and *C. reinhardtii* (Miura et al., 2004), respectively.

3.1. CO_2-RESPONSIVE GENES

When *C. paradoxa* cells are shifted form normal laboratory growth conditions (5% CO_2 in air) to ambient CO_2, growth stops and is reassumed (at a lower rate) after a lag period of about 3 h. During this period, induction of the CCM took place involving transcriptional activation of a total of 58 genes (Table 1), or repression of a total of 67 genes, respectively (Table 2). Low CO_2 stress triggers changes in metabolism as overall reduction of antenna size, photosynthetic electron transport, and Calvin cycle activity, whereas CA activity, starch biosynthesis, and uptake of Ci are enhanced (Burey et al., 2007). There is good correlation with macro- and microarray data for green algae and cyanobacteria, respectively (Tables 1 and 2). Rubisco activase is upregulated since size (in case of pyrenoid) or number (in case of carboxysomes) of the Rubisco microcompartments increase in order to compensate for the decrease in CO_2, and the amount of "Rubisco's catalytic chaperone" (Portis, 2003) parallels that of Rubisco. Granule-bound starch synthase is upregulated in *C. paradoxa*, resulting in increased amounts of cytosolic starch granules (Fathinejad et al., 2008) and in *C. reinhardtii*, where a starch sheath is deposited on the pyrenoid surface under low CO_2 (Moroney and Ynalvez, 2005). On the other hand, genes for phycobilisome linker polypeptides are downregulated under low CO_2] (Table 2), which also applies for biliprotein and linker genes in *Synechocystis* sp. PCC6803 (Wang et al., 2004). In *C. paradoxa*, electron micrographs indicate a shift of cyanelle ribosomes from the thylakoid region (involved in the biosynthesis of

Table 1. A selection of genes upregulated upon shift to low CO_2 in *Cyanophora paradoxa*.

Function in	Gene	Protein	Paralleled in
Photosynthesis (CCM)	*rca*	Rubisco activase	*C. reinhardtii*
		Granule-bound starch synthase	*C. reinhardtii*
CCM	*CAH3-1?*	beta-CA (mitochondrial)	*C. reinhardtii*
	CAH3-2?	beta-CA (mitochondrial)	*C. reinhardtii*
		CA (cytosolic?)	
	lciA	Bicarbonate transporter	*C. reinhardtii*
ROS-inactivating enzymes	*Prdx1*	Peroxiredoxin1	
	cat1	Catalase	
		Glutaredoxin	
Protein degradation	*UBC4*	Ubiquitin-conjugating enzyme E2	
		Ubiquitin/ribosomal protein S27a	
Chaperones		Protein disulfide-isomerase1	
		Peptidyl-prolyl cis-trans-isomerase	

Table 2. A selection of genes downregulated upon shift to low CO_2 in *Cyanophora paradoxa*.

Function in	Gene	Protein	Parallel in
Phycobilisome antenna	*cpcG*	Phycobilisome rod-core linker	*Synechocystis* sp. PCC6803
Calvin cycle		Sedoheptulose-1,7-bisphosphatase	*Synechocystis* sp. PCC6803, *C. reinhardtii*
	pgk	Phosphoglycerate kinase	*Synechocystis* sp. PCC6803, *C. reinhardtii*
	tktC	Transketolase	*Synechocystis* sp. PCC6803, *C. reinhardtii*
Photosynthetic electron transport	*psaC*	Photosystem I reaction center subunit II	*Synechocystis* sp. PCC6803, *C. reinhardtii*
	psaK	Photosystem I reaction center subunit X	*Synechocystis* sp. PCC6803, *C. reinhardtii*
	psaL	Photosystem I reaction center subunit XI	*Synechocystis* sp. PCC6803, *C. reinhardtii*
	petJ	Cytochrome c_6	
Protein synthesis		Translation elongation factor 1 beta 2	*C. reinhardtii*
		Translation elongation factor 1 alpha	*C. reinhardtii*
Cytoskeleton		Tubulin alpha-2	*C. reinhardtii*
		Tubulin beta-1	*C. reinhardtii*
		Tubulin gamma	*C. reinhardtii*

biliproteins) to the region surrounding the microcompartment (involved in the biosynthesis of Rubisco; Fathinejad et al., 2008).

3.2 ORGANIZATION OF RUBISCO IN GLAUCOCYSTOPHYTES

Morphology and location of the Rubisco microcompartments are variable among the four *bona fide* glaucocystophyte genera: they are central in *C. paradoxa* (Fig. 1), *Cyanophora biloba*, *Gloeochaete wittrockiana*, and *Cyanoptyche gloeocystis*,

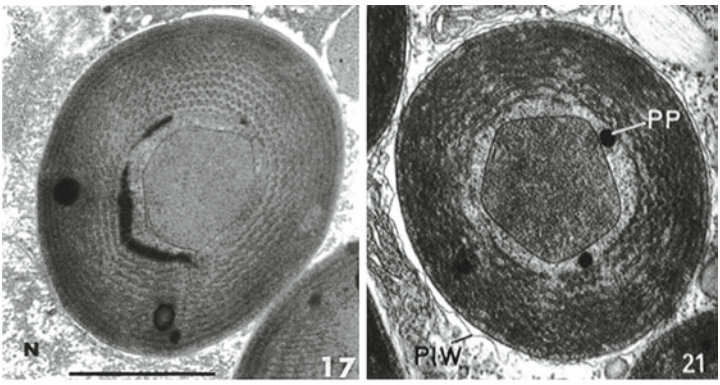

Figure 2. Cyanelles showing polyhedral microcompartments surrounded by an electron-dense, shell-like layer. (Modified from Kies, 1992.)

but peripheral (polar) in *Glaucocystis nostochinearum* (Kies, 1992; Kugrens et al., 1999). *G. nostochinearum* cyanelles also differ from the other coccoid muroplasts due to their elongated irregular shape resembling the clocks in the famous painting, "The persistence of time," of Salvador Dali. However, given that the peptidoglycan layer is thought to shape a bacterial cell, there is no significant difference in the muropeptide pattern between *C. paradoxa* and *G. nostochinearum* (Pfanzagl et al., 1996). *G. wittrockiana* and *C. gloeocystis* cyanelles, on the other hand, possess pentahedral (in the EM section) central bodies that are surrounded by an electron-dense (perhaps proteinaceous) layer resembling the shell of the (much smaller) cyanobacterial carboxysomes (Kies, 1992; Fig. 2).

3.3. PROTEOMIC INVESTIGATIONS OF ISOLATED MICRO-COMPARTMENTS OF *C. PARADOXA* AND ELECTRON MICROSCOPY OF CELLS GROWN UNDER HIGH AND LOW CO_2

A purification procedure based on Percoll step gradients was established. In order to reduce the close association between microcompartment, cyanelle DNA, and the innermost concentric thylakoid membranes, DNase and detergent treatment was indispensable (Burey et al., 2005). The resulting pellet contained the bulk of Rubisco and several protein bands, which were subjected to in gel digestion and mass spectrometric analysis: only Rubisco large and small subunit and Rubisco activase could be identified as *bona fide* constituents. All other bands were either contaminants or had no match in the databases. Especially, there was no indication of shell proteins or of a carboxysomal CA (Fathinejad et al., 2008).

A partial polyhedral appearance, possibly camouflaged through the close contact with DNA and thylakoids, can be observed under low CO_2, and the overall

size and electron density is higher (Fathinejad et al., 2008). However, a shell could never be seen around the *C. paradoxa* microcompartment, regardless of the fixation conditions used.

3.4. RUBISCO ACTIVASE

Rubisco activase is an enzyme/chaperone necessary for optimal activity of Rubisco and is ubiquitous in plants and algae and, as expected, associated with the pyrenoid in the latter (McKay et al., 1991). Corresponding mutants of *C. reinhardtii* are defective in the CCM (Pollock et al., 2003). Surprisingly, there is no *rca* gene in the genome of *Synechocystis* sp. PCC6803. The CCM associated with beta-carboxysomes seems to be efficient enough to ensure maximum activation of Rubisco at all times. However, *rca*-like genes are found in filamentous, nitrogen-fixing cyanobacteria (Li et al., 1999), which are considered the likely ancestors of plastids rather than unicellular ones (Martin et al., 2002). There is considerable sequence similarity in a central domain of about 300 amino acids. Plant activases possess a 70 amino acid N-terminal domain (in addition to the transit sequence) not present in the cyanobacterial counterparts. On the other hand, cyanobacterial activases are distinguished by an extra C-terminal domain with significant sequence similarity to the three or four repeat regions in the C-terminal domain of the largest cyanobacterial shell protein, CcmM (Ludwig et al., 2000). Whether this has implications for carboxysome assembly is not known at present.

The Rubisco activase of *C. paradoxa* appeared to be of plant type, i.e., contains the N-terminal extension and lacks the C-terminal extension found in cyanobacterial *rca* genes. The enzyme is localized in cyanelles, and is co-packaged with Rubisco (Mangeney et al., 1987) as evidenced through in vitro import of the precursor into isolated cyanelles and assembly into the microcompartment (Burey et al., 2005), proteomics of isolated central bodies, and western blotting of central body proteins with heterologous antisera (Fathinejad et al., 2008). Transcription is upregulated upon shift of *Cyanophora* cells to low CO_2 (Burey et al., 2007), likely like that of Rubisco as has been shown for cyanobacteria (Wang et al., 2004).

4. Eukaryotic Carboxysomes: The "Raison d'Être" for the Eukaryotic Peptidoglycan?

One hypothesis for CCM evolution is based on the geological record dating back 600 million years: in the phanerozoic period, about 400 million years ago, a decrease in atmospheric CO_2 accompanied by an increase in O_2 took place. This led to the development of a carboxysomal CCM in cyanobacteria and, independently, of a pyrenoidal CCM in algae (Badger and Price, 2003). Alternatively, earlier episodes of higher O_2 and lower CO_2 cannot be excluded. If this occurred

prior to the primary endosymbiotic event, the carboxysomal CCM was already established in the cyanobacterial ancestor of plastids. Glaucocystophytes are the sole group of algae that did not convert the inherited carboxysomal CCM into a pyrenoidal one. As a consequence, glaucocystophyte cyanelles had to retain the peptidoglycan wall, as a stress-bearing layer resisting the osmotic pressure of the thousandfold-enriched bicarbonate (Raven, 2003). All other plastids could abandon the peptidoglycan as the less pronounced bicarbonate accumulation associated with a pyrenoidal CCM was tolerable for plastid envelopes.

Glaucocystophytes are niche organisms. *C. paradoxa* is the best-investigated member due to its generation time of 20 h, *G. nostochinearum* grows slightly slower, and *G. wittrockiana* and *C. gloeocystis* grow very slowly. This indicates that keeping peptidoglycan rendered cyanelle division more complicated and rather argues against the carboxysome nature of the microcompartment, which would imply optimal CO_2 fixation and thus reasonable growth rates. On the other hand, Raven's hypothesis offers the most convincing explanation for the presence of the unique organelle wall.

In summary, the question "carboxysome or pyrenoid" (or a microcompartment with intermediate properties) cannot be answered at present. Unsolved problems are the extent of bicarbonate enrichment within cyanelles, the presence of a "shell," and the localization of cyanelle CA. At present, about 4,000 unique ESTs are known, an estimated quarter of the *C. paradoxa* transcriptome. Among the three CAs found, none shows the typical cyanelle stroma targeting peptide (Steiner and Löffelhardt, 2005). Two of them are tentatively assigned to mitochondria, the third is a cytosolic enzyme (Burey et al., 2007).

The delicate nature of the microcompartments implicates possible loss of components during purification. Proteomics of cyanobacterial carboxysomes yielded but three genuine components (Rubisco LSU and SSU and CcmM) plus more than 60 contaminating proteins (Long et al., 2005). "Carboxysome" preparations from *C. paradoxa* were inspected via negative contrast EM (S. Fathinejad and S. Reipert, unpublished): partial fragmentation became apparent and, after chemical fixation, collapse to the individual Rubisco molecules was observed, as found for cyanobacterial carboxysomes (Rodríguez-Buey et al., 2005).

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PART III:
AQUATIC SYMBIOSES

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Barneah
Brickner
Richier
Sabourault
Ferrier-Pagès
Merle
Furla
Allemand
Kawano
Irie
Kadono
Stambler

Biodata of **Takeshi Naganuma**, author of **"*Animal–Bacteria Endosymbioses of Gutless Tube-Dwelling Worms in Marine Sediments*"**

Dr. Takeshi Naganuma obtained his Ph.D. from the University of Tsukuba, Japan, in 1989, specializing in aquatic microbial ecology. Following the defense of his dissertation, he joined Japan Marine Science and Technology Center (JAMSTEC; currently Japan Agency for Marine and Earth Science and Technology) as a research scientist of Deep Sea Research Department. He also joined Institute of Physical and Chemical Research (RIKEN), Japan, in 1990 and Marine Science Institute, University of California, Santa Barbara, in 1991–1992, as a visiting researcher. In 1994, Dr. Naganuma became Associate Professor of Biological Oceanography, School of Biosphere Science, Hiroshima University, Japan. He has been also invited to JAMSTEC; Japan Atomic Energy Agency; National Institute of Radiation Studies; and, National Institute of Polar Research, as a visiting researcher. He has been appointed an editorial board member of academic journals *Marine Ecology Progress Series* (MEPS); *Marine Biotechnology* (MBT); *Aquatic Ecosystem Health and Management*; and *Polar Science*. He currently leads the International Polar Year (IPY 2007–2008) core activity, "Microbiological and Ecological Responses to Global Environmental Changes in Polar Regions" (MERGE), 2007–2009. He has written three single-authored books, contributed 16 book chapters, and published 90 reviewed articles in English.

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ANIMAL–BACTERIAL ENDOSYMBIOSES OF GUTLESS TUBE-DWELLING WORMS IN MARINE SEDIMENTS

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1. Introduction

Endosymbioses by autotrophic sulfur-oxidizing bacteria (thiotrophs) or methane-oxidizing bacteria (methanotrophs) occur in more than 200 marine invertebrate species, living in diverse ecosystems at intertidal to bathyal depths (McMullin et al., 2003; Stewart et al., 2005), and representing five or more phyla, depending on phylum classifications (Fisher, 1990; Cavanaugh, 1994). These endosymbioses-based communities often show higher population densities than the surrounding habitats (Fisher 1990; Van Dover, 2000; Levin and Michener, 2002; Dahlgren et al., 2004). Many of the host animals are notably derived from the lineages within typical non-symbiotic taxa, such as vesicomyid clams, bathymodiolid mussels, and gutless annelids (Distel et al., 1988; Peek et al., 1998; Halanych et al., 2001; Halanych, 2005).

It has been a common consensus that most host animals harbor a single thiotrophic or methanotrophic species. Thiotrophic symbionts carry out chemoautotrophic organic production via Calvin–Benson cycle where energy for CO_2 fixation by the enzyme RuBisCO (EC 4.1.1.39) derives from sulfide oxidation (Fisher, 1990). Generally, RuBisCO has two forms, namely I and II. It is hypothesized that the common ancestor of RuBisCOs was similar to the form II, since this form is more adaptive to high CO_2 concentration, a condition which is presumed to have been present on the primitive Earth (Jordan and Ogren, 1981). In contrast, the form I is believed to have evolved in response to the decline of CO_2 and emergence of oxygen as the Earth's atmosphere changed (McFadden et al., 1986). The symbiotic thiotrophs are mostly ascribed to Gammaproteobacteria, with a few exceptions in Alphaproteobacteria (Elsaied and Naganuma, 2001; Elsaied et al., 2002). In contrast, methanotrophic symbionts assimilate carbon derived not from CO_2 but from methane, and oxidize part of methane to gain energy for metabolisms (Fisher, 1990). Among the two major types, i.e., I and II, of methane-oxidizing bacteria belonging to Gammaproteobacteria, only the type I group has been found in symbioses (Fisher, 1990; Cavanaugh, 1994). In neither thiotrophic nor methanotrophic symbioses have the organic materials transferred from symbionts to hosts been characterized.

The most studied hosts are the gutless tube-dwelling siboglinid worms of vestimentifera (tube worms) and pogonophora (beard worms). The siboglinid worms have been reported from a number of hydrothermal vents (Fisher, 1990) and from cold seeps associated with hydrocarbon reservoirs, subduction zone accretionary prisms, landslides (McMullin et al., 2003), as well as whale carcasses (e.g. Feldman et al., 1998). The vestimentiferan and pogonophoran tubeworms, together with *Sclerolinum brattstromi* (e.g., Halanych et al., 2001) as well as the bone-eating worms *Osedax rubiplumus* and *Os. frankpressi* (Rouse et al., 2004), comprise the Family Siboglinidae within the Class Polychaeta under the Phylum Annelida (Winnepenninckx et al., 1995; McHugh, 1997; Rouse and Fauchald, 1997; Halanych et al., 2001; McMullin et al., 2003; Rouse et al., 2004; Struck et al., 2007). However, their convergent and homoplastic characters related to oligomerous deuterostomes are still argued (Salivan-Plawen, 2000). These worms share the same unique strategy for life, i.e., lack of digestive tract and dependence on endosymbionts for nutrition (Fisher, 1990), although the endosymbionts of the *Osedax* worms are heterotrophic (Rouse et al., 2004). Therefore the vestimentiferan and pogonophoran worms are referred collectively to "tubeworms" in this article.

2. Biological Interests of the Host Tubeworms

Siboglinids, i.e., vestimentiferans and pogonophorans (or frenulates), have a uniquely modified storage organ, namely, trophosome, for chemoautotrophic microbial endosymbionts (recent reviews by Halanych, 2005; Southward et al., 2005). Currently more than 150 siboglinid species are known and grouped into of four major lineages (Halanych et al., 2002; Rouse et al., 2004; Glover et al., 2005; Halanych, 2005). The best-known linage consists the hydrothermal vent species, including the hydrothermal vent species (e.g., *Riftia pachyptila*) and the methane-seep species (of the genera *Lamellibrachia* and *Escarpia*, for example). Physiological properties of vestimentiferans have been first and most studied (Arp and Childress, 1981, 1983; Felbeck et al., 1981, 2004).

Closely related to vestimentiferan are the moniliferan *Sclerolinum* worms, which feed on dead and decaying wood and other organic matter (Halanych et al., 2001). Basal to the vestimentiferan/*Sclerolinum* linage is the *Osedax* worms, the recently discovered whale-bone eaters that harbor not autotrophic but heterotrophic endosymbionts (Rouse et al., 2004; Goffredi et al., 2005; Glover et al., 2005; Fujiwara et al., 2007).

Pogonophorans, or frenulates (also known as perviates), are the greatest lineage of siboglinids, with nearly three quarters of all recognized siboglinid species. Pogonophorans are found throughout seafloors such as continental margins, slopes, fjords, trenches, and peripheries of vents and seeps (Ivanov, 1963; Webb, 1963; Southward, 1971, 1972, 1979, 1988, 1991; Imajima, 1973; Southward et al., 1981; Flügel and Langhoff, 1982, 1983; Schmaljohann and Flügel, 1987; Mayer et al., 1988; Black et al., 1997; Pimenov et al., 1999; Rouse and Pleijel, 2001; Halaynch et al., 2002; Levin and Michener, 2002).

The endosymbionts in the vestimentiferan trophosomes are transferred not vertically but horizontally (Feldman et al., 1997; Nussbaumer et al., 2006). However, there is still some debate with reference to early development of the tissue. Trophosome has been traditionally regarded as a modified gut or endodermal tissue (Southward, 1988). According to the traditional view, it has been imagined that vestimentiferan larvae take up ambient microorganisms through the short-living mouth-to-gut cavity, and subsequently select some for endosymbioses when converting gut to trophosome.

Recently, Nussbaumer et al. (2006) reported that trophosome is a mesodermal tissue formed de novo and that endosymbionts migrate across the epithelium into the mesodermal trophosome. Thus, the trophosome is hypothesized to be a mesodermal structure next to the gut. Future experiments demonstrating occurrence of mesodermal markers in trophosome or neighboring tissues may favor the hypothesis. Alternatively, future experiments showing expression of endodermal markers such as *Gata4-6* or *FoxQ* may oppose the hypothesis. This kind of evo–devo approaches could help resolving the debated issue of trophosome origin and endosymbiont acquisition.

Siboglinid evolution is likely associated with sulfide levels of the habitats ranging from soft sediments and methane seeps to hydrothermal vents (Schulze and Halanych, 2003). Vestimentiferans are generally known in high-sulfide vents and seeps (Fisher, 1990; Lutz and Kennish, 1993; McMullin et al., 2003), and associated with whale carcasses (Feldman et al., 1997; Di Meo et al., 2000; Baco and Smith, 2003; Smith and Baco, 2003; Rouse et al., 2004) and shipwreck (Dando et al., 1992; Williams et al., 1993). The northeastern Pacific vestimentiferans, *Escarpia spicata* and *Lamellibrachia barhami*, show opportunistic occurrence in vent and seep habitats and even on a decaying whale carcass (Black et al., 1997, 1998; Feldman et al., 1998). This opportunism may be explained by low geographical barriers between vent and seep habitats, and the similar opportunism should be found for the tubeworms of the western Pacific which has similar geological and geographical settings. In contrast, pogonophoran tubeworms have been mostly reported from low-sulfide habitats such as a Loihi Seamount hydrothermal vent and cold seeps (Flügel and Langhoff, 1983; Schmaljohann and Flügel, 1987; Dando et al., 1994; Black et al., 1997), fjord (Webb, 1963; Southward et al., 1981), landslide (Mayer et al., 1988), other shallow and deep muddy sediment (e.g. Ivanov, 1963; Southward, 1971, 1972; Imajima, 1973; Southward et al., 1981), and recently from a mud volcano off the Norwegian coast (Pimenov et al., 1999). The pogonophorans are likely to have wider distribution in non-vent habitats than expected. Tubeworms in seeps and muddy sediments have also greater longevity (~200 years or longer; Fisher et al., 1997; Bergquist et al., 2000; Cordes et al., 2003, 2005) than individual hydrothermal vents (~100 years; MacDonald et al., 1980; Killingley et al., 1981; Lalou and Brichet, 1982; Delaney et al., 1998), where focused fluxes of heat and chemicals sustain much larger masses of tubeworms. Compared with high fluxes at hot vents, cold seeps and muddy sediments provide only diffusive and slow fluxes, and the tubeworms need to develop the "life in the slow lane" of tubeworms (Fisher et al., 1997). Mud volcanoes may

supply high but pulsed fluxes of hydrocarbons (MacDonald et al., 2000). Whale carcasses are also ephemeral and able to supply only limited amounts of methane and sulfide (Smith and Baco, 2003).

The slow but long life of the seep tubeworms may be suitable for dissecting the process, where occasional bacterial intruders are selected for establishing functional endosymbioses. Bacterial endosymbionts are not heritable in a vent vestimentiferan (Cary et al., 1993), which is likely the common feature of other vestimentiferan and pogonophoran species. Each generation of the tubeworms has to introduce ambient microorganisms in their body and select useful ones as endosymbiont(s) for each individual worm. A speculation is that the rapid-growing vent worms (Lutz et al., 1994) may have a rapid process of symbiont selection, and thus tend to host single symbiont species that may vary on the habitat bases (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000). On the other hand, non-vent tubeworms living the "life in the slow lane" would have enough time for selecting their symbionts that may vary widely among habitats, individuals, and even symbiont-bearing cells. Thus, complexity and flexibility in the endosymbioses of the tubeworms inhabiting seeps and muddy sediment are focused in this mini-review, although the speculation has been unproven yet.

3. Biological Interests of the Endosymbiotic Bacteria

It is widely believed that most siboglinids host a single species of endosymbiont, upon which they obligately depend on for organic nutrition (Fisher, 1990). These endosymbionts are largely divided into two major energetic/anabolic groups: (1) thiotrophs (or sulfur-oxidizing chemoautotrophs) that obtain energy from oxidation of sulfide for autotrophic assimilation of CO_2 by the Calvin–Benson cycle; or, (2) methanotrophs that use methane as a single carbon source for energy generation and carbon assimilation (Fisher, 1990). An exception is the heterotrophic endosymbionts in *Osedax* species (Rouse et al., 2004; Goffredi et al., 2005; Fujiwara et al., 2007). In addition, some tubeworm species may also take up organic compounds from ambient environments (Southward et al., 1979), but this nutrition supply is regarded as only additional, not essential.

Phylogenetic analyses of the 16S ribosomal RNA gene (16S rDNA) have shown that siboglinid endosymbionts are mainly affiliated with Gammaproteobacteria (McMullin et al., 2003; Stewart et al., 2005). However, specimens of *Lamellibrachia* sp. harbor Alpha-, Beta-, and Epsilonproteobacteria in the form of endosymbiosis and/or intracellular association (Naganuma, et al., 1997a, b; Elsaied and Naganuma, 2001; Elsaied et al., 2002; Kimura et al., 2003b), although the stability and persistence of these associations is unclear (Naganuma et al., 2005). While most studies have mainly focused on endosymbionts associated with the larger vestimentiferans (recently reviewed in McMullin et al., 2003), only few studies have targeted at pogonophoran endosymbionts (Kimura et al., 2003a; Naganuma et al., 2005).

Modes of endosymbiont transmission and/or acquisition are an important subject in the ecology and evolution of tubeworm symbioses. If siboglinid worms had developed vertical transmission of endosymbionts to their larvae, it would have provided more ensured forms of endosymbioses as shown in other organisms such as insect–bacterial and plant–chloroplast associations (e.g., Dale and Moran, 2006). But, in reality, no examples have been reported for vertical transmission of siboglinid endosymbionts, and no bacteria has been observed in gonads, sperms, eggs, or newly hatched larvae (Cavanaugh et al., 1981; Cary et al., 1989, 1993). On the other hand, acquisition of endosymbionts from the surroundings is supported by the occurrence in the vestimentiferan *Riftia pachyptila* of a seemingly functional flagellin gene. The gene was probably derived from endosymbiotic bacteria, and may indicate a motile and thus transmittable stage in the endosymbiont life cycle (Millikan et al., 1999).

Endosymbionts of the Norwegian pogonophora *Siboglinum fjordicum* is probably a methanotroph, based on isotope and enzymatic analyses (Southward et al., 1981), and occurrence of a methanotrophic symbiont in the Japanese pogonophoran *Oligobrachia mashikoi* is also suggested by molecular technique (Kimura et al., 2003a), although a possibility of dual energetic metabolism by thiotrophy and methanotrophy is suggested (Schmaljohann et al., 1990; Schmaljohann, 1991). The seeming similarity between methanotrophic symbionts in two different pogonophoran species from geographically distant waters may suggest selection and even coevolution in these pogonophoran–bacterial symbioses, though this may not necessarily be a result of species-specific association between host animals and symbiotic bacteria.

Vestimentiferans also show no evidence of species-specific associations between the hosts and symbionts (Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000). Species-specific association and possible resultant coevolution would be expected only by vertical transmission of symbionts from parent to offspring (Clark et al., 2000; Degnan et al., 2004), which has been observed in the symbiosis of the vesicomyid clam *Clayptogenia* (Peek et al., 1998). It is thus concluded that siboglinid endosymbionts are newly acquired from the environment each generation, and that coevolution between siboglinid hosts and symbionts have rarely been an important process of siboglinid ecology and evolution (Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000).

Despite the seeming absence of coevolution, relatively a small group of environmental (free-living) bacteria are acquired for siboglinid symbioses (McMullin et al., 2003). There are patterns of endosymbiont distribution in terms of geographic locations and geological settings of the habitats, such as vents versus seeps, where phylogenetic groupings of vent- and seep-specific species are found (Feldman et al., 1997; Nelson and Fisher, 2000; Di Meo et al., 2000). Symbionts of vent vestimentiferans form a single distinct clade, and habitat-specific symbiotic 16S rDNA sequences occur in multiple species of the genera *Riftia*, *Oasisia*, and *Tevnia* (Di Meo et al., 2000; Nelson and Fisher, 2000). In contrast, symbionts of seep vestimentiferans are grouped into three clades (Nelson and Fisher, 2000;

McMullin et al., 2003), with the symbionts specific to (1) relatively deeper sites (1,800–3,300 m) of off-Oregon, off-Florida, and Fiji-Lau Basin areas; (2) intermediately deep sites (900–2,200 m) along the western coast of North America at intermediate depths; and, (3) shallower sites (550–650 m) in the off-Louisiana area. The seep-specific species of the *Escarpia* and *Lamellibrachia* worms hosted symbionts from all the three clades (McMullin et al., 2003).

4. Endosymbioses by Multiple Bacterial Species

The view of endosymbioses by single bacterial species has been challenged by multiple bacterial endosymbioses in seep/mud tubeworms and other vent/seep hosts (Table 1; Naganuma et al., 2005; Chao et al., 2007). Some vent mytilid mussels depend on dual symbioses of thiotrophic and methanotrophic bacteria (Distel et al., 1995;Robinson et al., 1998; Fiala-Medioni et al., 2002). The gutless oligochaete *Olavius algarvensis* has an endosymbiotic coalition of a sulfate-reducing bacterium (SRB) and a thiotroph (Dubilier et al., 2001). In the coalition, SRB supplies sulfide for oxidation by the thiotroph, but they should be separated in the host body to ensure anaerobic and aerobic microhabitats, respectively. The microhabitat segregation may be associated with a "physiological gradient" (de Burgh, 1986), which is obscure in *Ola. algarvensis* (Dubilier et al., 2001). The microhabitats may also be separated by a physical barrier, which has not been elucidated.

The undescribed lamellibrachid *Lamellibrachia* sp. L1 (Fig. 1) inhabits a methane seep, 1,167 to 1,170 m deep, in Sagami Bay, Japan (35°00.1′N, 139°13.6′E; Hashimoto et al., 1989; Masuzawa et al., 1992; Kojima, 2002), and hosts multiple symbionts (Figs. 2 and 3). At least two Epsilonproteobacterial cells and 16S rDNA sequences were observed in a specimen (Naganuma et al., 1997a, b). Many species of Epsilonproteobacteria are microaerobes, and the symbiotic ones may occupy a microaerobic niche in an oxygen gradient (Naganuma, 1998, 1999). However, another specimen of *Lamellibrachia* sp. L1 yielded a single 16S rDNA sequence related to the Alphaproteobacterium *Rhodobacter sulfidophilus* and two sequences of the RuBisCO form II gene (*cbbM*) related to that of the Betaproteobacterium *Thiobacillus denitrificans* (Elsaied and Naganuma, 2001; Elsaied et al., 2002). The third specimen demonstrated the endosymbiotic localization of alpha-, beta- and gammaproteobacterial 16S rDNA sequences by in situ hybridization (Kimura et al., 2003b). These studies suggest that the symbiotic 16S rDNA sequences in *Lamellibrachia* sp. L1 are highly variable. The varied symbioses should be ascribed not only to individual-to-individual (interindividual) variation but probably to part-to-part (intra-individual) variation within a single individual due to a physiological gradient in trophosome (de Burgh, 1986).

It is unclear whether those variable symbioses are already established forms or only transient forms during selection of symbionts acquired from ambient water and sediment. The view of environmental (occasional) acquisition of symbionts is suggested by the occurrence of related (not identical) 16S rDNA sequences in the *Lamellibrachia* trophosome and in the ambient sediment (Kimura

Table 1. Examples of observed and suspected multiple endosymbioses in marine invertebrates.

Host invertebrate		Endosymbionts	Reference
Seep pogonophoran	*Siboglinum poseidoni*	Thiotroph and methanotroph	Schmaljohann et al. (1990)
Mud pogonophoran	*Oligobrachia mashikoi*	Suspected thiotroph and methanotroph	Kimura et al. (2003a)
Seep vestimentiferan	*Lamellibrachia* sp.	Unidentified	Naganuma et al. (1997a)
Seep vestimentiferan	*Lamellibrachia* sp.	Epsilonproteobacteria	Naganuma et al. (1997b)
Seep vestimentiferan	*Lamellibrachia* sp.	Alphaproteobacterium (but two RuBisCO genes)	Elsaied and Naganuma (2001) Elsaied et al. (2002)
Seep vestimentiferan	*Lamellibrachia* sp.	Alpha-, Beta-, Gamma-proteobacteria	Kimura et al. (2003b)
Vent vestimentiferans	*Ridgiea* spp. (larvae)	"Large" and "small"	Southward (1988)
Vent vestimentiferans	*Ridgiea piscesae* *Ridgeia phaeophiale*	"Large" and "small"	de Burgh et al. (1989)
Vent vestimentiferan	*Riftia pachyptila*	"Variety of bacterial types"	Fisher and Childress (1984)
Vent vestimentiferan	*Ridgiea piscesae*	*Maorithyas hadalis* symbiont *Halothiobacillus*, etc.	Chao et al. (2007)
Gutless oligochaete	*Inanidrilus leukodermatus* *Olavius planus*	"Larger" and "smaller"	Giere and Langheld (1987)
Gutless oligochaete	*Olavius loisae*	Alphaproteobacterium, Gammaproteobacterium	Dubilier et al. (1999)
Gutless oligochaete	*Inanidrilus leukodermatus*	"Larger" and "smaller"	Krieger et al. (2000)
Gutless oligochaete	*Olavius algarvensis*	Gammaproteobacterium, Deltaproteobacterium	Dubilier et al. (2001)
Seep clam	*Maorithyas hadalis*	Two thiotrophic phylotypes	Fujiwara et al. (2001)

(continued)

Table 1 (continued)

Host invertebrate		Endosymbionts	Reference
Seep mussel	Unnamed mytilid	Methanotroph (Type I)	Brooks et al. (1987)
		Chemoautotroph (?)	Fisher et al. (1987)
			Distel and Cavanaugh (1994)
Vent mussel	Unnamed mytilid	Thiotroph, methanotroph	Distel et al. (1995)
Vent mussel	*Bathymodiolus* sp.	Thiotroph, methanotroph	Pond et al. (1998)
Vent mussel	*Bathymodiolus puteoserpentis*	Thiotroph, methanotroph	Robinson et al. (1998)
Vent mussel	*Bathymodiolus sp.*	Thiotroph, methanotroph	Trask and Van Dover (1999)
Vent mussel	*Bathymodiolus azoricus*	Thiotroph, methanotroph	Fiala-Medioni et al. (2002)
Vent snail	*Olgaconcha tufari*	Thiotroph, methanotroph	Gal'chenko et al. (1992)
Shipworm bivalve	*Lyrodus pedicellatus*	*Teredinibacter turnerae*, others	Distel et al. (2002)

et al., 1999). Unequivocal detection of endosymbionts as free-living forms in ambient environment is proved only for the lucinid bivalve *Codakia orbicularis* in shallow-water sea-grass bed (Gros et al., 2003). Environmental acquisition of symbionts has been also suggested in gutless oligochaetes (Giere and Langheld, 1987; Giere et al., 1991; Dubilier et al., 1995, 1999, 2001; Krieger et al., 2000) and vent vestimentiferans (Cary et al., 1993; Feldman et al., 1997; Laue and Nelson, 1997). This view is supported by the observations that (1) different species of vestimentiferan worms in the same habitats may bear identical endosymbionts, (2) the same species of vestimentiferans in different habitats may host different symbionts, and (3) vestimentiferans and endosymbionts have not necessarily coevolved (Feldman et al., 1997; Laue and Nelson, 1997; Di Meo et al., 2000), contrary to cospeciation of vesicomyid clams and symbiotic bacteria (Peek et al., 1998).

5. Possible Dual Thiotrophy–Methanotrophy in Pogonophorans

Previous studies have shown that pogonophoran endosymbionts are thiotrophs and/or methanotrophs, as demonstrated by TEM micrography, enzymatic activity, and carbon isotope ratio (Dando et al., 1986; Southward et al., 1986; Spiro et al., 1986; Schmaljohann and Flügel, 1987; Schmaljohann et al., 1990). Combined characterizations of 16S rDNA, the genes encoding RuBisCO forms I and II (*cbbL* and *cbbM*), particulate and soluble methane monooxygenase (*pmoA* and *mmoX*), methanol dehydrogenase (*mxaF*), and a sulfur-oxidizing enzyme (*soxB*) in pogonophoran symbioses have been done in only a few examples. One is the thiotrophic symbiosis of the undescribed pogonophoran worm from the world's deepest cold-seep (7,326 m) in Japan Trench (40°02.9′N, 144°16.5′E; Fujikura et al., 1999), which is first reported in this communication. Partial 18S rDNA sequences (accession numbers, AB070213 and AB070214) show that the Japan Trench worm is related to the pogonophorans *Oligobrachia mashikoi* (at 87% and 89% similarities, respectively), *Siboglinum fiordicum* (85% and 88%), and to a vestimentiferan *Ridgeia piscesae* (84% and 88%) as shown in Fig. 1. One symbiotic 16S rDNA sequence (AB070215; Fig. 2) is related to a free-living thiotroph from a shallow hydrothermal vent (AF170424) at 90% similarity, and to the thiotrophic symbionts of bivalves such as *Codakia orbicularis* (X84979), *Lucina floridana* (L25707), and *Solemya terraeregina* (U62131) at 88–90% similarities.

Partial sequence of the RuBisCO form I gene *cbbL* (620 bp; AB070216) is identified in the Japan Trench worm, and related to those of the Gammaproteobacteria *Thiobacillus* sp. (AF038430), *Hydrogenovibrio* sp. (D43622), and the pogonophoran *Oligobrachia mashikoi* endosymbiont (AB057772) at 74–76% nucleotide similarities. In contrast, a RuBisCO form II gene *cbbM* that occurs in *Lamellibrachia* sp. L1 and the genes *pmoA*, *mmoX*, and *mxaF* that are methanotrophic markers (MacDonald et al., 1995; Costello and Lidstrom 1999) have not been detected.

Pogonophorans such as *Siboglinum atlanticum*, *S. ekmani*, and *S. fiordicum* live in symbiosis with methanotrophic bacteria, contrary to the Japan Trench

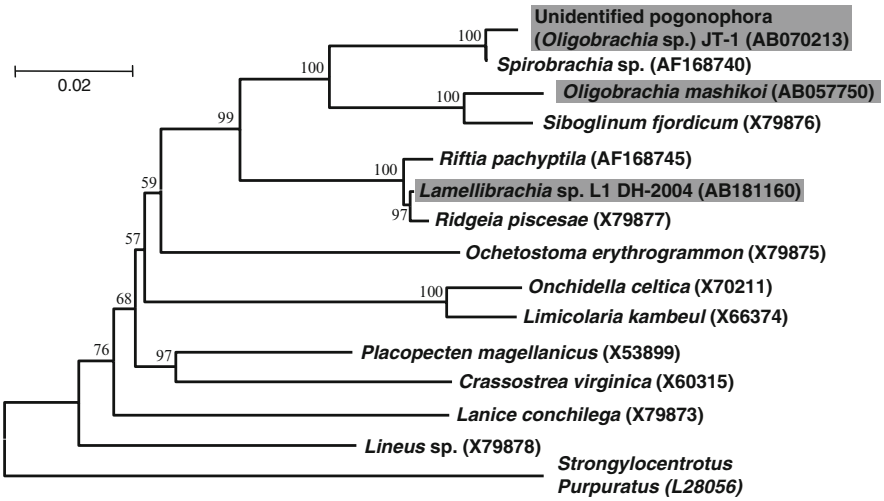

Figure 1. Phylogenetic tree based on 18 rDNA sequences of the studied pogonophoran and vestimentiferan hosts (shaded) along with representative marine invertebrates. The hosts focused in this study are *Lamellibrachia* sp. L1, an unidentified pogonophora (tentatively *Oligobrachia* sp. JT-1), and *Oligobrachia mashikoi*. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The branching pattern of the constructed tree was confirmed by reconstruction using the methods of maximum parsimony and maximum likelihood. Bootstrap values greater than 50 for 1,000 replicates are indicated at nodes, and values less than 50 are not reported. Scale bar, 0.02 substitutions per site.

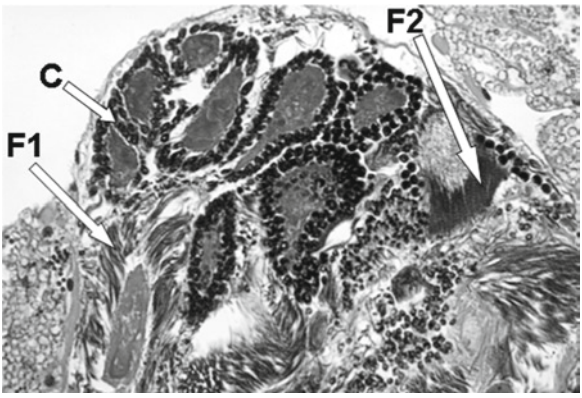

Figure 2. Light photomicrograph of the cross section of the vestimentiferan *Lamellibrachia* sp. L1 trophosome. Multiple forms of symbiotic microorganisms are stained with eosin-hematoxylin. C, coccoid form; and F1 and F2, two different filamentous forms. Photo width, 320 μm.

worm. However, the methanotrophic symbionts may possess RuBisCO, as revealed by culture experiments (Schmaljohann and Flügel 1987), very low stable carbon isotope ratios (Southward et al., 1981), and enzymatic activities (Spiro et al., 1986; Schmaljohann et al., 1990). The presence of RuBisCO in methanotrophic

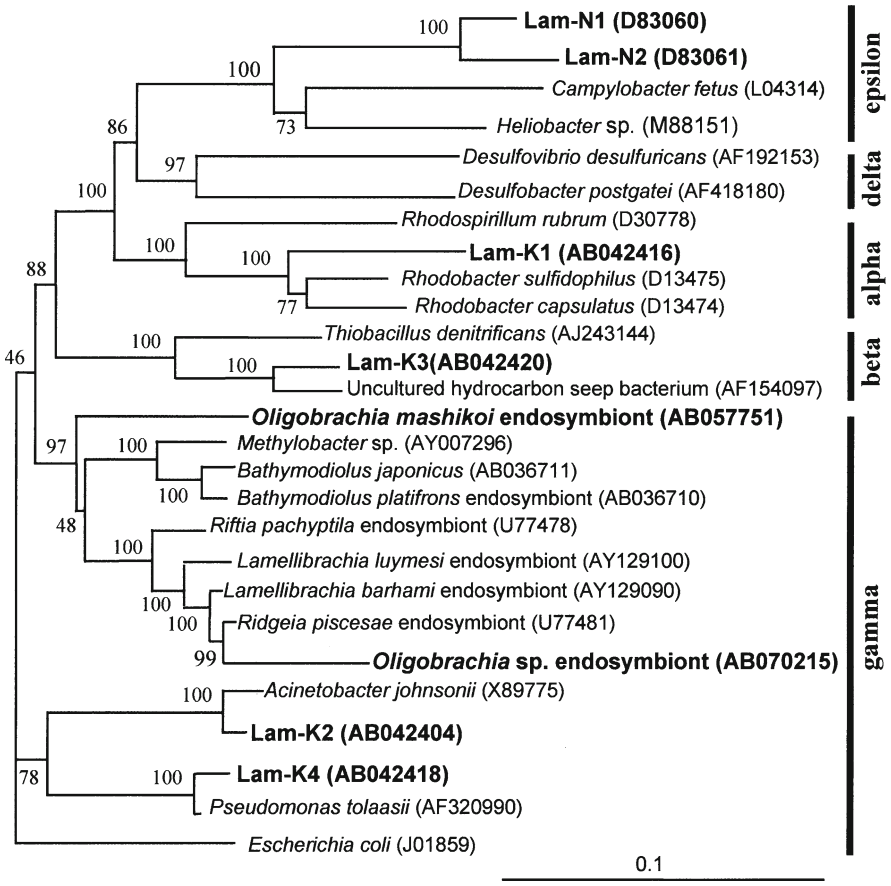

Figure 3. Phylogenetic tree based on 16 rDNA sequences of representative pogonophoran and vestimentiferan endosymbionts. The endosymbionts focused in this study are: *Lamellibrachia* sp. L1 endosymbionts Lam-N1 and -N2 (TW-1 and -2 of Naganuma et al., 1997b), Lam-K1, -K2, -K3 and -K4 (TW-2, -3, -5 and -6 of Kimura et al., 2003b), an *Oligobrachia* sp. JT-1 endosymbiont (this communication), an *Oligobrachia mashikoi* endosymbiont (Kimura et al., (2003a). The branching pattern of the constructed tree was confirmed by reconstruction using the methods of maximum parsimony and maximum likelihood. Bootstrap values greater than 50 for 1,000 replicates are indicated at nodes. Scale bar, 0.1 substitutions per site.

symbiosis is not surprising, as type X methanotrophs are capable of autotrophy via Calvin–Benson cycle (Colby et al., 1979; Hanson and Hanson, 1996).

The pogonophoran *Oligobrachia mashikoi* (18S rDNA, AB057750; Fig. 1) is the first described species of the genus *Oligobrachia* in Japanese waters (Imajima, 1973). It is closely related to vestimentiferans based on hemoglobin structure (Yuasa et al., 1996; Zal et al., 1998), but differentiated from the Phylum Annelid based on body wall muscular system (Matsuno and Sasayama, 2002). The studied *Oli. mashikoi* worm lives in shallow muddy sediment and harbors a single gammaproteobacterial symbiont (16S rDNA, AB057751; Fig. 2) related

to an uncultured bacterium from a hydrocarbon seep and *Methylobacter* sp., and to thiotrophic symbionts of the bivalves *Thyasira flexuosa*, *Codakia costata*, and *Lucina pectinata* (Fig. 3; Kimura et al., 2003a). Therefore, the *Oligobrachia. mashikoi* symbiont has not clearly been characterized as either thiotrophic or methanotrophic.

On the other hand, RuBisCO form I gene *cbbL* (AB257772) is identified in *Oli. mashikoi* and related to gammaproteobacterial *cbbL* of *Thiobacillus* and *Hydrogenovibrio* species (Kimura et al., 2003a). These 16S rRNA and RuBisCO form I genes are located in the *Oli. mashikoi* trophosome by *in situ* hybridization (Kimura et al., 2003a). It should be noted that the RuBisCO form II gene (*cbbM*), methanotrophic marker genes (*pmoA*, *mmoX*, and *moxF*), and a thiotrophic marker gene (*soxB*) have not been amplified by PCR from the *Oli. mashikoi* trophosome (Kimura et al., 2003a, unpublished). These data neither specify thio- or methanotrophic nature of the symbiont, nor rule out the possibility of dual thio- and methanotrophy known in type X methanotrophs (Colby et al., 1979; Hanson and Hanson, 1996). Symbiosis by a type X methanotroph has been suspected in pogonophorans (Southward, 1982) and vent bivalves (Robinson et al., 1998; Fiala-Medioni et al., 2002).

6. Strategies for Sulfide Exploitation

Thiotrophy of the tubeworms symbioses is predominantly seen in hydrothermal vent habitats with sulfide concentrations as high as ~111 mM (e.g., Shanks et al., 1995). Vestimentiferans take up sulfide and dissolved oxygen from the uppermost (anterior) "plume" standing into ambient water as high as tens centimeters to a few meters. In contrast, sulfide concentration in the bottom waters overlying cold seeps and muddy sediments varies from 20 mM (Girguis et al., 2002) to 1 μM (Julian et al., 1999). Sulfide is present only in interstitial water and virtually absent in the overlying water of the Gulf of Mexico seeps dominated by *Lamellibrachia* cf. *luymesi* (Freytag et al., 2001) and the so-called off-Hatsushima seep inhabited by *Lamellibrachia* sp. L1 (Masuzawa et al., 1992; Hashimoto et al., 1995; Gamo et al., 1988). In the sulfide-depleted water, *L.* cf. *luymesi* is known to take up sulfide from the posterior "root"; however, the relative importance of sulfide uptake via root versus plume is still unclear.

Pogonophoran worms may take up sulfide from the deep buried posterior part of the body, and the endosymbionts are mainly found in the posterior part of the trophosome (Southward, 1982). Similarly, seep vestimentiferans, particularly lamellibrachids, take up sulfide in the interstitial water from their posterior part, or "root," extended deeply into sediment (MacDonald and Fisher, 1996; Julian et al., 1999; Freytag et al., 2001). The root growth of lamellibrachids is similar to the "sulfide mining" observed in symbiotic thyasirid clams using the superextensile foot as the part of sulfide uptake (Dufour and Felbeck, 2003). The lamellibrachid "root hypothesis" (Julian et al., 1999; Freytag et al., 2001) is intriguing; however, lamellibrachids may not promptly reposition their roots in

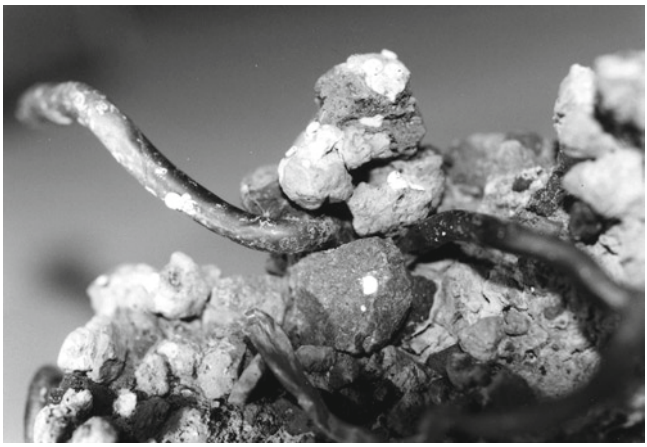

Figure 4. Tube of the living *Lamellibrachia* sp. L1 embedded in the authigenic carbonate concretion. Photo width, 7 cm.

the carbonate-cemented sediment to the upward/downward shift of the sulfide formation zone, namely, sulfate–methane interface (SMI; e.g., DeLong, 2000). Upward shift of SMI may lower sulfide uptake, because upper parts of worm tubes are thicker and thus less permeable to sulfide (Julian et al., 1999; Freytag et al., 2001), and the sulfide-permeable posterior part should grow upward to the shallower SMI. Downward shift of SMI is associated with lowered seepage and would force the roots to grow further downward. Tubeworms may thus keep up with SMI shifts by up- and downward growth of the roots within a lifetime, and worm tubes with several bends are often observed in the sub-seafloor part. In contrast, the vesicomyid clam *Calyptogena soyoae* adjusts the depth of "foot extension" according to the depth of SMI (Hashimoto et al., 1995).

Tubes of *Lamellibrachia* sp. L1 are buried fast in carbonate concretion in the off-Hatsushima seep (Fig. 4), and tubes grow upward to complement the fast burial. As lamellibrachid root grows downward, it penetrates the sulfide-forming SMI and reaches the sub-SMI zone with relatively high-methane but low-sulfide concentrations (Masuzawa et al., 1992). The depth of SMI is often recorded on the tubes of *Lamellibrachia* sp. L1 as shown by the blackish zone oxidized via sulfate reduction (i.e., "burnt" by sulfate), and the "roots" often extend to ten cm below the burnt zone (unpublished).

The root hypothesis implies enhanced regeneration of sulfide in the seep sediment in which interstitial sulfate has been already reduced to sulfide. Extra sulfate comes from overlying seawater via the worm's body fluid circulation and from endosymbiotic sulfide oxidation. The root hypothesis thus requires the coalition of endosymbiotic thiotrophs and epibiotic sulfate-reducing bacteria (SRB) in sediment, which contrasts the dual endosymbiotic coalition of a thiotroph and an SRB in *Olavius algarvensis* (Dubilier et al., 2001). Other explanation for

solving the sulfide deficiency problem is occasional dependence on thio- and methanotrophy as mentioned above.

7. Colonization and Fossilization of Seep Tubeworms

The off-Hatsushima seep is also colonized by the endobenthic vesicomyid clam *Calyptogena soyoae* (Hashimoto et al., 1989). Living *Calyptogena* clam moves in the soft muddy sediment to exploit sulfide and/or methane (Hashimoto et al., 1995), and thus rarely occurs in carbonate concretions that often exist beneath the superficial sediment or occasionally exposed on the seafloor (Ohta, 1990). In contrast, the *Lamellibrachia* worm anchors the posterior part to the carbonate concretions, and thus show habitat segregation with the *Calyptogena* clams.

The seep carbonates are mostly authigenic calcite derived from oxidation of methane in the seep fluids (Hattori et al., 1994). The off-Hatsushima seep represents modern seeps in the subduction zone of the Philippine Sea Plate against the North American Plate, and its Miocene counterpart (17.2–14.4 Ma) has been identified in the Miura Peninsula, 40 km east of the modern seep (Naganuma et al., 1995). Chemical compositions of the modern and Miocene carbonate concretions that contain worm tubes are closely similar and regarded as high-magnesium carbonate (Table 2). In contrast, the Miocene carbonate without worm fossils is clearly distinguished, even though it occurs next to the tube-containing carbonate.

The high magnesium content in the tube-containing Miocene and modern seep carbonates may be closely coupled with the colonization of lamellibrachid tubeworms. It is known that a high-magnesium carbonate, or possibly dolomite, is formed as a result of biological sulfate reduction (Vasconcelos et al., 1995). X-ray microanalysis of the Miocene worm tubes showed co-accumulation of sulfur and iron, indicative of iron sulfide (probably pyrite), inside of the tubes, at which soft body was positioned. Iron may have derived from hemoglobin of the worm's blood to yield iron–sulfur minerals such as iron sulfide (FeS; Naganuma

Table 2. Compositions of selected chemical species (weight %) in carbonate matrices determined by the energy dispersion fluorescent X-ray analysis. (From Naganuma et al., 1995.)

	Miocene carbonate (17.2–14.4 million years ago)		**Off-Hatsushima carbonate colonized by *Lamellibrachia* sp. L1**
	With no fossil tubes embedded	**With embedded fossil tubes**	
$CaCO_3$	66.6	38.7	39.3
FeO_2	28.4	4.6	5.1
SiO_2	<0.1	28.7	28.0
$MgCO_3$	<0.1	14.8	14.5
Al_2O_3	<0.1	10.0	9.7

et al., 1995). Iron sulfide may further react with sulfide to yield pyrite under highly sulfidic conditions, for example, tubeworm decay after death. The pyrite-in-tube is also observed in Cretaceous seep fossils (Beauchamp et al., 1989).

The Miocene worm tubes were embedded in calcite, and the co-occurrence of calcite and pyrite is an indicator of hypoxic oxygen level as low as 0.3–1.0 ml O_2 per liter (Brett and Baird, 1986). Tubeworms recharge the hypoxic to anoxic sediment with sulfate according to the root hypothesis. As a result, sulfate would be possibly precipitated on inner or outer surface of the worm tubes as calcium sulfate, which is the dominant form of sulfate in the deep-sea-reducing habitats. However, X-ray microanalysis on the Miocene and modern worm tubes showed no co-accumulation of sulfur and calcium (Naganuma et al., 1995, 1996), which may not be explained solely by the root hypothesis.

8. Biotechnological Perspectives and Metagenomics

Tubeworms live in chitinous tubes (Gaill et al., 1992) secreted in the expense of symbiont-fixed carbon (Felbeck and Jarchow, 1998; Bright et al., 2000). Chitin derivates, such as chitosan, have various applications in biotechnology and are used in novel drug delivery systems, wound healing, and anticoagulation (Hirano, 1996). Animals possess two forms of carbamylphosphate synthetase connected separately to the syntheses of arginine and pyrimidine nucleotides; however, the vent tubeworm *Riftia pachyptila* lacks enzymatic activities involved in the pyrimidine nucleotides synthesis (Simon et al., 2002; Minic et al., 2002). This feature, which may be shared by non-vent tubeworms, suggests that host enzymatic activities are repressed by symbiont and thus may be applied to develop a novel inhibition manner of enzyme activity or gene expression via bacterial symbioses. This aspect of host–symbiont interaction has also relevance to bacterial quorum-sensing auto-induction and signal transduction, and the signal kinase and signal regulator gene homologs are isolated from the *R. pachyptila* symbiont fosmid library (Hughes et al., 1997). Early stages of pathogen infections may also be studied from this symbiotic point of view.

Metagenomic libraries of endosymbiotic bacteria were constructed from two Pogonophoran specimens: an individual of the deep-sea tubeworm, *Lamellibrachia* sp. L1; and, an individual of the shallow-water beard worm, *Oligobrachia mashikoi* (Naganuma et al., unpublished). The metagenomic libraries were constructed using the Copy Control Fosmid Library Production Kit (Epicenture), which provides the fosmid vector allowing insertion of DNA fragments as long as 40 kilobase pairs (kb). As a result, 1,186 and 106 transformed *Escherichia coli* colonies (or clones) from the tubeworm and the beard worm, respectively, were obtained. These clone numbers may correspond to 24.3% and 8.5% of endosymbiont metagenomes from the tubeworm and the beard worm, respectively, despite low frequency of endosymbiotic bacterial genomes (i.e., high frequency of host animal genomes) in the retrieved bulk DNA pool. Low numbers of retrieved colonies should have been improved as well. None of the clones have so far shown expression of the genes that

encode the enzymes involved in degradation of host animal cells, tissues, and/or organs, namely, chitinase (EC 3.2.1.14), esterase (EC 3.1.1), amylase (EC 3.2.1), pectinase (EC 3.2.1.15), and laccase (EC 1.10.3.2), also known as, polyphenol oxidase. Efforts to detect and retrieve genes expressing enzyme activities of other industrial purposes such as cellulose, proteinase, or lipid metabolisms including synthesis of (particularly polyunsaturated) fatty acids have still been made. Development of more efficient hosts (*E. coli* and other species) and expression vectors (plasmids, phagemids, fosmids, etc.) is highly needed for more efforts.

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MULTIBIONT SYMBIOSES IN THE CORAL REEF ECOSYSTEM

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1. Introduction

Symbiotic systems are ubiquitous and play key roles in a variety of ecological systems (Douglas, 1995). Symbioses involving microorganisms underpin certain biological communities (coral–algae, plant–mycorrhizal fungi, plant–bacteria), and in these systems they are important to the flux of energy and nutrients (Douglas, 1995). The classic symbiotic systems (involving two partners) usually involve one large organism (host) and one/several smaller organisms (symbionts) that are located within the body of their host (Douglas, 1994). The original definition of symbiosis: "phenomena of dissimilar organisms living together," was coined by de Bary (1879) and has been frequently misinterpreted and used as a synonym of mutualism, mainly due to the fact that de Bary was engaged in research of the mutualistic relationship between algae and fungi in lichens (Castro, 1988). Much of the literature on symbiosis presupposes that the associations are mutualistic, but this view is not fully supported by direct experimental study (Douglas, 1995). Along with the continuous study of symbiotic systems and data accumulation, much effort has been invested by researchers to supplying a better and more accurate definition of the term "symbiosis." The study of various symbiotic systems by different researchers, who employed different tools for the task, led to difficulties and confusion (Smith, 1992). Several researchers saw the ecological consequences or outcomes of interspecies intimacy as the main factor in defining symbiosis (see Saffo, 1992), while others employed the presence or absence of metabolic dependency as the main criterion (Smyth, 1962; Castro, 1988). According to Smith (1992), for a large number of associations, the facts on the interactions between the partners were still too imperfectly understood to allow safe judgments about the existence of mutual benefit, and even in cases in which the "mutual benefit"-type definition is adopted, problems still arise, because "benefit" is a very difficult concept to define and measure (Smith, 1992). Moreover, even when the dynamics of certain symbiotic associations are fairly well understood, it is sometimes, nonetheless, too complex to pigeonhole into simple categories of mutualism, commensalism, and parasitism (Saffo, 1992).

While there are many well-studied symbioses of two interacting organisms, it is only recently that scientists have discovered more complex relationships involving three or more organisms from different taxonomic groups (Douglas, 1998; Hunter, 2006). When dealing with symbiotic systems that involve more than two partners, the above- mentioned difficulties of defining, categorizing and sorting of an association type are even more pronounced; indeed the possible relations between partners can include both antagonistically and mutualistically interacting pairs (Saffo, 1992) and can involve complex metabolic pathways in which not only symbiont–host interaction but also symbiont-symbiont interactions can be engaged. Along with the ongoing efforts to categorize symbiotic interactions, one can see that the vast variety of interactions, the complexity of physical and metabolic relations, as well as the number of unknown variables in several symbioses, inevitably leads to a retreat from strict classification back to a more broad and flexible terminology. Saffo (1992) suggested 'living together' as the appropriate interpretation for symbiosis, and that is the essence of the matter and at the root of most of our questions. Using such a broader definition of symbiosis facilitates investigations unprejudiced by preconceptions about outcomes (Saffo, 1992). Castro (1988) reported in his review of symbioses in coral reefs that a growing number of investigators have chosen to overcome the difficulties of categorizing symbioses by minimizing the need to classify the association in question, and merely referring to it as an instance of symbiosis.

Several multibiont symbiotic systems are already documented from the terrestrial environment. These include: (1) systems in which two of the partners are at the same organizational level (usually two bacteria), like the mutualistic relationship between the glassy-winged sharpshooter (*Homalodisca coagulata*) and two bacteria *Baumannia cicadellinicola* and *Sulcia muelleri* (discussed later) (Wu et al., 2006), or the colonization of many plant species with multiple genotypes of mycorrhizal fungi (De La Bastide et al., 1995; Clapp et al., 1995; Perotto et al., 1996); and (2) systems that include partners at different organizational levels. An example of the latter involves the relationship between a virus, a bacterium, and an insect: All aphids require a primary endosymbiont, the bacterium *Buchnera*, in order to synthesize the nutrients missing in their xylem food source. Some aphids, however, also contain a secondary bacterial symbiont, such as *Hamiltonella defensa*, which confers defense against other bacteria (Hunter, 2006). Moran et al. (2005) have identified an associated bacteriophage virus called APSE-2, whose genome contains a gene encoding cytolethal-distending toxins, which disrupt the eukaryotic cell cycle. The authors suggest that the phage-borne toxin provides defense against eukaryotic parasites for the aphid host. From the terrestrial environment it is also known that multibiont symbioses can involve many combinations of organisms of different sizes, in which one organism is host to another, while resident in a third (e.g., nematodes, bacteria, and insects; Hunter, 2006). Moreover, some of the symbioses involve interactions other than endosymbiosis. In some cases bacteria live outside their host, and have been given

the name "episymbionts" (Hunter, 2006). Interestingly, it was found that the reindeer lichen *Cladonia arbuscula* (which in itself is an association of fungi and photoautotrophs) is associated with bacterial cells of different taxonomic groups that are embedded in a biofilm-like layer (Cardinale et al., 2008). It would appear that the complexity and range of terrestrial multibiont symbioses is only just beginning to unravel. In this chapter, we focus on multibiont symbioses in the marine environment, and especially in the coral reef ecosystem.

Coral reefs are often described as the rain forest of the sea (Connell, 1978). As such, they include numerous organisms that interact with each other in a complex array of symbiotic associations (Paulay, 1997). A single coral colony may accommodate a variety of symbiotic organisms, including invertebrates and vertebrates, bacteria, archaea, viruses, fungi, protozoa, and algae, all living in close proximity and interacting with each other (Paulay, 1997; Wegley et al., 2007). The term "coral holobiont" was introduced by Rowan (1998) to describe the complex of the coral and its symbiotic algae. Later, Rohwer et al. (2002) expanded its meaning to include the complex and dynamic assemblages of the coral animal with its associating microbial eukaryotes (algae, fungi, and protozoa), bacteria, archaea, and viruses. The wealth and complexity of associations within a coral colony will serve here as the basis for our discussion of multibiont symbioses. The chapter will focus on multibiont symbioses involving corals and microorganisms such as zooxanthellae, cyanobacteria, and endolithic algae (microsymbionts), as well as associations that involve the coral and macroorganisms such as mussels, barnacles, and fish (macrosymbionts). Aspects of metabolic contribution, symbiont effectiveness, host gain/loss, and the establishment of multibiont symbioses will also be discussed.

The chapter is divided as follows:

1. Introduction
2. An overview of symbioses involving a coral colony and more than one microsymbiont type
3. An overview of symbioses involving a coral colony, its microsymbionts, and at least one type of macrosymbiont
4. Multibiont symbiosis during stress – the coral–algal adaptive bleaching hypothesis (ABH)
5. The establishment of multibiont symbioses – vertical and horizontal transmission of symbionts
6. Discussion

2. Coral Host and Microsymbionts

2.1. CORAL–ALGAL SYMBIOSIS AS A MULTIBIONT ASSOCIATION

The keystone symbiosis for coral reef existence is undoubtedly that between members of the phylum Cnidaria, such as corals and anemones, and their

photosynthetic dinoflagellate symbionts *Symbiodinium* spp. (also called zooxanthellae), which together form the trophic and structural foundations of coral reef ecosystems. Since the early days of zooxanthellae research and the description of the first *Symbiodinium* species, i.e., *Symbiodinium microadriaticum* Freudenthal (1962), dozens of studies have dealt with the diversity of these algal symbionts and provided sound evidence for the occurrence of high genetic variability among them (see Baker, 2003). The advent of molecular tools has resulted in a growing number of studies dealing with partner specificity, biogeography, and ecology of coral–algal symbiosis, and it was not long after the first molecular data were obtained that it was demonstrated that coral hosts can be associated with one or more genetically distinct algal symbionts (Rowan and Powers, 1991; Rowan and Knowlton, 1995; Rowan et al., 1997; Toller et al., 2001; LaJeunesse, 2001), hence negating the idea of uniformly strict specificity (in which all hosts exclusively contain only one symbiont type; Baker, 2003).

One of the first studies that demonstrated the relationship of a single coral host with more than one symbiont taxa dealt with two of the dominant corals of the Caribbean Sea (Rowan et al., 1997). Three distinct taxa of algal symbionts were found to be associated with *Montastraea annularis* and *M. faveolata*. Both coral species host members of *Symbiodinium* groups A, B, and C, with specificity dictated by the environment: corals at shallow-to-intermediate depths host two or three taxa of symbiont assemblages that map to the "sun" (*Symbiodinium* A and/or B) and "shade" (*Symbiodinium* C) patterns on colony surfaces. When such intracolony irradiance gradients are manipulated, the symbionts reestablish correct patterns of zonation (Rowan et al., 1997).

As molecular research has progressed, various examples of scleractinian species and other invertebrates hosting multiple algal taxa in a single individual/or colony have accumulated (see Baker, 2003). These served as the springboard for a range of new studies looking into the functional and physiological capabilities of the different symbiont types and their implications on the coral host during stress periods (see below). An implicit motivation behind much of this research has been to understand the role of symbiont diversity and/or flexibility in determining possible long-and short-term responses of coral reefs to environmental change and global warming (Baker, 2003).

The quest to understand the role of genetically different symbionts for their coral host during stress somehow preceded the search for a greater understanding of their role during "normal" times. It is only recently that this crucial question has been addressed by Loram and coworkers (2007), who explored how the nutritional function of *Symbiodinium* maps onto the molecular diversity of this genus. A thorough study was conducted on the giant sea anemone *Condylactis gigantea* that associates with members of two clades of *Symbiodinium*, either singly or in mixed infection. It was demonstrated that symbioses of *C. gigantea* with the dinoflagellate algae *Symbiodinium* of clades A and B are functionally different. The incorporation of algal photosynthetic carbon into animal lipids and amino acid pools was significantly higher in symbioses with algae of clade A than of

clade B (Loram et al., 2007). In the symbioses involving mixed infections, the metabolic indices either did not significantly differ from the monomorphic symbioses or were intermediate between them. A key question driving that study was that of whether the symbionts in mixed infections are less cooperative than those in single infections. The results suggest that the proportion of fixed carbon translocated to the host is not depressed in the mixed infections and that competition between co-occurring *Symbiodinium* taxa is suppressed in the symbiosis (Loram et al., 2007). The authors suggest three potential mechanisms by which the host might suppress such competition: (1) it might control the supply of limiting nutrients to its symbionts; (2) it might control the proliferation rates of symbionts; or (3) it might impose sanctions on any algal cells that release photosynthate at low rates. Furthermore, the authors point out a structural constraint of the symbiosis (the presence of each algal cell within a symbiosome inside the host cell) that might minimize the opportunity for inter-algae competition. The example presented above demonstrates for the first time the selective metabolic contribution to the host of two genetically different symbiont taxa.

2.2. CORAL, ALGAE, AND CYANOBACTERIA

The coral *Montastrea cavernosa* displays another three-party association that includes zooxanthellae and cyanobacteria as the symbionts. Both zooxanthellae and cyanobacteria are endosymbiotic. The latter were found to be genetically similar to either *Synechococcus* sp. or *Prochlorococcus* sp. within the order Chroococcales. In this system it was demonstrated that the cyanobacteria provide a source of nitrogen that is utilized by the zooxanthellae (Lesser et al., 2007). The pattern of nitrogen fixation is diurnal and confined to those times of the day when physiological hyperoxia or anoxia does not inhibit nitrogen fixation. Moreover, the availability of this novel source of inorganic nitrogen does not appear to affect the stability of the mutualistic association between the symbiotic zooxanthellae and the coral host (Lesser et al., 2007). This association involves two distinct endosymbiont types: zooxanthellae, which contribute photoassimilates to the coral host and cyanobacteria, which supply fixed nitrogen to the zooxanthellae. The symbionts in this consortium perform their "tasks" on the basis of alternating diurnal dominance. In this association it was demonstrated that a mixed infection could be beneficial to the host via an indirect pathway, in which one symbiont type contributes to another.

2.3. CORAL, ENDOSYMBIOTIC ALGAE, AND ENDOLITHIC ALGAE

A different multibiont association is that involving the ahermatypic stony coral *Oculina patagonica*, its endosymbiotic zooxanthellae, and its endolithic algae found within the coral skeleton (Fine and Loya, 2002). Although 80–90% of the colonies

of the Mediterranean encrusting coral *O. patagonica* bleach annually, surprisingly more than 90% of the bleached colonies recover (Kushmaro et al., 1996, 1998). One of the most intriguing questions being asked by coral reef researchers is that why some coral species survive bleaching events while others do not (Fine and Loya, 2002). Fine and Loya (2002) looked into the dynamics and photosynthetic pigment concentrations and biomass of endoliths in the skeleton of *O. patagonica* throughout a bleaching event, and demonstrated that during repeated summer bleaching events these endolithic algae receive increased photosynthetically active radiation (due to the loss of zooxanthellae), markedly increase in biomass, and produce increasing amounts of photoassimilates, which are transferred to the coral. It was therefore concluded that the endolithic algae serve as an alternative source of energy during coral bleaching (Fine and Loya, 2002). This case study demonstrates a situation in which stressful conditions cause a shift in symbiont abundance, which is directly followed by a shift in the energetic contribution from the symbionts to the host. It should be emphasized that this three-party association prevails throughout the coral's existence, with alternating metabolic dominance of the "algal player" as dictated by the bleaching severity.

3. Coral Host and Macrosymbionts

In the previous section, several multibiont symbioses involving corals and their unicellular photosynthetic symbionts were addressed. Additionally, a coral colony often provides shelter and food for diverse groups of macroorganisms such as polychaetes, molluscs, crustaceans, and fish (Castro 1988) (see Fig. 1). Several studies have dealt with coral macroorganism symbiosis (Goreau et al., 1970; Mokady et al. 1998; Simon-Blecher et al. 1996). This section is devoted to several case studies that demonstrate the metabolic contribution of the macroorganism to the coral colony via its photosynthetic symbionts.

3.1. BACKGROUND

Coral reefs are oligotrophic environments in which concentrations of inorganic nitrogen in the surrounding seawater are often <1 μmol/l (Muscatine and Porter, 1977; D'Elia and Wiebe, 1990). Consequently, zooxanthellae in the gastrodermal

Figure 1. (continued) The barnacles' shells are covered with the coral tissue except for the aperture, enabling the barnacle to breath and feed. Notice the deformation in the coral skeleton and the dark color of the coral tissue. (Photo by M. Fine.) Scale bar 10 mm. (**e**) The coral *Turbinaria* sp. with numerous acoel worms belonging to the genus *Waminoa* (black arrow). The worms contain both *Symbiodinium* sp. and *Amphidinium* sp. algal symbionts. (Photo by A. Shoob.) Scale bar 7 mm. (**f**) The polychaete worm *Spirobranchus giganteus* (Serpulidae) embedded in a faviid coral at Eilat (Red Sea). Notice the remains of coral tissue around the polychaete tube. (Photo by O. Ben-Tzvi.) Scale bar 18 mm.

Figure 1. Examples of coral hosts and their macrosymbionts: (**a**) A broken piece of the coral *Montipora erythrea* exposing the boring mussel *Lithophaga purpurea*. Additional burrows can be seen in the inner coral skeleton. (Photo by I. Brickner.) Scale bar 13 mm. (**b**) Open shells of the boring mussel *L. purpurea* taken from the massive stony coral *Montipora erythrea.* The arrow points towards a pair (male and female) of pea crabs *Pinotheres* sp. regularly, only one crab can be found within the *Lithophaga* mantel cavity. In cases where a pair of crabs does occur, they reside only in one side of the mussel mantel cavity, otherwise, the mussel could die due to damage caused to the gills. (Photo by I. Brickner.) Scale bar 8 mm. (**c**) *Trapezia* crab between the branches of *Stylophora pistillata*. Notice the algae on the crab's left arm, a result of grazing over the tissue-exposed area of the coral. (Photo by I. Brickner.) Scale bar 18 mm. (**d**) The massive stony coral *Porites* sp. infested with several barnacles.

cells of corals, sea anemones, and other zooxanthellate invertebrates are sometimes nitrogen-limited (Cook and D'Elia, 1987; Cook et al., 1988; Muscatine et al., 1989; Belda et al., 1993; Falkowski et al., 1993). Indirect confirmation of this comes from experiments in which increases in the concentration of external ammonia were shown to promote greater numbers of zooxanthellae (Stambler et al., 1991; Stimson and Kinzie, 1991; Belda et al., 1993; Muller-Parker et al., 1994). If zooxanthellae assist the host in meeting its energy requirements (Muscatine et al., 1981; Falkowski et al., 1993), then increased cell densities might be beneficial (Meyer and Schults, 1985). Conversely, raising the concentration of external nitrogen for prolonged periods encourages balanced growth within a population of zooxanthellae, which lowers the translocation of fixed carbon to the host (Falkowski et al., 1993).

Inorganic nutrients on coral reefs originate from several sources (Entsch et al., 1983). One of the suggested sources is the fertilization by associate organisms, e.g., coral symbionts of the waters surrounding coral colonies (Wielgus and Levy, 2006).

3.2. STONY CORALS AND BORING MUSSELS

One of the first prominent models for coral fertilization was that of highly modified mussel *Fungiacava* that lives within fungiid corals (Goreau et al., 1970). According to a model suggested by Goreau et al. (1970), this boring mussel releases nutrients (as ammonium) into the polyp coelenteron, enhancing the production of additional symbiotic algae, some of which, in turn, will be released as food for the *Fungiacava*. However, this model was never confirmed. Similarly, the boring bivalve *Lithophaga simplex* was found to inhabit the scleractinian coral *Astreopora myriophthalma* in high densities in the northern part of the Red Sea (Mokady et al., 1998). Ammonium production rate by the bivalves and its consumption rate by the coral (via the symbiotic algae) were measured in the laboratory. Ammonium production by the bivalves inhabiting the coral was found to be higher during daytime than at night. Under naturally occurring levels of ammonium, recycling of nitrogenous waste produced by the bivalves (ammonium) may supply a significant portion of the needs of the coral/zooxanthellae. Mokady et al. (1998) hypothesized that the association between *L. simplex* and *A. myriophthalma* may also represent an example of mutualistic symbiosis, contrary to the generally accepted view of boring bivalves as parasites of their coral hosts.

3.3. MASSIVE STONY CORALS AND CRUSTACEANS

Simon-Blecher et al. (1996) studied the spatial distribution of chlorophyll in three coral species carrying invertebrate symbionts, using spectral imaging techniques. The multipixel fluorescence map and the relative-intensity fluorescence ratios demonstrated a high concentration of chlorophyll A next to the pits of the pit crab

Cryptochirus coralliodytes in the stony coral *Favites halicora*. Spectral similarity maps of the fire coral *Millepora dichotoma* inhabited by the barnacle *Savignium milleporum* revealed relatively higher chlorophyll concentrations in these two corals next to the symbionts. Those researchers hypothesized that the invertebrate symbionts fertilize their immediate surroundings with their excreta, enhancing algal growth.

Cook et al. (1991) demonstrated that ^{32}P and ^{14}C ingested by the coral-inhabiting barnacle *S. milleporum* is mobilized and excreted, and subsequently taken up by the zooxanthellae of the hydrocoral host *M. dichotoma*. They suggested that uptake of excreted substances from symbiotic barnacles in the nutrient-poor waters of the Red Sea may be beneficial to *M. dichotoma*. However, the low density of *S. milleporum* on *M. dichotoma* colonies would not be sufficient to support the ammonium demands of the hydrocorals (Achituv and Mizrahi, 1996). Moreover, Achituv and Mizrahi did not find differences in zooxanthellae densities between *M. dichotoma* colonies or branches with or without barnacles.

3.4. CORALS AND POLYCHAETE WORMS

Ben-Tzvi et al. (2006) reported the presence of the polychaete worm *Spirobranchus giganteus* (Serpulidae) embedded in two faviid corals at Eilat (Red Sea; Fig. 1f). The authors observed a colony of the stony coral *Cyphastrea chalcidicum* that was almost completely dead and covered with turf algae, apart from three small area of living coral tissue that surrounded *S. giganteus* tubes. Only one of these living areas continued to grow around the polychaete tube, while the other areas died. In other instances, coral colonies belonging to the species *Favia favus* and *F. laxa* were damaged by bleaching and predation respectively. As in the former case, areas of coral tissue in close proximity to the polychaete tube showed no damage and the colonies quickly recovered. It was suggested that the corals benefit from increased availability of nutrients from waste materials excreted by the worm. *Astreopora*, *Cyphastrea*, *Echinopora*, *Leptastrea*, *Millepora*, *Montipora*, *Pavona*, and *Porites* at Eilat (northern Red Sea) have recently become infested with boring spionid polychaetes and there are indications that these infestations are correlated with anthropogenic nutrient discharges (Wielgus et al., 2006). Wielgus and Levy (2006) studied the influence of the infestation by the boring spionid polychaetes colonies on the reef-building coral *A. myriophthalma*. They used an active fluorescence technique to examine differences in the functional absorption cross-section of Photosystem II ($^{\sigma}$PSII) between areas of a coral colony that were infested with spionid worms versus areas lacking such worms.

The mean $^{\sigma}$PSII value in areas of the *A. myriophthalma* colony that were infested with spionid worms was significantly higher than in the areas that were not infested. The differences in $^{\sigma}$PSII between different areas of a coral colony reflect variations in photosynthetic activity. The researchers suggested that fertilization of the surrounding water by the boring spionid polychaetes can result in

zooxanthellae proliferation. Increases in zooxanthellae abundance will lead in turn to a rise in chlorophyll levels, and can also lead to further increase in $^{\sigma}$PSII. These results were followed by morphological changes in the infested area of the coral colony, including increased roughness and bumpiness in tangential-to-radial growth. Such morphological changes reflect a higher tissue growth/calcification rate that can only occur if the symbiotic association is provided with an increased amount of nitrogen (Wielgus and Levy, 2006).

3.5. CORALS AND FISH

Meyer et al. (1983) recorded the high level of ammonium excreted by haemulid fish schools resting over coral colonies. The schools feed in sea-grass beds at night and during daytime they rest over the coral heads, where they excrete substantial quantities of ammonium and particulate nitrogen and phosphorus into the nutrient-poor waters. The percentages of these nutrients contributed by the fish were comparable to those derived from other sources. Coral heads with resident fish schools grew faster than those without such schools.

4. Multibiont Symbiosis During Stress: The Coral–Algal Adaptive Bleaching Hypothesis

It is now well documented that different strains of *Symbiodinium* (symbionts of corals) exhibit variation in thermal tolerance and photosynthetic response to irradiance (Iglesias-Prieto and Trench, 1997; Warner et al., 1999; Savage et al., 2002; Rowan, 2004; Goulet et al., 2005). This variation has ecological implications and high temperatures and irradiance are thus considered the primary causes of coral bleaching (the breakdown of the symbiotic relationship between the coral and its zooxanthellae; Glynn 1996; Brown, 1997), a major threat to coral reef existence worldwide (Hoegh-Guldberg, 1999). The increase in frequency and severity of bleaching events worldwide (Wilkinson, 1999) and the emerging physiological differences between genetically distinct symbionts, gave rise to the ABH (Buddemeier and Fautin, 1993). The ABH posits that when environmental circumstances change, the loss of one or more types of zooxanthellae is rapidly, and sometimes unnoticeably, followed by the formation of a new symbiotic consortium with different zooxanthellae that are more suited to the new conditions in the host's habitat. Empirical data aimed at reinforcing the theory which demonstrated that stony corals taken from deep water and transplanted into shallow water experienced severe bleaching that resulted in the loss of their suboptimal low-light symbionts. Consequently, the newly vacant hosts allowed the proliferation of high-light algae (Baker, 2001, 2002). Such a result tends to favor the adaptive nature of bleaching,

but fails to prove that the new combination of symbionts in a host is indeed really new (Hoegh-Guldberg et al., 2002) and not just a phenotypic change caused by the increase in proportion of rare genotypes of symbionts that were always there. As the original ABH became more controversial over the last decade, new discoveries emerged and highlighted several crucial facts: laboratory cultures of zooxanthellae can become dominated by types of *Symbiodinium* that are not representative of the dominant symbiont in the host from which they were originally isolated (Santos et al., 2001; LaJeunesse, 2001). This indicates that algal symbiont communities *in hospite* include novel symbiont types whose relative numerical abundance is below the detection threshold of conventional PCR-based identification methods (Baker and Romanski, 2007; Thornhill et al., 2006). These "cryptic" symbionts may be critical in providing corals with greater capacity for symbiont "shuffling" in response to environmental change (Little et al., 2004; Baker and Romanski, 2007). Moreover, symbioses with a capacity for mixed infections/multiple genetically distinct symbionts may be at an advantage in times of rapid global climate change (Douglas, 1998; Loram et al., 2007). Therefore, although the ABH in its original context might not be accurate, experimental evidence suggests that bleaching can become adaptive in certain coral hosts that originally possessed a diverse set of algal symbionts. It should be kept in mind, however, that as molecular techniques improve (see below), the original hypothesis might be proven correct in other coral hosts.

5. The Establishment of Multibiont Symbioses: Vertical and Horizontal Transmission of Symbionts

The onset of symbiosis can occur at a variety of host life-history stages, depending on the host species. Symbionts can be transmitted horizontally, in which the host's sexual progeny acquire symbionts from the surrounding environment; or vertically being passed directly from host parent to offspring (Trench, 1987; Douglas, 1994). Horizontal transmission offers the host the opportunity to recombine with different symbiont types that are differentially adapted to the existing environmental conditions. In contrast, vertical transmission guarantees that a host is provided with a complement of symbionts, which are transmitted faithfully from parent to offspring (Douglas, 1998). Associations with horizontal transmission tend to be considered as more plastic and "ready to change," whereas those with vertical transmission are believed to be strict and inflexible, and hence are also termed "open" versus "closed" systems (Trench, 1987). When we think of multibiont associations, we are naturally drawn to think of them as open systems. However, surprisingly, this is not always the case. We present below several multibiont associations in which the symbionts were proven to be vertically transmitted, and introduce two case studies of horizontally transmitted symbionts that illustrate different symbiont makeup in juvenile versus adult hosts (of the same species).

5.1. VERTICAL TRANSMISSION

The marine sponge *Chondrilla australiensis* (Demospongiae) contains unicellular cyanobacteria with an ultrastructure resembling that of *Aphanocapsa feldmannii*, which occur in the cortex, and bacterial symbionts, which are located throughout the mesohyl. In *C. australiensis*, the developing eggs are distributed throughout the mesohyl and are surrounded by nurse cells attached to them by thin filaments. The nurse cells form cytoplasmic bridges with the eggs, apparently releasing their contents into the egg cytoplasm. The presence of cyanobacterial and bacterial symbionts inside developing eggs and nurse cells in 25% of female *C. australiensis* was confirmed using transmission electron microscopy, suggesting that these symbionts are sometimes passed on to the next generation of sponges via the eggs (Usher et al., 2001).

Waminoa brickneri, a newly discovered species from the reefs of Eilat (Red Sea; Figs. 1e and Fig. 2a) is epizoic on living corals (Ogunlana et al., 2005). Similar worms belonging to the genus *Waminoa* were detected there on 14 species of stony and soft corals at a depth range of 2–50 m (Barneah et al., 2007a). The worms possess two distinct types of dinoflagellate algal symbionts within their cells: small symbionts 5–10 μm in diameter, which were identified as belonging to the genus *Symbiodinium*; and larger symbionts 12–20 μm in diameter, which were identified as belonging to the genus *Amphidinium* (Barneah et al., 2007a; Barneah unpublished data). The initial hypothesis that the worms receive their *Symbiodinium* algal symbionts from their coral hosts was examined using denaturing gradient gel electrophoresis (DGGE) profiles of the ITS2 region of *Symbiodinium* derived from coral hosts and resident worms (Barneah et al., 2007a). However, it was found that the corals and the worms possess different phylotypes of *Symbiodinium*, thus suggesting different sources for their symbionts. Histological sections performed on sexually mature worms (Fig. 2b) showed an ovary with oocytes containing the two distinct types of algal endosymbionts within their ooplasm (Fig. 2d, e). Transmission electron microscopy corroborated the presence of algal symbionts within the developing embryos (Barneah et al., 2007b). These findings offer the first definitive evidence of simultaneous maternal transmission of two distinct taxa of dinoflagellate algal symbionts in a triploblastic organism (Barneah et al., 2007b).

The following example is taken as a comparative example from the terrestrial environment. The sharpshooter (*Homalodisca coagulate*) is an important plant parasite that feeds on the xylem fluids of the plant. It harbors two distinct symbiotic bacteria, *B. cicadellinicola* and *Sulcia muelleri*, which are responsible for the selective synthesis of vitamins and cofactors and essential amino acids, respectively (Moran et al., 2005; Wu et al., 2006; Hunter, 2006). The sharpshooter provides the bacteria with the raw carbon-based ingredients that they need. This three-party symbiosis was discovered as obligatory, and the bacteria are often found to coexist in the same cell in adult sharpshooters (Wu et al., 2006). The symbionts are vertically transmitted together in eggs and are housed in a dedicated “bacteriome” within developing sharpshooter nymphs. Phylogenetic studies have shown that the three partners are coevolving (Hunter, 2006).

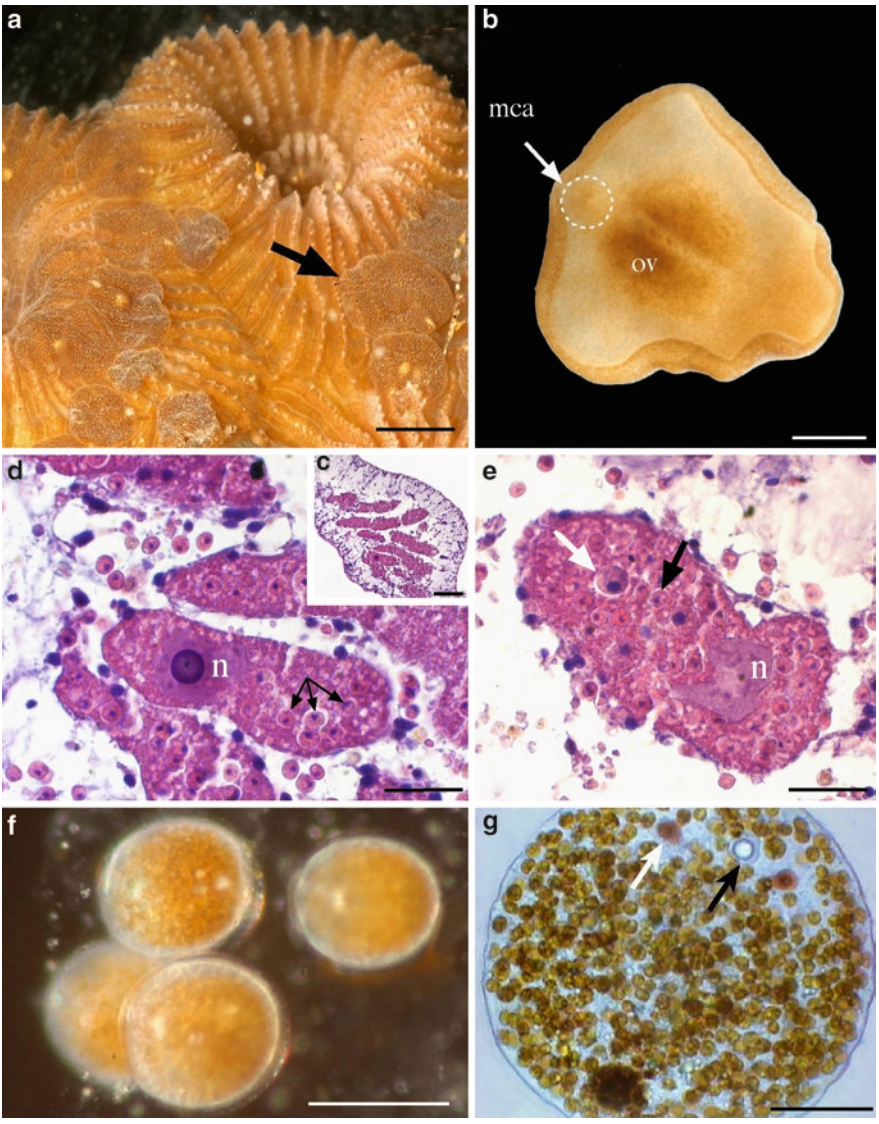

Figure 2. (**a**) The stony coral *Plesiastrea laxa* with *Waminoa brickneri* worms. Scale bar 3 mm. (**b–f**) Stages of sexual reproduction in *W. brickneri*. (**b**) Ventral view of a sexually mature specimen with paired ovary (ov) and the male copulatory apparatus (mca) circled with dashed line. Scale bar = 1 mm. (**c**) Histological section of worm containing gonads, 5 days prior egg laying, showing elongate oocytes. Scale bar = 110 μm. (**d**) Oocyte containing nucleus with prominent nucleolus and algal symbionts (arrows). Scale bar = 30 μm. (**e**) Oocyte containing two symbionts types: *Symbiodinium* sp. (black arrow) and single larger symbiont (white arrow). Scale bar= 30 μm. (**f**) Gelatinous egg mass. Scale bar = 200 μm. (**g**) Worm hatchling containing numerous algal symbionts, statocyst (black arrow), and eyespot (white arrow). Scale bar = 45 μm. (From Barneah et al., 2007b.)

5.2. HORIZONTAL TRANSMISSION

The corals *Acropora tenuis* and *A. millepora* are broadcast-spawning corals that as adults express different specificities for *Symbiodinium* strains. At Magnetic Island (Great Barrier Reef, Australia), adult colonies of *A. millepora* contain *Symbiodinium* strain D, whereas *A. tenuis* adults contain *Symbiodinium* strain C1 and occasionally strain C2 (Little et al., 2004). The production of aposymbiotic larvae by both species provides the opportunity to observe natural patterns of zooxanthellae infection and dynamics (Little et al., 2004). Larvae of *A. tenuis* were raised from spawned gametes, settled onto tiles that were attached to the reef, and were monitored up to 9 months after settlement. Contrary to expectations, it was found that the apparent specificity for strain C1 observed in adult populations of *A. tenuis* was not present in the early stages of the infection. Two distinct *Symbiodinium* strains, D and C1, were acquired by juveniles in the first month. In the subsequent 4 months, the relative abundance of these two strains within the symbiosis changed, with a clear increase in the number of juveniles harboring strain D. The opposite dominance of strains D and C1 in juveniles and adults of *A. tenuis* in the Magnetic Island populations suggests that there may be "active" selection by the host (Little et al., 2004). Similar results were reported by Weis et al. (2001), who examined host–symbiont specificity during symbiosis onset in the planula larvae of the solitary Hawaiian scleractinian *Fungia scutaria*. Such a selection was suggested as a mechanism by which to maximize the symbiont effectiveness, which varies with differences in physiological requirements between juveniles and adult corals (Little et al., 2004). For example, corals may have a higher demand for nutrients when they reach reproductive maturity, leading to a preference for one type to meet increased energy requirements. It is possible that strain C1 persists in the symbiosis at very low densities and is maintained as an undetectable "background" strain (Little et al., 2004). This case study demonstrates that the dynamics of coral–zooxanthellae associations may vary with the changing physiological needs of the host in response to life-history stage requirements (Little et al., 2004). The suggested difference in symbiont effectiveness for the host was proven correct in a different symbiotic system: that of the flatworm *Convoluta roscoffensis* and the prasinophyte algae *Tetraselmis* (Douglas, 1985). The worms reproduce sexually and produce larvae that are aposymbiotic and become infected by feeding on the algae (Douglas, 1985). At one site in the United Kingdom, each animal bears algae of either subgenus *Tetraselmis* or *Prasinocladia*. In natural populations, animals bearing *T.* (*Prasinocladia*) sp. are smaller and less fecund than animals with *T.* (*Tetraselmis*) sp. It was found that *T.* (*Tetraselmis*) sp. releases four times more photosynthate than *T.* (*Prasinocladia*) sp. to the animal (Douglas, 1985), and correlated with this is thus a less-effective symbiont than *T.* (*Tetraselmis*) sp. Under laboratory conditions, juvenile *C. roscoffensis* can form a symbiosis with any species of *Tetraselmis* (*Platymonas* and *Prasinocladius*). When exposed to a mixed suspension of different *Tetraselmis* species, all the algal species are ingested and start to proliferate. However, only one species is retained,

while the others are lost from the animal over a period of up to 2 weeks. The transient mixed infection reduces animal growth over 30 days and thus appears to be costly to the animal (Douglas, 1985, 1998).

6. Discussion

Multibiont symbioses are undoubtedly more common than the few examples discovered so far both in the terrestrial and the marine environment. The variety of organisms involved in such symbioses is immense and the types of associations are assorted. A host can be associated with two symbionts at the same organizational level (e.g., coral–algal symbiosis) or different organizational levels (e.g., coral–algae–mussel). The association can be classified as endosymbiosis or can include one endosymbiotic and one ectosymbiotic partner. Furthermore, some associations represent even higher degrees of complexity, like that involving the coral *Montipora erythraea* (and its endosymbiotic algae); the burrowing mussel *Lithophaga purpurea*, found within its skeleton; and the pea crab, *pinnotheres* sp., which infests the mussel and harms its reproduction (Brickner, personal comm.). This association highlights the occurrence of simultaneous mutualistic and parasitic interaction contained in one multibiont symbiosis.

In several symbiotic systems, mainly those in which the symbionts belong to the same organizational level (bacteria, algae, etc.), such as coral–algal symbiosis, the magnitude of the presence of more than one symbiont genotype is only recently gaining gradual exposure, a process directly linked to the progress in molecular techniques. This process has undoubtedly also affected the ability of different researchers to analyze and draw conclusions regarding crucial issues such as the capacity of coral–algal symbiosis to cope with changing environmental conditions. A recent publication (Goulet, 2006) dealing with the ability of corals to change their algal symbionts stated that out of 442 coral species only 23% host multiple zooxanthellae clades, while 77% exhibit fidelity to a narrow subset of a single zooxanthellae clade or to a specific algal genotype. Baker and Romanski (2007) critically reevaluated the same data with the exclusion of coral species that were either under sampled or not adequately defined, and showed that when the analysis is restricted to the species for which sampling has been more significant, over two thirds of all coral species (and almost three quarters of scleractinian coral species) host multiple algal symbiont types at the clade level or below. Moreover, upon looking into within-clade diversity, they found that 20% of the corals that had been documented as hosting only one clade of *Symbiodinium* actually contained multiple types within that clade. These two latter publications, deriving nearly opposite conclusions from one data set, further highlight the obstacles and setbacks still occurring in the research of algal symbiont diversity in coral hosts. It is now evident that technical constraints hinder the efforts to reveal the true diversity of zooxanthellae within a host (Carlos et al., 2000; Kinzie et al., 2001; Baker and Romanski, 2007). Although extensive sampling of

invertebrate host and algal symbionts (see Goulet (2006) and Baker and Romanski, (2007)) have been undertaken worldwide, it is assumed that most field surveys published to date are extremely likely to have underestimated within-colony *Symbiodinium* diversity at all taxonomic levels (Baker and Romanski, 2007). If this is indeed the case, then as molecular techniques continue to improve and the "background symbionts" become more easily detectable, we foresee that symbioses currently identified as monomorphic could be reclassified as actually being comprised of multiple genetically different algal symbionts. Thus, it is possible that multibiont associations in coral–algal symbiosis are far more widespread than previously assessed.

The multibiont symbioses that were presented in Section 3 share a common feature: the role of the macrosymbiont (fish, crustacean, polychaete, or bivalve) as an ammonium donor. The contribution of ammonium is beneficial to the coral holobiont via its microsymbionts, which are the actual recipients. Overall, it seems that such multibiont symbioses enable the recycling of nitrogen through the flow of ammonium ions in one direction (to *Symbiodinium*) and amino acids in the other (to the coral host), thus conserving nitrogen in the otherwise nutrient-poor tropical oceans (Muscatine and Porter, 1977).

Established symbioses might respond to environmental changes by switching partners (Buddemeier and Fautin, 1993; Rowan and Knowlton, 1995; Lewis and Coffroth, 2004) or by "shuffling" of existing partners (quantitative change in the relative abundance of existing symbiont communities within colonies; Baker, 2003), as was suggested for the role of bleaching in coral–algal symbiosis. Interestingly, recent literature concerning the coral holobiont and its associated microbial community suggests that the coral animal can also adapt to differing ecological niches by "switching" its microbial partners (Wegley et al., 2007). The metabolic roles of the microbial community associated with specific coral hosts are beyond the scope of this chapter. However, recent experimental data suggests that coral-associated bacteria take part in carbohydrate, protein, sulfur, and nitrogen cycling in the coral animal, and thus are important to the functioning of the holobiont (Wegley et al., 2007). Further studies concerning the metagenomic analysis of the microbial community associated with different coral species (Wegley et al., 2007) will undoubtedly shed new light on the specificity, physiology, stability, and significance of such associations to the coral reef dynamics.

The occurrence of symbioses with multiple genotypes of symbionts raises the question of whether a host with a symbiont population of multiple genotypes derives the same benefit as a host with a genetically uniform population of symbionts (Douglas, 1998; Frank, 1996). Frank (1996) assumed that in the former state, each symbiont genotype is predicted to exhibit more competitive traits, including increased proliferation rates and elevated acquisition of host-derived nutrients, resulting in a depressed performance of the host. There might not, however, be any decisive answer to this question, as every symbiotic system has its own unique characteristics (structural, metabolic, and physiologic). Moreover, it was demonstrated that in juvenile *Convoluta roscoffensis*, the presence of more than one symbiont can cause a transient state of reduced host growth for up to

30 days, by which time only one symbiont will be retained in the worm (Douglas, 1998). Such a scenario might indicate the occurrence of a competitive process. A different scenario is presented by Loram et al. (2007), who showed that the proportion of fixed carbon translocated to the sea anemone *C. gigantean* is not depressed in the mixed infections (of two co-occurring *Symbiodinium* taxa) and suggest that competition between the symbionts seems to be suppressed (Loram et al., 2007). In support of the complexity of this issue, is some experimental data that show evidence for competition between mycorrhizal fungi, resulting in reduced plant performance (Pearson et al., 1993). On the other hand, Newsham et al. (1995) have demonstrated that individual plants may derive distinct benefits from different fungal genotypes.

Browsing through the multibiont symbioses discussed in this chapter and from those taken from the terrestrial environment, the rarity of vertical transmission of symbionts is prominent. Several researchers have argued that mutualistic symbioses evolved from parasitic relationships, and that vertical transmission played a key factor in the reduction of symbiont virulence (Ewald, 1987). Surprisingly, in several mutualistic associations vertical transmission is completely absent, such as in those between plants and mycorrhizal fungi, legumes and rhizobia, and some corals and dinoflagellates. It is expected that all mutualisms must have evolved a perfect vertical transmission if the relationship is truly mutualistic, because hosts may fail to acquire symbionts if these are not vertically transmitted (Genkai-Kato and Yamamura, 1999). Douglas (1995) stated that vertical transmission of symbionts is advantageous to the host, in that the host is assured of gaining a compatible symbiont. Vertical transmission is an obvious trait during asexual reproduction of hosts, whether mediated by fragmentation, binary fission, or specialized asexual propagules. Two factors may limit the incidence of vertical transmission: structural barriers in the host and the cost of vertical transmission. The costs can be two-fold: space and nutrition. Potentially there is also a long-term cost of vertical transmission. The host, presumably, lacks access to a variety of alternative symbiont taxa. Nevertheless, a recent study has demonstrated for the first time the uptake of heterologous zooxanthellae by zooxanthellate primary polyps (with maternally-derived zooxanthellae) of the soft coral *Litophyton crosslandi* (Zurel et al., 2008). Based on a mathematical model, Genkai-Kato and Yamamura (1999) suggested that mutualistic symbiosis without vertical transmission should evolve only when (1) vertical transmission involves some costs in the host, (2) the symbiont suffers direct negative effects if it exploits the host too intensively, (3) the host establishes the ability to make use of waste products from the symbiont, and (4) the mechanism of vertical transmission is controlled by the host. Coral–dinoflagellate relationships have persisted since the Triassic period and are likely to have contributed to the longevity, diversity, and success of this group (Stanley, 2006). From the coral's perspective, horizontal transmission and complex mixtures of symbionts might provide a short-term ecological flexibility to cope with fluctuating physical conditions that outweighs the possible costs of evolutionary conflicts among symbionts (Herre et al., 1999). From all the above-mentioned findings and factors, it seems that the modes of symbiont transmission

can not serve as a criterion for classifying symbiotic (mutualism, parasitism, etc.) or multibiont symbiotic systems. In support of this assumption comes the study by Marlow and Martindale (2007) of *Symbiodinium* localization and mode of gastrulation in two species of scleractinian embryos: *Fungia scutaria* (with horizontal transmission) and *Pocillopora meandrina* (with vertical transmission). Those researchers determined that both species, independent of whether or not they "seed" their oocytes with symbionts, undergo a "nutritive" stage before gastrulation, wherein lipid-rich cells (*F. scutaria*) or membrane-bound cellular fragments (*P. meandrina*) are passed to the blastocoel, from where they are subsequently taken up by the definitive endoderm. This emergent property of anthozoan development appears to have been co-opted to facilitate the movement of *Symbiodinium* to the blastocoel (future site of the endoderm) in the seeded species, where they are later phagocytosed by the newly formed definitive endoderm. Unfortunately, data concerning the modes of transmission of other symbionts taxa in the coral holobiont are still lacking. Future studies focusing on morphological adaptations and the recognition process among the partners taking part in multibiont symbioses may lead to a better understanding of the true nature of symbiosis.

Multibiont symbioses constitute an extremely complex phenomenon and therefore are hard to categorize with simplified titles. Currently it would appear that "digging deep enough" could eventuate in identifying more and more partners in one symbiotic system, much like a Russian "Babushka" doll. On top of structural complexity, metabolic and physiologic aspects can not be ignored. The resolution of the search will also affect its complexity; hence if one looks for phage/virus inside bacteria, inside algae, inside a worm that is living on a coral. The intricacies of such associations appear endless. Multibiont symbioses, the puzzle of nature, are fascinating, intriguing, and challenging.

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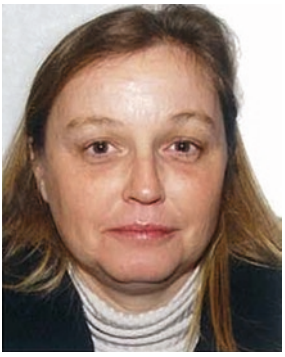

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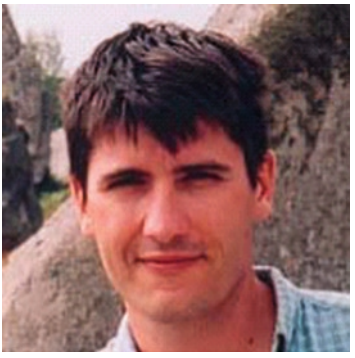

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CNIDARIAN–DINOFLAGELLATE SYMBIOSIS-MEDIATED ADAPTATION TO ENVIRONMENTAL PERTURBATIONS

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1. The Mutualistic Cnidarian–Zooxanthellae Symbiosis

Photosynthetic symbionts are widespread in cnidarians although they occur mostly in anthozoans and scyphozoans. Cnidarians contain two types of symbiotic photosynthetic protists. One is the unicellular green algae *Chlorella* sp. (Chlorophyceae), called zoochlorellae, found in the freshwater hydrozoan *Hydra* and in the sea anemones *Anthopleura xanthogrammica* and *A. elegantissima* (Muscatine, 1971). The second is the dinoflagellate, *Symbiodinium* sp., generally called zooxanthellae, which is the major symbiont of cnidarians. In a few cases, such as in the sea anemone *Anthopleura* zooxanthellae may share their hosts with zoochlorellae, the relative proportion of each symbiont depending on the seawater temperature (Verde and McCloskey, 2002). Symbiosis is particularly well studied in scleractinian corals, as this beneficial relationship plays a major role in the establishment of coral reef ecosystems in nutrient-poor tropical waters. About half of the scleractinian species harbor zooxanthellae (Cairns, 1999). While this symbiotic relationship is thought to be obligatory, some sea anemones such as *Aiptasia* sp. or *A. elegantissima* can prosper without their symbionts under conditions where alternative sources of nutrition are available (Coffroth and Santos, 2005; Rodriguez-Lanetty et al., 2006). Except in some scyphozoans, localization of symbionts is restricted to endodermal cells, where they settle in membrane-bound vacuoles derived from the animal cell wall (Wakefield and Kempf, 2001), known as the symbiosome. This intracellular location suggests

that the host may control its symbionts by regulating the transfer of organic or inorganic compounds right through this symbiosome membrane.

Initially considered as a single species, called *Symbiodinium microadriaticum* (Freudenthal, 1962), the zooxanthellae have since shown great diversity: to date, eight clades (called A to H) containing hundreds of distinct genotypes have been described on the basis of sequence variations of the nuclear and chloroplast rRNA genes (Pochon et al., 2006). Associations of these different clades with their host did not evolve randomly, and members of the same host species generally harbor the same *Symbiodinium* clade(s) (see review by Coffroth and Santos, 2005). Symbionts may be transmitted either vertically, directly from parents to progeny ("closed" system), or horizontally ("open" system) through the environment. This last mode of transmission, which seems to be used by about 85% of zooxanthellate cnidarians (Schwarz et al., 2002), offers the opportunity for the host to become associated with zooxanthellae that are better adapted to a particular environment (Baker et al., 2004) but could also alter the cost–benefit ratio of the symbiotic association, according to Sachs and Wilcox (2006). However, the reservoir of horizontally transmitted symbionts remains to be determined. A controversial theory on virulence predicts that horizontal transmission of symbionts may promote the evolution of parasitism, altering the cost–benefit ratio of the symbiosis (Fine, 1975; Sachs and Wilcox, 2006).

The roles of cnidarian symbionts, particularly their nutritional roles, are well studied and have been reviewed several times (see for review Muscatine, 1990; Muller-Parker and Davy, 2001). Here, we consider only the main aspects of these roles that are directly related to an enhanced tolerance of environmental stress. Symbionts play a major role in host feeding and up to 90% of photosynthetically fixed carbons can be transferred, typically as glycerol and other simple molecules, from the algae to the host for its own needs (Muscatine et al., 1984; see review by Muscatine, 1990). Furthermore, nitrogen, a limiting resource in tropical ecosystems, is better assimilated and conserved in symbiotic cnidarians. The presence of symbionts thus triggers the inorganic nitrogen uptake by the host, both as ammonium and nitrate (Grover et al., 2002, 2003), or the uptake of organic nitrogen, such as urea or amino acids (Al-Moghrabi et al., 1993; Grover et al., 2006). In corals, the presence of symbionts increases the rate of host calcification by a mechanism known as the "light-enhanced calcification," the physiological bases of which are still debated (Barnes and Chalker, 1990; Gattuso et al., 1999; Allemand et al., 2004). Conversely, the host transfers to its symbionts essential nutrients such as phosphorus-, nitrogen-, or sulfur -containing compounds. The host also offers a shelter as well as a constant supply of inorganic carbon (CO_2) to the symbionts used by the latter's chloroplasts via carbon-concentrating mechanisms (Allemand et al., 1998; Leggat et al., 2002).

Nevertheless, the presence of photosynthetic symbionts imposes specific conditions on the animal host, such as the obligation to live close to the sea surface to optimally expose algae to sunlight. Such conditions may be detrimental to the host by exposing it to high levels of ultraviolet radiation (UVR) and rapid changes in environmental parameters. Another important side effect of this symbiosis is the daily hyperoxia induced by symbiont photosynthesis. However, in contrast to

non-symbiotic species, the evolutionary process in symbiotic cnidarians has selected some mechanisms that allow both partners to circumvent these drawbacks.

2. Symbiosis-Mediated Tolerance to Oxidative Stress

One of the most prominent features of cnidarian symbiosis is the host tolerance to the hyperoxic conditions induced by symbiont photosynthesis (Dykens and Shick, 1982). For example, O_2 partial pressures vary from less than 1% to as much as 60% (i.e., threefold normoxia) in the cœlenteric fluid of the tentacles of the sea anemone, *Anemonia viridis* (Richier et al., 2003). Similarly in the coral *Favia favus*, the O_2 level reaches 3.5-fold normoxia close to the tissue surface (Shashar et al., 1993). Such high concentrations of O_2 can lead to the overproduction of reactive oxygen species (ROS), which may cause protein oxidation, lipid peroxidation, and DNA degradation (Halliwell and Gutteridge, 1999). However, no damage increase has been recorded in symbiotic cnidarians tissues although pro-oxidant conditions have been observed in the light (Richier et al., 2005).

2.1. LARGE AND DIVERSE ANTIOXIDATIVE DEFENSE IS ONE CONSEQUENCE OF SYMBIOSIS

This extreme resistance of an animal to ROS production was initially thought to be due to a high activity of antioxidant enzymes. Indeed, in light compared to dark conditions, Dykens and Shick (1982) found higher activity in superoxide dismutase (SOD), one of the major enzymes involved in ROS scavenging. The use of electrophoresis gels in non-denaturating conditions allowed Richier et al. (2003) to find up to seven SOD isoforms within the *A. viridis* symbiotic host cells. This result, which was not due to cross-contamination of the animal tissues by zooxanthellae extracts, is exciting because such a diversity in SOD isoforms is generally a plant feature. By contrast, only three SODs were found by these authors in the non-symbiotic sea anemones *Actinia schmidti* (Richier et al., 2005), a result that is in agreement with standard SOD diversity observed in animal cells (Halliwell and Gutteridge, 1999; Alscher et al., 2002). A similar diversity (6 SOD isoforms) was observed in zooxanthellae of temperate clade A that inhabits sea anemone tissue (Richier et al., 2005). This result suggests that the high resistance of symbiotic cnidarians to oxidative stress is due to enhanced oxidative defense in terms of both intensity and diversity. In addition, Richier et al. (2003) also demonstrated that, by contrast with other metazoans, the three SOD classes (FeSOD, CuZnSOD, and MnSOD) were all present in the tissue of sea anemones. Later, the presence of an additional extracellular CuZn-SOD was also demonstrated (Plantivaux et al., 2004).

Interestingly, this original pattern is not restricted to temperate sea anemones. Shick and Dykens (1985) found a good correlation between activities of both SOD and catalase, and chlorophyll concentration within 34 species of symbiotic invertebrates. Similarly, a high diversity in SOD patterns was also demonstrated in

three scleractinian corals (*Stylophora pistillata*, *Plerogyra sinuosa*, and *Pocillopora verrucosa*) and in a tropical sea anemone (*Entacmea quadricolor*) (Richier et al., 2005, 2008; Furla et al., 2005) as well as in other zooxanthella clades (Richier et al., unpublished data). Therefore, the variability in SOD, either in terms of activity or isoform diversity between symbiotic and non-symbiotic hexacorallians suggests that the diversification of SOD in animal host cells could be a consequence of symbiotic relationships rather than a unique feature of the Phylum Cnidaria.

2.2. ACQUIRED RESISTANCE TO HYPEROXIA AND ASSOCIATED OXIDATIVE STRESS

It is reasonable to assume that an organism experiencing daily transitions from dark hypoxia to diurnal hyperoxia for millions of years has evolved strategies to resist oxidative damage. It has thus been demonstrated that no significant changes are observed in terms of lipid peroxidation (measured by MDA assay) or protein carbonylation during natural light-induced hyperoxia in both host and symbionts (Richier et al., 2005). This suggests a host adaptation to symbiont photosynthesis, possibly involving constitutive expression of several SOD isoforms, as is the case in plants (Gillham and Dodge, 1987). It should be noted, however, that levels of lipid peroxidation and protein carbonylation measured during the day are higher in a symbiotic compared to a non-symbiotic sea anemone. A similar high basal value of oxidative stress biomarkers has also been observed in photosynthetic organisms (Asada and Takahashi, 1987).

The efficient enzymatic protection from oxidative stress in symbiotic sea anemones may protect them against experimental hyperoxia. Indeed, incubation of the symbiotic sea anemone *A. viridis* in seawater bubbled with pure O_2 (100%) induced neither qualitative changes in SOD patterns nor significant increases in cellular damage. In contrast, a similar treatment of pure O_2 led to both qualitative changes in SOD expression and a tenfold increase in protein oxidation in the non-symbiotic sea anemone *A. schmidti* (Richier et al., 2005).

Together with antioxidant enzymes, cnidarians have also non-enzymatic antioxidants, such as ascorbic acid or glutathione (GSH), which are water-soluble (Lesser, 2005). Carotenoids and tocopherols are the major lipid-soluble antioxidants that scavenge ROS (Edge et al., 1997). The carotenoids, peridinin, dinoxanthin, diadinoxanthin, and diatoxanthin are found in symbionts while β-carotene is present in both animal host and symbionts (Mobley and Gleason, 2003). It has been shown that a large part of host carotenoids are derived from zooxanthellae metabolism (Mobley and Gleason, 2003). However, carotenoids are also found in non-symbiotic cnidarians, as the animal host may also obtain them by feeding on carotenoid-rich zooplankton (Sebens et al., 1996). In all cases, the production of carotenoids depends on environmental conditions and increases in corals subjected to high levels of solar radiations (Ambarsari et al., 1997), which enhances the holobiont's ability to withstand oxidative stress (Brown et al., 1999).

For example, during a bleaching event, the proportion of β-carotene increases in a similar way as observed in higher plants (Ambarsari et al., 1997).

Green-fluorescent protein (GFP) is a spontaneously fluorescent protein (FP) that absorbs blue light and reemits it as green fluorescence (Tsien, 1998). GFP-like proteins include both fluorescent and nonfluorescent pigments, which give pink, purple, or blue colors to the organism (Dove et al., 2001). Referred to as pocilloporins or FPs, these pigments have been identified in both symbiotic and non-symbiotic cnidarians (Matz et al., 1999; Dove et al., 2001; Wiedenmann et al., 2004). Although FP concentrations do not vary along a depth gradient (Mazel et al., 2003), they are predominant in symbiotic species living in photic environments, but they are encoded only by the host genome. The sequence has been cloned in the non-symbiotic anthozoan *Cerianthus membranaceus* (Hexacorallia: Ceriantharia) and shows a poor homology with the *Aequorea victoria* GFP (17.8% of identical residues; Wiedenmann et al., 2004). Their functions are not fully understood, but have been described as photoprotective in high-light conditions by scattering the light reaching the coral (Salih et al., 2000), or conversely, by enhancing photosynthesis in low-light conditions (Schlichter and Fricke, 1990; see section 3.3). In addition, it has been shown that GFPs might display antioxidant activity. Indeed, it has recently been reported that GFP from the hydromedusa *Aequorea victoria* could quench superoxide anion radicals ($O_2^{\cdot-}$) and exhibit SOD-like activity by competing with cytochrome c for reactions with $O_2^{\cdot-}$ (Bou-Abdallah et al., 2006).

Usually considered as an ultraviolet (UV) screen, mycosporine-like amino acids (MAAs) are low-molecular-weight molecules that absorb UVR and thus play a major role as UV protectants (Shick and Dunlap, 2002; see below). However, some MAAs may protect organisms not only against UVR, but also against scavenging ROS. The oxo-MAA mycosporine-glycine plays this role (Dunlap and Yamamoto, 1995). This MAA is predominant in phototrophic symbioses (Dunlap et al., 2000). Its functional analogue, mycosporine-taurine, plays the same role, but is found only in sea anemones from the genus *Anthopleura* (Stochaj et al., 1994). MAAs that are not redox active nevertheless diminish UV-induced ROS production, because they absorb and dissipate harmful UV energy (see fig. 22.3 in Shick et al., 2000). Yakovleva et al. (2004) found that mycosporine-glycine may protect corals from thermal stress-induced oxidative stress by providing a rapid protection, before the effective induction of enzymatic antioxidant defenses.

3. Symbiosis-Mediated Tolerance of Solar Irradiation

3.1. COOPERATIVE SYNTHESIS OF MYCOSPORINE-LIKE AMINO ACIDS

MAAs are UV-absorbing compounds (λ_{max} = 309–360 nm), which include 13 different structures identified in reef-building corals (Shick and Dunlap, 2002). In those corals and in reef organisms in general, MAAs mainly act as natural

sunscreens that absorb and dissipate solar UVR. Their concentrations in animal and algal tissues increase when UVR intensities are experimentally enhanced (Shick et al., 1999), or when corals are transplanted from greater to shallower depths (Shick et al., 1996). Using the coral *Stylophora pistillata* it was experimentally shown that UVB (but not UVA) was the main stimulant of the MAA accumulation (Shick et al., 1999). The presence of detectable amounts of MAAs in deepwater colonies or in corals maintained without UVR under laboratory conditions suggests that the biochemical pathway leading to MAA synthesis is constitutively expressed or is very sensitive to UVR (Shick et al., 1999).

It has been assumed that MAAs in symbiotic anthozoans are synthesized via the shikimate pathway (Shick et al., 1999). Indeed, addition of glyphosate, an inhibitor of several enzymes of the shikimate pathway, was shown to repress MAA production in the coral *Stylophora pistillata* exposed several days to UVR (Shick et al., 1999). Since this pathway is usually lacking in animals (Bentley, 1990), and therefore in aposymbiotic corals (Banaszak and Trench, 1995b), zooxanthellae have been assumed to be the main MAA-synthesizing partner. Despite this evidence, few studies have investigated the effect of UVR on the MAA synthesis by isolated zooxanthellae, and contrasting data are present in the literature. The sea anemones *Anthopleura elegantissima*, which may harbor, simultaneously or otherwise, two taxonomically different unicellular algae (zoochlorella/zooxanthella, see above), always contain the same types of MAAs (Shick et al., 2002). While Banaszak and Trench (1995a, 2001) found no production of MAAs either by freshly isolated zooxanthellae of the sea anemone *Anthopleura elegantissima* or by cultured zooxanthellae (*Symbiodinium muscatinei*) from this sea anemone, at least four MAAs were produced by cultured zooxanthellae isolated from different coral species: mycosporine-glycine, shinorine, porphyra-334, and mycosporine-2-glycine. They were described as primary or *Symbiodinium* MAAs (Shick et al., 2005). There might therefore be clade-specific differences in the production of MAAs. It is also noticeable that isolated zooxanthellae, cultured under UVR, generally produce a more restricted source of MAAs than is found in corals (up to ten in *S. pistillata*) or in free-living dinoflagellates (Jeffrey et al., 1999). This might be due to a lower production of MAAs when zooxanthellae are separated from the host (but no experiment has tested this assumption directly), or to an action of the host itself, which can modify the primary MAAs to produce secondary ones (Shick et al., 1999).

MAAs are usually more concentrated in the host tissues than in symbionts (Stochaj et al., 1994). In the temperate sea anemone, *Anemonia viridis*, MAAs are preferentially found in ectodermal tissues free of zooxanthellae (D. Allemand and J. M. Shick, unpublished data). Therefore, MAAs, probably originating from the symbionts, are translocated to the host tissues and then metabolized. In the coral *Stylophora pistillata*, up to seven different MAAs were found in host tissues, while zooxanthellae did not produce in vitro MAAs (Shick et al., 1999). Recently, a bioinformatic study of the genome of the non-symbiotic sea anemone, *Nematostella vectensis*, challenged this traditional view by revealing

the unexpected presence of genes encoding enzymes for the shikimate pathway (Starcevic et al., 2008). Molecular evidence led the authors to suggest horizontal transfer of ancestral genes of the shikimic acid pathway into the genome of the sea anemone. The donors were identified as unknown bacteria and two dinoflagellates (*Oxyrrhis marina* and *Heterocapsa triquetra*). It remains to be determined if these genes are expressed and active in *Nematostella vectensis*.

Another way to acquire MAAs, especially for non-symbiotic anthozoans, is through the diet (Banaszak and Trench, 1995b), because most of them, including corals, are able to feed on a wide range of organisms, from bacteria to zooplankton (Houlbrèque et al., 2004). The relative contribution of auto- and heterotrophic sources of MAAs remains unclear, and it may also vary from species to species. As a consequence of the genotypic diversity in zooxanthellae and hosts, there is also a considerable diversity among corals in their MAA complement (Gleason and Wellington, 1995; Shick et al., 1995; Teai et al., 1998). This might explain the different tolerance and performance of corals to irradiance. LaJeunesse (2002) indeed showed that zooxanthellae from clade A were particularly restricted or absent in host living in shallow waters, where UVR fluences were high.

3.2. SYMBIONT-DERIVED MYCOSPORINE-LIKE AMINO ACIDS LEAD TO ULTRAVIOLET SUNSCREENING

The sun-screening role of MAAs in corals was reviewed in Shick and Dunlap (2002). The UV-screening properties of MAAs were shown to protect photosynthesis of the algae (Shick et al., 1991; Ferrier-Pagès et al., 2007), which would be otherwise inhibited, as demonstrated with freshly isolated zooxanthellae (Shick et al., 1991) or *in hospite* zooxanthellae (Ferrier-Pagès et al., 2007). UVR indeed directly affects photosynthesis by causing molecular damage or inducing ROS formation (Lesser, 2005). In the mucus layer (Teai et al., 1998), MAAs may also reduce the amount of UVR that reaches the coral tissue and therefore protect DNA, RNA, and protein from damage (Jagger, 1985).

3.3. MUTUAL PROTECTION INDUCED BY HOST AND SYMBIONT PIGMENTS

In order to colonize habitats close to the sea surface, symbiotic cnidarians have had to tolerate high solar irradiation. Obviously, this implies good adaptation and acclimatization of the photosynthetic pigments within the zooxanthellae. As discussed in the last part of this chapter, under high irradiance, mechanisms of photoprotection such as photoinhibition have been reported to thwart the vulnerability of algal photosynthesis. However, acclimatization and adaptation to high illumination also requires a certain plasticity of the holobiont. This later implicates the expression by the host tissue of a photobiological system that could, at

least partly, regulate the light environment of coral tissue. In fact, the expression of many different types of pigments, giving their vivid and diverse colors, is one of the best-known features of many coral species, although the precise roles of such pigments still remain an exciting field of investigation.

Apart from the brownish pigmentation caused by the photoactive compounds of the dinoflagellate symbionts, most of the reef anthozoan colors come for the animal host tissues (Oswald et al., 2007). These pigments could be globally depicted either as an antenna or, contrarily, as an umbrella (see below for details). However, the list of their potential roles is much longer as they may also act as scavengers, chemical defenses, moonlight photoreceptors, or just brilliant decorations. It should be added that anthozoan pigments also represent a great field of research, both intrinsically fascinating to better understand cnidarian physiology, and also tremendously valuable in the field of biotechnological development of new fluorescent chromophores.

Most of the compounds responsible for coral colors have been named pocilloporins (=FP), which is a generic term classically associated with homologues of the GFP. Due to their large diversity, they cover a great range of absorption and emission spectra (Prescott et al., 2003). Most of them are fluorescent molecules, without intrinsic bioluminescence capacity, but some only display intense absorption without emission and are more precisely called chromoproteins (Miyawaki, 2002). Finally, other pigments have recently been identified to absorb blue light and are photoreceptor proteins named cryptochromes, playing important roles in synchronizing mass spawning events according to the lunar irradiance (Levy et al., 2007). As mentioned above, the first classical role of the GFP pigments would be to amplify the levels of photosynthetic active radiation (PAR) available for zooxanthellae harbored by colonies growing under low-light habitats (Schlichter and Fricke, 1990). Some FPs could indeed capture short-wavelength light (including UV) and reemit it into longer wavelength in the PAR region of the spectrum, susceptible to be absorbed by the primary photosynthetic algal pigments. This statement implies that host-based compounds act as accessory pigments for zooxanthellae photosynthesis, which potentially represents a "brilliant" example of symbiotic partnership. However, it has been shown that there is inefficient energy transfer from the FP to the zooxanthellae chlorophyll, at least in shallow corals (Gilmore et al., 2003). By contrast, the second role attributed to symbiotic cnidarian pigments is to act as photoprotectants that shield the zooxanthellae photosynthetic machinery of corals settled in high-light conditions (Salih et al., 1998; Dove, 2004). This single role can be connected to different modes of action. First, due to their reflection and absorption properties, some of these pigments could act as an active sunscreen. In addition, parts of the deleterious UV radiation could also be neutralized by these pigments, which could reinforce the MAA-UV-protecting capacity.

Salih et al. (1998) and Dove et al. (2001) have made interesting observations that could offer a unified explanation for the apparently opposite roles of FPs. These authors provided evidence that at cellular levels, pigments are located in the host

tissue either above the zooxanthellae, in high-light environments, or below the symbionts, in low-light conditions. The regulation mechanisms of such pigment distribution remains to be discovered, but this can explain how, according to their position above or under the algae, the same type of absorbing and reflecting molecules could act to respectively, scatter or amplify high- or low-light irradiance.

The photoprotecting role attributed to coral pigments particularly raises exciting questions considering the fact that increased light is acknowledged to be one major cause of bleaching induction. On the one hand, Salih et al. (2000) observed a correlation between bleaching resistance and GFP-like contents. The mechanism of such a protection is not defined, but it has been proposed that these pigments, as described for MAA or xanthophylls, could also act as ROS scavengers, helping to reduce the pro-oxidant imbalance of stressed cells (Mazel et al., 2003). On the other hand, host pigment expression is suspected to be altered under increased temperature conditions, resulting in a reduced protection against solar radiation in the case of a thermal stress, a situation that could lead to major cellular damage for both partners (Dove 2004). Other observations have confirmed that bleached sea anemones also displayed a reduced content of GFP pigments, which could further reinforce the pale color of the host tissue (Leutenegger et al., 2007). To date it is not known whether the decrease in host pigments is a cause or a consequence of zooxanthellae loss, but the fact that pigments are reduced in bleached sea anemones suggests major disruption in the aposymbiotic host physiology. Nevertheless, these photoactive roles cannot be generally applied to all corals, firstly because some non-symbiotic cnidarians also express GFP homologues, demonstrating that the presence of symbiotic algae is not the unique cause for host pigment expression. Secondly, several symbiotic species have been reported to lack stratification in GFP-like protein content as a function of either depth or light. Furthermore, in contrast to the classic point of view, spectral analyses of the pigments of these coral species revealed no influence of GFP photon absorption either to enhance (Gilmore et al., 2003) or reduce the photosynthetic activity (Mazel et al., 2003). Additional observations, concerning the macroscopic distribution of pigments in different corals, demonstrate that the fluorescent compounds are mainly distributed in rapidly growing parts, like branch tips or plate-colony edges, as well as in skeletal ridges regions, which could be considered as areas where the animal cells are highly vulnerable and where zooxanthellae density is low (Salih et al., 1998; Mazel et al., 2003). This further suggests that host cells are the first beneficiaries of their pigment protection, with minimum interactions with photosynthetic activity of the symbiotic algae. Finally, Mazel et al. (2003) raise the fact that corals have other mechanisms of photoprotection (like MAA or non-photochemical quenching; see Lesser and Gorbunov, 2001) that are significantly more effective than those afforded by GFPs. This last statement could be balanced by the fact that, although the scavenging properties of pigments might be considered minor in some cnidarians under basal conditions, they may however become crucial in stressful conditions, for instance when antioxidant defenses get close from their tolerance limits.

It could be further speculated that additional roles that have not been extensively studied so far may be revealed as crucial. At least in the case of pigments visible under daylight illumination, they could play ecological roles in the attraction of some coral commensal organisms or alternatively by visually warning aggressors, displaying their intrinsic toxicity. They could also function in prey attraction (Haddock et al., 2005; Schnitzler et al., 2008) or to attract zooxanthellae during uptake in larval corals (Hollingsworth et al., 2005). More directly, Dove et al. (1995) mentioned that pigmented morphs may have enhanced resistance to fouling or predation, and that these morphs could also use their pigments as chemical defense systems for territorial competition at contact sites between non-similar colonies.

However, an exciting aspect concerning the alternative roles of pigments is correlated to the growing body of evidence suggesting their implication in the immune defense of the holobiont-facing virulent aggressors. For instance, the presence of a red FP, responsible for a non-normal pigmentation, was reported macroscopically in areas of wound healing, suggesting that such FPs may be a part of a generalized defense response to localized stress (Palmer et al., 2008).

Clearly, further studies are required to better estimate the physiological implications of pigments in the precise regulation of the symbiosis partnership. In summary, it could be philosophically remarked that the number of GFP homologues and their potential roles, as well as the common interspecific differences in morph pigmentation are a perfect and kaleidoscopic illustration of the undersea diversity and complexity.

3.4. CONTROL OF PHOTOINHIBITION

As mentioned above, because of the wide range of irradiances offered by the photic zone where the symbiosis occurs, the partners of the association can be exposed to high-light conditions. One requisite of this photosynthetic symbiosis is the ecological niche location within the photic zone, which requires the association to be exposed to high-light conditions. However, algal photosynthesis is very vulnerable to high solar irradiance, which may cause photoinhibition. Photoinhibition is only one of the daily challenges faced by most photosynthetic organisms (Long et al., 1994, Hoegh-Guldberg and Jones, 1999). This phenomenon is defined as any decrease in the capacity of a photosystem to capture and process photons that is caused by incoming light (Long et al., 1994; Osmond, 1994). Classically, photoinhibition has been interpreted in terms of damage sustained to photosynthetic systems and was measured originally as a long-lasting decrease in photosynthetic rate (oxygen flux) at high irradiances (Koh, 1956). More recently, changes in photosynthetic conversion efficiencies, in response to light or temperature stress, have also been associated with an array of photo-protective phenomena (Osmond, 1994). The latter has been referred to as “dynamic” (downregulation) as opposed to “chronic” (damage) photo-inhibition (Osmond, 1994; Osmond and Grace, 1995).

Damage occurring during chronic photo-inhibition is primarily associated with photosystem II (PSII), particularly with the increasing loss of the D1 protein of the PSII reaction center (Telfer and Barber, 1994). By contrast, the photoprotective mechanisms associated with dynamic photoinhibition span a wide range of phenomena that are reversible over a range of physiological scales. Energy-dependent quenching of PSII is thought to achieve photoprotection by changing the aggregation of light-harvesting complex II (LHCII) particles, or by reversibly deactivating individual PSII reaction centers within seconds (Duysens and Sweers, 1963; Schreiber and Bilger, 1987). Other changes may take minutes (e.g., xanthophylls cycle interconversions; Long et al., 1994), hours (e.g., changes in the expression of oxygen-scavenging components), or days (e.g., developmental changes; Long et al., 1994). These changes decrease redox pressure at various points in photosynthetic electron transport, and act as uncoupling mechanisms between incoming light energy and subsequent redox outcomes.

By measuring the rate of O_2 production, it was initially shown that the host may prevent photoinhibition of their symbiont as freshly isolated and cultured zooxanthellae exhibit reduced rates of net photosynthesis at high-light levels, while no photoinhibition was observed in the holobiont (Muller-Parker, 1984; Goiran et al., 1996). However, using the PAM-fluorimetry technique, it has more recently been shown that for numerous scleractinian corals, a marked reduction in the photochemical efficiency by zooxanthellae is apparent under high-light conditions (Brown et al., 1999; Jones and Hoegh-Guldberg, 2001; Winters et al., 2003; Hoogenboom et al., 2006). In such corals, the photoinhibition phenomenon is reported as a causative agent of bleaching (i.e., loss of most of the symbiotic zooxanthellae; Walker, 1992; Jones et al., 1998).

Correspondingly, avoidance of excessive light levels is a determinant of morphology in coral colonies and many species generate self-shading morphologies in high-light habitats (Oliver et al., 1983; Titlyanov, 1991; Muko et al., 2000). As mentioned above, because high levels of light cause photodamage and photoinhibition in photosynthetic symbionts (Long et al., 1994), it has been suggested that fluorescent pigments may reduce the susceptibility to photoinhibition of fluorescent corals by filtering out damaging UVA and excessive photosynthetically active radiation (Salih et al., 2000). Finally, genetic variants in zooxanthellae associated with the host may affect photoinhibition susceptibility of the symbiosis. As described above, coral colonies can act as hosts to several different lineages of endosymbiotic dinoflagellates, with the composition of these communities following gradients of solar irradiance (Rowan et al., 1997). Thus, the genetic diversity within *Symbiodinium* is likely to correlate with an equally diverse range of physiological properties in host–symbiont assemblage.

Previous studies have indicated that *Symbiodinium microadriaticum* has both sun and shade-loving genetic variants, and that the distribution of these is dependent upon the host species (Jokiel and York, 1982). Observed patterns of bleaching where shaded portions of colonies tend to bleach first could be explained by different susceptibilities of different genetic strains of zooxanthellae to photoinhibition

(Buddemeier and Fautin, 1993; Rowan and Knowlton, 1995). An alternative possibility is the susceptibility of zooxanthellae to photoinhibition dependent upon their respective light history, as cells that live under continuous low irradiance are much more susceptible to photoinhibition, and its subsequent effects (Richter et al., 1990). This susceptibility is possibly related to the light-dependent repair of PSII as observed for plants that are grown in shaded or low-light environments that appear to have a lowered capacity for repair than plants grown in full sunlight (Foyer et al., 1994). Moreover, it has been recently shown that some clades of *Symbiodinium* might be less beneficial to the coral host than others and in this latter case host–symbiont interactions may be closer to parasitism than mutualism (Stat et al., 2008).

4. Symbiont Protection Against Chemical Stress

4.1. SYMBIONT PROTECTION AGAINST SALT STRESS

Living close to the sea surface, symbiotic cnidarians are more easily subjected to environmental changes than organisms living at deeper depths. If temperature or UV stresses have been much studied in the perspective of global warming, these animals are also subjected to osmotic (=salt) stress. Indeed, high precipitation, freshwater runoff, and storms, all lead to hypoosmotic stress and, conversely, periods of prolonged drought to hyperosmotic stress. Even in normal conditions, they have to maintain their intracellular osmotic pressure identical to that of seawater, i.e., about 1,100–1,200 mOsmoles (Willmer et al., 2000). As the intracellular ionic content is lower than that of seawater, osmoconformers generally accumulate osmotic solutes in the form of low-molecular-weight organic molecules such as amino acids. This raises the issue of how symbiotic organisms may adapt to salt stress. It has been suggested that MAAs could act as osmolytes (Oren and Gunde-Cimerman, 2007). If such a role is possible in cyanobacteria, where their concentration approaches that of free amino acids (i.e., ~100 mM; see for a discussion Shick and Dunlap, 2002), this hypothesis is not compatible with the low concentration measured in cnidarians. For example, in sea anemones, MAAs represent only 3% of the amino acid concentration (Shick and Dunlap, 2002), disproving any major role in osmoregulation.

Compatible organic osmolytes (COOs) are molecules synthesized by most marine invertebrate cells that fluctuate in response to osmotic stress and do not disrupt cellular function. COOs are typically either polyols (including glycerol), free amino acids, methylammonium, and methysulfonium solutes or urea, and are synthesized or degraded in order to alter intracellular osmolarity (Mayfield and Gates, 2007). Up to 98% of glycerol, the main carbon source synthesized by zooxanthellae, is translocated into host cytoplasm (Muscatine, 1967), and further used for host respiration and growth (see review by Muscatine, 1990). However, some glycerol is maintained in cellular pools under normal conditions. Gates and Edmunds (1999) have shown that the host tissue of the coral *Montastrea franksi* has glycerol level around 200 µg/mg proteins. For a water space of 2.58 µl/mg

protein (Bénazet-Tambutté et al., 1996), this gives a concentration of glycerol around 840 mM in host tissue. Consequently, there is no doubt that symbiont-produced glycerol may play an important role as osmolyte during hyperosmotic stress (Mayfield and Gates, 2007).

4.2. DIFFERENTIAL METAL ACCUMULATION AND DETOXIFICATION

Heavy metals, from both industrial sources and antifouling paints, are common marine pollutants. Bleaching has also been associated with anthropogenic perturbations, including heavy metals (see Brown et al., 2000). Field studies have demonstrated that symbiotic anemones (*Anemonia viridis* and *Anthopleura elegantissima*) accumulate more cadmium than aposymbiotic specimens (Harland and Nganro, 1990; Mitchelmore et al., 2003a). Laboratory experiments have also demonstrated a differential metal uptake (cadmium, copper, zinc, or nickel), depending on the symbiotic state (Mitchelmore et al., 2003b). While symbiotic *A. elegantissima* accumulated more metals (at least Cd, Ni, and Zn) during exposition period (42 days), a fast removal of metals occurred during recovery, which suggested specific detoxification mechanisms in symbiotic species. No evidence was found for a differential metal accumulation by the zooxanthellae. One of the hypotheses is that metals taken up by symbionts are subsequently deposited in host tissue, as observed in giant clam–dinoflagellate symbioses (Benson and Simmons, 1981). Similarly, sensitivity of cnidarians to heavy metals is also dependent on the symbiotic state: it was shown that the aposymbiotic form of *Hydra viridissima* was more sensitive to low Cu concentrations than the symbiotic form, while the toxicity of highest concentration of Cu was similar for both groups (Karntanut and Pascoe, 2005). Several defense mechanisms for regulating metals have been demonstrated in symbiotic cnidarians, such as metal binding in external mucus, metal sequestration into granules, and metal detoxification. Regarding metal binding, it has been demonstrated in cnidarians that mucus production is a response to metal exposure (Harland and Nganro, 1990), reducing metal availability, and that symbiotic specimens appeared to produce more mucus that aposymbiotic ones. Furthermore, various detoxification mechanisms have been identified. Symbiotic *A. elegantissima* exposed to cadmium showed far more metal-binding GSH than aposymbiotic ones (Mitchelmore et al., 2003a).

4.3. FATTY ACID PRODUCTION AND STRESS TOLERANCE

Fatty acids form up to 40% of reef-building coral dry biomass (Yamashiro et al., 1999). They are essential constituents of cell membrane lipids as well as an important source of metabolic energy, and corals contain large amount of storage lipids. Furthermore, fatty acid composition is specific to particular groups of organisms and is a good index of their physiological state. Though some of them are obtained from food sources (zoo- and phytoplankton), lipids are mainly derived

from symbiotic zooxanthellae (Harland et al., 1993; Papina et al., 2003). There is good evidence that the lipid production is light-dependent and driven by photosynthesis of zooxanthellae. Furthermore, an active transport of saturated and several unsaturated (PUFA) fatty acids from dinoflagellates to the host have been demonstrated (Harland et al., 1993; Papina et al., 2003).

Changes in lipid amount in coral tissues have been correlated with stress, especially thermal stress and bleaching (Glynn et al., 2001; Yamashiro et al., 2005). Again, the authors have pointed out the role of symbionts in supplying lipids to the host cells: bleached corals showed a decreased storage lipid content, correlated to the reduced photosynthesis. This decrease of lipid content was most probably explained by the reduction in wax content, as a consequence of the decreased lipid supply from zooxanthellae. Thus, the susceptibility of corals to bleaching has been proposed to be related to the extent in the reduction of storage lipid level. Furthermore, there is another issue for zooxanthellae fatty acids: thermal sensitivity in symbiotic hosts has been correlated with the degree of saturation of the lipids in the zooxanthellae thylakoid membranes (Tchernov et al., 2004). The critical threshold of temperature separating thermally tolerant from sensitive species of zooxanthellae is determined by the saturation of the lipids. In thermally sensitive corals, membrane integrity is affected and membranes are energetically uncoupled, thus leading to ROS production and, finally, zooxanthellae expulsion. Thermally tolerant corals harbor zooxanthellae with a markedly lower content of the major PUFA (Δ6,9,12,15-*cis*-octadecatetraenoic acid, 18:4) in relation to Δ9-*cis*-octadecatetraenoic acid (18:1). These data suggest that the higher relative concentration of 18:1 PUFA enhances thermal stability of thylakoid membranes and reduces the susceptibility of membrane lipids to ROS attack. Therefore, both storage lipids and cell membrane lipids are crucial components for the survival of a bleaching event.

5. Changing Symbionts as a Way to Adapt to Environmental Stress: The Adaptative Bleaching Hypothesis

As mentioned above, there is a great diversity in dinoflagellate symbionts (Coffroth and Santos, 2005). Based on the assumption that different host–symbiont combinations may harbor different tolerance against thermal stress (see, e.g., Rowan, 2004), Buddemeier and Fautin (1993) hypothesized that bleaching may be an adaptive response to environmental changes by providing an opportunity for thermal-adapted zooxanthellae to repopulate corals host, "resulting in a new holobiont better suited to the altered environmental circumstances." This change in the symbiont population may thus produce non-Darwinian adaptation, resulting in an immediate phenotypic variability. It can be the result of either symbiont switching or symbiont shuffling. While symbiont switching is the acquisition of new symbionts from the ambient seawater, symbiont shuffling corresponds to a change of abundance of the different clades (or sub-clades) already present within the host (see Baker, 2003). The latter hypothesis, known as adaptive bleaching hypothesis

(ABH; see also Fautin and Buddemeier, 2004), was experimentally strengthened by Baker (2001), who has performed transplant experiments in different depths. He showed that transplanted corals might adjust abundance of algae from specific clades when transplanted in shallower waters. Similarly, Rowan (2004) showed that corals might adapt to higher temperatures by hosting specifically adapted symbionts. Loram et al. (2007) subsequently gave a mechanistic basis of this observation by showing that incorporation of symbiont photosynthates into the sea anemone *Condylactis gigantea* lipids and amino acid pools was significantly higher in symbioses with zooxanthellae of clade A than of clade B.

However, this hypothesis has been the object of a large and still open debate (see, e.g., Hoegh-Guldberg et al., 2002) and it is out of the scope of this review to summarize all the arguments used by the different authors. While zooxanthella transmission is supposed to occur mainly by horizontal transfer (Schwarz et al., 2002), Goulet (2006) showed that only 23% of the coral species studied (442 species) host multiple zooxanthella clade. She therefore suggests that less than one quarter of coral species may have the ability to adapt to climate change by switching symbiotic algae, thus refuting the generalization of the adaptative bleaching hypothesis. This assumption, however, has been widely criticized (Baker and Romanski, 2007). In addition, Mieog et al. (2007) showed, by using a newly developed real-time PCR technique, that the diversity of symbionts within the host is higher than previously expected. Even if bleaching is not an adaptative response, probably it gives opportunity to cnidarians for new combinations, as stated by Fautin and Buddemeier (2004). However, this repopulation remains dependent on the presence of different clades in the environment, which is at present not clear, zooxanthellae having been found until now in the sand interstitial waters (Carlos et al., 1999) and not in the water column, suggesting that free-living populations are small and transient. Also, the specificity of the mechanism of zooxanthella clade recognition by the host is unknown. A lectin, suspected to play a role in symbiont selection, was characterized in a soft coral (Jimbo et al., 2000, 2005; Koike et al., 2004) and later in the coral, *Fungia scutaria* (Wood-Charlson et al., 2006).

However, it is important to mention that a growing number of studies have demonstrated that many symbioses are extraordinarily stable and do not switch or shuffle their symbionts seasonally or under different environmental conditions (Kirk et al., 2005).

6. Potential Mechanisms of Symbiont-Induced Stress Tolerance

6.1. CORAL ACCLIMATIZATION: ZOOXANTHELLAE INDUCED PRECONDITIONING

6.1.1. Host-Adaptation to Hyperoxia Induces Thermotolerance

We showed above that symbiotic cnidarians acquired resistance to oxidative stress. Could this resistance contribute to host adaptation to other environmental stressors

such as increased temperature? A thermal stress applied during 5 days to the non-symbiotic *A. schmidti* resulted in both a time-dependent increase in damage biomarkers (a threefold increase in MDA production and a tenfold increase in protein oxidation) and an increase in global SOD activity, together with the expression of a novel stress-inducible CuZn-SOD isoform. By contrast, in the symbiotic species *A. viridis*, similar thermal stress had no effect on lipid peroxidation or resulted in less than a 3.5-fold increase in protein oxidation without any modification of the SOD activity pattern (Richier et al., 2005).

This comparative study strongly suggests that while the non-symbiotic species appeared sensitive to elevated temperatures, the sympatric symbiotic species was tolerant. These observations suggest that the symbiotic state plays a role in host cell adaptation to thermal stress. This resistance could be the result of the presence of the symbionts that acclimate the animal cell to high pO_2 on a daily basis. The greater diversity of SOD isoforms induced by the presence of the photosynthetic symbionts within animal tissue could contribute to this adaptation. Hyperoxic adaptation may also be a preconditioning step that could prevent cellular damage during thermal stress, implying that symbiosis may confer host resistance to multiple stresses. Moreover, interactions at the antioxidant defense level between both symbiotic partners could also be a basis for protecting cells following environmental changes. As a result of physical interactions or possible molecular communication between both species, the presence of the zooxanthellae may induce increases in SOD activity and may contribute to the adaptation to stress encountered by the animal partner.

A similar relationship has been already demonstrated in associations between fungi and plants. In this symbiosis, the host plant acquires a thermotolerance because fungal endophytes produce melanin that may dissipate heat and/or complex with oxygen radicals generated during heat stress. Alternatively, the endophyte may act as a "biological trigger" allowing symbiotic plants to activate stress-response systems more rapidly and strongly than non-symbiotic plants (Redman et al., 2002). The Mediterranean demosponge *Petrosia ficiformis* also shows comparable adaptation with enhanced antioxidant defenses in symbiotic specimens in response to photosynthetically produced ROS (Regoli et al., 2000, 2004). The potential for symbiont-induced stress adaptation was supported by transplantation experiments, comparing aposymbiotic *versus* symbiotic specimens. While aposymbiotic sponges did not survive when moved from a cave to a light-exposed cliff, symbiotic ones remained healthy after transplantation from reduced to elevated solar irradiance (Regoli et al., 2000). Authors concluded that the different sensitivity to sunlight exposure might be explained by the higher levels of antioxidants in the symbiotic sponges that guarantee greater protection against sunlight-mediated photooxidative stress. In lichens, the symbiotic partnership mutually enhances resistance to ROS in the context of dessication stress (Kranner et al., 2005).

The molecular basis of preconditioning has been well studied during ischemia/reperfusion injury. It was shown indeed that after a period of ischemia,

tissue necrosis occurs not during ischemia itself, but during subsequent reoxygenation (Li and Jackson, 2002). Brief episodes of ischemia may prevent ischemia/reperfusion injury (see review by Halestrap et al., 2007). It was shown that H_2O_2 generated within mitochondria during hypoxic condition is the potential cell mediator of the initiation of preconditioning (Van den Hoek et al., 1998). Exogenous H_2O_2 can induce cell protection, while SOD inhibition abolishes the protective effect of preconditioning (Van den Hoek et al., 1998). Thus, although free radicals are toxic for the cells, they may also act as intracellular messengers, eliciting protective responses. We can therefore suggest similar mechanisms in symbiotic cnidarians, which have, however, to be tested.

6.1.2. Symbiont-Derived Mycosporine-Like Amino Acids Lead to Thermotolerance

Similarly, several in situ observations and laboratory experiments tend to suggest that exposure to UVR (and the correspondent synthesis of MAAs) induces some thermal resistance in symbiotic corals. Indeed, Hoegh-Guldberg and Salvat (1995) were the first to observe that shallow water corals were more thermally resistant than deeper living ones due to their constant exposure to high solar radiation levels. Then, it was also observed that parts of colonies regularly exposed to high solar radiations were more thermally resistant than shaded parts (Brown et al., 2002). By exposing colonies of different scleractinian coral species to UVR at a non-stressful temperature, Ferrier-Pagès et al. (2007) demonstrated that UVR-exposed colonies presented a reduced photoinhibition under elevated temperatures. This experiment indeed consisted in culturing half of the coral colonies under UVR during 14 days, while the other half was protected from UVR. The UVR-exposed colonies synthesized three to ten different MAAs and contained four to eight times more MAAs than the control corals. Seawater temperature was then increased for both control and UVR-exposed corals from 27°C to 34°C. While control corals significantly decreased their photosynthetic efficiency (measured as F_v/F_m), the UVR-exposed corals did not show any significant change in F_v/F_m, suggesting some thermal protection of the photosystem. The mechanism underlying this acquired thermotolerance is presently unknown, but may be related to the antioxidative properties of some MAAs (see above). This observation has implications in coral bleaching, and suggests that the bleaching susceptibility of corals depends on their previous history as well as on their capacity of upregulating their defenses.

6.2. ORIGIN OF SYMBIOSIS-INDUCED ADAPTATION

As highlighted in the previous paragraphs, living in symbiosis brings multiple physiological and biochemical adaptations to both partners that help them to resist to environmental changes. These adaptations not only reinforce some biochemical ways as development of antioxidant defenses and UV screens, but also

create new biochemical capacities as carbon or nitrogen absorption in animal cells (see Furla et al., 2005). Although the origin of these new properties is still unknown, coevolution and gene transfer could be hypothesized.

Coevolution is a direct consequence of the species' interaction with one another. Moreover parallel evolution of phenotypes is due to similar environmental constraints that act on them, for example, resistance from hyperoxia or UV. Through coevolution, host and symbiont improve their own arms against the same external changes. By consequence, though phylogenetically distant, the two partners gain similar mechanisms of adaptation. The high diversity in number and classes of SOD isoforms in symbiotic cnidarian hosts could then be explained by selection pressure driven by symbiont photosynthesis-induced hyperoxia and ROS production.

Another way of acquiring new biochemical properties to better resist external changes is sharing molecular tools. Horizontal gene transfer (HGT) is well known in prokaryotes, where it has been demonstrated as an important mechanism of acquisition of new metabolic capacities. Raymond and Blankenship (2003) even consider them as evolutionary leaps creating variability and adaptation far more rapidly than natural selection. In eukaryotes, intracellular HGT (from mitochondria and chloroplasts to the nucleus) have been extensively studied (see Timmis et al., 2004). Less-investigated HGT between eukaryotes living in symbiosis have been described in plants (Mower et al., 2004; Davis and Wurdack, 2004) and animals (Steele et al., 2004; Hirt et al., 2002). By intricate association of phylogenetically distant organisms, symbiosis could affect genome evolution by facilitating gene transfer from one genome to another (Moran, 2007). Moreover, HGT are not random events but are often linked to environmental factors (Simonson et al., 2005), appearing in organisms sharing the same constraints. In this context, cnidarian symbiosis could be a preferential place where HGT could occur. The origin of unexpected genes involved in stress resistance (e.g., shikimate pathway genes or antioxidant genes; see Starcevic et al., 2008) could then be related to HGT between the two partners or between ancestral symbionts. This hypothesis is supported by the analysis of cnidarian genomes where several non-eukaryotic genes have been identified (Kortschak et al., 2003; Technau et al., 2005) like in other symbioses (i.e., insect–bacteria symbioses; Dunning Hotopp et al., 2007; Gladyshev et al., 2008).

7. Conclusions and Future Research

Symbiotic association has deeply modified the physiology of both partners. The symbiotic host cells have acquired mechanisms for CO_2 absorption and concentration (Allemand et al., 1998), inorganic and organic nitrogen absorption (Grover et al., 2002, 2003, 2006), and ROS detoxification (Richier et al., 2003, 2005). The present review shows that symbiosis also elicits mechanisms for stress tolerance with oxidative, thermal, PAR and UV radiation, salts and metal stresses.

However, our understanding of the benefits of symbiosis is likely to be incomplete, and more unexpected advantages will be discovered. Recently, Rosenberg et al. (2007) have pointed out the role of secondary symbiosis (including bacteria and archaea) within cnidarians. These microorganisms could confer benefits on their host by various mechanisms, including infection prevention. This new aspect of the symbiosis needs more investigation but seems to corroborate the conclusions of Haine (2008), who pointed out that symbionts (especially vertically transmitted ones) contribute significantly to invertebrate resistance to natural enemies. In symbiotic cnidarians, the dinoflagellate symbionts could play a predominant role by secreting antimicrobial substances or toxic compounds like free-living dinoflagellate species.

Although presently being investigated by a large number of scientists, cnidarian symbiosis remains poorly understood as most of these studies have been primarily focused on trophic exchanges between the two partners. This focus could have hidden other molecular exchanges necessary for the holobiont growth and resistance to environmental stress. Crosstalk mechanisms between the two partners in normal or stress conditions also remains to be discovered.

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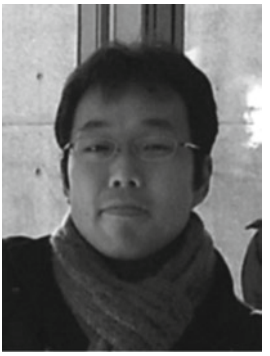

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OXIDATIVE STRESS-MEDIATED DEVELOPMENT OF SYMBIOSIS IN GREEN PARAMECIA

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1. Introduction

Due to the presence of endosymbiotic green algae in its cytoplasm, *Paramecium bursaria* is often referred to as a green paramecium (Fig. 1). The symbiotic green algae are morphologically similar to the genus *Chlorella*, and the phylogenetic analysis based on the ribosomal DNA sequence suggested that symbiotic algae are highly close to *C. vulgaris* group (Nakahara et al., 2003; Hoshina et al., 2004). The unique ecological status of *P. bursaria* being considered as a protozoan cell and also as a membrane-enclosed gathering of green algae, lately made this species listed as one of good model organisms for testing the ecological impacts of various chemical pollutants (Kadono et al., 2006; Kadono and Kawano 2007; Riediger et al., 2007).

Some groups have shown that the hosting ciliate and endosymbiotic algae can be separated from naturally growing *P. bursaria* cells, and they can be freely cultured independently (Hosoya et al., 1995; Nishihara et al., 1998; Gerashchenko et al., 2000) as illustrated in Fig. 2. The alga-free cell strains of *P. bursaria* can be readily produced by treating the stocks of green paramecia with photosynthesis-related herbicides such as DCMU ((3-(3,4-dichlorophenyl)-1,1-dimethylurea); Reisser, 1976) and methylviologen known as paraquat (Hosoya et al., 1995). In addition, aposymbiotic *P. bursaria* strains lacking algae can be occasionally found in natural environments (Tonooka and Watanabe, 2002). Generation of such aposymbiotic "green paramecia" *via* natural processes (without using any chemical) can be reproduced by the classical protocol in which the green paramecia are maintained and propagated in continuous darkness with excessive supplementation of food bacteria (Siegel, 1960).

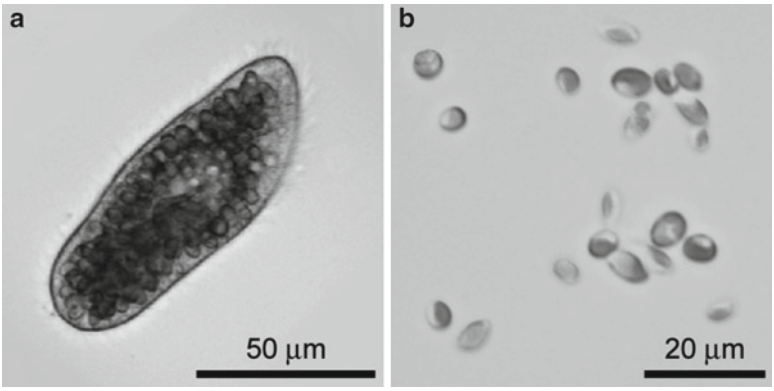

Figure 1. Light microscopic images of *P. bursaria* and its ex-endosymbiotic algae. (**a**) Matured cell of *P. bursaria* harboring the symbiotic green algae. (**b**) Ex-symbiotic algae isolated from *P. bursaria.*

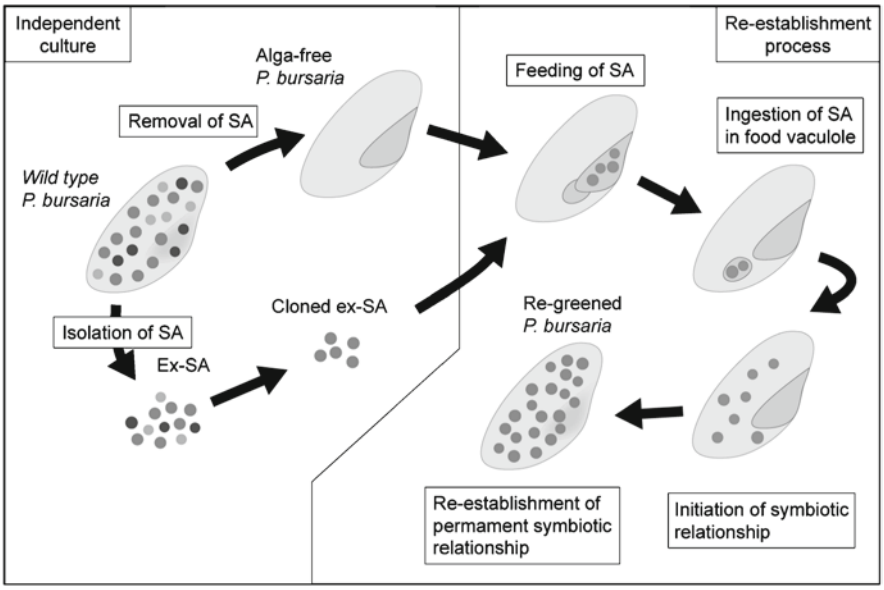

Figure 2. Schematic diagram showing the reestablishment of symbiotic relationship in green paramecium. Alga-free hosting ciliate can be prepared from paraquat-treated *P. bursaria*. Symbiotic algae (SA) can be readily isolated from the homogenates of *P. bursaria*. Experimentally, ex-SA can be reincorporated into the alga-free aposymbiotic host cells of *P. bursaria*. Although only a few SA survive in the perialgal vacuoles at early phase, SA regain its growth to reassociate and reestablish the symbiosis. (Adopted and modified from Gerashchenko et al., 2000.)

Treatment with a protein synthesis inhibitor cyclohexamide, also results in the removal of algae from the host cells (Weis, 1984). On the other hand, the clones of the ex-symbiotic algae can be isolated from green paramecia by crushing the host cell. Kinetic analysis based on the flow-cytometric cell counting revealed that the growth

capability of freely cultured ex-symbiotic algae is similar to that of non-symbiotic *Chlorella* species (Nishihara et al., 1998). When appropriate conditions are set, these independently cultured two organisms can reassociate and reestablish the symbiotic relationship (Nishihara et al., 1998). Interestingly, some strains of non-symbiotic *Chlorella* species can be introduced into aposymbiotic green paramecium cells as novel symbionts, when the combinations of algal cell lines and host cells are compatible (Gerashchenko et al., 2000).

According to Omura et al. (2004), reestablishment of the symbiosis from aposymbiotic algae and host cells of *P. bursaria* could smoothly proceed with high frequency if the culture was maintained free of bacteria. Probably this is partly due to the competition of bacteria with algae during the early infection processes. Infections of aposymbiotic *P. bursaria* (algae-free ciliate) with bacteria and yeasts can occur in nature, while the infection experiments showed that *Chlorella*-bearing *P. bursaria* cannot be infected with either bacteria or yeast (Görtz, 1982). The bacteria and the yeast cells taken up were shown to be located in the perisymbiont vacuoles (equivalent to perialgal vacuole, in case of algae).

2. Host–Symbiont Chemical Communications

P. bursaria is an excellent experimental model for studying the nature of endosymbiosis in which one species propagates inside the cells of other species. Since this organism attracted the attention of biologists, biochemists, and ecologists, the chemical communications between the host and symbiont such as recognition of the partners, chemical exchanges, and regulation of metabolic processes have been well documented (Kadono and Kawano 2007; Brown and Nielsen, 1974; Reisser et al., 1986; Tanaka and Miwa, 1996). Figure 3 illustrates the metabolic interactions between the hosting and symbiont cells. The symbiotic algae are capable of photosynthesis by fixing the carbon dioxide provided through the host respiration while producing oxygen as a consequence of algal photosynthesis. The oxygen evolved from the algae, in turn, is available for host respiration (Kadono and Kawano, 2007). In addition, symbiotic algae often release various mono- and disaccharides (mainly maltose), and then these saccharides are likely consumed by the host cells (Brown and Nielsen, 1974; Muscatine et al., 1967). Therefore, under light condition, the relatively high population of *P. bursaria* in the culture can be maintained for long term without feeding of food bacteria (Kadono et al., 2004a). However, this internal nutrient supply may not fully cover all the nutritional demands of the host cells especially during active propagation. When the host cells propagate explosively, the host demands an external food supply such as bacteria even under optimal light conditions.

The sexual interactions (mating) among the cells of *P. bursaria* can be observed when mixing the cells of complementary mating types if the cells are in the stationary phase and sexually matured (Sonneborn, 1954). When alga-harboring *P. bursaria* are

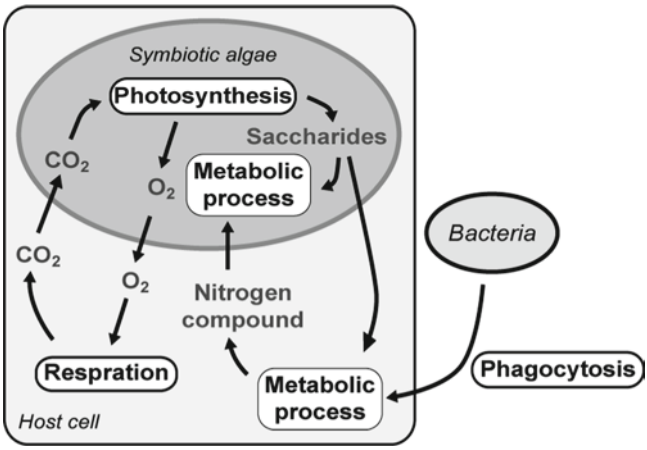

Figure 3. The metabolic interactions between the host cell and symbiotic algae. During the photosynthesis of the symbiotic algae, the CO_2 provided by the host respiration is utilized. Instead, host cells accept the O_2 provided by the symbiotic algae. In addition, symbiotic algae release various saccharides, often maltose. These sugars are consumed by the host cells as one of the energy sources. Due to algal photosynthesis in the presence of light, quite high population of *P. bursaria* can be maintained in the culture for long period without feeding any food bacterium. The metabolites derived from digestion of food bacteria are good sources of nitrogen compounds for symbiotic algae. (Adopted and modified from Kadono and Kawano 2007.)

transferred to continuous darkness, paramecium cells often lose their circadian rhythm in mating reactivity, while the circadian mating rhythm in the alga-free *P. bursaria* is insensitive to the shift to continuous dark condition, suggesting that communication between host and algae alters the biological clock response in the hosting cells (Tanaka and Miwa, 1996). This was attributed to the circadian rhythmic supply of maltose by symbiotic algae through daytime photosynthesis. This mechanism somehow regulates the rhythmic conditioning of mating reactivity. This is the best-known example that symbiotic algae play an important role in controlling of the host's behavior.

A difference in response to light is also observed between alga-harboring and alga-free cells of *P. bursaria*. The alga-harboring *P. bursaria* positively migrates and accumulates in the light field, while the alga-free *P. bursaria* favors the dark field (Iwatsuki and Naitoh, 1981). This interesting phenomenon can be interpreted to a metaphor that endosymbiotic algae drives their host cells as vehicles to reach the bright environment which is surely better for photosynthesis (this is obviously good for the host's energy balance sheet also since more returns from the algae could be expected). In fact, the alga-hosting paramecium and related species are efficient locomotive machinery and their driving can be readily controlled artificially under electrical fields (Ludloff, 1895). Therefore, aposymbiotic cells of *P. bursaria* can be utilized as electrically controllable bio-micromachines for transportation of bulky particles in the capillary system (Furukawa et al., 2009). Our latest study illustrated that such directed migration in *P. bursaria* is finely geared by cellular signaling events such as involving ion channels sensitive to inhibitors of T-type

Ca^{2+} channels such as NNC55-0396, 1-octanol, and Ni^{2+}, but insensitive to the L-type channel inhibitors such as nimodipine, nifedipine, verapamil, diltiazem, and Cd^{2+} (Aonuma et al., 2007).

On the other hand, some key influences on the symbiotic algae by the hosting paramecia are also known. Microscopic observation has revealed that the synchronization is imposed on the algal cell division by the hosting paramecia (Weis 1977). However, the detailed symbiotic associations between hosting ciliate *P. bursaria* and symbiotic algae are unknown. In our recent study, we focused on the impacts of the host's cell cycle and growth status on the life cycle of endosymbiotic algae (Kadono et al., 2004b). Populational analysis with flow cytometry has revealed that the life cycle of symbiotic algae is largely affected by the growth status of the hosting cells. The propagation of host actually alters the algal cell size and DNA contents. Such changes could not be observed when the host cells were in the resting phase. Number of the cells (algal spores) in the envelope structures of so-called sporangia was shown to be highly synchronized during the symbiosis.

Interestingly, the number of autospores in a single sporangium actually differs between endosymbiotic and ex-symbiotic (freely cultured) algae. In the endosymbiotically propagating algae, two or four autospores can be found in a single sporangium, while over 4 (up to 16) autospores are observed in each sporangium of the ex-symbiotic algae as revealed with the flow-cytometric analysis by monitoring the changes in the endogenous chlorophyll level, DNA content, and cell size of the algal complexes. In addition, microscopic analysis suggested that the algal cell division occurs only "before" and/or "during" but not "after" the host cell division. We found that the cell division in the symbiotic algae occurs only prior to completion of the host cell division and newly doubled algal population could be evenly redistributed to the two daughter cells of hosting paramecia (Fig. 4). These results strongly suggested that there were exchanges of signals or interactions mutually affecting the life cycles between the hosting ciliate and symbiotic algae. In addition, several genes in algal cells showed drastic changes in their expression patterns according to the presence and absence of host cells, further confirming the nature of symbiosis (Kadono et al., 2005).

3. Oxidative Stress

While the host cells in *P. bursaria* receive and utilize the photosynthetic products (carbohydrates) secreted from the symbiotic green algae, unbeneficial flow of stress-related chemical species from algal cells to its hosting cells (cytosolic space) has been suggested (Kawano et al., 2004). The alga-derived chemical species most apparently threatening the host cells are the members of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), and singlet oxygen ($^1O_2$). In green plant species including green algae, ROS members, namely H_2O_2, O_2^-, and $^1O_2$, are produced as the photosynthetic and/or photochemical by-products *via* three different mechanisms, namely photorespiration, Mehler reaction, and photodynamic action, respectively (Asada, 1999). In addition, hydroxyl

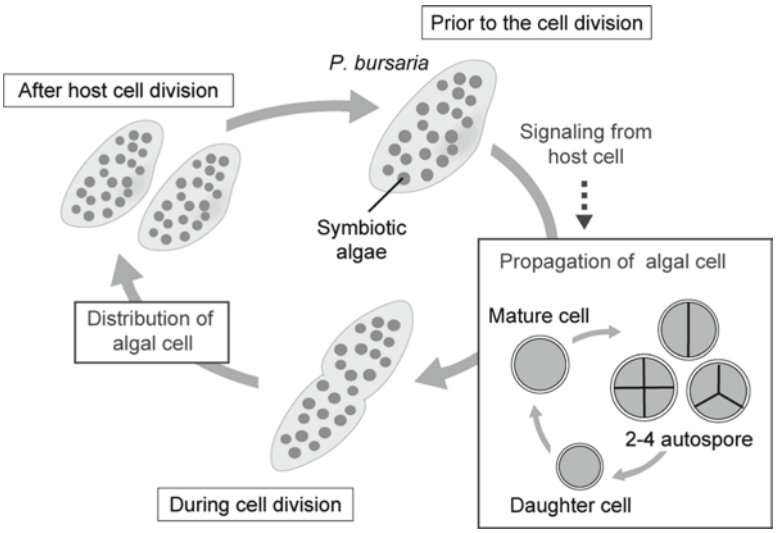

Figure 4. The relationship between the host cell division and algal cell division. The algal cell division leading to an increase in sporangia with 1–4 autospores in the paramecia might be stimulated prior to or during the division of the host cells. After the host cell division, symbiotic algae were distributed almost equally to the two daughter cells. (Adopted and modified from Kadono et al., 2004b.)

radicals ($HO^{\bullet}$), the most violent member of ROS are formed from other ROS such as H_2O_2 and O_2^-. It is well known that the cells and organelles in green plants and algae are rich in antioxidants such as ascorbate and glutathione, and that the ROS-detoxifying protective enzyme networks consist of glutathione reductase, ascorbate peroxidase, catalase, SOD, etc. (Maughan and Foyer, 2006; Tausz et al., 2004; Noctor and Foyer, 1998); therefore, green plant species are safely protected from the photochemically produced ROS (Patterson and Myers, 1973; Ishikawa et al., 1993; Collen et al., 1995). Note that *Chlorella vulgaris* (which can be experimentally introduced into *P. bursaria*; Gerashchenko et al., 2000) are also rich in ROS-detoxification enzymes (Takeda et al., 1997).

In addition, the intracellular ROS detoxification mechanisms, a variety of aquatic algae simply excrete the excess of H_2O_2, the only membrane-permeable ROS member in order to avoid the oxidative damage. Similarly in some symbiotic algae, production and release of ROS may proceed through the above processes. In turn, the alga-harboring hosts are internally exposed to the oxidative stress due to the release of ROS through algal photosynthesis, possibly resulting in oxidative damages to the host cells. When the median lethal concentrations (LC_{50}) of H_2O_2 were determined and compared among *Paramecium* species covering non-symbiotic (*P. caudatum* and *P. trichium*), symbiotic (*P. bursaria*), and aposymbiotic (alga-free *P. bursaria*) paramecia, the most H_2O_2-tolerant species was shown to be *P. bursaria* both in the symbiotic and aposymbiotic states, suggesting that symbiotic capability should be largely attributed to host cells' tolerance to the oxidative

stress (Kawano et al., 2004). According to a model proposed by our group (Kawano et al., 2004), the symbiotic relationship between the algal cells and the ciliate in *P. bursaria* has been developed as a consequence of host cell's adaptation to the oxidative stress due to ROS production *via* algal photosynthesis (Fig. 5). Based on the hypothesis and supportive experimental data, we proposed two models explaining the evolution of green paramecia. One model is merely a summary of the observation, showing that ciliates with higher tolerance to ROS successfully acquire endosymbiotic algae (Fig. 5a), and the other model proposed for explaining the evolutional processes claims that the oxidative stress due to the primitive symbiosis with algae (loaded to ancestral green paramecia) plays a key role in the natural selection of ROS-tolerant species during evolution (Fig. 5b).

The first model (Fig. 5a) propounds the correlation between the (re)greening ability of the paramecia and the tolerance to ROS, whereas the second model (Fig. 5b) emphasizes the cause of evolutionary selection, leading to the emergence of ROS-tolerant *Paramecium* species. In both models, the free-living algae might have been taken up by the paramecia by chance (as we can reproduce in the laboratory conditions), and then the majority of algae might have undergone immediate removal processes since the ancestral *Paramecium* species might dislike the presence of ROS-generating non-self particles. The possibility for the algal settlement in *Paramecium* cytoplasm must be higher in the paramecia with higher tolerance to ROS. Actually, the regreening process of the ROS-tolerant aposymbiotic cells

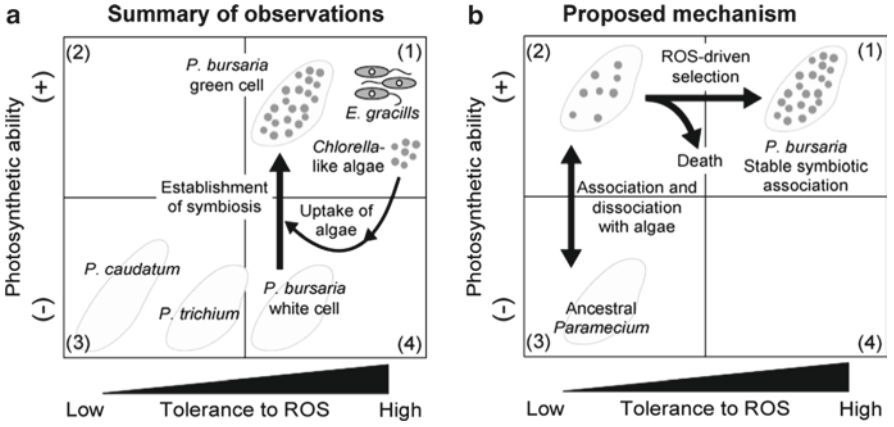

Figure 5. Possible origin of oxidative symbiosis in *P. bursaria*. (**a**) Summary of observations. Relationship between the ROS tolerance in *Paramecium* species and successfulness in experimental introduction of algae was implied by a series of experiments examining the symbiotic capability and tolerance to H_2O_2. This implies that emergence of ROS-tolerant paramecia enabled the symbiosis with algae. (**b**) Proposed model. Since the model suggested by the summary of observations never explain why and how the ROS-tolerant paramecia emerged out in the course of evolution, oxidative stress due to algae incorporated into ancestral paramecia (by chance) was considered as the driving force for the natural screening of the ROS-tolerant species. In this model, cohabitation of the ancestral paramecia with algae was followed by the selection of ROS-tolerant species. The quadrants in (**a**) and (**b**) are numbered.

of *P. bursaria* (white cells) in model 1 can be experimentally demonstrated with extremely high reproducibility (Nishihara et al., 1998; Gerashchenko et al., 2000). However, with the evolutionary time span, this model does not explain the nature of driving force required for emergence of the ROS-tolerant *Paramecium* species. This model assumes that the ancestral paramecia and its descendants had to experience the photosynthetic ROS-driven evolutionary selection for some eras, once a single cell of ancestral paramecia had successfully acquired the endosymbiotic partners by chance. This natural screening finally resulted in formation of the ROS-tolerant host species with stable association with symbiotic green partners as illustrated (Fig. 5b).

There would be another possible view that some derivatives of ancestral endosymbiotic algae causing less oxidative damages to the host might be selected in the course of coevolution. However, this is unlikely since it has been experimentally shown that the ex-symbiotic free host cells favor the chlorophyll-rich photosynthetically active free algae (but not the safer algae with less chlorophyll content) as the partners for re-association of the symbiosis (Gerashchenko et al., 2000). Since the photosynthetic activity due to the presence of chlorophylls and the level of photo-oxidative stresses are tightly related, it can be said that the less-stressful algae are less active in photosynthesis, and thus, less attractive as the source of energy, suggesting that the host cells in *P. bursaria* surely favor the risky but highly beneficial symbiotic partners.

4. Effect of Paraquat on Algal Bleaching

In our ongoing study, the ROS production by the symbiotic algae was experimentally promoted in the presence of paraquat, a commonly used herbicide, to exaggerate and highlight the role of the algae as the sources of stressful ROS. It is well known that paraquat induces the generation of ROS such as $HO^{\bullet}$ (most violent ROS member), in the chloroplasts under sunlight (Babbs et al., 1989). When the ROS production by the symbiotic algae was experimentally promoted by paraquat, the excretion of algae from the host cells was highly stimulated as discussed below. This result tells us a likely model in which the host cell bodies in *P. bursaria* positively eliminate the algae that are the sources of photochemical ROS production. By doing so, the host cells could survive by avoiding or lowering the risk of internal oxidation when the levels of oxidative stress were high enough to damage the host cells. This type of loose symbiosis between algal cells and the ciliate regulated by oxidative stress might be still in the course of coevolutional process leading to creation (selection) of really tolerant host species capable of gaining energy from the oxidative symbiosis. This view must be tested in the model experiments by taking the advantage of *P. bursaria*, the only material available for use in such tests.

Paraquat has been demonstrated to be a highly toxic compound for humans and animals by acting through oxidation of cellular NADPH, and thus inducing the disruption of NADPH-requiring key biochemical processes (Suntres, 2002).

Paraquat is known to induce much severer oxidative damage to plants since it produces O_2^- through interaction with photosynthesetic apparatus in plants. In chloroplasts in green plants and algae, paraquat and related compounds are known to extract electrons from photosystem I and transfer them directly to molecular oxygen to produce O_2^- and other ROS (Babbs et al., 1989). Thus, under light exposure, the amount of paraquat-stimulated O_2^- production in plant species must be obviously greater compared to the non-photosynthetic organisms. By expecting such herbicidal action of paraquat against green algae but not against the host cell bodies in *P. bursaria*, paraquat has been used as a key reagent to prepare the alga-free host cells from the intact green paramecia (Hosoya et al., 1995). The absence of algal genomes in the white (apparently aposymbiotic) cells derived from the paraquat-treated *P. bursaria* has been confirmed by diagnostic PCR (Tanaka et al., 2002). However, the mechanism how algae could be eliminated from the host in the presence of paraquat is not fully understood.

In case of aquatic algae, excess ROS could be excreted to exterior water environment in the form of H_2O_2 for minimizing the oxidative damages as discussed above. In symbiotic algae, certain level of ROS production may naturally proceed *via* photosynthetic paths and thus threaten the host cells. As *P. bursaria* was shown to be the most oxidative stress-tolerant species among other *Paramecium* species that were non-symbiotic paramecia (Kawano et al., 2004), the host cells must be able to cope with such stressful by-products under normal ecophysiological conditions. By analogy, we proposed an alternative hypothesis explaining the paraquat action for algal removal from *P. bursaria*. Here, we tested the possibility that algae were excreted rather than being simply killed by the herbicide action within the host cells. The likely driving force for stimulating the algal excretion was assumed to be the ROS released from the paraquat-exposed algae. Here, the hypothesis was tested by monitoring the changes in the number of symbiotic algae in the culture medium, which were excreted from the host cells. The changes in algal population within the host cell after addition of paraquat to the culture of *P. bursaria* were also monitored. As described below in detail, the number of excreted algae from the host cell to the culture medium in the presence of light was shown to be greater than that in the dark condition. In addition, scavengers of ROS significantly minimized the level of algal excretion. These results are in support of our view that oxidative damages to host cell originating from symbiotic algae results in excretion of symbiotic algae behaving as the source of oxidative stress, as an emergency action for survival.

For this experimental demonstration, green paramecium strain F1-1b (syngen 1, mating type I; provided from Prof. T. Kosaka, of Hiroshima University, Japan) was used. The cells were cultured in yeast extract-based nutrition mixture EBIOS (1 tablet/L; Asahi Food & Healthcare, Tokyo, Japan) medium inoculated with a food bacterium *Klebsiella pneumoniae*, under a light cycle of 12 h light and 12 h dark with ca. 3,500 lux (30 cm from the light source) of natural-white fluorescent light at 23°C. The bacterized medium was prepared by inoculating the EBIOS medium with *K. pneumoniae* 1 day prior to the use in ciliate culture.

The effect of paraquat on the survival of the host paramecia and free-living ex-symbiotic algae, and also the number of endosymbiotically growing algae were assessed (Fig. 6). As expected, the action of paraquat was drastically affected by the presence of light. Under light condition, the apparent LC_{50} values for paraquat in *P. bursaria* host cells and freely cultured ex-symbiotic algae cultured in vitro were 110 and 2.4 μm, respectively, while the apparent LC_{50} of paraquat against endosymbiotically growing algae was shown to be much lower (0.9 μm; Table 1, Fig. 6a). In the dark condition, LC_{50} of paraquat against *P. bursaria* and freely cultured ex-symbiotic algae were largely higher, 230 and 11 μm, respectively (Table 1, Fig. 6b). The apparent LC_{50} value of paraquat against endosymbiotic algal population was also higher in the dark condition (120 μm) suggesting that paraquat toxicity was drastically lowered in the darkness (ca. 130-fold). Although the treatments with extremely high concentrations of paraquat (over 300 μm; under light condition, 1 mM; dark condition) completely killed the host paramecia, we concluded that host cells are highly tolerant to paraquat treatment compared to the symbiotically growing algae showing very high sensitivity to paraquat.

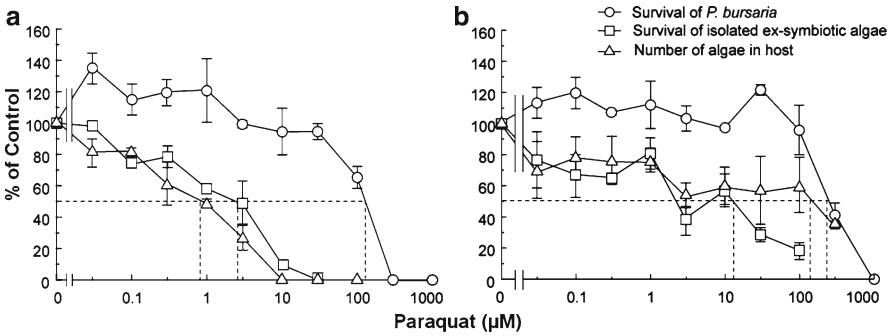

Figure 6. Effect of paraquat on the population of the host paramecia, freely cultured ex-symbiotic algae, and endosymbiotically growing algae in the host cell. Graphs show the relative survival rates of host cell (circle), freely cultured ex-symbiotic algae (square), and the relative number of endosymbiotically growing algae found in the host cells (triangle) in the presence of paraquat under light (**a**) or dark (**b**) conditions. Broken lines help determining the apparent LC_{50}. Bars stand for S.E. Paramecia or freely cultured ex-symbiotic algae in the stationary phase were treated with paraquat and incubated for 3 days at 23°C under constant light condition (ca. 3,500 lux) or constant dark condition.

Table 1. LC_{50} of paraquat against *P. bursaria* host cells, freshly isolated algae cultured in vitro and endosymbiotic algal population.

	Light condition (μM)	**Dark condition (μM)**
P. bursaria	110	230
Freshly isolated algae	2.4	11
Endosymbiotically growing algae	0.9	120

5. Algal Excretion

Effect of paraquat on the emergence of colorless paramecia (which possess no algal cell) was examined. Data suggested that light is required for the induced algal elimination. The likely factors required in this phenomenon are members of ROS since paraquat are known to generate ROS in the light-exposed chloroplasts. The absence of algae in the ciliates was readily judged by the lack of chlorophyll fluorescence as previously reported (Tanaka et al., 2002). Emergence of colorless paramecia was shown to be dependent on the concentration of paraquat and the presence of light (Fig. 7a).

Under light condition, the colorless cells appeared in the presence of moderate concentration of paraquat (1 μm), while in the dark, higher concentrations of paraquat (>30 μm) were required for the emergence of colorless cells. Figure 8b compares the number of algae excreted from a single host cell and the number of algae retained in the host. In the presence of paraquat under light condition, algal excretion was shown to be enhanced compared to the dark condition.

In the presence of relatively higher concentrations of paraquat, host cells are all punctuated and no single host cell survived (≥100 μm, light condition; 1 mM, dark condition). Thus, the number of algal cells in the medium counted in the presence of such high paraquat concentrations simply reflected the original size of symbiotically grown algal population. When *P. bursaria* cells were treated with 0.1–1.0 μm paraquat, the number of symbiotic algae in culture medium was at maximal levels, suggesting that these concentrations of paraquat (often used for preparation of alga-free *P. bursaria*) stimulated the excretion of algae. Interestingly, the algae excreted from the paraquat-treated green paramecia regained their growth after resuspension in a fresh paraquat-free media (data not shown). These results suggested that elimination of algae from the *P. bursaria* is mostly due to the excretion of live algae, rather than killing of the algae inside the host cells by the herbicidal action of paraquat.

6. ROS as Signaling Molecules

Some studies reported that *Chlorella* spp. maintained in the presence of paraquat can be used as an excellent solar-assisted biocatalyst for production of H_2O_2, which is considered as a chemical energy applicable for launching rockets (De la Rosa et al., 2001), suggesting that paraquat at micrometer levels is not lethal to the algae since passive but efficient movement of paraquat-induced ROS could be achieved through excretion of H_2O_2 out of the algal cells. It is highly likely that symbiotic algae (*Chlorella* spp.) within *P. bursaria* exposed to paraquat might also release H_2O_2 out of algal cells, thus oxidatively stimulating the host cells from inside.

To test the possibility that ROS derived from the paraquat-fed algae actually drives the algal excretion process, we examined the effects of ROS scavengers namely

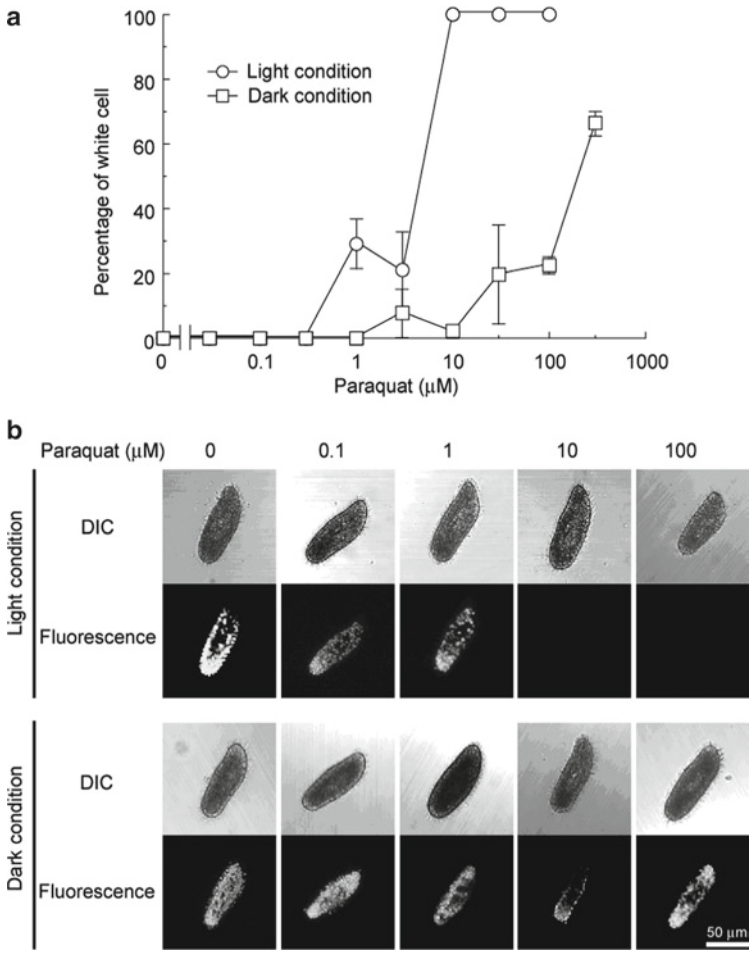

Figure 7. Emergence of colorless (white) paramecium after treatment of *P. bursaria* with paraquat. (**a**) Graph shows the percentage of colorless paramecium that emerged under the light (circle) and the dark (square) conditions. Note that no intact host paramecium was observed in the presence of high concentrations of paraquat (over 100 μm, under light condition; 1 mM, in dark condition). (**b**) Microscopic images of *P. bursaria* with nomarski differential interference (DIC) and the typical chlorophyll fluorescence images of *P. bursaria* treated with various concentrations of paraquat under light or dark condition. Bars stand for S.E. The cells were fixed in 3% (w/v) formaldehyde at room temperature for 5 min. Images were acquired using a laser scanning confocal microscope (Radiance 2100; Bio-Rad, CA).

Tiron (known to remove O_2^-), dimethylthiourea (DMTU, known to remove HO•), and *N*-acetylcysteine (NAC, known to act against a variety of ROS). These chemicals are supposed to remove ROS and protect the living organisms from oxidation by ROS. Figure 8b shows the effect of ROS scavengers on the paraquat-induced excretion of symbiotic algae from the host cell. ROS scavengers significantly decreased the number of algae excreted in the medium. However, the decrease in

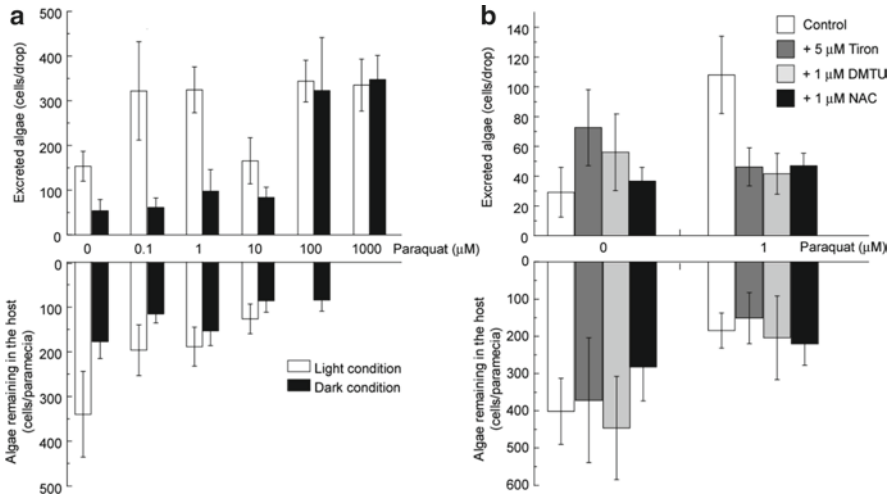

Figure 8. Effects of paraquat on algal excretion. (**a**) Effect of paraquat concentration. (**b**) Effect of ROS scavengers on the paraquat-enhanced algal excretion. Number of algae excreted from a single host cells kept in a separate drop and that remained in an intact host cell.

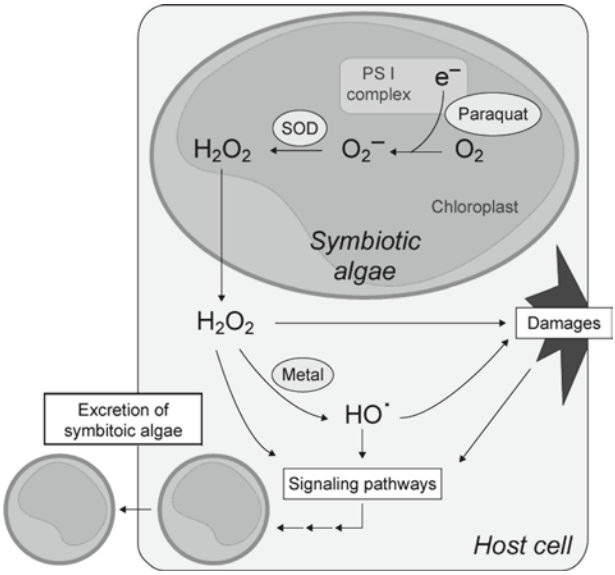

Figure 9. The relationship between the oxidative stress and the symbiotic association in green paramecium. In green algae, ROS is continuously produced under normal ecophysiological processes. The unicellular green algae can excrete membrane-permeable ROS (most likely H_2O_2) for passive protection from the oxidative stress. As a consequence, *P. bursaria* cells harboring algae are internally exposed to ROS potentially resulting in cellular damages. The oxidative stress possibly triggers the signaling processes involved in the symbiotic associations between green paramecia and symbiotic algae. (Adopted and modified from Kawano et al., 2004.)

intra-paramecium algal population was hardly prevented by extracellular applications of ROS scavengers. One possible explanation to this phenomenon is the limited accessibility of the ROS-scavenging chemicals. ROS scavengers could have reached the host cell membrane to minimize the oxidative stress to the hosting cells, and thus ROS-driven algal excretion could be prevented, but the action of ROS scavengers could not cover the events occurred in the photosynthetic organelles within algae and therefore paraquat-mediated death in algae could not be blocked. If it is the case, the paraquat-dependent decrease in intra-paramecium population of symbiotic algae should be attributed to both ROS-mediated algal excretion and paraquat-induced algal cell death. This should be clarified in the future study.

Figure 9 shows the putative cellular signaling pathways leading to the paraquat-induced excretion of symbiotic algae. In chloroplasts within the symbiotic algae, O_2^- is produced in the presence of paraquat by coupling with the photosynthetic reactions. Detoxification (disproportionation) of O_2^- is catalyzed by superoxide dismutase (SOD), and as a consequence H_2O_2 could be released. Since H_2O_2 can migrate in and out of the living cells across the bio-membranes (Kawano et al., 2004), H_2O_2 can be readily released out of algal cells and dispersed within the host cytoplasm. Eventually, H_2O_2 and $HO^{\bullet}$ (generated from H_2O_2 *via* Fenton-type reactions) possibly contribute to the oxidative damages to the host cell membranes. Then these ROS members and/or oxidative chemicals produced in a damage-dependent manner may act as the signals for triggering the algal excretion by the host cells.

7. Perspectives

For studying the mechanism of symbiotic relations between different organisms, we have a tendency to use the materials showing successful symbiosis. Apart from such successful model materials, we would like to describe a case of symbiosis distortion-causing unregulated growth of algae (not frequently observed, but sometimes reproducible under specific conditions).

Recently, we have screened some cell lines, from the mass of *P. bursaria* cells treated with paraquat and exposed to the passive regreening processes (during which algae rarely survive or those reintroduced from the media occasionally regain their growth after incubating the culture in the paraquat-free media for certain length of time). The resultant cell lines show novel and unusual morphological features with heavily darker-green color distinguishable from the original pale-green-colored cells (Irie et al., unpublished results).

In this type of isolates (designated as KMZ cells, Fig. 10), endosymbiotic algae propagate in a unique manner in which algal cells are restricted within one or two dense spots at the center of the host cells' cytoplasm, suggesting that the algal cells are encapsulated as a group in each of membrane compartment possibly made of digestive vacuole-like membranes derived from the oral groove or other membrane-enclosed structures. It seems that the dense algal community encapsulated in the

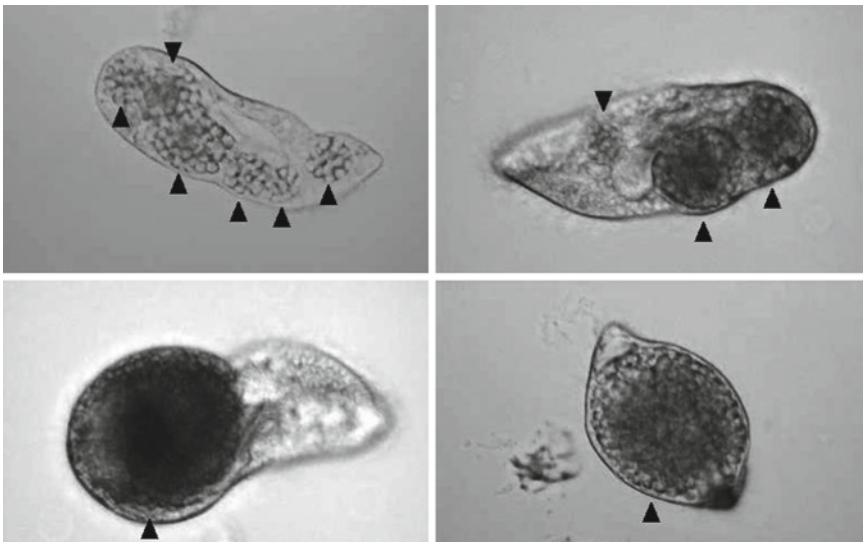

Figure 10. Newly isolated model cell line (KMZ cells) for studying the cell biological basis for the alga–ciliate symbiosis in *Paramecium bursaria.* Snapshots show the presence of dense mass of algae overgrowing in the host cells. Such KMZ cells often harbor single giant dense-colored compartment of algae (bottoms), while some pale-green cells are harboring several small-sized algal compartments (tops). Arrow heads indicate the positions of the masses of aggregated algae.

intracellular compartments failed to communicate with the host cells in order to synchronize their cell cycles. As a consequence, the dark-green compartments likely overgrow, obviously exceeding the original size of the normal host cells.

Due to overgrowth of the algal compartment within paramecia, such *P. bursaria* cells harboring encapsulated mass of algae often burst out or struggle to form the constriction during cell division, and thus the growth of paramecium population in KMZ culture are likely lower than that in native green paramecium culture (we also observed occasional release or excretion of free algae from this type of paramecia possibly avoiding the burst of the host cells). In addition, the paramecium cells with a single algal compartment often bear two unevenly divided daughter cells, one with algal compartment and the other without algae (thus colorless). One may expect that the culture originated from a single dense green cell and likely became full of colorless cells when this type of uneven cell division was repeated for several generations. However, as it seems to us, the dense green cells surely manage their propagation by chance with slow rate. In addition, some colorless daughter cells are likely regaining the algae from the media to initiate the growth of algal compartment in the identical manner. We are now examining the nature of such symbiotic distortions (i.e., lack of algal size regulation) observed in these KMZ isolates. We are expecting that the use of such materials that failed to maintain the normal symbiosis may contribute to uncover the secret of the successful symbiosis made up through tight communications between two symbiotic partners.

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CORAL SYMBIOSIS UNDER STRESS

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1. Introduction

Coral reefs of the world are in decline as a result of exposure to an increasing number of major stress agents (Wilkinson and Buddemeier, 1994; Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007). Stress causes bleaching and, in many cases, leads to the death of coral colonies and of entire reefs (e.g., Rosenberg and Loya, 2004). Coral reef ecosystems are one of the largest remaining reservoirs of biodiversity and are among the most diverse in the world. The reefs are based on the symbiotic relationship between the coral animal host to endocellular dinoflagellate microalgae, commonly referred to as zooxanthellae (yellow-brown algae, Brandt, 1883) embedded in their tissues (e.g., Karako et al. 2002).

Zooxanthellae are found to be in symbiosis with most of the reef organisms – from the coelenterates (stony corals, soft corals, zoanthids, sea anemones, and jellyfish) to sponges, foraminifera, ascidians, and mollusks. The most widespread zooxanthellae belong to the dinoflagellate genus *Symbiodinium*, mainly to the different genotypes (clades) in the species *Symbiodinium microadriaticum* Freudenthal. Other algal symbionts belong to additional dinoflagellate genera, to diatoms, or even to cyanobacteria (in the latter, the symbiont is called zoocyanellae; Trench, 1987).

The plant–animal symbiotic relationship is based on the energy provided by the algae to the animal as photosynthetic products, and in return, the supply of nutrients, mainly nitrogen and phosphorus, provided by the animal's available metabolic waste products to the algae (Muscatine et al., 1981).

2. Stressors

Any physical, chemical, and/or biological stressors (Figs. 1 and 2) can affect one of the symbiosis partners: the animal, the zooxanthellae, both partners, or the relationship between them (e.g., Douglas, 2003; Coles and Brown, 2003; see below). Stress can affect the growth rate, reproduction, and/or survival of the holosymbiont. Respiration, the process of using chemical energy to drive all ongoing life processes in the coral colony,

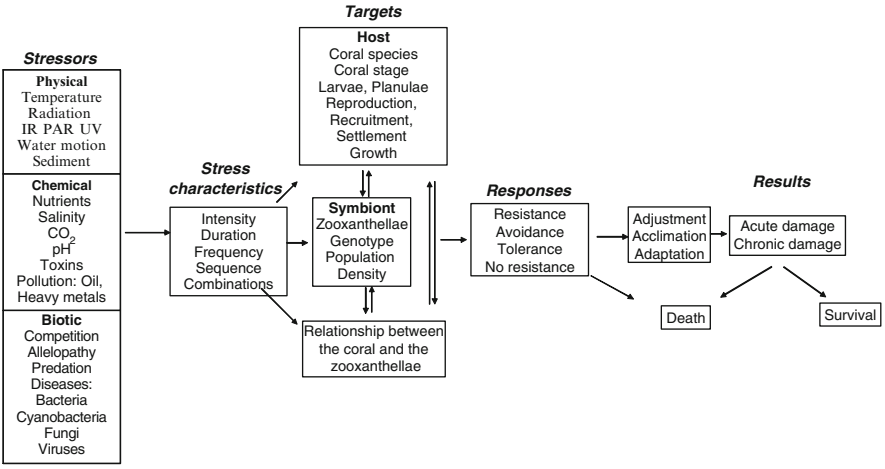

Figure 1. Different stress effects on coral symbiosis. (Based on Gaspar et al., 2002.)

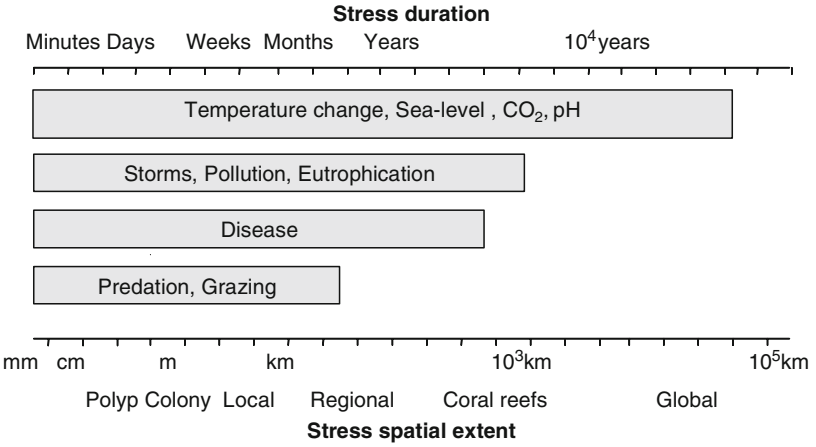

Figure 2. Range and duration of coral stresses. (Based on Jackson, 1991 and Nyström et al., 2000.)

is usually the most sensitive, and, as such, is a good indicator of stress. Photosynthesis, the main energy source of symbiosis, is one of the first processes affected by stress on the zooxanthellae (in most cases, there is damage to the photosystem, the thylakoid membrane, or proteins such as D1 (Figs. 3 and 4), or on the coral host due to reactive oxygen species (ROS) damage (see Stambler and Dubinsky, 2004). The major bleaching mechanism involves expulsion of zooxanthellae in response to environmental stress in in situ degradation followed by exocytosis. Other options are pinching off, programmed cell death (apoptosis), cell death resulting in loss of zooxanthellae (necrosis), and host cell detachment (Gates et al., 1992; Weis, 2008). In some cells detachment is probably unlikely (Sandeman, 2007). The biochemical mechanism of zooxanthella expulsion was described by Perez and Weis (2006; Fig. 3).

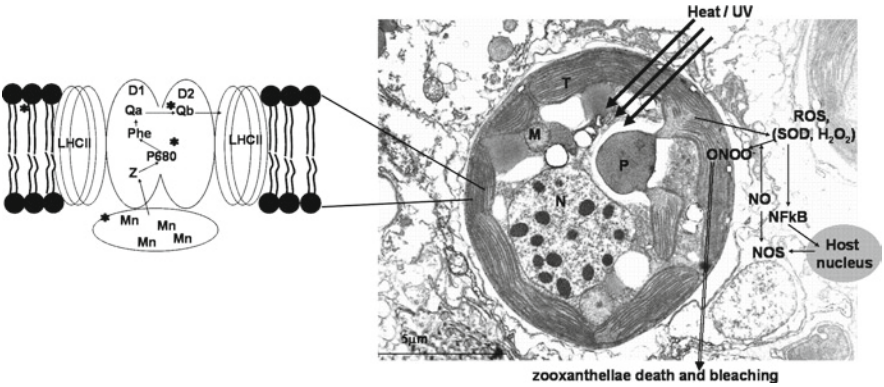

Figure 3. Heat and temperature effects on coral symbiosis. Transmission electronic micrograph of zooxanthellae (*Symbiodinium* sp.) T = thylakoid, N = nucleus, M = mitochondria, p = pyrenoid, including possible schematic for PSII site (*) damaged by heat stress. (Based on Iglesias-Prieto, 1997; Tchernov et al., 2004; and the model of Perez and Weis, 2006.) The zooxanthellae-created reactive oxygen species (ROS), including superoxide (SOD) and hydrogen peroxide (H_2O_2), to which the host cell responds by producing free radical nitric oxide (NO) through signaling leading to the upregulation of nitric oxide synthases (NOS). This signaling could involve the transcription factor, nuclear factor kB (NFkB). The reaction of superoxide with NO produces the reactive nitrogen species peroxynitrite (ONOO–), with additive deleterious effects leading to cell death and bleaching.

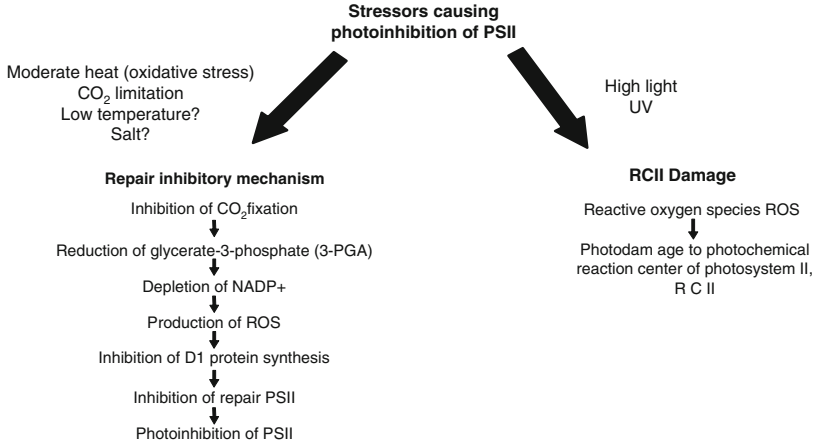

Figure 4. Stress-enhanced photoinhibition of PSII. (Based on Murata et al., 2007.)

The effect of stress depends on severity, duration, and frequency, i.e., repetition and duration of intervals between exposures to stress, as well as the combination of stresses (Figs. 1 and 2). Examples: How many times during summer did the temperature increase above normal and for how many days? When did the oil spill occur? Was it during low tide at high-sun radiation or not? Among the most serious effects of stress is the result of the combination of high temperature with high light intensity.

Response to stress can shift from avoidance to adjustment by genetic adaptation or physiological acclimation, through survival with acute or chronic damage, to death (Fig. 1). The response of marine calcifying organisms to increasing sea-surface temperature (SST) may depend not only on the absolute temperature increase but also on the rate of that increase. Long-term changes in environmental factors such as increased pollution and sedimentation might have acted synergistically with elevated SST to bring about coral bleaching in recent years, while explaining its absence during the 1940s–1960s (Barton and Casey, 2005). In nature, corals are exposed to seasonal variation and they adapt and acclimate to it (e.g., Brown et al., 1999; Fagoonee et al., 1999; Fitt et al., 2000; Warner et al., 2002; Winters et al., 2006; Gupta et al., 2007).

2.1. DURABILITY OF CORALS UNDER STRESS

The following mechanisms contribute to durability of corals under stress:

1. Acclimation and adaptation of algal symbiosis by increasing their thermal tolerance by 1.0–1.5°C.
2. Some corals acquire a new and more tolerant assemblage of symbionts from the environment; these symbionts increase the survival of the coral (e.g., Buddemeier and Fautin, 1993; Baker, 2001, 2003; Baker et al., 2004; Rowan, 2004; Baker and Romanski, 2007; Jones et al., 2008; Maynard et al., 2008), and some do not (Goulet, 2006, 2007). But these clades may be less productive (see Lesser, 2007).
3. Corals change their nutrition and reach carbon energy from phytoplankton, mainly zooplankton. While bleached and recovering, *Montipora capitata* corals met more than 100% of their daily metabolic energy requirements by markedly increasing their feeding rates and CHAR (percent of contribution of heterotrophically acquired carbon to daily animal respiration). *Porites compressa* and *Porites lobata* corals could not (Grottoli et al., 2006).

Different stresses both directly and indirectly affect the coral community reef taxa. The results include chronic effects on coral individuals, catastrophic mortality leading to extinction of entire reefs, and changes in pathogens (diseases), predators, and competitions (Lasker and Coffroth, 1999; Lesser et al., 2007). The degree, geographic extent, and duration of the Indo-Pacific coral decline have been significantly underestimated. Analysis of 6,001 quantitative reef surveys indicates that the Indo-Pacific coral significantly declined between 1968 and 2004 (Bruno and Selig, 2007).

It is clear that under major stress, long duration of stress, combinations of different kinds of stress, and/or fast environmental changes such as the rate of CO_2 in the atmosphere, the genotypes and phenotypes of corals do not appear to have the capacity to adapt fast enough (Hoegh-Guldberg et al., 2007).

3. Temperature Effects

3.1. HIGH TEMPERATURE

High temperature causes bleaching. Zooxanthellae are lost from corals by exocytosis, apoptosis, programmed cell death, necrosis, or host detachment. Time duration causes bleaching change: short-term exposure for 1–2 days at temperature elevations of 3–4°C above normal summer ambience and/or long-term exposure, i.e., several weeks at elevations of 1–2°C cause bleaching and loss of symbiotic zooxanthellae (Jokiel and Coles, 1990; Fig. 3). Two defense mechanisms exist against high temperature: (1) changing the amount of heat-shock proteins (HSPs) and (2) oxidative enzymes, including copper/zinc superoxide dismutase (SOD), manganese SOD, iron SOD, ascorbate peroxidase, and catalase. These prevent subsequent cellular damage from active species of oxygen (Gates et al., 1992; Fang et al., 1998; review by Coles and Brown, 2003; Fig. 3). For example, the exposure of *Montastraea faveolata* to temperatures of up to 35°C for 2 h, followed by tolerance-mechanism-induced synthesis of HSPs (Black et al., 1995). In *Agaricia tenuifolia* from the Caribbean, high temperature causes the production of reduced oxygen intermediates, or toxic oxygen, in the dinoflagellate symbionts and host tissues. These subsequently caused cellular damage and expulsion of symbionts. A decrease in photosynthesis was followed by bleaching (Lesser, 1997).

Photosynthesis of cultures of *Symbiodinium microadriaicum* from jellyfish *Cassiopeia xamachana* is impaired at temperatures above 30°C and completely cease at about 34°C. Algae themselves are adversely affected by elevated temperatures (Iglesias-Prieto et al., 1992; Fig. 3). Growing at 32°C for 2 months, the thylakoid membrane lipid composition of symbiotic algae is damaged. The thermally damaged membranes are energetically uncoupled but remain capable of splitting water. The fraction of the photosynthetically produced oxygen is reduced by photosystem I (PSI) through the Mehler reaction to form ROS. Elevated temperatures cause uncoupling of photosynthetic energy transduction. The accompanying proton leak and loss of ATP restrict photosynthetic carbon assimilation; however, O_2 generated by photosystem II (PSII) can react with the photochemically generated electrons in PSI to form ROS, which in turn oxidizes membrane lipids. The oxidized lipids initiate positive feedback of ROS production accelerated by high light. Ultimately, the ROS kill the intracellular algal symbionts and damage the host cells. The symbiotic algae are literally bleached and/or expelled from their hosts (Tchernov et al., 2004; Fig. 3). It should be noticed that zooxanthellae expelled from the corals *Cyphastrea serailia* and *Pocillopora damicornis* at 33°C have healthy effective quantum yields after 8 h under bleaching conditions. Zooxanthellae are unaffected in their photosynthesis and could be heated even to 37°C before photosynthetic destruction. Temperature-induced expulsion of zooxanthellae involves a dysfunction in the interaction of the zooxanthellae and the coral host tissue, and not a dysfunction in the zooxanthellae per se (Ralph et al., 2001, 2005). PSII function is represented by the ratio of variable fluorescent, F_v ($F_v = F_m - F_0$, where F_0 and F_m are the minimum and maximum yields of chlorophyll fluorescence, respectively) to maximum yields of chlorophyll fluorescence,

F_m, F_v/F_m. PSII functioning, the overall photosynthesis, and the rate of electron transport through PSII (ETR) of zooxanthellae released from polyps of *Galaxea fascicularis* at elevated temperature (30°C and 32°C) are intact when compared with those retained within the polyps. Polyps exposed to elevated temperature release healthy-looking zooxanthellae. Zooxanthellae are released by the coral non-selectively with respect to their PSII functioning. The host is primarily affected by acute temperature stress, and host-cell necrosis or detachment may lead to nonselective release of healthy zooxanthellae (Bhagooli and Hidaka, 2004b). Corals exposed to temperatures of 32–34°C show a decrease in the quantum yield of photosynthesis (dark-adapted F_v/F_m) and loss of functional D1 photosynthetic reaction center protein (Warner et al., 1999).

High temperature causes photoinhibition of photosynthetic electron transport, consequent photodamage to PSII, and the production of damaging ROS in the zooxanthellae (*Symbiodinium* spp.). These might lead to perturbations of the metabolic processes in the zooxanthellae and/or their host cells, which could trigger events leading to bleaching. Production of ROS by the thylakoid photosynthetic apparatus in the zooxanthellae plays a major role in the onset of bleaching resulting from photoinhibition of photosynthesis. Hydrogen peroxide generated in the zooxanthellae may play a signaling role in triggering the mechanisms that result in the expulsion of zooxanthellae from corals (Smith et al., 2005). The host *Stylophora pistillata* protects its zooxanthellae *in hospite* and increases oxidative stress tolerance by raising SOD activity (Richier et al., 2005).

A response to high temperature is the induction of antioxidant defenses exclusively localized within the gastrodermal cells. This induction is likely to counteract the increase in cellular damage. There is a slight increase in the activity of the antioxidant enzymes SOD and ascorbate peroxidase in zooxanthellae. There is a decrease in zooxanthella antioxidant defenses during thermal stress: (1) dysfunction of zooxanthella metabolism induced by necrosis or programmed cell death; and (2) decrease in antioxidant defenses following chlorophyll decrease. In the first hour of stress, the gastrodermal cells undergo oxidative stress, which is rapidly followed by apoptotic events and, finally, by the occurrence of bleaching. Gastrodermal cell death is then hypothesized as being responsible for zooxanthella expulsion and/or gastrodermal cell detachment (See in Shick et al., 1995; Lesser, 1996; Dunn et al., 2002; Richier et al., 2005, 2006). In the *Anemonia viridis* (sea anemone), the gastrodermal cells undergo oxidative stress acting at the molecular level. This is rapidly followed by apoptotic events and, finally, by the occurrence of bleaching. Oxidative stress is followed by induction of caspase-like activity in animal host cells. Gastrodermal cell death is then hypothesized as being responsible for zooxanthella expulsion and/or gastrodermal cell detachment (Richier et al., 2006). The exposure of *Aiptasia pallida* (sea anemone) to elevated temperatures induces symbiotic anemones to produce high levels of nitric oxide (NO). The increase in NO leads to the collapse of symbiosis (Perez and Weis, 2006; Weis 2008; Fig. 4).

There are positive correlations between the accumulation of oxidative damaged products and bleaching in corals from Florida Keys during one season. High levels of antioxidant enzymes and small HSPs are negatively correlated with levels of oxidative-damaged products. Corals that experience oxidative stress have higher chaperonin levels and protein turnover activity (Downs et al., 2002).

The bleaching threshold temperature of Hawaiian corals is ~2°C less than that of congeners from the tropical Pacific (see Jokiel and Brown, 2004). Some coral species are more resistant than others to environmental factors that cause bleaching and bleaching-related mortality. Species with low mortality rates generally have higher densities of zooxanthellae per square centimeter and a low rate of release of degraded zooxanthellae. Low-mortality species also have more total coral tissue per coral surface area (Stimson et al., 2002). The coral host species plays a significant role in recovery and resilience (Grottoli et al., 2006).

Blue, cream, and green color morphs of *Acropora asper* have different green fluorescent protein (GFP) homologues. These host pigments are photoprotective at normal temperatures less than 32°C. However, loss of symbionts and reduction in the quantum yield of photosynthesis (dark-adapted F_v/F_m), which are observed after exposure to elevated temperature above 32°C, are most severe in the blue morph. Loss of animal-soluble protein results in coral mortality (Dove, 2004).

Temperature affects the growth and reproduction of corals. Decline in mean-annual calcification rate of the *Montastraea* species is observed during periods of elevated SST. There is a doublet in the high-density bands (dHDB) in the coral skeletons (Wórum et al., 2007). Larvae of the Caribbean coral *Porites astreoides* are the largest and have the highest population densities of *Symbiodinium* sp. between 26.4°C and 27.7°C, and are smallest and have the lowest population densities between 25.8°C–28.8°C. Larval size and symbiont population density are elevated slightly at the temperature extremes of 25.1°C and 30°C (Edmunds et al., 2005).

Zooxanthella clades may change under high temperature. *Pocillopora* spp. grow at warm habitats more than 31.5°C host only *Symbiodinium* D, while in cooler habitats, they host *Symbiodinium* C. Symbiosis recombination may be a mechanism by which corals adapt to global warming (see Rowan, 2004). As a result of change in the symbiont type dominating their tissues from *Symbiodinium* type C to D, shuffling existing types already present in coral tissues and/or the environment, corals are capable of acquiring increased thermal tolerance (e.g., Baker, 2001, 2003 and Berkelmans and van Oppen, 2006). Temporal and spatial variability can support the coexistence of diverse symbionts within a host, despite the potential for destabilizing competition among them. Corals will replace the symbionts as a response to stress only in some cases (Buddemeier and Fautin, 1993; Rowan et al., 1997; Baker, 2001, 2003; Baker et al., 2004; Goulet, 2006, 2007; Baker and Romanski, 2007). Bleaching of corals results in significant coral reef degradation.

3.2. HIGH TEMPERATURE AND LIGHT ACCLIMATION

Different morphs of *Montipora monasteriata* respond differently to 32°C and 650 μmol quanta m^{-2} s^{-1}. There is a reduction in peridinin, xanthophyll pool, chlorophyll c_2, and chlorophyll *a*, with no change in zooxanthella densities. Chlorophyll allomerization probably results from the interaction of chlorophyll with singlet oxygen (O_2) or other ROS. At early stages of thermal stress, thylakoid membranes are intact. Heavily pigmented coral hosts taken from a high-light environment show reductions in GFPs such as homologues, whereas non-host pigmented high-light morphs experience a significant reduction in water-soluble protein content. Based on chlorophyll fluorescence data, shade-acclimated cave morphs are less thermally stressed than high-light morphs (Dove et al., 2006).

3.3. LOW TEMPERATURE

Under low temperature (12°C), there is a decrease in photosynthetic efficiency (F_v/F_m), loss of symbiotic dinoflagellates, and changes in photosynthetic pigment concentrations of *Montipora digitata*. Corals growing under high-light regimes are more susceptible to cold-temperature stress. Moderate-cold stress results in photoacclimatory responses, but severe cold stress results in photodamage, bleaching, and increased mortality (Saxby et al., 2003).

3.4. HIGH AND LOW TEMPERATURE

Subtropical non-reef coral communities of *Oulastrea crispata* grow at high and low temperatures, from 12°C in winter to 35°C in summer. The nature of hosting a stress-tolerant symbiont, *Symbiodinium* clade D, may play a key role in the ability of *Oulastrea crispata* to achieve such physiological adaptability (Chen et al., 2003). *Pocillopora damicornis* releases intact host endoderm cells containing zooxanthellae at 32°C (16 h) and 12°C (4 h). Most of the released host cells are viable, but they soon disintegrate in the seawater, leaving behind isolated zooxanthellae. The detachment and release of intact host cells suggest that thermal stress causes host cell adhesion dysfunction. The adhesion is due to denaturation of protein or membrane thermotropism (Gates et al., 1992).

4. Light Intensity Effects

4.1. HIGH AND LOW LIGHT

The protective mechanism against high light includes (1) dissipation of radiation as heat via the xanthophyll cycle in a process termed nonphotochemical quenching (NPQ). Heat dissipation is achieved by the reversible interconversion of the

xanthophylls, diadinoxanthin, and diatoxanthin; (2) fluorescent coral pigments; and (3) oxidative enzymes (review by Coles and Brown, 2003; Levy et al., 2006).

Photoinhibition of photosynthesis is caused when coral is exposed to high light, >1,500 μmol q m^{-2} s^{-1}, for several hours during low tide when the coral is exposed to direct sunlight. In addition, ROS trigger death and expulsion of the endosymbiotic algae (Anthony et al., 2007). When *Turbinaria mesenterina* is exposed to ten times higher light than the acclimation irradiance, the daily costs of photoinhibition are negligible. In the long term, photoacclimation reduces daily energy acquisition due to decreased chlorophyll concentrations. Changes in the photosynthetic activity of symbiotic dinoflagellates over a diurnal irradiance cycle do not cause a measurable decline in net oxygen evolution for coral colonies. Repeated exposure to excessive irradiance can reduce energy acquisition per unit surface area, and, hence, influence the upper limit of depth distribution of scleractinian corals (Hoogenboom et al., 2006).

Corals that fall to deep water or are covered by sediments or kept in the dark die (personal communication). In cave corals, no zooxanthellae exist on the side of the coral facing the cave, however, the colony survives (Dubinsky and Jokiel, 1994).

4.2. LIGHT AND TEMPERATURE

Effects of light are less dramatic under optimal temperature (Coles and Jokiel, 1978). However, the following responses to light depend on temperature increase, duration, coral species, and zooxanthellae: (1) physiological bleaching and changes on the steady state of zooxanthella density; (2) algal stress bleaching that can be either chronic photoinhibition, i.e., releasing the zooxanthellae by exocytosis, or dynamic photoinhibition – reversible photostress by the algae using the xanthophyll cycle; and 3) animal stress – susceptible to thermal stress more than the algae (Fitt et al., 2001). Elevated temperature and irradiance differentially affect different coral species and algal clades. *Monastraea franksi* clade B, *Favia fragum* clade C, *Agaricia* sp. clade C, *Porites astreoides* clade A, *Porites porites* clade A, *Monastraea franksi*, and *Favia fragum* are bleached by loss of 50–80% of their algal cells, with no significant impact to chlorophyll or peridinin in retained algal cells. *Agaricia* sp. shows no reduction in algal cells at elevated temperature and irradiance, but loses substantial amounts of chlorophyll *a* and carotenoid pigments, presumably through photo-oxidative processes. Two coral species (*Porites astreoides* and *Porites porites*) are not bleached. The levels of the photoprotective xanthophylls (dinoxanthin and diatoxanthin, Dn + Dt) and β-carotene vary among the corals in both pool size and xanthophyll cycling, and are not correlated to coral-bleaching resistance (Venn et al., 2006).

High light intensity at both upper and lower sublethal temperatures leads to loss of zooxanthellar pigment, higher mortality rates, reduced carbon fixation, and lowered growth rate (Coles and Jokiel, 1978). In *Agaricia agaricites* and *Porites porites* growing at 30°C, 32°C, or 34°C and 850, 1,250, and 2,000 μmol q $m^{-2}s^{-1}$,

thermal bleaching is related to the breakdown of the Ca^{2+} exclusion system. Irradiance bleaching, which takes place at lower temperatures and is driven by light, is the result of a buildup of photosynthetically produced hydrogen peroxide in the tissues. Under high light and temperature, pieces of gastrodermis round off, zooxanthellae move to the surface, protrude from the surface and, after a delay, detach, surrounded by a thin layer of host cytoplasm, inclusions, and plasma membrane. Higher temperature and light level shorten the delay and increase the rate of algal detachment. Fragmentation by the ballooning-out and detachment of small spheres of cytoplasm take place at the same time. This is probably due to oxidation by hydrogen peroxide (H_2O_2) of –SH groups on the cytoskeleton and its attachment to the plasma membrane. Isolated zooxanthellae and whole corals are shown to release H_2O_2 in the light, a mechanism limiting algal populations in the gastrodermis. At above-normal temperatures, under the synergistic effect of light and temperature, the rate of production of H_2O_2 exceeds the rate at which it can be lost by diffusion or destroyed, and H_2O_2 accumulates. This results in damage to the calcium exclusion system, detachment of zooxanthellae into the coelenteron, and fragmentation of the gastrodermis (Sandeman, 2007).

Light intensity for photoinhibition varies for different coral species. Chronic photoinhibition is recorded under 520 and 1,015 µmol quanta m^{-2} s^{-1} at 34.8°C in *Platygyra ryukyuensis*, under 1,015 µmol quanta m^{-2} s^{-1} at 32.8°C, and under all light levels at 34.8°C in *Stylophora pistillata*. High temperature reduces the threshold. Chronic photoinhibition of *Stylophora pistillata* causes paling and high mortality, while species of *Platygyra ryukyuensis* look healthy (Bhagooli and Hidaka, 2004a).

Colonies of *Acropora*, *Porites*, *Faviidae*, *Mussidae*, *and Pocilloporidae*, which are exposed at low tides to high solar radiation, are bleached or suffer partial mortality (Anthony and Kerswell, 2007).

4.3. UV RADIATION (UVR), 290–400 NM

UVR causes coral bleaching independently and is an important synergistic factor in bleaching caused by thermal stress. Corals produce UVR-absorbing compounds such as mycosporine-like amino acids (MAAs) in order to avoid some of the damage. UVR-induced DNA damage: (1) direct effects on DNA photoproducts; (2) indirect effects, increased production of ROS, causing oxidative stress and damage to lipids, proteins, and also DNA (Shick et al., 1995, 1996; Lesser and Farrell, 2004; Torregiani and Lesser, 2007; Figs. 3 and 4).

Short-term (3 days) exposure of *Montipora verrucosa* to UV radiation leads to decreases in quantum yields of PSII fluorescence. DNA photoproducts are produced. DNA damage is observed more at the shallow depths. Corals show increases in MAA concentrations. The coral levels of tolerance depend on previous light history. Originally, deep (10 m) corals showed the highest MAA concentration and lowest DNA damage in response to exposure to UVR (Torregiani and Lesser, 2007).

4.4. UV RADIATION AND TEMPERATURE

Short-term temperature (5 h) that increases to 34°C significantly decreases the F_v/F_m in *Stylophora pistillata* (clade A zooxanthellae) and *Montipora aequituberculata* (clade A). Increased UV radiation alone significantly decreases the F_v/F_m in the *Stylophora pistillata* (clade A zooxanthellae), *Montipora aequituberculata* (clade A), *Acropora* sp. (clade C), and *Pavona cactus* (clade C). UV radiation and temperature of 27°C reduce F_v/F_m in all corals by 25–40%. During long-term (5 h every day for 17 days) exposure to UV radiation, the F_v/F_m is significantly reduced after 3 days. There is no recovery to the initial values after 17 days. The corals synthesize mycosporine-like amino acids (MAAs). More are synthesized by corals containing clade A. Prolonged exposure to UV radiation at the nonstressful temperature of 27°C confers protection against independent, thermally induced photoinhibition in the four species (Ferrier-Pages et al., 2007).

Zooxanthellae probably harbor latent viruses. Upon exposure to stress and elevated temperatures and/or UV, zooxanthellae *Symbiodinium* sp. strain CCMP 2465 and corals produce virus-like particles (VLPs). There is a latent infection caused by zooxanthella filamentous virus 1 (ZFV1) (Lohr et al., 2007).

5. Salinity and Metals, Herbicides, Cyanide, Oil, and Nutrients

5.1. SALINITY AND METALS

Salinity narrows the range of tolerable temperature and also reduces the ability of the coral to survive (Coles and Jokiel, 1978). In Hawaii, storm floods, which cause reduction in salinity to 15‰, lead to the death of coral reefs (Jokiel et al., 1993).

Zooxanthellae play an important role in trace-metal regulation in corals. They are continuously renewed and lost in healthy corals and, in this way, reduce the metal content in the coral. Stress from exposure to high-metal concentrations causes zooxanthella loss in corals (Reichelt-Brushett and McOrist, 2003; Nystrom et al., 1997). In *Plesiastrea versipora*, which is exposed to increasing amounts of copper ($CuSO_4$) for periods of 12 (0–5.04 μm) to 36 days (two cycles of 0–7.55 μm), algae are not expelled and there is no decrease in photosynthesis or chlorophyll *a*. Photosynthesis inhibiting factor (PIF) activity, which partially inhibits photosynthetic carbon fixation, is slightly higher after exposure, but host release factor (HRF) activity is always similar to seawater control corals. Oxidative damage is evident in coral tissues (Grant et al., 2003). Copper (0–200 μg l^{-1}) has an inhibitory effect on fertilization success in spawning corals *Acropora surculosa*. The duration of exposure has a negative impact on the number of potential larvae that may be produced from the fertilization process (Victor and Richmond, 2005). Respiration rate of *Porites lutea* from the Gulf of Thailand remains the same when the corals are exposed to 0.33, 14.16, and 30 mg l^{-1} copper ($CuSO_4$) for 14 h; however, the primary production rate is reduced (Nystrom et al., 2001).

5.2. HERBICIDES

Herbicides cause major damage to corals. Colonies of *Porites cylindrica* are exposed to 10 and 100 μg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and diuron and to 10, 50, and 100 μg l^{-1} diuron [3-(30, 40 dichlorophenyl) -1,1-dimethylurea] for 48 h. Coral gross primary production rate, gross primary production to respiration ratio, and effective quantum yield are significantly reduced when exposed to 100 μg l^{-1} 2,4-D and 50 and 100 μg l^{-1} diuron, while respiration seems unaffected (Raberg et al., 2003). *Madracis mirabilis* that is exposed for a short time (8 and 24 h) to acute irgarol 1051 (photosystem II inhibitor), shows significant increases in total dinoflagellate multixenobiotic resistance (MXR), dinoflagellate Cu/Zn SOD (produced as response to oxidative damage), dinoflagellate chloroplast HSPs, and cnidarian protoporphyrinogen oxidase (PPO). *Madracis mirabilis* also responds to irgarol 1051 by decreases in cnidarian glutathione peroxidase, GPx, cnidarian ferrochelatase, cnidarian catalase, cnidarian cytochrome, and CYP 450-3 and -6 classes (Downs and Downs, 2007).

5.3. CYANIDE

In *Stylophora pistillata*, exposure to cyanide results in termination of photosynthetic electron transport rate. The observed tissue bleaching is caused by loss of zooxanthellae from the coral tissues, representing a sublethal stress response of the coral. *Stylophora pistillata* and *Acropora aspera* display marked decreases in dark-adapted F_v/F_m, the ratio of variable fluorescence (F_v) to maximal fluorescence (F_m; Jones et al., 1999).

5.4. CLOVE OIL

Clove oil is an anesthetic that is widely used to catch demersal fish. Low concentrations (0.5 ppt, 1–60 min) do not affect *Pocillopora damicornis* coral color or its photosynthetic efficiency. Colonies exposed to high concentrations (50 ppt) of clove oil solution die immediately, including those colonies that are exposed briefly (1 min). Intermediate concentrations (5 ppt) of clove oil solution produce variable results: a 1 min exposure has no effect, a 10 min exposure causes bleaching and reduces photosynthetic efficiency, and a 60 min exposure causes total mortality (Frisch et al., 2007).

5.5. OIL SPILL

Oil pollution cause decrease in colony viability, damage to the reproductive system of corals and complete lack of colonization by hermatypic corals in reef areas chronically polluted by oil (Loya and Rinkevich, 1980).

5.6. EUTROPHICATION

In response to eutrophication, zooxanthella density in *Pocillopora damicornis* and *Stylophora pistillata* increases, chlorophyll per area increases, photosynthetic efficiencies are reduced, and coral growth rate decreases (Dubinsky et al., 1990; Stambler et al., 1991; Dubinsky and Stambler (1996); Ferrier-Pages et al., 2001). In *Acropora pulchra*, nutrient enrichment causes unbalanced growth between organic tissue and the carbonate skeleton (Tanaka et al., 2007). High nitrate concentrations enhance zooxanthella volume and chlorophyll contents per *Pocillopora damicornis* and *Porites lobata* cells. Variations between species: 30% of *Pocillopora damicornis* colonies remain healthy in contrast to 90% of *Porites lobata*. The branching *Pocillopora damicornis* is significantly affected by the addition of nitrate, whereas *Porites lobata* is significantly influenced by water temperature (Schloder and D'Croz, 2004).

Terrestrial runoff causes an increase in dissolved inorganic nutrients, enrichment with particulate organic matter, light reduction from turbidity, and increased sedimentation, which have direct effects on the growth and survival of hard-coral colonies, coral reproduction, and recruitment. Secondary effects on organisms that interact with coral populations: crustose coralline algae, bioeroders, macroalgae and heterotrophic filter feeders, as well as on coral diseases, pathogens, and coral predators (review by Fabricius, 2005).

6. Water Motion and Breakage

Bleaching is prevented under moderate-to-high water-flow rates when corals are subjected to high SSTs (up to 33°C) and high irradiance (Nakamura and Van Woesik, 2001). Corals at wave-exposed sites are largely unaffected and <1% of the corals are bleached by spring low tide, while in wave-protected sites, 40% and 75% of the colonies are damaged – they are either bleached or suffer partial mortality (Anthony and Kerswell, 2007).

Skeletal breakage occurs on branching corals due to the mechanical destruction of corals by divers, anchors, and hurricanes. Although some of the fragments might survive, they and the colony might be more sensitive to bacterial infection and predators (Zakai and Chadwick-Furman, 2002). Massive, sub-massive, and encrusting corals are more resistant and resilient to the direct impact of tsunamis than branching, tabulate, and foliose life forms, whereas the latter are more tolerant of temporary coverage by sand. Sub-massive corals are the most tolerant overall and survive sand coverage, breakage, and overturning. Live coral cover recovers in 3 months (Worachananant et al., 2007).

The ecology dogma is that sex reproduction occurs under stable conditions, while asexual reproduction occurs under stress. However, in *Montastraea annularis*, 70% of multicolony genets observed are formed by asexual reproduction due to physical breakage, consistent with storm damage and greatest wave exposure (Foster et al., 2007).

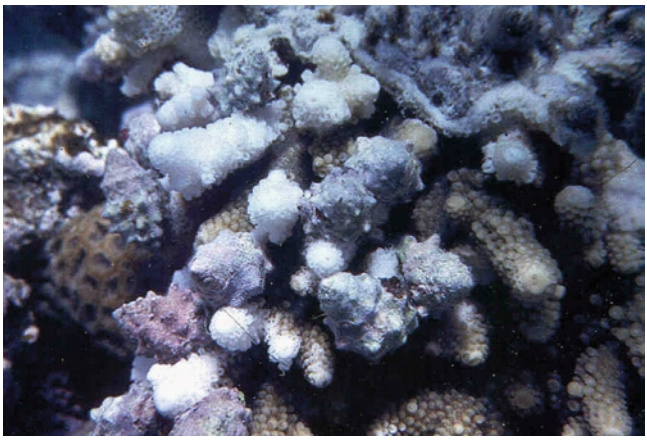

Figure 5. *Acropora* in the Red Sea being devoured by several corallivorous snails of the genus *Drupella* sp. Note the bare coral skeleton remaining after the snail's attack. (Photograph by Zvy Dubinsky.)

7. Grazers and Predators

Montastraea spp. corals that are previously grazed by the parrotfish show a reduction in symbiont density compared to intact colonies. Grazed corals exhibit greater diversity in the genetic composition of their symbiont communities, changing from uniform ITS2-type C7 *Symbiodinium* prior to bleaching to mixed assemblages of *Symbiodinium* types post-bleaching. Parrotfish grazing may be considered chronic stress and can alter coral response to other stressors (Randi et al., 2006). High densities of the sea urchin *Acanthaster* cause mortality of corals, reduction of corals, and death of entire reefs (Glynn, 1973; McClanahan, 1990). *Drupella cornus* (gastropod), which regularly occurs on *Acropora* colonies, can cause motility of the whole coral. *Drupella* numbers have increased considerably during the last 2 decades in the northern Red Sea, probably due to the heavy impact of anthropogenic factors (e.g., Zuschin and Stachowitsch, 2007; Fig. 5).

8. Competition with Macroalgae, Cyanobacteria, and Sponge

Competition with sponge: 18 h exposure of corals to secondary metabolites of some sponge species causes a decrease in the photosynthetic potential of the symbiotic algae and bleaching of the coral tissue (Pawlik et al., 2007). The macroalgae *Lyngbya* spp., *Dictyota* spp., and *Lobophora variegata* damage *Porites astreoides* larvae and cause inhibition of coral recruitment (Kuffner et al., 2006). The red alga *Corallophila huysmansii* settles on, overgrows, and kills live coral tissue, perhaps due to allelochemical production (Jompa and McCook, 2003). The macroalgae *Chlorodesmis fastigiata* cause polyp retraction, but have little other noticeable effect on coral tissue (Jompa and McCook, 2003). The coral *Montastraea faveolata* is sensitive to mixed

turf algae and bacteria. The algae directly stress the coral as a superior competitor. Zooxanthellae density, chlorophyll *a* per square centimeter, and tissue thickness are reduced (Quan-Young and Espinoza-Avalos, 2006). The macroalgae *Lobophora variegate*, *Dictyota pulchella*, and *Porites cylindrica* around a coral colony reduce the growth rate of juvenile corals, decreasing their number to 60% that of the control corals, without causing mortality. Shading by *Dictyota pulchella* results in 99% growth inhibition (Box and Mumby, 2007). It should be mentioned that acidification has a profound impact on the development and growth of crustose coralline algae, which affect the corals (Jokiel et al., 2008).

The algae can indirectly cause coral mortality by enhancing microbial activity via the release of dissolved compounds (Smith et al., 2006). Competition causes increased post-settlement mortality in coral due to reduced light, flow, or growth rate. Under other stresses that cause mass coral mortality events, greater competitive effectiveness of macroalgae relative to corals leads to algal colonization (Hoegh-Guldberg et al., 2007).

9. Sedimentation

Sedimentation is a major cause of mortality in scleractinian coral recruits. Sediment composition and deposition affect survival of coral juveniles. Coral *Acropora willisae* recruits survive short-term (43 h) exposure to low levels of transparent exopolymer particles (TEP) and low levels of muddy sediments, but sediments enriched with TEP at concentrations recorded at some of the inshore stations prove to be detrimental (e.g., Fabricius et al., 2003). Different sediments exert greatly contrasting levels of stress on the corals. In *Montipora peltiformis*, it yields changes in the photosynthetic yield. Photophysiological stress is measurable after 36 h of exposure to most of the silt-sized sediments, and coral recovery is incomplete after 48–96 h recovery time (Weber et al., 2006).

In control colonies of *Montipora peltiformis*, maximal quantum yields of PSII F_v/F_m range from 0.67 to 0.71, while maximum yields of sediment-covered fragments (79–234 mg cm^{-2}) are below 0.1 in most colonies after 36 h. Maximal quantum yield declines linearly in relation to both the amount of sediment deposited per unit surface area and exposure duration. Zooxanthella densities and chlorophyll concentrations per unit area of sediment-treated corals decrease in the same manner. Sedimentation stress of colonies exposed to large amounts of sediment for short periods of time is similar to that exposed to low amounts of sediment for prolonged periods of time. Colonies are recovered from short-term or low-level sedimentation within <36 h, whereas long-term exposure or high levels of sedimentation kill exposed colony parts (Philipp and Fabricius, 2003). Red soil affects gene expression in *Pocillopora damicornis*. Nine candidate PCR fragments are derived from the differentially expressed genes. One of the clones, pPd9-1, shows a high similarity to a member of the HSP. Red soil may cause protein denaturation in the coral. The expression of pPd9-1 is also increased by elevated temperature, but not by reduced salinity (Hashimoto et al., 2004).

9.1. TEMPERATURE, LIGHT, AND SEDIMENT LEVELS

High temperature (30°C) increases the mortality risk of *Acropora intermedia* (Great Barrier Reef) at all light (up to 372 μmol q m^{-2} s^{-1}) and sediment levels (up to 10 mg L^{-1}). High sediment potentially reduces mortality under high temperature and/or high light by alleviating light pressure and by providing an alternative food source for bleached corals (Anthony et al., 2007).

10. Increased CO_2

Increased CO_2 lowers the aragonite saturation state of seawater, thus lowering carbonate saturation state, and carbonate ions are less available for calcification. As a result, there is low coral cover at the reefs. Under high CO_2, coral reef habitats might make the transition from being dominated by corals and coralline algae to being dominated by seaweeds and fleshy macroalgae (Hoegh-Guldberg, 2005; Andersson et al., 2005). "The rate of atmospheric CO_2 concentration change is critical given that modern genotypes and phenotypes of corals did not appear to have the capacity to adapt fast enough to sudden environmental change" (Hoegh-Guldberg et al., 2007).

10.1. TEMPERATURE AND pCO_2

Stylophora pistillata were grown at temperatures of 25°C or 28°C, with pCO_2 values of ca. 460 or 760 μatm for 5 weeks. The chlorophyll c_2 and protein content remained constant throughout the experiment, while the chlorophyll *a* content was significantly affected by temperature and was higher under high-temperature–high-pCO_2 conditions. Cell-specific density was higher at high pCO_2 than at normal pCO_2 (1.7 versus 1.4). The net photosynthesis normalized per unit protein was affected by both temperature and pCO_2, whereas respiration was not. Calcification decreased by 50% when temperature and pCO_2 were both elevated.

Calcification under normal temperature does not change in response to increased pCO_2 (Reynaud et al., 2003). Increased CO_2 causes declining *Porites* from the Great Barrier Reef calcification: the coral shows reductions in linear extension rate of 1.02% $year^{-1}$ and in skeletal density of 0.36% $year^{-1}$ during the past 16 years (Cooper et al., 2008).

11. pH

Mediterranean coral species *Oculina patagonica* and *Madracis pharensis* are grown in low pH 7.3–7.6 (compared to the ambient 8.0–8.3) and after 1 month, morphological changes are noted: initially polyp elongation, dissociation of colony form,

and complete, skeletal dissolution. After 12 months: biomass of the solitary polyps under acidic conditions is thrice as high as that of the control polyps. All pH fragments maintain their algal symbionts, and gametogenesis develops similarly during the spring and summer months. All skeleton-free coral fragments survive. When transferred back to ambient pH conditions, soft-bodied polyps calcify and reform the colonies (Fine and Tchernov, 2007). Coral reef calcification shows decline in calcification as pH declines (Kleypas et al., 1999). Coral calcification decreases between 15% and 20% under acidified conditions. However, under acidified conditions, larvae of the coral *Pocillopora damicornis* are able to recruit and *Montipora capitata* produce gametes (Jokiel et al., 2008). Acidification affects coral calcification, probably by decreasing the availability of the CO_3^{2-} substrate for calcification. However, the decrease in coral calcification can be attributed either to a decrease in extra- or intracellular pH or to a change in the buffering capacity of the medium, impairing supply of CO_3^{2-} from HCO_3^- (Marubini et al., 2008).

12. Disease, Bacteria, Viruses

Eukaryotic algae, cyanobacteria, bacteria, viruses, and archaea grow in the mucus layer of skeletons and in the tissues of healthy corals. Under stress, some of these microorganisms cause coral bleaching and other diseases. Corals can adapt to higher environmental temperatures and develop resistance to specific pathogens (review by Rosenberg et al., 2007). On high (50%) cover reefs of the Pacific, there is a significant relationship between the frequencies of warm-temperature anomalies and of white syndrome, an emerging disease. Host density plays an important role as a threshold for white syndrome outbreaks. Corals might survive and recover their symbionts after mild thermal stress, but they can exhibit reduced growth, calcification, and fecundity, as well as experience greater incidences of coral disease (Bruno et al., 2007; Harvell et al., 2002). Three coral pathogens tend to increase in warmer seas. Heat-induced viruses can also be involved in temperature-induced coral bleaching (Kushmaro et al., 1998; Wilson et al., 2001; Harvell et al., 2002). *Pavona danai*, *Acropora formosa*, *Stylophora pistillata*, and *Zoanthids–Zoanthus* sp. produce numerous virus-like particles (VLPs) that are evident in the animal tissue, zooxanthellae, fresh isolated zooxanthellae (FIZ), and the surrounding seawater (Davy et al., 2006). Bacteria from the environment, the host, or the coral mucus layer have become opportunistic pathogens when the coral is exposed to physiological stress such as elevated temperature, which results in reduced host resistance (Lesser et al., 2007).

Temperature stress increases one component of sea-fan resistance, of *Gorgonia ventalina* (sea-fan coral) against *Aspergillus*. Temperature causes increase in activity of host-derived antifungal compounds. Temperature stress and infection induce higher levels of resistance. However, pathogen growth rate also increases over the same temperature range, providing an opportunity for pathogen establishment before host resistance becomes maximal (Ward et al., 2007).

13. Stress Recovery

Recovery is the acquisition of symbionts of either similar genotypes of the original population and/or the acquisition of different symbionts from the external environment. Changes in the relative abundances of different algal types, after coral bleaching, are a possible mechanism to select for thermally tolerant genotypes and genotypes capable of surviving and propagating under the extreme high-light environment of a non-pigmented coral. Even though the biomass increases in endolithic algae after bleaching due to less competition with the zooxanthellae, it may provide partial protection to the surviving symbionts from excessive radiation by reducing the reflectivity of the skeleton during the early stages of the recovery process. The zooxanthella symbiont population exhibits photosynthetic responses and characteristics consistent with acclimation to higher irradiance relative to fully recovered corals (Buddemeier and Fautin, 1993; Baker et al., 2004; Rodriguez-Roman et al., 2006; Goulet, 2006, 2007; Baker and Romanski, 2007). Some symbiont communities change in reef-building corals, suggesting a population-wide acclimatization to increased water temperatures, creating new, more thermally tolerant, holobionts (Jones et al., 2008; Maynard et al., 2008).

Growth rates of juvenile corals of reef flats at the surface-to-30-m depth from multiple locations in the Caribbean and Indo-Pacific, show an exponential decrease within a 32-year period (1973–2005), indicating a possible decline in the ability of coral communities to recover from disturbances (Nystrom et al., 2000; Bellwood et al., 2004; Edmunds, 2007). The coral community in Tiao-Shi Reef, southern Taiwan, collapsed as a result of chronic anthropogenic impacts and typhoons. The coral reef community shows dynamic recovery: (1) area which still retains dominance with the branching *Acropora* corals; (2) zone recovery with a significant increase in branching *Montipora stellata*, as it is recruited and grows faster than branching *Acropora* corals; and (3) zone is occupied by anemone, *Condylactis* sp., and demonstrates a stable phase of coral deterioration without recovery (Tkachenko et al., 2007).

14. Reef Stress and Evolution

Collapses and recoveries are postulated to correlate symbiont loss and symbiotic renewal, respectively. There is a survival of aposymbiotic corals. Phanerozoic reefs frequently collapse during mass extinctions, with eclipses lasting 8 to 20 million years. Global warming, cooling cycles, sea-level changes, acid rain, eutrophication, and sunlight reduction, all of which have been proposed as accompanying Mesozoic reef extinctions, appear inimical to zooxanthella coral symbiosis. High nutrient levels interpreted for some Paleozoic reefs do not fit this model but post-Paleozoic nutrient-limited reef settings might (Stanley, 2006). Ocean acidification outcomes of anthropogenic and carbon dioxide increase have the potential to trigger a sixth mass extinction of corals (Veron, 2008).

15. The Future

In the event that the models for the years 2050 to 2100 prove to be correct, then the atmospheric CO_2 level will be 500 ppm, the oceans will be acid, global temperature will increase by more than 2°C compared to today, and, in addition, local stresses will cause a decline in water quality. The results will be less-diverse reef communities and carbonate reef structures that fail to be maintained. Reefs moving toward their maximal stage of survival will collapse, and then become extinct. In other words, corals will become increasingly rare on reef systems (Hoegh-Guldberg et al., 2007).

Direct and indirect effects of pollution from agriculture and land development, including overfishing, have been the major forces of massive and accelerating decreases in the abundance of coral reef species, causing widespread changes in reef ecosystems over the past 2 centuries. Climate change, mainly temperature increase, has so far caused bleaching and disease. Changes in ocean chemistry due to higher atmospheric carbon dioxide may cause weakening of coral skeletons and reduce the accretion of reefs, especially at higher latitudes (Hoegh-Guldberg et al., 2007). Increase in frequency and intensity of hurricanes, tropical cyclones, and typhoons may shorten recovery time between recurrences (Hughes et al., 2003). Coral reefs exist in stable states by ecological feedback controls that are exacerbated by stressor and climate change (Hoegh-Guldberg et al., 2007).

Further stress resulting from global climate change may modify forever the way coral reefs exist (e.g., Done, 1999; Hoegh-Guldberg, 1999, 2005; Gardner et al., 2003; Buddemeier et al., 2004; Sala and Knowlton, 2006; Lesser, 2007; Edmunds, 2007; Hoegh-Guldberg et al., 2007; Day et al., 2008; Carpenter et al., 2008; Veron, 2008).

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Biodata of **Francisco Carrapiço** author of ***"Azolla as a Superorganism: Its Implication in Symbiotic Studies"***

Dr. Francisco Carrapiço was born in Lagos (1951), Portugal, and has a B.Sc. in Biology from the University of Lisbon, a Ph.D. in Cell Biology (1985) from the same university and a post-doc from the Arizona State University, USA. Being an Assistant Professor at the Faculty of Science of the University of Lisbon, and researcher of the Centre for Environmental Biology, his main field of research is Symbiomics, namely the *Azolla*-*Anabaena*-bacteria symbiotic system. Currently, he is part of the research team based at Utrecht University in The Netherlands studying the *Azolla* discovered in the Middle Eocene marine sediments from the Arctic and incorporated in the Azolla Darwin Project (http://www.bio.uu.nl/~palaeo/Azolla/Azolla.htm). He also the position of Secretary of the International Symbiosis Society (ISS) during the last 3 years.

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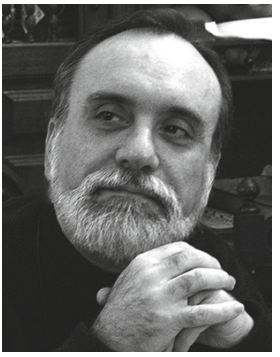

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AZOLLA AS A SUPERORGANISM. ITS IMPLICATION IN SYMBIOTIC STUDIES[1]

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1. Introduction

The symbiosis history begun many million years ago, probably even before the first manifestation of life arose in our planet (Carrapiço et al., 2007). But it was only in the nineteenth century with the presentation in 1867, by the Swiss botanist Simon Schwendener, of the "dual hypothesis" related to the lichens structure, that this "real story" had a scientific starting point for society (Boucher, 1985; Sapp, 1994; Honegger, 2000). Although Albert Bernhard Frank introduced the term "symbiotismus" in 1877, the word "symbiosis" was credited to Anton de Bary who, 1 year later, defined it as "the living together of unlike named organisms" (Frank, 1877; De Bary, 1878; Sapp, 1994; Sapp et al., 2002). This concept was presented in a communication entitled "Ueber Symbiose" (On Symbiosis) during a meeting of the Congress of German Naturalists and Physicians, at Kassel in Germany (De Bary, 1878). One of the biological materials used by this author to explain and characterize the symbiotic phenomenon was the *Azolla-Anabaena* association (De Bary, 1878, 1879).

Although this symbiotic association was previously studied by the German botanist Eduard Strasburger in 1873 (Strasburger, 1873), De Bary noted that no stage of the fern's life cycle was free from cyanobacterium and that the latter was in no way harmful to *Azolla*. He considered this association as an example of a mutualistic case applied to the Plant Kingdom and based on the definition introduced in 1875 by the Belgian zoologist Pierre-Joseph van Bénéden (De Bary, 1878; Sapp, 1994, 2003). In 1895, the Danish botanist Eugenius Warming published "Plantesamfund" (Oecology of Plants), considering the *Azolla-Anabaena* association as an example of mutualism and as an exception to the normal behavior in plant communities – "in plant community egoism reigns supreme" (Sapp, 1994).

[1] This article is dedicated to **Prof. Maria Grilli Caiola** life's work and her contribution to the construction and development of the modern symbiotic studies, namely on the *Azolla-Anabaena*-bacteria research.

All these ideas and studies, at the beginning of the symbiotic research, reflect the importance of this fern and their symbionts for a more complete understanding of organisms' biology. All this, reinforced by the research developed nowadays and by the new data obtained, allows us to have a more broad and dynamic vision of the characterization of the *Azolla-Anabaena*-bacteria symbiotic system.

2. Un Peu d'Histoire

The genus *Azolla* is referred to have been established by the French naturalist Jean-Baptiste Lamarck in 1783 based on a specimen collected by the French botanist Philibert Commerson and his assistant Jeanne Baret in the Magellan region, during his voyage around the world in the Bougainville's expedition (1766–1769) (Svenson, 1944; Lamarck, 1783; Monnier et al., 1993; Schiebinger, 2003). However, Lamarck included it as a new genus of the family Naiadaceae (Lamarck, 1783), which is a family of flowering aquatic plants and not of pteridophytes. Recent data obtained from the Herbarium of the Botanical Garden of Lyon (Jardin botanique de la ville de Lyon, herbier LYJB, France) seems to indicate another possibility. It shows one specimen collected in Argentine (Buenos Aires region) by Commerson and incorporated in 1779 in the herbarium of Claret de la Tourrette, which was later identified as *Azolla magellanica* (currently *Azolla filiculoides*) by another researcher, and was probably the first to be collected (Fig. 1). This supposition is based on the journey on board the vessel the *Étoile*, where Commerson had made the voyage. Is it possible that Lamarck was not the first to

Figure 1. The *Azolla* sample collected by Philibert Commerson in the Buenos Aires region and existing in the Herbarium of the Botanical Garden of Lyon (LYJB) (photo courtesy of Frédéric Danet).

identify and to describe the genus? We do not have a final answer, but in the original description made by Lamarck in the Encyclopédie Méthodique (1783) about *Azolla filiculoides*, he refers: "C'est une petite plante aquatique, qui paroit flotter à la surface des eaux à la manière des Lenticules, (Lemna) avec lesquelles elle semble avoir beaucoup de rapports, et qui a néanmoins l'aspect d'une très petite fougère." (*It is a small aquatic plant, which appears to float on the water surface in the manner of Lenticules, (Lemna) with whom it seems to have a great relation, and which nevertheless looks like a very small fern*).

All these data are correct, but how could Lamarck have all this information, considering that he was not on the trip, that he only saw the plant in the dried form, and *Azolla* did not exist in Europe at that time? The answer can probably be found in the information included by Commerson with the collected plant about its ecology, which was observed by this naturalist *in loco*. Curiously, there is a reference to *Asplenium* with a question mark in the *Azolla* specimen label existing in the Herbarium of the Botanical Garden of Lyon (LYJB). It seems that Commerson considered that the plant was probably a fern, but ignoring the correct genus he decided to do further studies later, considering that the plant resembled an *Asplenium*. In the 1783 work, Lamarck did not refer to the origin of the name, only saying that "Cette plante a été rapportée de Magellan par M. de Commerson" (*This plant was brought from Magellan by Mr. Commerson*).

Traditionally, the term *Azolla* is referred to be formed by two Greek words: *azo*, to dry, and *ollyo*, to kill, alluding to death from drought (Lumpkin, 1993). However, another possibility can be considered, that the term was adapted from a word used by the local population when Philibert Commerson collected it in Argentine or Chili. The first description and crude illustration of this plant in the taxonomic literature was made in 1725 by the French priest and naturalist Louis Feuillée from a Peruvian specimen in the book *Journal des Observations Physiques, Mathematiques et Botaniques* (Fig. 2). The plant under the name of *Muscus squamosus aquaticus elegantissimus* was mentioned to be used for improving chicken egg production (Feuillée, 1725; Evrard and Van Hove, 2004).

3. The Basic Biology

Azolla is a worldwide heterosporous floating or semi-aquatic pteridophyte, presenting overlapping scale-like bilobed leaves covering a slander and branched stem (rhizome) that floats horizontally on the water surface, with single or fasciculate pendulous roots (Carrapiço et al., 2000). This genus is placed in the Azollaceae family and includes monoecious plants that possess dimorphic sporocarps, whose micro- and megaspores develop in a leptosporangiate way (Carrapiço et al., 2000), with a fossil record dating back to the mid-Cretaceous. *Azolla* usually reproduces vegetatively by fragmentation of the abscission layer, at the base of each branch. Sexual reproduction is not very common and seems to be influenced by environmental factors. Sporocarps occur in pairs of either microsporocarps, megasporocarps,

Figure 2. The *Azolla* drawing presented by the naturalist Louis Feuillée in the year 1725 and under the name of *Muscus squamosus aquaticus elegantissimus* (courtesy of the Bibliothèque Centrale du Muséum National d' Histoire Naturelle, Paris).

or one of each, with the exception of *Azolla nilotica* that shows tetrads, in the place of the first leaf lower lobe of the sporophyte branch. Megasporocarps are much smaller than microsporocarps and contain only one megaspore (Carrapiço et al., 2000).

The gametophytic structures of *Azolla* show an endosporic development and are formed by the megaspore and microspore. These female and male structures produce an archegonium and antheridia. Fertilization occurs in the archegonium, where the egg-cell is fertilized by an antherozoid produced in antheridia, originating a zygote. The development of this structure gives an embryo and, by further development, a new sporophyte plant (Becking, 1987). The microspores remain embedded in a structure called massula, where occurs the development of the male gametophyte. The female gametophyte (megametophyte) also remains completely concealed in the megasporocarpic complex and fertilization is facilitated by the close proximity of both structures (male and female gametophytes). In fact, massulae show glochidia, a specialized structure for anchoring to the megasporocarpic complex that facilitates the fertilization process, enabling the massulae with microspores to become entangled in the filamentous appendages of the epispore wall of the megaspore (Becking, 1987).

The leaves are sessile, alternate, often imbricate, in two ranks along upper side of the stem, 0.6–2 mm wide (Lumpkin, 1993). Each leaf has an emerged, thick, greenish or reddish, and photosynthetic dorsal lobe and a very thin, immersed hyaline ventral lobe (Fig. 3). The dorsal lobe has an ellipsoid cavity, measuring approximately 0.15×0.30 mm, that opens to the external environment

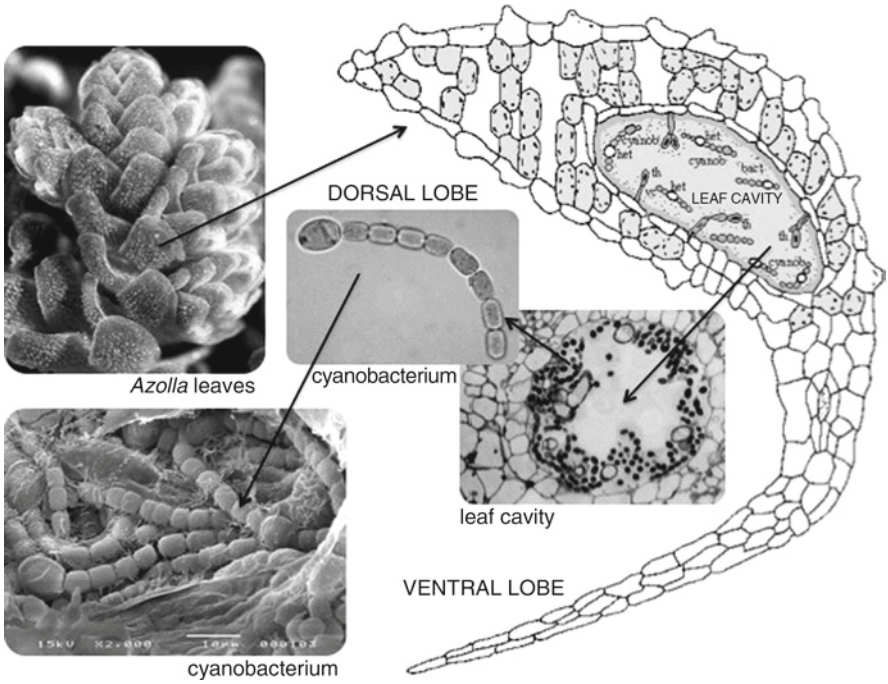

Figure 3. Location of the leaf cavity in *Azolla filiculoides* and *Anabaena azollae* (cyanobacterium).

through a pore, surrounded by two cell layers, located in the adaxial epidermis of the leaf cavity (Braun-Howland and Nierzwicki-Bauer, 1990; Veys et al., 1999; Lechno-Yossef and Nierzwicki-Bauer, 2002). This cavity, an extracellular compartment formed by an infolding of the adaxial epidermis during development (Peters and Meeks, 1989), contains an endosymbiotic community composed of two types of prokaryotic organisms: a heterocyst-forming, N_2-fixing filamentous cyanobacterium – *Anabaena azollae* Strasburger – (first described by Strasburger as *Nostoc* in 1873 and re-named *Anabaena azollae* in 1884, and probably it may well belong to this genus rather than *Anabaena*) (Peters and Meeks, 1989; Adams, 2000) and a variety of bacteria strains mainly identified as members of the genus *Arthrobacter*, *Corynebacterium,* and *Agrobacterium*, associated with other bacteria showing the presence of nitrogenase (Carrapiço, 1991; Lindblad et al., 1991; Serrano et al., 1999; Lechno-Yossef and Nierzwicki-Bauer, 2002). These microsymbionts are specific of this association and live immobilized in a mucilaginous fibrillar network, which fills the peripheral area of the cavity. This mucilaginous material is delimited by two envelopes, an internal and external one, leaving the center empty and probably filled with gas or liquid (Carrapiço, 1991, 2002; Lechno-Yossef and Nierzwicki-Bauer, 2002).

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 2 (one primary and one secondary). Since the *Azolla* leaf cavity has no direct connection with the vascular system of the fern, 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 (Braun-Howland and Nierzwicki-Bauer, 1990; Pereira and Carrapiço, 2007). A mixture of lipids, unsaturated lipids, polysaccharides, polyphenols (*o*-dihydroxyphenols, phenols with free –OH groups and tannins), and alkaloids or alkaloid-like compounds were detected in the vacuoles of simple hairs (Pereira and Carrapiço, 2007). The function of these bioactive metabolites in the *Azolla* symbiosis is still not well understood, but may play a role in the selection of the microorganisms that are not useful to the fern, in the control of the endosymbionts in the cavity and in the establishment and maintenance of the symbiosis (Pereira and Carrapiço, 2007). These data also suggest an exchange of chemical compounds at the level of the host–microbionts–host system, functioning as a biological and chemical communication language in this dynamic association (Pereira and Carrapiço, 2007). As we referred previously, the interior of the leaf cavity is lined by an outer and an inner envelope, creating a narrow space close to the periphery of the cavity where the bacteria, the cyanobacterium, and the trichomes are located. This results in an intimate contact between all the partners, helping in the recognition process as well as in the exchange of metabolites and efficient use of the nitrogen fixed by the cyanobiont (Carrapiço, 2002).

4. *Azolla*, a Scientific Curiosity?

For many years, this pteridophyte was seen as a botanical curiosity by Western researchers, since the presence of cyanobacterium inside the leaf cavity allowed it to be considered as a classical example of a mutualistic symbiosis. However, the complexity of the relationship between the host and the symbionts was later recognized as a new level of biological organization. In fact, the *Azolla* leaf cavity behaves as both the physiological and dynamic interface unit of this symbiotic association where the main metabolic and energetic flows are located, and where molecular recognition between the symbionts and the host occur. In this sense, it can be considered as a natural microcosm, a special micro-ecosystem, which reveals a self-organization and an ecological defined structure (Carrapiço, 2002). This micro-ecosystem can also be considered as a natural photobioreactor (Shi and Hall, 1988), with millions of years of evolution, where the symbionts are immobilized and driven by the fern into increasing some of its own physiological and metabolic activities.

Azolla has been used as green manure for rice cultivation and animal feed in China and in Vietnam, during several centuries, and more recently in Africa (Carrapiço et al., 2000) and in Central and South America. The use of *Azolla* as

biofertilizer can avoid the adverse effects of chemical fertilizers on long-term soil fertility, thus improving soil productivity and environmental safety. The new advances in the investigation of this symbiosis have contributed to a more comprehensive perception of the complexity between the host and the symbionts. This complexity has been translated into new models of knowledge and new areas of application. The biotechnology and environmental engineering are some of these main fields, which have and can still profit with these new data. New bioreactors can be developed if we consider these results, namely those related with the living conditions existing in the leaf cavity of the fern. The latter enable high performance for some specific metabolic reactions of the symbionts, namely nitrogen fixation, ammonium and hydrogen production by the immobilized cyanobiont. Further, the use of *Azolla* as wastewater biofilter (Costa et al., 1999; Forni et al., 2001) and in biologically based life-support systems (BLSS) incorporated in bioregenerative space devices are now in progress in several laboratories (Carrapiço, 2002).

Recent data from the 2004 Arctic Coring Expedition (ACEX) cores, drilled in the central Arctic Ocean near the North Pole, show the presence of fossil *Azolla* in Eocene sediments (~48,5 Ma) (Brinkhuis et al., 2006). The plant's remains occur as laminations, reflecting seasonal or longer cycles and they have also been observed in more than 50 Arctic wells from northern Alaska, the Canadian Beaufort, and the Chukchi Sea (Bujak, 2007). According to this author, these data can be used to determine the maturation level (amount of heating) and type of hydrocarbons produced by the *Azolla* remains, suggesting that the *Azolla* interval may be an Arctic-wide petroleum source rock (Bujak, 2007). The presence of repeated *Azolla* laminations in the central Arctic Ocean also indicate that the *Azolla* plants grew *in situ* on freshwater layers that repeatedly developed on the surface of the Arctic Ocean, rather than being transported from freshwater bodies, such as lakes, on the surrounding land. Brinkhuis et al. (2006) and Bujak (2007) also suggested that the enormous quantities of *Azolla* inhabiting the Eocene Arctic Ocean for almost a million years may have triggered the initial shift from the Mesozoic greenhouse world towards our present icehouse state. According to their model, CO_2 absorption by the fern resulted in an abrupt reduction in this atmospheric gas with critical consequences in the climatic change and implications for the global biogeochemical cycles (Brinkhuis et al., 2006; Bujak, 2007). A very recent work presented by Collinson et al. (2009, p. 155) indicates that the Arctic *Azolla* can be included in a new fossil species, *Azolla arctica*.

5. *Azolla-Anabaena*-Bacteria Association, a New Level of Biological Organization

Although traditionally considered as a lower vascular plant, *Azolla* exhibits symbiotic characteristics more evolved than the other vascular plant–cyanobacterial symbioses – cycads (Cycadophyta) and *Gunnera* (Anthophyta). There appears to

be no direct correspondence between the fern's evolutionary phylogeny and the complexity of the symbosis. In fact, this symbiotic system is sustained throughout the fern's life cycle, where the cyanobacterium and bacteria are always present (Fig. 4), either in the dorsal lobe leaf cavities or in the sexual structures (sporocarps) (Carrapiço, 1991, 2002). The *Azolla* plants are never infected de novo, since the cyanobiont is transferred between generations as akinete inocula. The presence of *Anabaena* and bacteria throughout the life cycle of the fern favors the obligatory nature of the symbiosis and suggests a parallel phylogenetic evolution of both partners, and can be considered a successful co-evolved system. Bacteria was first observed and described to be present in the *Azolla* leaves cavities by Grilli (1964). The presence of these prokaryotes in the leaves and also in the cavity below the megasporocarp's indusium in association with cyanobacterium cells suggests a behavior pattern similar to the cyanobiont and can be considered the third partner of this symbiotic system (Carrapiço, 1991).

The *Azolla* leaf cavity can also be considered as the basic physiological unit of this symbiotic association (Grilli Caiola and Forni, 1999), where complex ecological communities of permanent microorganisms co-exist with the fern to maintain the whole. New novel metabolic and organic capabilities are acquired and developed by the partners to establish a new level of organization, extending beyond the capability of each individual forming the association. This information is

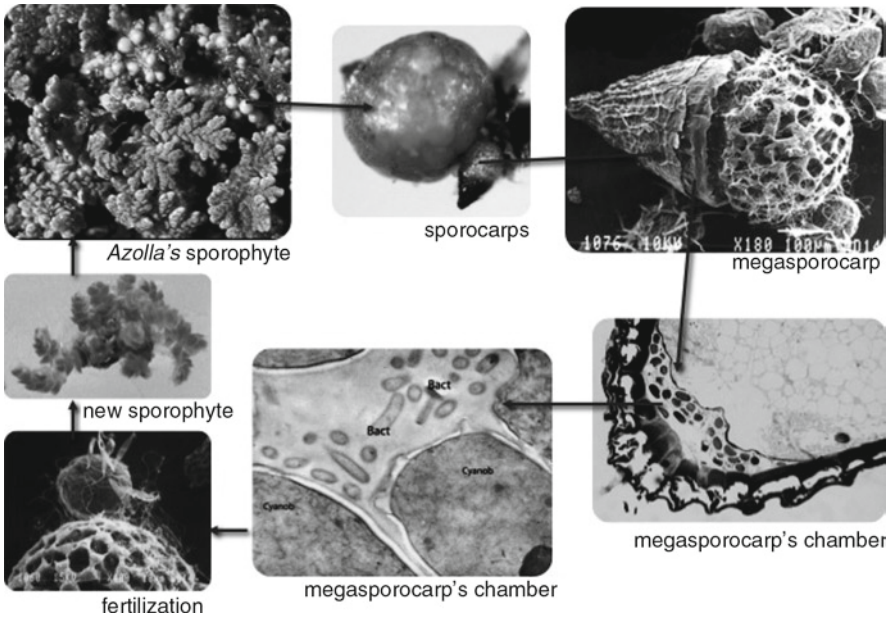

Figure 4. *Azolla*'s life cycle, showing the permanent presence of bacteria (bact) and cyanobacteria (cyanob) throughout the fern's life cycle.

supported and agrees with the concept introduced by René Dubos and Alex Kessler in 1963 related to the creative manifestations of symbioses, where the nutritional effects of symbiosis are not its most interesting manifestation. More important is the fact that many symbiotic systems produce substances and structures that neither one of the two components produces when growing alone (Dubos and Kessler, 1963). This is emphasized by Angela Douglas in her book "Symbiotic Interactions" referring that the common denominator of symbiosis is not mutual benefit but a novel metabolic capability, acquired by one organism from its partners (Douglas, 1994). Also, Douglas Zook reinforces these principles referring that symbiosis is the acquisition and maintenance of one or more organisms by another that results in novel structures and metabolism. Some symbiotic evolutions may involve partner genetic exchanges (Zook, 1998).

These ideas can be found and translated in a clear way in the metabolism of nitrogen associated to this symbiotic system shared by the host and partners. The atmospheric N_2 fixed by the cyanobacterium through the heterocysts is converted into ammonia and released into the leaf cavity. It has been shown that intracellular ammonia pools of symbiotically associated *Azolla* are five times greater than those of endophyte-free *Azolla* (Braun-Howland and Nierzwicki-Bauer, 1990). The activities of ammonia-assimilating enzymes in the isolated trichomes of the dorsal leaf cavity were much higher than those in *Azolla* leaves, while the activities in the *Anabaena* filaments were repressed to very low levels. For example, it was shown that the host accounted for at least 90% and 80% of the total glutamine synthetase and NADH-dependent glutamate dehydrogenase activities, respectively. These results suggest that hair cells play an important role in the assimilation of nitrogen, which the cyanobiont fixes and releases into the cavity and it is transferred to the pteridophyte (Uheda, 1986), and it was acquired during the development of the symbiotic process. Recent data published by Papaefthimiou et al. (2008) indicate the existence of different cyanobacteria strains or ecotypes inhabiting the fern species. These results reinforce our belief that the leaf cavity behaves as a micro-ecosystem or as a natural microcosm (Carrapiço, 2002) with a self-organization and an ecological defined structure, where natural selection acts to evolve different cyanobacteria ecotypes.

All this information agrees with the concept of superorganism referring that, in ecological terms, each plant and animal must be considered as "superorganism" – symbiome, which includes its own genes, those of cellular organelles (mitochondria and/or chloroplasts), as well as the genetic information of symbiont bacteria and virus living within the organism (Sapp, 2003). It is also important to take into consideration the relevance of the fitness and how we validate it in terms of symbiotic prevalence. It goes beyond the reproductive view of each individual and reinforces the ecological behavior of the symbiotic system as a whole (Bouchard, 2007).

These ideas and concepts, especially the superorganism one, can be applied to *Azolla* and its symbiotic association, a good example of a synergistic biological system. In this association, complex ecological communities of permanent microorganisms co-operate along with the fern in the maintenance of the whole.

New metabolic and organic capabilities are acquired and developed by the partners, which establish a new level of organization that goes beyond the individual capabilities of any individual partner, suggesting that the synergies associated to symbiosis had and have a leading role in the morphological, reproductive, physiological, and metabolical complexification of the organisms.

The *Azolla-Anabaena*-bacteria symbiotic association can also be considered as a successful co-evolved system, with the symbionts always present in the fern's life cycle, indicating a phylogenetic parallel evolution of the relation partners, and a typical example of a hereditary symbiosis.

6. *Azolla* in Stress

As we referred previously, *Azolla* can develop as a free floating or semi-aquatic plant. These two ecotypes are present in different but complementary ecological conditions. The first one is present in freshwater aquatic environment, where *Azolla*'s sporophyte shows a horizontal growth with normal rate of sporocarps formation, and roots growing freely in the aquatic medium. The second one, more rare, can be found in sand wet banks, when the water level goes down and where *Azolla* is fixed to the substrate by the root system. It shows a sporophyte vertical growth with a high rate of sporocarps formation, and turning reddish very quickly due to the anthocyanins production and accumulation in the vacuoles of the epidermal cells of the leaves. The presence of these phenolic compounds in the *Azolla* sporophyte leaves is a normal consequence of a stress condition related to high temperatures and phosphorus deficiency (Tung and Watanabe, 1983) and to light intensity. The sporophyte does not present the typical growth it has in water, but resembles a kind of small "bunches," to prevent water losses and to maintain the humidity (Fig. 5). The root system is interconnected and forms a kind of complex network, where the sand particles are trapped. Finally, the higher sporulation rate improving the fitness of the symbiotic system as a whole (symbiome) is a response to the survival in dry environments. This stress condition is a unique situation that needs to be further studied for a full understanding of the plant behavior.

Azolla can grow in natural and artificial media without the presence of a nitrogen source, forming extended, colored mats that cover the freshwater surfaces, where phosphorus is the main limiting factor to its growth. When a phosphorus level over 0.4 mg $\cdot$ l^{-1} occurs in nature, an *Azolla* bloom can be the result. It was what happened in April 1993, when a massive fern bloom occurred along several kilometers of the international Guadiana river in Portugal (Fig. 6). In this situation, phosphorus acted as an environmental stress factor and the main trigger for the uncontrolled growth of this plant in the river. In the period 1990–1993, southern Portugal experienced low rainfall with long dry seasons. This factor, combined with several dams along the river, caused low water flow (3.64–1.13 $m^3 \cdot s^{-1}$) during 1993. Moreover, farming and industrial activity in the upper area of the Guadiana, together with untreated domestic effluents from several towns and

Figure 5. *Azolla*'s sporophyte living in the wet sand banks of the Golegã lagoon (Portugal) and submitted to natural stress conditions, shows small bunch arrangements to prevent water losses (detail in the lower right corner).

Figure 6. Photo of the *Azolla* mat taken in a boat placed in the middle of the Guadiana river, Portugal.

villages, contributed to the organic contamination of the river that year. Lower flows also promoted very high nutrient concentrations. At different river sites during the first months of 1993, the phosphorus levels changed, with maximum concentration values in April between 5.36 and 0.63 mgP $\cdot$ l^{-1}. This massive *Azolla*

bloom represented the first occurrence in Portugal and Europe of such a large scale uncontrolled growth of this fern in a river (Carrapiço et al., 1996).

Salinity is another stress condition for *Azolla* growth. The presence of 10 mM NaCl in the environment affects the growth of *Azolla pinnata*, becoming lethal at 40 mM (Rai et al., 2006). Plants exposed up to 30 mM NaCl exhibit longer roots than the control and their number is reduced. The salinity sensitivity associated with *Azolla-Anabaena* association results from the inability to maintain low Na and high Ca level under salt stress, since this symbiotic system lacks mechanisms of regulating ion transport, level of Na and NaCl-induced deficiency of Ca, when stressed with NaCl (Rai et al., 2006).

The different abundance of proteins in *Azolla* and in *Anabaena azollae* with functions related to protein assembly, modification and degradation (chaperones), and stress-related proteins, such as superoxide dismutase and peroxiredoxins, probably reflects the radically different growth conditions experienced in the symbiosis (Ekman et al., 2008). Some of these proteins, such as superoxide dismutase located in the heterocysts of the cyanobiont, suggests a role in protecting nitrogenase from superoxide radicals generated via respiration (Canini et al., 1992). Probably, the first stress conditions can be found in the cyanobacterium lifestyle present in the *Azolla* leaf cavity due to the accommodation in this symbiosis, with high heterocyst differentiation and nitrogen fixation elicited by N deprivation (Ekman et al., 2008).

7. Concluding Remarks

We believe that it is important to reconsider symbiosis as a general mechanism in heredity and development, in addition to gene mutations and recombination, as a source of evolutionary innovation, and to hold symbiotic processes as one of the main bases of biodiversity and evolution on Earth. This idea implies also the central role of interactions, in which individuality (new entity) emerges through incorporation. It involves horizontal mergers, which can be rapid, and, usually, discontinuous, creating permanent and irreversible changes, the ground for evolutive novelty. Something new arises through merging, being a unique or new metabolism or structure(s), which was not present before symbiosis (Carrapiço et al., 2007). In this context, we can consider this entity as a new taxonomic novelty or even as a new level of biological organization. Thus, biology must take into account this reality, integrating symbiosis, not only as a factor of evolutionary change, but also as a taxonomic element in the organization of the living world. A good example is the symbiotic system *Azolla-Anabaena*-bacteria, a successful co-evolved system that also makes important contributions to the ecological, biofertilization, and biotechnological fields. The importance of symbiosis must be understood in a global perspective, with consequences not only at the scientific level, but also to develop and improve the adequate tools for teaching symbiomics

in schools and universities. As Joseph Seckback refers in the preface of his book "Symbiosis: Mechanisms and Model Systems," we must have an "open-minded and science-orientated reader to the global importance of symbiosis and to new aspects of symbiotic relationships among living organisms" (Seckback, 2002).

8. Summary

Symbiosis is one of the main processes responsible for the biodiversity and evolution on Earth, which has had a leading role in the morphological, physiological, and metabolical complexification of organisms. *Azolla*, a heterosporious floating or semi-aquatic fern, constitutes a good example of a synergistic symbiotic system. In the chlorophyllous dorsal lobe leaf, there is an ellipsoid cavity with a filamentous nitrogen-fixing cyanobacterium, usually referred to as *Anabaena azollae*, and several genera of bacteria. This leaf cavity behaves both as the physiological and dynamic interface unit of this symbiotic association where the main metabolic and energetic flows occur, and as a natural microcosm. This symbiosis is sustained throughout the fern's life cycle, where the cyanobacteria and bacteria are always present. In the *Azolla-Anabaena*-bacteria association, complex ecological communities of permanent microorganisms cooperate along with the fern in the maintenance of the whole, which leads to the idea that this symbiotic system can be considered as a superorganism in ecological terms. New metabolic and organic capabilities are acquired and developed by the partners, which establish a new level of organization that goes beyond the individual capabilities of any individual partner, suggesting that the synergies associated to symbiosis had and have a leading role in the morphology, reproduction, physiology, and metabolic complexification of the organisms.

9. Acknowledgments

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PART IV:
TERRESTRIAL SYMBIOSES

Little
Jurkevitch
Applebaum
Ichelczik
Humber
Altenbach
Böhmer
Gitter
Läuchli
Wieczorek
Koltai
Kapulnik
Ruiz-Lozano
Aroca
Hirsch
Belkin
Qvit-Raz
Soto
Lostroh
Nishiguchi
Grilli-Caiola
Canini
Berg
Egamberdieva
Lugtenberg
Hagmann
Rodrigues
Woodward
Redman
Torres
White
Bar
Avni
Grube
Stocker-Wörgötter
Chapman R.L.
Chapman M.R.
Freystein
Reisser

Biodata of **Ainslie E.F. Little**, author of **"*Parasitism Is a Strong Force Shaping the Fungus-Growing Ant-Microbe Symbiosis*"**

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PARASITISM IS A STRONG FORCE SHAPING THE FUNGUS-GROWING ANT–MICROBE SYMBIOSIS

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1. Introduction

Parasitism is a biological stress that is relevant to organisms in every niche imaginable. There is ample evidence documenting the effects parasites have on individual hosts and on host populations, yet there has been little work done to review the importance or frequency of how a parasite influences the organisms it's host interacts with – it's host's immediate community. There is, however, a shift occurring in the way scientists think about mutualisms, and people are beginning to move away from considering interactions that occur in isolation, or strictly a binary manner and toward considering symbioses as interactions embedded in a more complex community (Althoff et al., 2004, 2005; Currie et al., 1999a, b; Little and Currie, 2008). In this chapter, I discuss parasitic and mutualistic symbioses as important determinants of organismal diversity, radiation, and fitness, and I use the fungus-growing ant–microbe symbiosis as a model system in which to study ways that parasites shape the ecological and evolutionary dynamics of mutualistic organisms. I conclude with the hypothesis that mutualism may be a viable solution to parasitism and, in turn, discuss the possibility that parasitism may in fact contribute to evolutionary stability of persistent mutualisms.

1.1. SYMBIOSIS

Symbiosis was first defined by Anton de Bary in 1879 as "the living together of unlike named organisms." More recently, the definition has been refined to be a prolonged physical association between two or more "differently named" organisms (Margulis and Sagan, 2002). Regardless of definition, symbiosis includes a vast assemblage of interactions. Partner integration may vary in intimacy at any or all levels of organization (e.g., behavioral, metabolic, genetic), and partner relationships range and shift between beneficial and antagonistic.

Symbiosis is a fundamental source of innovation in evolution (Margulis and Fester, 1991; Moran and Telang, 1998), and arguably the most dominant life habit on the planet; almost every multicellular organism has a symbiotic association with a microorganism. In the early 1900s scientists began to hypothesize that the eukaryotic nucleus had a symbiotic origin (Mereschowsky, 1905; Wilson, 1928). More recent research suggests that the nucleus has a chimeric archaeal/eubacterial origin (Horiike et al., 2001; Margulis et al., 2000). It is now well accepted that mitochondria and chloroplasts, both double-membrane organelles involved in energy production, were primitive proteobacteria and cyanobacteria, respectively, that were "captured" by engulfing hosts. Further, the flagella of many protists, which provided the first multicellular organisms motility options, are believed to be derived from ancestral spirochetes (Li and Wu, 2005; Margulis et al., 2000). From an evolutionary perspective, the acquisition of microbial symbionts, such as these, has mediated many major breakthroughs in the increased complexity of eukaryotic organisms. Current research is teasing apart how coevolved microbes influence the development of their hosts and their host's immunity over time (Hooper, 2004; McFall-Ngai, 2002; Rakoff-Nahoum et al., 2004).

The acquisition of microbial symbionts has also been a strong force in shaping the diversity of species on earth. The reason for this is that symbionts allow their host organisms to invade and adapt to new niches, that aposymbiotic (without symbionts) would not successfully invade. This is exemplified by the colonization of land by plants, which was facilitated by microbes. One of the major barriers to life on land for plants was the acquisition of phosphorous, which is often tightly bound to iron in soils. Symbiosis with mycorrhizal fungi, capable of efficient phosphorous uptake, allowed plants to inhabit many new niches in terrestrial soils (Pirozynski and Malloch, 1975). Mycorrhizal symbionts remain an important determinant of current plant species diversity and functional ecosystems (Van der Heijden et al., 1998). Species diversity within Animalia has also been mediated by symbiotic associations. Insects, the most speciose animal group, have experienced vast radiations in their geographic range and instances of specialization largely because of their role as pollinator and seed-dispersal symbionts. The same can be said for their plant partners. As mentioned earlier, many symbionts acquire and/or process nutrients that would otherwise limit the fitness of their hosts, which provides opportunities for diversification. Further, macrophages, and macrophage-like cells, a keystone feature of innate immunity in animals, are hypothesized to be the descendents of free-living amoeba (Janeway et al., 2004). The development of innate immunity has allowed animal hosts to recognize self from non-self and beneficial symbionts from antagonistic microbes, thus permitting animals to survive in niches despite the presence of microbial competitors and pathogens.

The symbiotic lifestyle has become dominant in all types of environments, despite the additional challenges and selective pressures symbionts encounter. Unlike free-living organisms, symbiotic organisms directly interact with their hosts and thus must either become securely attached to that host or develop traits that allow a continuous relationship. Symbionts must also overcome physiological defenses of their host, and cope with potential predators, competitors, and host

parasites to maintain an intimate relationship. Furthermore, most symbionts must be able to survive in both their immediate host niche and the macroenvironment of their host during transmission. The numerous and strong selective pressures facing symbionts require additional and often rapid adaptations, which can be costly. However, symbiotic relationships remain common. Why? The answer is simply stated: *symbiotic groups have found solutions to stresses caused by various selective pressures where free-living individuals have not*. Data to support this statement are robust. Animals that persist in extreme environments do so as a result of their relationship with symbiotic microbes. Marine invertebrates aptly survive in deep-sea hydrothermal vents and methane seeps because of their relationships with sulfur-oxidizing and methylotrophic bacteria (Cavanaugh, 1983; Cavanaugh et al., 1987). Similarly, lichenized fungi are able to grow in harsh arctic deserts due to their photosynthetic partners (green algae or cyanobacteria). None of these organisms would successfully inhabit extreme niches in the absence of their symbionts.

2. Parasites and Mutualists

Loosely, symbiosis can be divided into three types of interactions, which exist in a continuum from parasites to mutualists (Fig. 1). Parasites (Greek, *para* – near; *sitos* – food) are organisms that live on or in another organism, obtaining all or part of their necessary nutrients at the expense of their host. Commensals (Latin, *com* – together; *mensa* – table) are relationships in which one partner derives benefit from the other, while the other partner is neither harmed nor benefits from the association. Mutualism (Latin, *mutualis* – reciprocal) is an association in which both organisms derive benefit from one another.

Purely commensalist relationships may not exist. More likely those organisms are either beneficial or harmful to their hosts, depending on the community

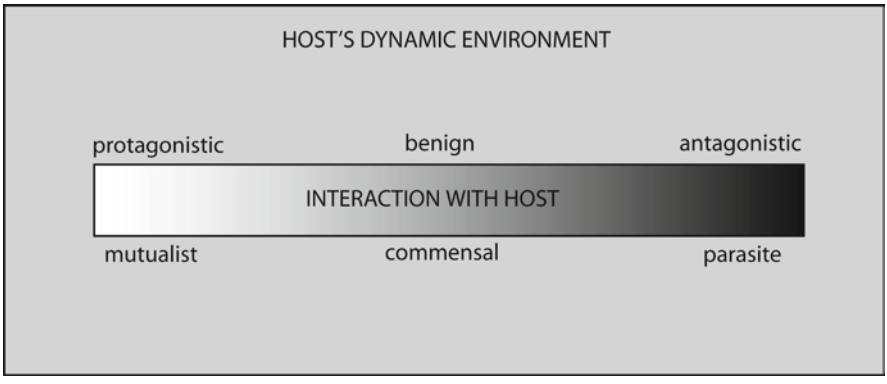

Figure 1. Scheme of the continuum of symbiotic interactions. The effect symbionts have on their host can shift along the spectrum from beneficial interactions (mutualism, far left) to detrimental (parasitism, far right) depending on the dynamic nature of the biotic and abiotic pressures in both their host, and their host's environment.

dynamics in the niche, but researchers have yet to delimit and quantify the costs and benefits exchanged between the host and symbiont. For example, many microbes in the human gut historically termed "commensal" are now recognized as critical factors in gut and immunity development, nutrient uptake, and homeostasis of the system (Hooper, 2004; Hooper et al., 2002; Rakoff-Nahoum et al., 2004). In fact, many symbioses can be regarded as dynamic interactions that shift along a continuum between parasitism and mutualism in response to various abiotic factors, such as light, pH, and temperature, and biotic factors, such as the macro- and microbiological communities they live in. Good examples of the dynamic nature of symbionts are exhibited by opportunistic pathogens. These organisms live within their hosts without detectable negative effects; they may even have beneficial effects we are unaware of, but the nature of their relationships with their hosts is context-dependent. When conditions change, opportunistic pathogens become antagonistic to their hosts. Interestingly, a common hypothesis for the evolutionary origin of mutualistic relationships is that they began as parasitic interactions. Because parasites that kill their hosts lose their niche, it is recognized that natural selection can favor the evolution of lower virulence (Thompson, 1994), which can lead to parasites evolving to become benign or beneficial symbionts. This is especially true in cases of high partner fidelity, the pairing of specific individuals over a long series of exchanges (through vertical transmission), and those with a low availability of alternative partners (Axelrod and Hamilton, 1981; Bull and Rice, 1991).

2.1. EFFECT OF PARASITES ON HOST AND POPULATION BIOLOGY

Most of our knowledge of host–microbe interactions comes from pathogenic studies. Much is understood about how pathogens infect, reproduce, and transmit between hosts, and how each of those stages influence host biology (e.g., behavioral or physiological modifications). A host that is able to survive long enough to reproduce despite the challenges of parasites will pass on its genetic traits, whereas individuals who succumb to parasites prior to reproduction will not. Thus, traits that confer resistance or tolerance to parasites can become fixed in populations. For instance, plants and fungi have multiple biosynthetic pathways that produce complex chemical compounds used to deter pathogens and parasites. Animals have evolved innate immunity to respond to changes in their immediate environment particularly invading microbes. Vertebrates have evolved adaptive immunity to specifically protect themselves from local pathogens and parasites. There are also phenotypic traits that visibly confer information about parasite load, such as extreme sexual ornamentation and display by male animals that is sometimes attributed to "healthy mate selection" (Hamilton and Zuk, 1982). This theory suggests that certain male traits have evolved to indicate that the individual has low levels of parasites. For example, in some male turkeys, the larger the snood (a strange beak ornament), the lower the internal load of coccidian parasites

(Buchholz, 1995); females prefer males with larger snoods. Each of these traits, selected by the pressure of parasitism, is adaptive. Because these heritable traits increase an organism's fitness, they have become fixed in populations.

Since the 1920s, when Lotka and Volterra formulated their host–parasite model (predator–prey equation), it has been recognized that parasites are an important regulator of their hosts population dynamics. Indeed, parasite–host interactions are similar to predator–prey relationships in many ways but with one major exception; the generation time of predator and prey are usually similar, whereas the generation time of host and parasite often differs by magnitudes. If this asymmetry allows parasites to evolve improved attack mechanisms faster than the host can evolve defense strategies, the host's best defense may be genotypic diversity. This discrepancy in host–parasite generation time led Hamilton (Hamilton, 1980; Mailton, 1980) to suggest that genetic recombination in hosts through sexual reproduction helped defense against parasites with quick generation turnover.

Parasites act as direct agents of natural selection, and are considered a major driver of host evolution because the genotypes and phenotypes favored in the presence of parasitism are different from those that would be favored in the absence of parasitism. Something less frequently considered is how parasites shape the interactions between species such as competition, predator–prey and mutualistic interactions. Good examples of the ways parasites shape mutualistic relationships have been developed in the fungus-growing ant–microbe symbiosis.

3. The Fungus-Growing Ant–Microbe Symbiosis

The fungus-growing ant symbiosis is an ideal model system to investigate parasite–mutualist dynamics for several reasons. First the symbionts are widely distributed in the New World tropics, and conspicuous and populous enough to allow for adequate collection. Second, symbionts are amenable to laboratory maintenance, and they are all readily culturable, which permits us to study and manipulate each symbiont separately and in combination. Third, an entire tribe of ants culture fungi for food. Each lineage in the tribe cultivates specific fungi, which each host a specialized mycoparasite, each ant lineage hosts mutualistic bacteria, and all genera tested have amplifiable black yeast symbionts that antagonize mutualistic bacteria. This phylogenetic diversity allows hypothesis testing to be done on an evolutionary scale; by comparing basal, intermediate, and derived symbiont lineages, adaptive patterns that have evolved over the past 50 million years can be identified.

3.1. ANT–FUNGUS MUTUALISM

Fungus-growing ants, in the New World tribe Attini, are distinguished within the formicids by their unique ability to cultivate fungus for food (Hölldobler and Wilson, 1990; Wilson 1971; Fig. 2). Fungiculture by attine ants is believed to have

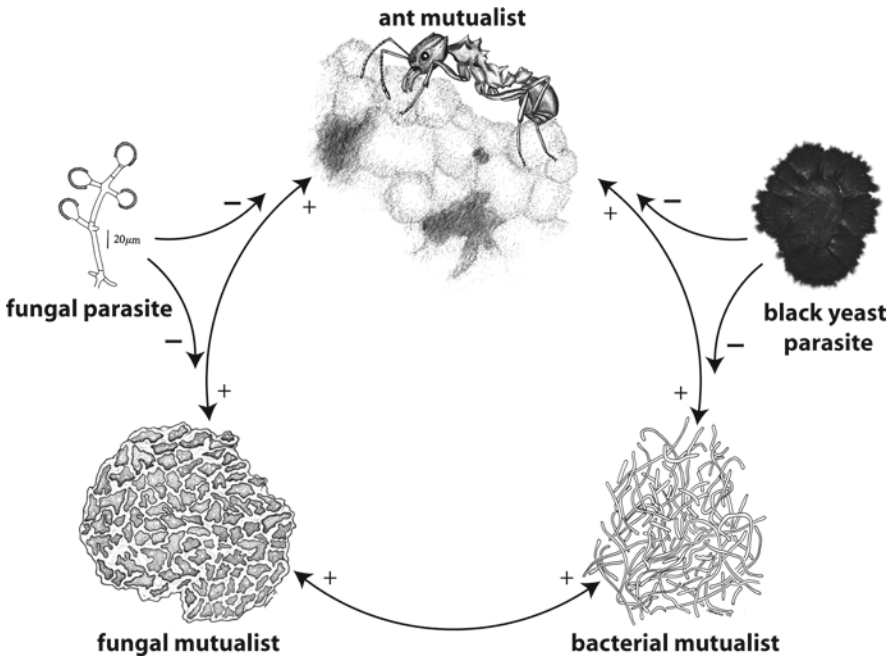

Figure 2. The fungus-growing ant–microbe symbiosis is comprised of five players. Ants in the tribe Attini that cultivate fungi for food. The ants' fungal cultivar is attacked by a fungal parasite (*Escovopsis*). To protect their food source from the fungal parasite, ants engage in a second mutualism with bacteria (*Pseudonocardia*), which produce antibiotics that inhibit the parasite. The mutualistic bacteria are inhibited by black yeast parasites, which can indirectly increase the virulence of the parasite *Escovopsis*.

a single origin about 50 million years ago in the Amazonian rainforest (Mueller et al. 1998, 2001; Wilson, 1971). Subsequently, the monophyletic group of fungus-growing ants has evolved into 13 genera comprising more than 200 described species, and radiated south to the cold deserts of central Argentina and north to the pine barrens of New Jersey (Weber, 1972). Most fungus-growing ant species belong to one of seven genera known as the lower attines (*Apterostigma*, *Cyphomyrmex*, *Mycetarotes*, *Mycetosoritis*, *Mycetophylax*, *Mycocepurus*, and *Myrmicocrypta*). Most lower attine ants are inconspicuous, have relatively small colonies (12–1,000 individuals), and propagate relatively small fungus gardens (Hölldobler and Wilson, 1990; Weber, 1972). The remaining attines are part of four genera, *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta*, and are referred to as the "higher" attines. These ant lineages have larger colonies (millions of workers) and propagate much larger fungus gardens.

Attine ants continuously manure their cultivar with substrate for growth and, in return, the fungus serves as the ants' primary food source. Lower attines are

characterized by providing plant detritus and insect feces to their cultivars as manure, while higher attines provide fresh vegetation to their cultivars. The most recently derived genera, *Acromyrmex* and *Atta*, known as leaf-cutter ants, have scissor-like mandibles that allow them to cut leaf material, which they use to support their fungi. This adaptation has helped make leaf-cutter ants the most dominant pest in the neotropics – a single *Atta* colony only 3 years old will have gathered approximately 6 t of leaf material to cultivate their fungus garden (Hölldobler and Wilson, 1990). This also makes leaf-cutter ants very important contributors to nutrient cycling in tropical forests, as their fungal cultivars break down significant amounts of leaf material. The fungus gardens of higher attines are distinguished from lower attine gardens by special nutrient-rich hyphal structures called staphylae (clusters called gongylidia), which are produced for the ants, particularly larvae, to feed upon.

The most derived lineages of attines, *Atta* and *Acromyrmex*, have a very sophisticated caste system that has further allowed them to become the most dominant herbivores in the new world tropics. Each colony member is part of a physical caste, some of which are further divided into task-based castes. Foragers are medium-sized ants that cut leaves and bring them back to the colony. At nest entrances are soldier ants with very large heads and strong mandibles that protect the nest from predators. In the nest are minima workers whose tasks are age-dependent. The youngest workers tend to the larvae, then to the fungus garden, and finally they move to the refuse heap where they manage waste materials from the fungus garden.

Ants cultivate their fungi vegetatively (asexual clones within nests) and maintain this via vertical transmission of the fungus during mating flights. As such, the clones have coevolved with their ant hosts. Most fungal mutualists are cultivated as mycelium (multicellular), although some are cultivated as yeasts (unicellular). Most are members of the Lepiotaceae (Agaricales; Mueller et al., 1998, 2001) with the exception of some members of the genus *Apterostigma*, which cultivate fungi closely related to the Pterulaceae (Munkacsi et al., 2004). Although the ants generally propagate identical clones of their fungus, domestication of multiple cultivars has occurred over the 50-million-year relationship. The acquisition of novel cultivar strains is an ongoing process, and is perhaps used as a means to replace the fungi after accidental loss or loss to pathogens, or as a means to acquire new and improved cultivar strains (Mueller et al., 2001).

3.2. ESCOVOPSIS: A SPECIALIZED MYCOPARASITE

In addition to general microbial competitors, attine-ant nests are attacked by a specialized microfungal parasite belonging to the genus *Escovopsis* (Ascomycotina: anamorphic Hypocreales; Fig. 2; Currie, 2001b; Currie et al., 1999a, 2003b). These parasites have been isolated from the fungus gardens of all attine genera studied, but have not been isolated from any other substrate (e.g., leaf litter or soil

in the vicinity of nests). *Escovopsis* is horizontally transmitted between nests by an unknown vector and is abundant in mature fungus-growing ant colonies (Currie, 2001c). Infections of *Escovopsis* result in a significant decrease in the growth rate of gardens and, indirectly, a reduction in the production of workers (Currie, 2001c). *Escovopsis* infection is lethal to the garden if not controlled (Currie et al., 1999a; Currie and Stuart, 2001).

Like the association between ants and their fungal cultivar, the association between *Escovopsis* and attine fungus gardens has an ancient origin. *Escovopsis* constitutes a monophyletic group that is divided into four major parasite lineages, each of which is associated with a group of ants and their mutualistic fungi (Currie et al., 2003b). At the broad scale, *Escovopsis* exhibits parallel cladogenesis with ants and fungi, indicating a closely coevolved relationship between the symbionts over evolutionary time. However, at a finer phylogenetic scale there is evidence of host switching by *Escovopsis* (Gerardo et al., 2006; Taerum et al., 2007).

3.3. BACTERIAL MUTUALISTS (*PSEUDONOCARDIA*)

To further protect their food source from the parasite *Escovopsis*, ants form a second mutualism with bacteria (Actinomycetales: *Pseudonocardiaceae*) that produce antibiotics with specific activity against *Escovopsis* (Currie, 2001b; Currie et al., 1999b; Fig. 2). *Pseudonocardia* sp. are filamentous gram-positive organisms in the same family as the genus *Streptomyces*, which contains the organisms that we exploit to produce more than half of commercially available antibiotics. Most *Pseudonocardia* spp. inhabit the soil where they play an important role as decomposers.

The mutualistic bacterium is transmitted vertically between colonies as queens carry it on their cuticle during mating flights (Currie et al., 1999b). Vertical transmission ensures an efficient defense for newly founded gardens, which are prone to infection (Currie et al., 1999b). Within colonies, the abundance of the bacterium is dependent on ant age and caste (Currie et al., 2003a, 1999b; Poulsen et al., 2002, 2003), and bacterial growth varies under stresses such as infection (Currie et al., 2003a). Individual strains of bacteria are associated with a single ant garden; however, multiple strains and/or species can occur within a single species or population of ants. The antibiotics produced by this bacterium are specific to the garden pathogen *Escovopsis*, and if removed, *Escovopsis* infection in the garden increases significantly (Currie et al., 2003a). The presence of actinomycetous bacteria on garden workers is an integral component of garden hygiene in attine ants (Currie et al., 2003a).

The relationship between the ants and their bacterial mutualist is also ancient. The ants have structures on their exoskeleton that house bacteria, and exocrine glands below their cuticle to support bacterial growth (Currie et al., 2006). The location and morphology of these structures vary across fungus-growing ant species. In fact, examination of these structures has revealed several broad evolutionary patterns including increased complexity in the most recently

derived lineages. Collectively, these data suggest that there is coevolution between the ants and their bacterial mutualists at a broad evolutionary scale (Currie et al., 2006).

3.4. BLACK YEAST ANTAGONISTS (*PHIALOPHORA* SP.)

A second antagonistic microbe plays an important role in the fungus-growing ant mutualism: black yeast in the genus *Phialophora* (Little and Currie, 2007). Black yeasts are widely distributed in nature, occurring in soil, plants, water, and decaying wood, where they act as secondary saprophytes and oligotrophs, but are also known to be parasites of humans and human crops (Agrios, 1997; de Hoog et al., 2000). Black yeasts are commonly associated with fungus-growing ants. The black yeasts have been isolated from ants collected throughout their geographic distribution in Central and South America. The association is also very common within ant populations, being found in between 50% and 100% of ants sampled per population (Little and Currie, 2007). Black yeasts grow on the ants' cuticle, specifically localized to the area below the ants' forelegs, which is also where the ants' mutualistic bacteria are cultured. Molecular phylogenetic analyses reveal that the black yeasts form a derived monophyletic lineage associated with the phylogenetic diversity of fungus-growers. Since black yeasts are commonly associated with fungus-growing ants, occurring across broad geographical and phylogenetic distributions of the ants, and form a monophyletic group whose growth is concentrated on the specialized location on the ants' cuticle, they are now considered symbionts of the attine ant–microbe symbiosis rather than transiently associated microbes.

Microbial bioassay challenges have shown that the symbiotic black yeasts can utilize ant-associated *Pseudonocardia* as a nutrient source for growth, consequently suppressing bacterial growth (Little and Currie, 2008). Experimental manipulation of ant colonies and their symbionts shows that ants infected with black yeasts are significantly less effective at defending their fungus garden from *Escovopsis*, the prevalent and specialized fungus garden pathogen (Little and Currie, 2008). Together, these data indicate that black yeasts are parasites of the ant–bacterial mutualism that indirectly benefit the fungus garden parasite *Escovopsis* (Fig. 2).

4. Parasites Impose Forces on Special Characteristics of Mutualist Biology

An area of interest in symbiosis biology that has not been well addressed is the effect that parasites, microbes in particular, have on mutualists. This is not to be confused with the occurrence of cheaters in mutualisms, an interaction that has been well documented in several systems (e.g., non-pollinating fig wasps or ants occupying acacia plants without providing defense; Marr et al., 2001; Yu and Pierce, 1998). Cheaters are symbionts derived from members of a mutualistic

lineage that acquire benefits from their partner without paying the cost of providing a benefit in return. There has been intense interest in how cheating evolves and persists without breaking down mutualisms, and, conversely, how cheaters are restricted in ancient mutualisms (for a review, see Bronstein, 2001). Similar questions are relevant to parasites that are not derived directly from mutualists. Such "external" parasites likely infect many mutualistic partners, although not many have been recognized as important selective forces in the relationship.

Parasitism can affect mutualists at multiple levels (Table 1). For instance, each organism must contend with the natural enemies their free-living counterparts face. For example, fungus-growing ants are attacked by entomopathogens such as *Metarhizium* sp. and *Beauveria* sp. These filamentous fungi are necrotrophic, i.e., they utilize the insects as a nutrient source, killing them in the process. In the case of attines, a worker ant infected with *Metarhizium* or *Beauveria* would likely be less efficient at foraging for food, or carrying out its tasks in the fungus garden, which is also a detriment to the ant's fungal mutualist. Also, if a symbionts host mounts an immune response against it's natural enemy, the symbiont may be affected by the response as well. Parasites may inflict additional or different selective forces on mutualists than on non-mutualistic organisms, given the additional characteristics required to maintain mutualisms. Several facets of mutualist biology, discussed below, represent alternative niches with unique environmental parameters for parasites to invade. Furthermore, specific behaviors of mutualists, and cooperative dynamics of partners have the potential to be shaped by the stress of parasitism.

4.1. RESOURCE EXCHANGE BETWEEN SYMBIONTS

The most obvious mutualistic trait that could be exploited by parasites is the resource(s) that is exchanged between partners. Examples of such exploitation are numerous. Piper ant-plants in tropical forests provide lipid- and protein-rich food cells and shelter for *Pheidole bicornis* ants. In return, the ants remove small herbivores and vines from Piper foliage. In contrast to all other ant-plants, Piper ant-plants produce food bodies only when *P. bicornis* is present in the plant. This relationship is exploited by *Phyllobaenus* beetles, which stimulate the plants to produce food bodies as if ants were present (Letourneau, 1990). The beetles then inhabit the plant, exploiting nest sites and food produced by the plants for ants, preying on ant broods, and depriving the plants of resources and services provided by the ants. Nectar robbers are bees that visit flowers but, unlike most pollinators, bypass features of flowers that promote pollination (long, narrow corolla tubes), by making incisions at the base of the corolla tube to "rob" the nectar (Inouye 1980). In this case the benefit provided for the pollinator symbiont by the plant (nectar) is exploited by nectar robbers.

In fungus-growing ants there are two instances in which resource exchange is exploited by parasites. The nutrient benefit that fungal mutualists provide ants is directly exploited by the specialized mycoparasite *Escovopsis*. *Escovopsis* is a

Table 1. Characteristics of mutualist biology affected by parasitism.

Mutualist characteristic	Affected by parasitism	Model system Example	Reference
Association with other organisms	Yes	The presence of *Escovopsis* may drive the ants' mutualistic association with *Pseudonocardia*	(Currie, 2001a)
Behavior	Yes	Fungus-grooming, weeding, and infrabuccal pellet pile formation are all specialized behaviors to deter *Escovopsis* infection	(Currie and Stuart, 2001; Little et al. 2006)
Coevolution	Hypothesized	It has been suggested that *Escovopsis* may be driving the tight coevolution of the ant–cultivar mutualism	(Currie et al., 2003a)
Genetics	Unknown		
Geographic distribution	Unknown		
Mode of transmission	Hypothesized	It has been hypothesized that vertical transmission may be selected for in the presence of parasite pressure, but thus far there is no empirical evidence to support the hypothesis.	(Little, 2007)
Morphology	Yes	Fungus-growing ants have specialized crypts in their exoskeleton that house mutualistic bacteria	(Currie et al., 2006)
Physiology	Yes	Fungus-growing ants have specialized glands located below crypts that house bacteria, which hypothetically produce nutrients to support mutualistic bacteria	(Currie et al., 2006)
Reproduction	Unknown	*Escovopsis* directly utilizes the fungal nutrients that cultivars provide to ants. Black yeasts indirectly inhibit resource exchange between ants and their mutualistic bacteria by hypothetically inhibiting antibiotic production	(Little and Currie, 2008; Reynolds and Currie, 2004)
Resource exchange	Yes		

necrotrophic parasite, which obtains nutrients from the fungus garden (Reynolds and Currie, 2004). Garden sections infected with *Escovopsis* become unpalatable to ants, thus eliminating their benefit as nutrition for ants. Thus, resource exchange between ants and their mutualistic fungi is the target of exploitation.

Additionally, the relationship between ants and their mutualistic bacteria is exploited by black yeast that colocalize with *Pseudonocardia* on the surface of ants. Unlike *Escovopsis*, which directly parasitizes the ants' food source, black yeasts indirectly inhibit the effectiveness of bacterial antibiotic defense, which is the main benefit *Pseudonocardia* provide to ants. Black yeasts are capable of utilizing *Pseudonocardia* as a nutrient source, and as such it indirectly inhibits antibiotic protection of the fungus garden during *Escovopsis* infection (Little and Currie, 2008). Although indirect, black yeast exploitation of *Pseudonocardia* remains important to the attine ant–microbe symbiosis because the obligate resource exchange between ants and their cultivar is inhibited.

4.2. SYMBIONT TRANSMISSION

Symbiont transmission occurs in one of two ways: vertically, parent to offspring, or horizontally, between unrelated individuals. Bacterial endosymbionts are transmitted maternally during reproduction, whereas bacterial symbionts in other systems, such as the human gut, are acquired anew each generation from the environment. Vertical transmission is postulated as a mechanism that aligns the interests of mutualists, and promotes long-term stability of the relationships because it promotes partner fidelity and limits partner choice (Bull and Rice, 1991). Another reason vertical transmission of symbionts may be successful is that it provides greater protection to the symbiont from parasites. In the aforementioned case, vertical transmission is maternal via the mother's gametes, leaving little opportunity for exposure to exploitation by parasites. In other cases vertically transmitted symbionts are physically protected from parasites because they are housed in specialized host structures, such is the case for fungus-growing ants.

New fungus-growing ant nests are established by foundress queens who select a healthy piece of fungus garden to carry with them in their infrabuccal pocket during nuptial flights (Fig. 3; Hölldobler and Wilson, 1990; Weber, 1972). The queen will find a suitable male, mate, and then search for a suitable nesting site on the ground. Once she has dug a tunnel and cavern fit for her new nest, she regurgitates the fungal inoculum and manures it with fecal material, and adds small amounts of vegetative materials, until the first brood is reared 40–60 days later (Hölldobler and Wilson, 1990). New workers then begin foraging for vegetation or insect feces to support the growth of the fungal cultivar. Carriage of the cultivar in the infrabuccal pocket is good physical protection from parasites. Cultivar is also potentially protected by the bacterial symbiont during transmission as *Pseudonocardia* has been isolated from the infrabuccal pocket of several species (Little et al., 2006).

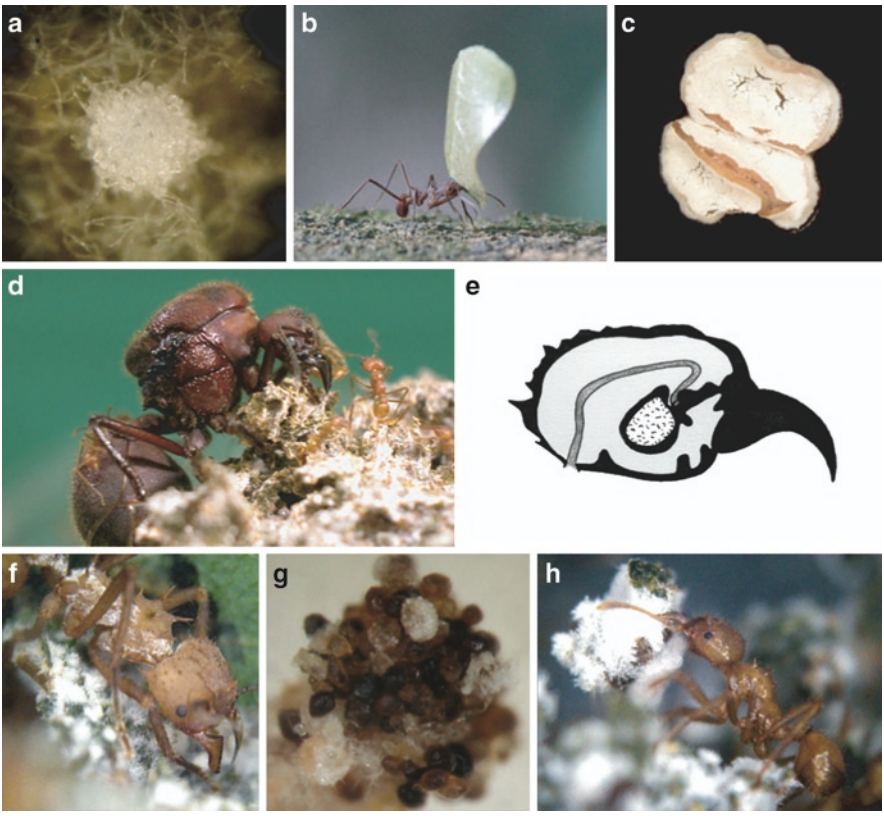

Figure 3. Features and behaviors of fungus-growing ant mutualists that are influenced by the stress of parasitism. (**a**) The nutrient resources cultivar provide to ants, shown here in special nutrient-rich hyphal structures called gongylidia, are the target of exploitation by the parasite *Escovopsis*. (**b**) The leaves ants used to feed their fungal mutualist are thoroughly licked clean before addition to the garden, thus preventing contamination by foreign microbes. (**c**) Bacterial mutualists, shown growing in culture, produce antibiotics specific to the specialized parasite *Escovopsis*. (**d**) Queen ants vertically transmit their fungal mutualist by carrying a small piece of the garden on their nuptial flight in their infrabuccal pocket (**e**). The fungal inoculum is used to start a new colony. To protect their gardens from *Escovopsis*, ants use three specialized behaviors: (**f**) grooming, in which ants remove parasitic hyphae from mutualist hyphae using their mandibles; (**g**) the infrabuccal pocket is also used to filter and sterilize foreign microbes found in the garden, then the contents of the pocket are expelled as pellets and piled near the garden; and (**h**) weeding, where ants excise chunks of unpalatable cultivar from their garden.

Theoretically, horizontally transmitted symbionts may be more likely to experience parasitic exploitation than vertically transmitted symbionts, because they are less protected from natural enemies. There are multiple examples of horizontally transmitted symbionts that are exploited by parasites (Inouye, 1980; Letourneau, 1990) and few examples of parasites that exploit vertically transmitted symbionts

(but see details on exploitation in the fungus-growing ant–microbe symbiosis). For example, ants are important symbiotic vectors for seed dispersal, but in some cases stick insects lay eggs that mimic the phenotypic features of seeds. Consequently, ants take the stick insect eggs to their nests, instead of seeds, where they are protected from environmental fluctuations and predators, until the eggs hatch, robbing the plant of its symbiotic benefit (Compton and Ware, 1991). It would be interesting to directly test the hypothesis that vertically transmitted symbionts suffer less from parasitism than their horizontally transmitted counterparts in a system (or phylogenetically similar systems) in which both methods of transmission occur.

4.3. BEHAVIORS THAT SUPPORT A SYMBIOTIC LIFESTYLE

To protect their mutualistic partners from the threats of natural enemies, many symbiotic hosts have developed behaviors to protect their mutualists (Fig. 3). A major threat to the health of fungus gardens is the potential invasion of the garden by microbial competitors and pathogens. Most species of attine ants maintain their fungus gardens in the soil, amidst myriad highly competitive and potentially parasitic microbes. The ants' fungal cultivar is also exposed to the ubiquitous bacteria and fungi present on the substrate added to the garden to support the growth of the cultivar. Accordingly, attines have developed various behaviors to protect their cultivars from microbial threats. When founding a new colony, queen ants usually place their fungal inoculum on either a small rootlet that they have exposed and clean with their mouth parts, or on a substrate that they have cleaned, such as their wings, thus effectively separating their nest from immediate contact with soil microbes (Fernandez-Marin et al., 2005). All substrates used to manure the fungus garden are thoroughly licked by worker ants, and most often fecal droplets, which have antimicrobial properties, are added to the substrate when it is incorporated into the garden matrix.

Fungus-growing ants have developed several behaviors to specifically protect their gardens from the specialized parasite *Escovopsis*. The infrabuccal pocket, a filtering device in the ants' oral cavity, is an integral part of the mechanisms that highly derived attines use to prevent infection of their fungus garden. Ants groom their garden, collecting debris and spores of *Escovopsis* in their infrabuccal pocket, the contents of which are later expelled in refuse heaps. A diverse collection of attine ants construct and maintain infrabuccal pellet piles in the vicinity of their gardens (Little et al., 2003). Microbial analyses of the ants' infrabuccal pellets reveal that the ants' infrabuccal pockets function as a specialized sterilization device. The pocket houses *Pseudonocardia* that kills spores of the garden parasite *Escovopsis* (Little et al., 2006). Leaf-cutter ants (the two most derived genera of fungus-growing ants) use additional specialized hygienic behaviours called fungus grooming and weeding to remove *Escovopsis* spores and infected garden material (Currie and Stuart, 2001). During fungus-grooming, ants separate parasitic hyphae and tissue from that of their fungus garden using their mandibles and dispose of the material via infrabuccal pellets. Additionally, waste–management tasks of the

fungus garden are partitioned to prevent the spread of potentially harmful microbes from the refuse into the garden (Hart and Ratnieks, 2001, 2002).

5. Balancing Symbiosis: Can Mutualism Be a Solution to Parasites, and Can Parasites Allow Mutualism to Persist?

One category of mutualistic interactions is "protective" mutualisms, in which one partner protects the other from a natural enemy (such as a predator, herbivore, pathogen, or parasite). The ant–plant mutualism is a classic example of protective mutualism. As mentioned earlier, some plants provide nutrient rewards to ants that protect them from herbivores. Thus, the mutualistic association with ants is an effective solution to herbivory for some plants. In the fungus-growing ant symbiosis, there are similar patterns. Because the mutualism is tripartite, there are two defensive mutualisms. First, there are ants that defend their fungus gardens from fungivores. In the absence of ant mutualists, the fungus garden would need an alternative (perhaps more costly) strategy to survive fungivory (e.g., sexual reproduction or chemical defenses). However, defense via mutualism has evolved as the primary defense. Second, bacterial mutualists defend the fungus garden from microfungal parasites through antibiosis. Without antibiotic protection, the fungus garden would suffer significant mortality and, consequently, so would their ant mutualists. In each case, forming mutualistic associations with phylogenetically distant partners has been an effective solution to the threats posed by natural enemies.

Another hypothesis is that the presence of a natural enemy, such as a parasite, may serve to maintain fidelity between mutualists. For example, in protective mutualisms the natural enemy can be viewed as a selective pressure that helps maintain the mutualism rather than to break it down. In the African Savannah, *Acacia* trees provide housing and nectar to mutualistic ants and, in return, ants deter herbivory of the *Acacia*. In the absence of large herbivores, the *Acacia* decrease their efforts to support symbiotic ants, which lead to the breakdown of the mutualism and, indirectly, a decrease in health of the *Acacia* (Palmer et al., 2008). In the case of protective mutualisms, the stress of parasitism (or herbivory or predation) allows the mutualism to persist throughout evolutionary time. Similarly, it seems possible that the external force of parasitism may play a role in stabilizing cooperation between fungus-growing ants and their fungal cultivars and fungus-growing ants and their bacterial mutualists. This would be possible if the selective pressure caused by the parasite(s) was such that it could align the selfish interests of ant–fungal and ant–bacterial partners.

6. Conclusions

Parasitism is a stress that shapes individual organisms, mutualistic pairs, and symbiotic webs of species. The specialized coevolved parasite of the ant–fungal mutualism is a strong selective factor that has resulted in specialized behavioral,

morphological, physiological, and symbiotic adaptations by fungus-growing ants. Parasitism is likely an agent of selection in many other beneficial symbioses, and I suspect that investigations into the presence of specialized and/or coevolved parasites in ancient mutualisms such as the fig-fig wasp, yucca-yucca moth, and fungus-growing termite systems would be both interesting and informative in this regard.

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Biodata of **Edouard Jurkevitch**, author of ***"Evolution and Consequences of Nutrition-based Symbioses in Insects: More Than Food Stress"***

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EVOLUTION AND CONSEQUENCES OF NUTRITION-BASED SYMBIOSES IN INSECTS: MORE THAN FOOD STRESS

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1. Introduction

"Stress" has many definitions but according to the endocrinologist Hans Selye, the first to use the word in a biological sense, this term referred to "the consequences of the failure of a human or animal to respond appropriately to emotional or physical threats to the organism, whether actual or imagined" (Selye, 1956). He also saw stress as a nonspecific response of the body to a demand. Other definitions are "the sum of the biological reactions to any adverse stimulus, physical, mental or emotional, internal or external, that tends to disturb the organism's homeostasis, should these compensating reactions be inadequate or inappropriate, they may lead to disorders. The term is also used to refer to the stimuli that elicit the reactions" (http://www.biology-online.org/dictionary/Stress) or "an organism's total response to environmental demands or pressures" (http://www.answers.com/topic/stress), or "any activity that puts pressures on living things and threatens to reduce their numbers or range" (http://www.pima.gov/cmo/sdcp/kids/gloss.html). These definitions may differ in their broadness, with the broader ones including biotic as well as abiotic parameters, or the notion of homeostasis. For example, abiotic changes may come in the form of temperature fluctuations that impose a burden on biological activities such as feeding or reproducing. Biological stresses may express, very commonly, as deficiencies in food supply. These deficiencies, in turn, may be quantitative (not enough food), qualitative (not enough nutritious food) or both.

It is stipulated that stress has the potential to negatively impact on organisms and that stress can cause a reduction in the organism's fitness. However, stress may also be positive, and enhance function (e.g. immunological reactions; Dhabhar and McEwen, 1999). One should also consider that the length of the period an organism is under "stress" may lead to different short-term and long-term outcomes. Transient stress may require transient adaptation while recurrent or permanent stress may exert direct selective pressure. These concepts are relevant to bacterial–insect interactions, and as will be

discussed, various types and magnitudes of stress may have led to evolutionary adaptations in these symbiotic systems.

The digestive tract is the organ or the suite of organs within which processes of food digestion and assimilation occur. Food enters this tract, its nutritional fraction is extracted, leaving waste products that are secreted. In most animals, and in all insects, energy, minerals, and essential (not endogenously synthesized) biochemicals are obtained only through the digestive tract. Usually, a large part of the digestive tract is composed of the intestine, which can be further compartmentalized. Nutrient assimilation starts with food intake and continues to the last segments of the hindgut, just ahead of waste secretion. In addition, bacteriomes, structures containing bacteriocytes (or mycetocytes), specialized cells holding symbiotic bacteria can be found in body cavities of aphids and other insects, or associated with a gut compartment, as in tsetse flies (*Glossina* sp.). These structures that may not necessarily be part of the digestive tract itself contain large numbers of bacterial symbionts that may contribute nutrients to their hosts (Fig. 1). Probably, all animals but a few have prokaryotic cells associated with their digestive tract or with the structures related to nutrient supply (bacteriocytes), but the composition of these communities is very variable.

Since our discussion will center on insect digestive tract- and bacteriocyte-associated bacteria, reproductive parasitism by prokaryotes such as *Wolbachia* will not be treated here.

2. A Synopsis On Insect Bacteria

Many insects bear specific prokaryotic partners. Endosymbionts are enclosed within bacteriocytes, and ectosymbionts remain extracellular. Moreover, symbionts are described as "primary" (P) if they are essential and as secondary (S) if they are dispensable or even deleterious (Hypša and Nováková, 2009). P-symbionts usually share long evolutionary histories with their hosts and are mainly transmitted vertically from parent to progeny, while S-symbionts appear to be more recently associated to their hosts, and are often transmitted horizontally (Moya et al., 2008). In general, heritable associations tend to become mutualistic, and in most cases, the host cannot survive without the endosymbiont, or the elimination of the endosymbiont has a deleterious effect (Baumann et al., 2006).

An S-symbiont can evolve to become an obligate partner, and establish a microbial consortium with the P-symbiont, as in the aphid *Cinara cedri* where the P-symbiont *Buchnera aphidicola* BCc co-exists in the bacteriome with the S-symbiont *Serratia symbiotica* SCc (Gomez-Valero et al., 2004). Further, long co-evolutionary relationships may lead to co-primary symbiosis as found in leafhoppers (Takiya et al., 2006) and sharpshooters (Wu et al., 2006). However, in *Acyrtosiphon pisum*, three S-symbionts (*Serratia symbiotica, Hamiltonella defensa,* and *Regiella insecticola*) with various effects on their hosts have been identified (Moran et al., 2005b, and see below).

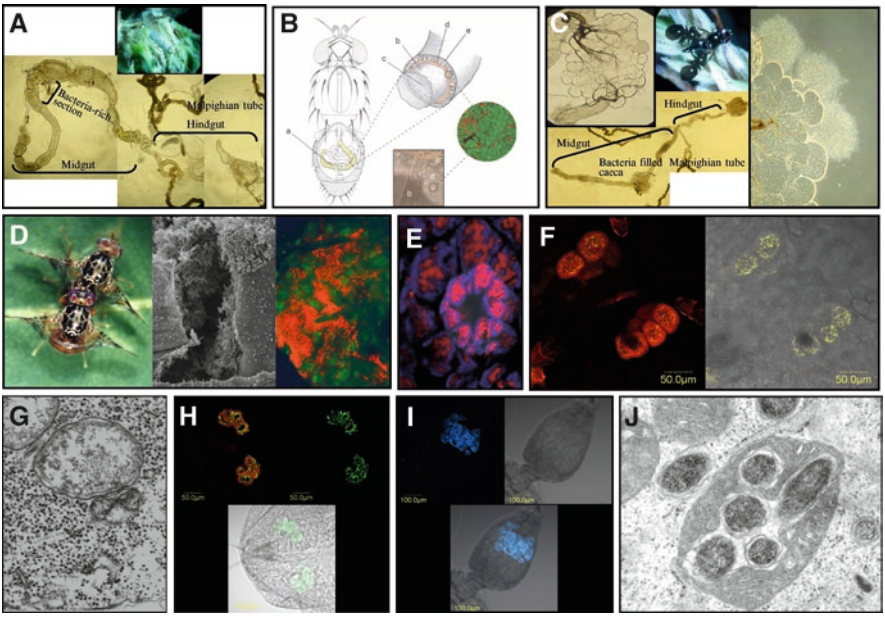

Figure 1. Associations between bacteria and insects are diverse and widespread. (**A**) *Tephritis praecox*. Lower picture: An isolated digestive system. Upper picture: an adult *T. praecox* (M. Ben Yosef, 2007, unpublished). (**B**) Gut symbionts in *Tephritis matricariae*. The bacteria are found in the yellow-colored midgut section (**a**). Resident bacteria are found in the interstitial gap (**d**) that runs between the peritrophic tube (**b**) and the outer midgut epithelium (**e**). Lumen (**c**). Fluorescence-based viability assay: live cells (green); dead cells (red) (Mazzon et al., 2008, by permission). (**C**) *Oxyaciura tibialis*. Upper left: midgut caeca. Middle: an *O. tibialis* adult. Below: an isolated digestive tract. Right: bursting caeca releasing bacterial cells (M. Ben Yosef, 2007, unpublished). (**D**) *Ceratitis capitata*, the Mediterranean fruit fly. Left: a mating pair. Center: a large mass, possibly a biofilm developing above the peritrophic membrane (C. Lauzon, California State University). Right: Fluorescent *in situ* hybridization of gut bacteria with a 16S rRNA probe (red) showing large conglomerates of metabolically active bacteria, and gut cells (green). (**E**) *In situ* hybridization with 16S rRNA probes showing symbiotic betaproteobacteria '*Candidatus* Tremblaya princeps' (blue) of *Planococcus citri* bearing gammaproteobacterial endosymbionts (red) (von Dohlen et al., 2001, by permission). (**F**) *In situ* hybridization with 16S rRNA probes showing the secondary *Arsenophonus* (yellow) symbiont together with the primary *Portiera* symbiont (red) in a whitefly (*Benesia tabaci*) larva (Y. Gotlieb, 2007, unpublished). (**G**) Transmission electron micrograph of '*Candidatus* Cardinium' in *Encarsia pergandiella* (E. Zchori Fein, 2007, unpublished). (**H**) As in (**F**) but with the secondary symbiont *Hamiltonella* (green), and *Portiera* (red). (**I**) *Portiera*, the whitefly's primary symbiont, visualized by 16S rRNA hybridization (blue), in a female. (**J**) Transmission electron micrograph of '*Candidatus* Midichloria mitochondrii', a bacterium that preys on mitochondria in *Ixodes* ticks (Luciano Sacchi, University of Pavia).

Tsetse flies harbor two symbionts, the *Wigglesworthia* P-symbionts, and the commensal S-symbiont *Sodalis glossinidius* (as well as *Wolbachia* but see above). The former are hosted in a bacteriome, and display concordant evolution with their host species (Chen et al., 1999) while the latter is found in midgut cells, the hemolymph, and other tissues, excluding the ovaries (Baumann et al., 2006) and

most likely represents a transition phase between a free-living to a mutualistic life style (Toh et al., 2006). It may also increase the flies' susceptibility to infection by the trypanosome parasite (Welburn and Maudlin, 1999). Both *Wigglesworthia* and *Sodalis* belong to the Enterobacteriaceae and are transmitted to the intrauterine progeny through the milk gland secretions of the viviparous female (Pais et al., 2008).

It thus can be difficult to draw a clear line between primary and secondary symbionts. Yet, primary symbionts are subjected to genome erosion, through an accelerated rate of molecular evolution, AT-biased nucleotide composition, the loss of open reading frames, including of DNA repair functions, and of regulatory sequences, resulting in genomes of strongly reduced sizes (McCutcheon and Moran, 2007; Tamas et al., 2002; Moran, 1996). This degeneracy may eventually lead to symbiont replacement (Lefevre et al., 2004; Koga et al., 2003). On the other hand, some P-symbionts have access to the germ line, and it is conceivable that they may become organelles in the specialized structures they inhabit. Sequence data of their host genomes may provide clues to such possible scenarios by revealing if gene transfer occurs from the bacterium to the host's nucleus. This was actually demonstrated by the massive lateral gene transfer between *Wolbachia* and several of its hosts (Hotopp et al., 2007). A recent paper shows that at least two genes found in the aphid *Acyrthosiphon pisum* originate in bacteria and that these genes are used to maintain the *Buchnera* symbiont (Nikoh and Nakabachi, 2009).

Most of the symbiotic lineages of P- as well as of S-symbionts can be traced to originate in Gammaproteobacteria, and more specifically in the Enterobacteriaceae (Hypša and Nováková, 2009). However, because of the peculiar evolution of P-symbiont genomes, the monophyly of these symbionts (which anyway does not imply a single unique transition from free-living to symbiotic status in the various insect (super)families where such symbionts are found) or their polyphyly cannot yet be established with certainty (Hypša and Nováková, 2009). In addition, a number of P- and S-symbionts belong to the Alphaproteobacteria and to the Bacteroidetes. It may be expected that the known diversity of symbionts will increase as more symbionts are characterized.

Many bacterial populations associated with insects such as termites, bees, tephritid fruit flies, and drosophilids have been defined as bacterial partners, but they were not attributed to the P- or S-symbiotic types. Maybe the best known of the symbiotic systems is the one between prokaryotes and termites, and their associations with protozoa in the gut of the insects. However, these fascinating relationships will not be covered here. For more details, the reader is invited to consult Brune (2006).

Some of the many genera that are comprised within the family Tephritidae are important agricultural pests (e.g. *Bacterocera, Ceratitis, Rhagoletis*, *Anastrepha,* and *Dacus*). The association of bacteria with tephritid fruit flies was first recognized at the beginning of the twentieth century with the description of a symbiotic relationship between the olive fly *Bactrocera oleae* and a microorganism (Petri, 1909). Albeit until recently, little knowledge was gained on the functional basis of the association between bacteria and fruit flies. The Mediterranean fruit fly (*Ceratitis capitata*) is a polyphagous, cosmopolitan, and invasive member of

this family. Its guts are heavily colonized by diverse *Enterobacteriaceae* species. Indeed, *Citrobacter freundii, Enterobacter* spp., *Klebsiella oxytoca, Pectobacterium cypripedii* quantitatively dominate the bacterial community during the whole life cycle of the insect (Behar et al., 2005, 2008a). These taxa appear to be widespread in Tephritidae as at least *Enterobacter*/*Pantoea* spp. and *Klebsiella oxytoca*/*pneumoniae* were found in 15 of 20 Tephritidae species tested (Behar et al., 2009). In contrast to the P- and many S-symbionts mentioned above, possibly the majority of the medfly gut community bacteria can live without their insect host and can be put in culture (A. Aharon and E. Jurkevitch, 2008, unpublished data).

An extraordinary and striking example of multilevel symbiosis is seen in mealybugs: The *Tremblaya princeps* betaproteobacterial P-symbiont, which is enclosed in bacteriomes, contains an gammaproteobacterium within its cytoplasm (von Dohlen et al., 2001). While in some of the cases mentioned above, functional relationships between the symbionts and their hosts have been shown, details of the latter relationship are not known. The diversity of bacteria–insect associations is exemplified in Fig. 1 that depicts intracellular symbionts of bacterial symbionts, predatory symbionts, recently described, and hitherto uncharacterized associations.

In the next sections, relationships between selected, well-known, and less-known gut bacteria–insect host systems will be examined through the prism of stress.

3. Abiotic Stress

Temperature is the sole abiotic stress that has been substantially studied in relation to insect-associated bacteria.

A point mutation in the *Buchnera aphidicola* P-symbiont that became fixed twice in laboratory lines of the aphid *Acyrthosiphon pisum* diminished the reproductive capabilities of the host under high temperatures but improved reproduction under cool conditions (Dunbar et al., 2007). This mutation was mapped to a homopolymeric region in the sequence of the heat-shock transcriptional promoter *ibpA* (Dunbar et al., 2007). This gene encodes a small heat-shock protein, the transcription of which is abolished in the mutant lines. When co-cladogenesis occurs between P-symbionts and their hosts, as between aphids and *Buchnera aphidicola* (Clark et al., 2000) and plataspid stinkbugs and their gut symbionts (Hosokawa et al., 2006), the bacterial genes can effectively be treated as alleles of the insect population, enabling population genetics analyses to be performed. About 20% of field populations of the aphid were shown to bear the mutation, revealing that it may be beneficial under certain environmental conditions.

Changes in *A. pisum* S-symbionts exhibited effects opposite to those of the *ibpA* mutation. Under heat shock, the number of *Buchnera* bacteriocytes decreases (Montllor et al., 2002; Russell and Moran, 2006). However, the presence of the S-symbionts *S. symbiotica* or *H. defensa* led to increased bacteriocyte persistence, thus presumably increasing *Buchnera* survival. In contrast, the *R. insecticola* S-symbiont did not provide such a benefit (Russell and Moran, 2006). The two

latter symbionts (*H. defensa* and *R. insecticola*) are closely related (Moran et al., 2005b). However, their effects on aphid fitness can be quite different, and appears to be related to environmental conditions. These studies suggest that maternally transmitted microbes play a role in adaptation to the abiotic environment. These effects also appear in the field, as secondary symbionts conferring heat tolerance have higher prevalence after periods of summer heat (Montllor et al., 2002) and are present in 100% of pea aphids in hot desert sites (Harmon et al., 2009), consistent with selection for heat-shock tolerance.

In the tsetse fly *Glossina morsitans* subsp. *morsitans*, from which the *Wigglesworthia* symbionts had been removed with antibiotics, an increase in temperature from 24°C to 27°C significantly increased the mortality rate over the controls (Pais et al., 2008).

The pentatomid stinkbug *Nezara viridula* transmits its gastric caeca bacterial symbionts by smearing them on the surface of eggs during oviposition.(Prado et al., 2009). When the insects were maintained at 20°C, 25°C, or 30°C, 100%, 84%, and 8.3% of the individuals contained symbionts, respectively. Surface sterilization of the egg mass resulted in increased nymphal developmental and generation times, in greater longevity and in the complete suppression of egg laying by their progeny. Whether compensatory mechanisms for the loss of symbionts exist at high temperature is not known.

Climate change may induce changes in the range, phenology, and interactions of insect populations with predators, prey, and plant resources (Parmesan, 2006; Walther et al., 2002). These first studies point at another level of interactions that should be considered in climate change evaluations: the bacterial symbiotic partners.

4. Nutrient Stress

The struggle for food may represent the dominant of the stresses that organisms are exposed to. Not only should an organism obtain a sufficient quantity of food, its quality should also be high to avoid deficiencies. However, starvation and nutrient deficiencies are common for most life forms, whether periodically or chronically. For example, many organisms have had to adapt to the oligotrophic conditions prevailing in soil and in the ocean. Similarly, insects that feed on plant sap experience shortages in essential amino acids. Phloem sap (the nutritive resource of aphids psyllids, whiteflies, mealybugs, and stinkbugs) is relatively rich in sugars but deficient in essential amino acids (Sandstrom and Pettersson, 1994), and xylem sap (used by sharpshooters) is nutrient poor, with even lower levels of carbohydrates and amino acids. Therefore, the utilization of such deficient resources entails, if possible, adaptations to, or ways to circumvent these limitations. In the latter case, shortages should be compensated for through synthesis (e.g. amino acids or vitamins), or through acquisition (e.g. nitrogen) of the missing nutrient by microbial symbionts. Nutrient upgrading was suggested by Buchner (1965) who observed that endosymbionts in insects are associated with deficient diets. Metabolic, gene expression, and genomic data, mainly obtained from studies of members of the *Sternorrhyncha* and

of the *Auchenorrhyncha,* support the assumption that P-symbionts provide essential, missing nutrients to their hosts. Stinkbug symbionts may also provide their hosts with nutritional supplements (Futkatsu and Hosokawa, 2009).

4.1. NUTRIENT COMPLEMENTATION

As exposed above, the ability of insects to feed on nutrient sources largely deficient in some essential nutrient that cannot be acquired by ingesting larger quantities of the resource is dependent on the presence of symbiotic partners. Many such interactions have been described (for an exhaustive list, see Braig et al., 2009). Plant sap feeding insects have a diet that is low in amino acids, including the essential amino acids that the insects need to incorporate preformed since they cannot synthesize them. The *Buchnera*–aphid relationship is the most studied of the plant-sap-based symbiotic interactions.

The use of synthetic diets has shown that some species of aphids can grow without added essential amino acids but that growth is strongly impaired by the addition of antibiotics to the medium, clearly suggesting that the bacterial symbionts provide the missing nutrients. In such experiments, the omission of any one essential amino acid in the diet reduced growth and survival of aposymbiotic *Myzus persicae* aphids, in comparison to symbiotic control insects (Mittler, 1971). Douglas and Prosser (1992) detected tryptophan synthetase activity in both symbiont-bearing pea aphids (*Acyrthosiphon pisum*) and in isolated *Buchnera* symbionts but not in chlortetracycline-treated aphids. Further support for the provision of essential amino acids by the symbionts was brought forward through the tracking of radio-labelled compounds. *Buchnera* symbionts of *M. persicae* reduced $^{35}S0_4^{2-}$ supplemented to a synthetic diet to hydrogen sulfide and incorporated it into methionine and cysteine, which could be traced to the insect tissue (Douglas 1988). *Acyrthosiphon pisum* supplemented with ^{14}C-labelled arginine, threonine, isoleucine and lysine or with ^{15}N-glutamine, and treated with rifampicin, showed a reduction in, or an elimination of, the synthesis of these and other essential amino acids (Liadouze et al. 1996; Sasaki and Ishikawa, 1991). Nakabachi and Ishikawa (1999) further demonstrated the importance of the symbiosis for provisioning riboflavin (vitamin B2).

The importance of nutrient complementation is further underlined by the phenomenon of symbiont replacement (see above). For example, *B. aphidicola* strain BCc can neither synthesize tryptophan nor riboflavin. At least, tryptophan biosynthesis appears to have been taken over by the *Serratia symbiotica* SCc secondary symbiont (Moya et al., 2008). This understanding was gained through the analysis of genomic data (Perez-Brocal et al., 2006). Further insights based on genome analyses are detailed in the next section.

Nutrient compensation may also be driving the association of members of the Tephritid fruit flies family and their gut microbiota. Many insects, including

the medfly and other tephritid flies, feed on nitrogen-deficient diets (Slansky, 1985; Waldbauer and Friedman, 1991) and it has been difficult to quantify where from and how much nitrogen they obtain (Waldbauer, 1968; Slansky, 1993). In effect, nitrogen is often a limited resource (Dixon and Kahn, 2004). The Enterobacteriaceae-dominated gut community of *Ceratitis capitata* is composed of taxa that are potentially diazotrophic. Behar et al. (2005) showed that all field-caught medflies carried high levels of diazotrophs in their guts. Moreover, it was shown that these bacteria express the *nifH* gene encoding for dinitrogenase reductase, the enzyme responsible for converting atmospheric dinitrogen into ammonia, *in vivo* in the insect gut. Finally, the enzymatic reaction was shown to occur in live flies as well as in surgically isolated guts, thus demonstrating that nitrogen is fixed in fruit fly guts. This supports the hypothesis that the bacteria provide this essential nutrient to their hosts. The mechanism by which this is achieved has yet to be shown. As mentioned in section II, many fruit flies and other insect species bear similar enterobacterial populations, or other potential diazotrophs (Kneip et al., 2009) and feed on carbon-rich, nitrogen-poor diets, suggesting that nitrogen fixation may be quite widespread in insects.

Finally, another study puts forward a role for *Wolbachia* in host-nutrition (Brownlie et al., 2009). *D. melanogaster* females infected with the *Wolbachia* ωMel strain and reared on low iron diets were more fecund than uninfected females. A similar advantage was shown with diets overloaded with iron. *Wolbachia* may then improve iron homeostasis over a large range of environmental conditions. Evolutionary pressure on this function may be reflected by the identification of positive selection on genes that encode components of the heme biosynthetic pathways in the *Wolbachia* ωMel genome (Brownlie et al., 2007). For a detailed and recent review on the impact of microbial symbionts on insect nutritional ecology, see Douglas (2009).

4.2. GENOME ANALYSES: CLUES FROM MISSING PARTS

Nutrient complementation can be further elucidated by genome analysis. At the time of writing (April 2009), there were 19 completed genomes of insect endosymbionts, and seven others were incomplete (http://www.genomesonline.org/), not including *Wolbachia* strains. Another few genomes appearing under "insect gut, cockroach hindgut, mosquito, or termite" were also completed or were under analysis.

The first sequenced insect endosymbiont genome was that of *Buchnera* sp. APS (Shigenobu et al., 2000). This genome comprises one circular chromosome 640,681 base pairs (bp)-long, encoding for 583 open reading frames, and two circular plasmids. It confirmed the previous knowledge on small genome size, AT content, and the limited abilities of *Buchnera* for DNA repair and recombination. Strikingly, and in accordance with physiological studies, genes for biosynthesis of the amino acids essential for the aphid host were present, whereas those for the

nonessential amino acids were almost completely missing. "Genomic dependence" of symbiont and host could be shown in that the precursors of some essential amino acids are nonessential amino acids, such as glutamate and aspartate, which the symbiont cannot synthesize. Similarly, conversion of pantothenate to pyruvate may be accomplished by the symbiont but it is missing the genes for its conversion to coenzyme A, a function that may be provided by the host.

In total, six *Buchnera* sp. genomes have been completed, and four have been published (Perez-Brocal et al., 2006; Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003). Excluding *B. aphidicola* strain BCc, the genome of which is greatly degenerated, encoding 362 ORFs and having lost most of its metabolic functions, the size and gene content of the other genomes differ only slightly. This indicates that variations in host lifestyle are differentially affecting the need for some genes (Gil et al., 2006). These interpretations were substantially confirmed by the use of transcriptomics in an analysis of bacteriocytes mRNAs by Nakabachi et al. (2005). Genes for amino acid metabolism, including those for biosynthesis of amino acids that *Buchnera* cannot produce, and those for the utilization of amino acids that *Buchnera* can synthesize were strongly enhanced in the bacteriocyte, as well as genes encoding for the transport of amino acids.

Another compelling feature of the *Buchnera* genomes is their lack of genes coding for phospholipid biosynthesis, except that for cardiolipin synthetase, implying that *Buchnera* is unable to synthesize its own cell membrane. These components should therefore be supplied by the host, and upregulation of a Ras-like Rab GTPase, which regulates vesicular transport of proteins and lipids between compartments in eukaryotic cells supports this assumption (Nakabachi et al., 2005).

Another informative absence is that of regulatory systems, including two-component signaling systems, leader sequences, transcriptional attenuators, and catabolite repressor proteins, with only two sigma factors and *dnaA* being present (Shigenobu et al., 2000). Moran et al. (2005a) demonstrated this lack of regulatory capabilities in a transcriptomic study of the *Buchnera* genome. Only the *metE* gene, a major component in the methionine biosynthesis pathway, was regulated. This gene is also the only one known for which an ancestral regulator is retained.

These alterations, brought about by a Muller's ratchet-like process leading to genome reduction (Moran, 1996), may be seen as beneficial to the host, bringing the symbiont "under control" through "domestication," thereby increasing homeostatic control of host metabolism. Alterations of these regulatory properties bring about a shift from energy conservation in the presence of end-products to overproduction of metabolites such as essential amino acids, even under conditions where the end-products accumulate (Baumann, 2005). On the other hand, and although regulation can still be achieved at other levels (transcript longevity, translation, protein stability), the irreversible loss of transcriptional regulators surely constrains the flexibility of the bacterial symbiont to react to environmental fluctuations, possibly reducing allostatic control.

A fascinating story has been uncovered by genome, phylogenetic and distribution analyses of "*Candidatus* Sulcia muelleri" (Bacteroidetes) and of "*Candidatus*

Baumannia cicadellinicola" (Gammaproteobacteria) symbionts associated with sharpshooters. Their long-term coinheritance during the evolution of their hosts has made them co-primary symbionts (Takiya et al., 2006). Their genomes point at nutritional complementarity as the symbionts provide nutrients lacking from the extremely poor xylem sap-based nutrition of their host. This complementarity is all the more striking as the functions are not perfectly partitioned between the two genomes. For example, *S. muelleri*'s tiny genome (245 kbp) encodes the biosynthetic functions of most essential amino acids but it is unable to make neither histidine nor cysteine. In turn, *B. cicadellinicola*'s genome (also small, at 646 kbp) retains the biosynthetic pathways for amino acids lacking from *Sulcia*, such as histidine and cysteine as well those required for vitamin synthesis, but it cannot make homoserine, a capacity found in *Sulcia* (Wu et al., 2006; McCutcheon and Moran, 2007). These findings led Moran (2007) to suggest that *Sulcia* started to feed on primitive vascular plants during the late Permian and only later, during the Eocene, was *Baumania* acquired at approximately the same time as xylem sap began to be exploited.

Nutrient complementation also appears to be of central importance in the relationship between tsetse flies (Diptera: Glossinidae) and their *Wigglesworthia* P-symbionts (Nogge and Ritz, 1982). Tsetse flies feed on blood, a resource low in vitamins of the B complex as well as in several cofactors. Genetic, physiological, and metabolic studies have established that *Wigglesworthia* exhibits "classical" features of an obligate endosymbiont, such as a small genome size (700 kbp<), high AT bias, and loss of repair functions (Akman et al., 2002). Its genome has retained many biosynthetic pathways required for cofactor and vitamin biosynthesis, with about 62 genes involved in the biosynthesis of cofactors, prosthetic groups, and carriers (Akman et al., 2002; Zientz et al., 2004). On the other hand, it has lost most of its amino acid biosynthetic pathways. Consistent with this lack of biosynthetic capabilities, amino acid transporters are among the few transporters that were kept in the genome (Zientz et al., 2004). The tsetse's S-symbiont, *S. glossinidius,* exhibits large genetic erosion with a reduced coding capacity of 51%. It has apparently retained many of the capabilities of free-living bacteria, such as functional pathways for glycolysis, gluconeogenesis, the tricarboxylic acid cycle, the pentose phosphate pathway, as well as parts of a type III secretion system. However, it has few phosphoenolpyruvate sugar transport systems and no galactosidase and glucosidase genes, possibly because of the low carbohydrate content of the blood-based diet of its hosts. On the other hand, pathways required for the synthesis of all amino acids except alanine are predicted, while many of the genes required for amino acid degradation are missing. (Toh et al., 2006).

5. Direct and Indirect Effects of Nutrition-based Symbiosis on Host Life History

5.1. REPRODUCTIVE AND ONTOGENETIC EFFECTS

The use of antibiotics to remove bacterial partners from insects to study their effect on their host is widespread (Table 1 in Wilkinson, 1998). Aposymbiotic

aphids grown on a diet lacking essential nutrients exhibited a considerable reduction in larval growth, and reproduced very poorly (Douglas, 1996; Mittler, 1971; Sasaki et al., 1991). In the tsetse, elimination of intracellular *Wigglesworthia* compromised fecundity, and *Sodalis* removal decreased longevity in the progeny (Pais et al., 2008). In an attempt to understand the contribution of gut bacteria to Mediterranean fruit fly fitness, Ben Yosef et al. (2008b) separately treated male and female flies after eclosion with a mixture of piperacillin and ciprofloxacin, thereby strongly reducing gut bacterial populations. In addition, the flies were fed a diet containing peptides, sugar and minerals, or a sugar diet, lacking peptides. Secondary effects of the antibiotic treatment were few, as diet consumption, dry weight, or nutritional reserves did not differ between antibiotic-treated and control flies fed with the same diet. Yet, females feeding on the full diet without antibiotics exhibited increased lipid levels. Significant alterations in reproductive behavior were detected following exposure to antibiotics in females fed the sugar diet and in males fed the full diet. In the former, the oviposition rate was accelerated. In the latter, the latency to mate was increased. A similar setting was used to test the effect of the gut bacteria on longevity (Ben Yosef et al., 2008a). Nutritionally stressed flies (i.e. fed a sugar diet) treated with antibiotics enjoyed a prolonged lifespan but no such effect was seen on flies fed a full diet.

The large and diverse community of Enterobacteriaceae associated with the medfly was shown to be life-stage-dependent (Behar et al., 2008a). During the larval stages, pectinolytic *K. oxytoca* and *Pectobacterium* spp. dominated. Moreover, the majority of the larva's bacterial community was diazotrophic as well as pectinolytic. At least part of this community was shown to be inoculated to the fruit and to grow within it, causing its decay, and to be transferred to the next generation of flies. Bacteria-assisted pectinolysis may rapidly provide additional, readily metabolizable sugars for the growing larva, helping it graduate to the next ontogenetic stage. Furthermore, nitrogen fixation may occur in larvae as evidenced by detection of *nifH* transcripts (Behar et al., 2008a). Pectinolysis may thus provide an ample supply of readily available carbohydrates to fuel the energy-demanding nitrogen-fixation process. Lastly, maceration of the fruit cell walls may reduce frictional forces within the fruit, reducing the energetic costs of movements. These findings suggest a complex relationship between the medfly and its gut bacteria. The fly may benefit from a supply of carbon, nitrogen, and possibly other factors, and the bacteria from a secure and stable environment, periodic exposure to large resources, and dissemination. At least under certain circumstances, a cost may be incurred to the fly.

In plastatid stinkbugs, the obligate gut symbiont "*Candidatus* Ishikawaella capsulata" (Gammaproteobacteria) is vertically transmitted through a bacterial capsule that is laid along with the eggs, and consumed by the nymphs as they emerge (Hosokawa et al., 2006). Removal of the capsule before egg hatching resulted in aposymbiotic nymphs, in a strong reduction of adult emergence, and in nonreproducing adults with abnormal phenotypes (Hosokawa et al., 2006). Moreover, removal of symbiotic capsules altered the behavior of nymphs from a

resting behavior as found in normal nymphs to a wandering behavior (Hosokawa et al., 2008). In *Nezara viridula*, symbiont acquisition occurs during the first instar. After hatching, nymphs aggregate, and this behavior may be at least driven by the need to acquire their symbionts from the eggs' surface (Prado et al., 2009). Thus, behavior may contribute to the maintenance of these mutualistic relationships, and it may have evolved for this purpose (Fukatsu and Hosokawa, 2009).

5.2. METABOLIC EFFECTS

The exposure of aphids to antibiotic-containing diets may not only remove the symbiotic partners and thereby their contribution to nutrition, it may also help uncover contributions of the symbionts to homeostasis control. Wilkinson's study (1998) showed that a number of parameters such as the mitochondrial complement, the assimilation of dietary amino acids, the incorporation of amino acids into proteins, osmoregulation, feeding rate, and the capacity to penetrate plant tissues were not impaired in aposymbiotic aphids. It was concluded that the antibiotic itself was not the cause of the general malaise associated with these insects, such as their low growth rates and sterility. Rather, cumulative secondary effects of symbiont loss may be the source of these changes. As an example of such effects, the total concentration of amino acids (mainly glutamine –Wilkinson and Douglas, 1995) is higher in aposymbiotic than in symbiotic pea aphids, but the level of essential amino acids is lower (Douglas, 1996). The latter may obviously result from the absence of supply by the symbiont. Lower availability of essential amino acids may slow protein synthesis in aposymbiotic aphids, resulting in total higher levels of free amino acids (Douglas and Prosser, 1992). While symbionts may act as a sink for ammonia, in contrast, in aposymbiotic insects, waste ammonia is incorporated into glutamine as a means of detoxification. However, high ammonia may also result from an increase in ammonia production from the degradation of amino acids in these insects and not from bacteria acting as sink (Wilkinson, 1998).

Recently, Pais et al. (2008) investigated the impact of *Wigglesworthia* removal from its *Glossina* host. In addition to the effects already mentioned in previous sections (reproduction, longevity, temperature sensitivity), *Wigglesworthia* was shown to improve the digestion of hemoglobin and to increase trypanosome vectorial competence in older adults, suggesting that it is highly integrated with its host, impacting on important physiological processes.

Another interesting example is metabolism of GroEL, a ubiquitous chaperone protein. Much as in other endosymbionts and pathogens, GroEL is constitutively expressed at high levels in *Buchnera* (Baumann et al., 1996). Being a chaperone, GroEL binds to and stabilizes newly translated polypeptides, mediates their functional folding and assembly, as well as repairs damaged proteins in an ATP-dependent manner (Hartl, 1996). It has been suggested, using *Escherichia coli* as an experimental proxy, that high levels of GroEL in the symbiont may counterbalance the deleterious effects of genome erosion (Fares et al., 2002).

Buchnera GroEL is not restricted to the bacteriocyte, it is also found in the saliva (Filichkin et al., 1997) as well as in the hemolymph of symbiotic insects (Filichkin et al., 1994), where its presence was demonstrated to be essential for the persistence of luteoviruses (van den Heuvel et al., 1997). It may be used as a carrier protein for transferring viral particles from the hemocoel to the accessory salivary gland and, finally, into the saliva (Filichkin et al., 1997). It should be noted that in the whitefly (*Bemisia tabaci*), GroEL appears to originate in the S-endosymbiont (Morin et al., 1999). The high level of GroEL expression both in P- and S-symbionts suggests an essential role. Whatever its role within symbionts, its presence in the hemolymph may serve other purposes than being a helper protein for infectious viruses. Clearly, aposymbiotic aphids are not "merely aphids with their symbionts removed" (Wilkinson, 1998).

5.3. RESISTANCE AGAINST PATHOGENS AND PARASITES

Nakabachi et al. (2003) explored whether aposymbiotic aphids were colonized by other microorganisms than their known symbionts. They found that high levels of histidine present in aposymbiotic aphids were correlated to high levels of fungi and bacteria. Half of the identified bacteria (through 16S rRNA gene sequencing) belonged to the gammaproteobacteria – a phylum known to produce large amounts of histidine in spoiled food (Nakabachi et al. 2003), 24% belonged to the Alphaproteobacteria (a taxon containing many symbionts, parasites, and pathogens) and 15% clustered within the Bacteroidetes (previously the CFB group). In control symbiotic insects, almost all of the bacteria present appeared to be the P-symbiont, as determined by real-time quantitative-PCR. *Buchnera* spp. P-symbionts form extremely dominant populations both in natural and laboratory populations of aphids but secondary/accessory, more diverse, and variable bacteria can also be found, depending on the aphid species or line. However, this diversity was found to be rather limited, containing very few taxa belonging almost exclusively to the gamma and the alpha proteobacteria (Haynes et al., 2003). Harada et al. (1996) surgically removed the guts of symbiotic aphids, isolated bacterial and fungal colonies, and analyzed their phylogeny with using the 16S and 18S rRNA genes, respectively. Different fungi and an even more diverse bacterial community were detected. The dominant bacterial group belonged to the Enterobacteriaceae, and its members were closely related to *Enterobacter, Serratia, Klebsiella,* and *Erwinia*. These taxa are often found in insect guts, for example in various fruit flies (Behar et al., 2008a; Lauzon et al., 2000; Murphy et al., 1994), in termites (Ohkuma et al., 1999), and in trips (de Vries et al., 2001) (also see above). The discrepancy between the Harada et al. (1996) and the Haynes (2003) studies may be due to the use of laboratory lines vs. natural populations or to the analytical methods: in the former, bacteria were isolated and characterized, while in the latter, noncultured bacteria were detected by analyzing 16S rRNA gene fingerprints of PCR amplicons obtained from total insect DNA. Since bacteriocyte-associated symbionts are largely dominant, other

populations may not be detected, as PCR fingerprinting-based methods usually do not enable the detection of minor populations (Muyzer and Smalla, 1998). It is possible that gut bacteria are transient, i.e. ingested with food and lost, as aphids raised on sterile food appear not to contain any gut bacterium (Douglas, 1998). If this is indeed the case, it would be interesting to understand how the aphid prevents colonization of its intestine.

However, the presence of secondary endocellular symbionts in many aphid lineages, including yeast-like symbionts, suggest that the aphid is exposed to exogenous microorganisms that can gain hold within the insect. Certainly, pathogenic microbes, including microoganisms that may interfere with the symbiotic relationship, have a negative impact on the host. However, gut bacteria may represent a source of genes – probably through lateral gene transfer to S-symbionts, as P-symbionts are genetically isolated- or a reservoir of novel secondary symbionts from which new functions as well as "replacements" for defective P-symbionts may be selected. The large increase in microbial diversity in aposymbiotic insects hints at a defense mechanism dependent on the presence of *Buchnera* symbionts to control proliferation of foreign microorganisms (Nakabachi et al., 2003). In this respect, the situation in whiteflies is very interesting. In addition to the *Portiera* P-symbiont, many other species can be found within the bacteriocyte. These include *Hamiltonella, Arsenophonus, Cardinium, Wolbachia*, and *Rickettsia* (Gottlieb et al., 2008; Fig. 1). In contrast to the situation in aphids, these bacteria are located together with the primary symbiont within the bacteriocyte where they occupy different niches. Their functions are unknown but the *Rickettsia*-like symbionts were shown to increase their host's sensitivity to insecticides (Kontsedalov et al., 2008).

Interestingly, the most abundant transcripts in *A. pisum* bacteriocytes were significantly similar to invertebrate-type lysozymes (Nakabachi et al., 2005), enzymes that degrade the sugar backbone of bacterial peptidoglycan and are thus antimicrobial agents. Early microscopic studies had shown that *Buchnera* as well as secondary symbionts were degraded in bacteriocytes by what appeared to be lysosomal activity (Hinde, 1971; Griffiths and Beck, 1973). This mechanism may help the aphid host control homeostasis as it can regulate the size of its symbiont populations, remove dead symbionts, eliminate microbial intruders, or harvest *Buchnera* cells for resource allocation (Nakabachi, 2009).

There is evidence to suggest that bacteriome and gut bacteria modulate insect host responses towards pathogens and parasites. The vertically transmitted aphid secondary symbiont *Regiella insecticola* was shown to increase its host's resistance to the entomopathogenic fungus *Pandora neoaphidis* as well as to lower the rate of transmission of the fungus (Scarborough et al., 2005). How this response is mediated in not known. Other studies have shown that the structure of insect gut microbiota has an important role in keeping their host's health. Harada et al. (1996) observed that a "bacterium X," when reinfected in microbe-free aphids grew indefinitely in the host's gut causing its death. Since aphids found in nature contained a large number of bacterium X but were normal, it

was supposed that the balance of various groups of bacteria may be very important in keeping the host insect healthy. In the medfly, although they clearly dominate, Enterobacteriaceae are not the only bacteria found in the insect's gut. By selectively removing enterobacterial 16S rRNA gene templates from DNA extracted from medfly guts, Behar et al. (2008b) detected a very low level of pseudomonads. Representatives were isolated on a specific medium. An isolated *P. aeruginosa* strain was shown to strongly reduce fly longevity when introduced into adults by feeding (10 cells.μl^{-1}). In contrast, a mixture of enterobacteria at a much higher level (10^5 cells. μl^{-1}) extended the fly's life. In a recent study (Ryu et al., 2008), the interplay between the host defense immune system and the composition of the commensal gut community was deciphered in *Drosophila melanogaster*. It was shown that the inhibition of the homeobox gene *Caudal* led to an increase in antimicrobial peptides. Normally, dominant populations of *Acetobacteraceae* EW911 were reduced, while those of *Gluconobacter* sp. strain EW707, normally found at low levels, were strongly increased, leading to gut cell apoptosis and host mortality.

The role of symbionts in increasing resistance against deleterious organisms is not limited to offensive microorganisms. *A. pisum*'s secondary symbionts *Serratia symbiotica* and *Hamiltonella defensa* were shown to confer to their host increased resistance against the parasitoid *Aphidius ervi* (Oliver et al., 2003). The degree of resistance achieved not only varied between the different symbionts but also between isolates (tested with *H. defensa*; Oliver et al., 2005). These latter differences may be due to the presence of lysogenic bacteriophages in the bacterium's genome (Oliver et al., 2005).

In conclusion, the microbiota associated with the gut/bacteriocyte affects the heath and the resistance of the host to pathogens. These effects, which are brought about by alterations of immune system responses, other signaling pathways and metabolic functions appear to play an important role in keeping homeostasis.

5.4. NICHE EFFECTS

Many symbionts provide nutrient complementation and thus they may have an effect on the niche their host occupies. The evolution of nutrient-complementation-based symbiotic relationship may have occurred as a response to phloem sap or blood becoming nutritionally deficient to limit exploitation by parasites (Perotti et al., 2009). Alternatively, a loss in the ability to synthesize, take up, or utilize certain nutrients from a rich diet and the acquisition of complementing symbionts could enable the exploitation of deficient diets such as phloem or blood (Perotti et al., 2009). A more subtle effect may come as changes or expansion of host range. The presence of the secondary symbiont *Regiella* was shown to increase the fitness of its host aphid *A. pisum* on white clover (*Trifolium repens*) all the while not decreasing its fitness on vetch (*Vicia sativa*) when compared to aphids lacking the symbiont (Tsuchida et al., 2004). A putative mechanism explaining

these results is detoxification by *Regiella* of bioactive secondary metabolites present in white clover (Tsuchida et al., 2009). The main host plants of the stink-bugs *Megacopta punctatissima* and *M. cribaria* are the wild vines, *Pueraria lobata* and *P. montana*, respectively. In addition, *M. punctatissima* can infest soybean, pea, and other legumes while *M. cribaria* is barely known to do so (Fukatsu and Hosokawa, 2009). Indeed, on soybean, *M. cribaria*'s egg hatch rate was much lower than that of *M. punctatissima*. However, when the symbiont-containing capsules were exchanged between egg masses of the two species, egg hatch rates were inverted, with *M. punctatissima* nymphs failing to escape from the egg, and *M. cribaria* hatching at a high rate (Fukatsu and Hosokawa, 2009). Whether this difference stems from the ability of the *M. punctatissima*'s symbiont to detoxify a plant derived-toxic compound, to provide a specific but lacking nutrient, or from a factor enabling utilization of a nutrient is not known.

Other ecological effects can affect the range of an insect host through their interrelation with symbionts. Chandler et al. (2008) demonstrated that the aphid *Aphis fabae*'s low growth rate on *Lamium purpureum*, which may be due to low nitrogen content of its phloem, was exacerbated by the presence of secondary symbionts. It is noteworthy that the effect was not specific to a single symbiont species and that it was expressed as an abnormally high density of the bacteria within the aphids.

These examples show that the presence or the absence of specific symbionts may have significant adaptive consequences by altering the biotic and abiotic ranges in which the host insect can perform.

6. Concluding Remarks

Insect and their associated gut or bacteriocyte bacteria have been partners for eons. In some cases, including the aphid-microbial symbionts models, phylogenetic, anatomical, ecological, physiology, and molecular studies have yielded a lot of insights and understanding on the evolution and the functioning of their relationships. The biological systems that are investigated are relevant to important human activities such as agriculture, health, and ecosystem preservation.

We indeed have learned a lot and, this is not breaking news, we still have to learn a lot. To paraphrase Copernicus, we do not even know what we do not know. A perspective of what we do not know may be grasped in the following figures: Over one million insect species have been described, (Chapman, 2006, http://www.environment.gov.au/biodiversity/abrs/publications/other/species-numbers/index.html). While it is anyone's guess how many more species there are, possibly over ten million insect species may live on our planet. At least 10% of the insects and other arthropods are known to harbor symbiotic relationships, many of them co-evolving, and therefore composed of distinct microbial species (Amann et al., 1995).

Yet, the following questions may be addressed in the near future: What are the specificities that render the Gammaproteobacteria, and maybe more

specifically the Enterobacteriaceae such good colonizers of insect digestive systems? What are the reservoirs new bacteria associated with guts are selected from, how is this achieved? Are there "flows" of genes or of symbionts between conspecific and heterospecific insects sharing the same habitats, e.g. in aggregating ovipositioning insects, or at larval growth sites shared by different insect species? Finally, signaling between gut bacteria, and between them and their hosts, is still a frontier. Yet, great advances have been made in this domain, with classical models in which the host is genetically amenable, like *Drosophila.* With the newest high throughput sequencing technologies becoming largely available, one can be certain that it will not be long before signaling and molecular responses in other insect-symbiont models are also much better appreciated.

I would like to end with a truly anthropocentric perspective, i.e. one which projects human values on natural phenomena: there is great beauty in these tiny animals living with even tinier organisms. The beauty of nature, the thoughtfulness of observation, the intellectual pleasure of deduction, and this strange, fleeting feeling of getting a glimpse at "how it works."

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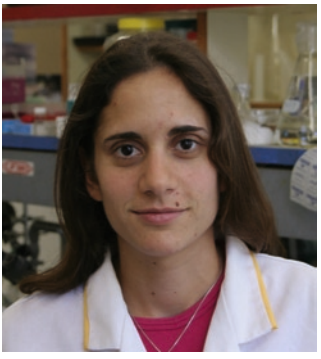

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THREE IN A BOAT: HOST-PLANT, INSECT HERBIVORE, AND FUNGAL ENTOMOPATHOGEN

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"We must not think of the things we could do with, but only of the things that we can't do without"

-Jerome K. Jerome

1. Prologue

This review of open-ended tritrophic relations deals with the interactions of several major pathogenic fungal species and their insect hosts, resident on different plants and how such interactions are affected by the physiology of these organisms in a changing environment.

To survive, develop, and accumulate essential resources for functioning under such constraints, insect herbivores have co-evolved sensory interactions with plant-derived semiochemicals that may be detrimental to other potential recipients. Appropriate hosts are then those that additionally supply the recipient insects with essential nutrients for developmental processes, while secondary metabolites may be co-opted unchanged, or undergo further metabolism to products that are used by the insect for protection or communication. The insect species may be a "generalist" (of broad host specificity) or a specialist (of restricted host specificity) and this impacts on how the specific insect will exploit primary nutrients and tolerate secondary compounds. To compound matters even more, the physiology of the host-plant and recipient insect are each independently affected by abiotic environmental factors, such as photoperiod, temperature, and moisture. These environmental variables may compromise the response of the insect directly, or as a consequence of changes in the chemical composition of the host plant.

The interactions of entomopathogenic fungi with their potential hosts are not less complex. Fungal entomopathogenicity has apparently evolved independently in many major taxa of fungi. This may explain the variation in degree of

specificity and total or only partial reliance on the presence of an appropriate host for their survival. In any case, once encountering a suitable host, an intricate relationship commences between the insect and the fungus, beginning with invasion, then evasion, culminating in proliferation and dispersion of conidia to new potential hosts. Such convergent evolution may also be the reason that evasion from host defence by the mode of avoiding recognition as "nonself," a strategy employed by many entomopathogenic fungi, is based on variable characteristics amongst the various species.

Finally, this sequence of events may be affected, directly or indirectly, by an array of biotic and abiotic factors, including the insect's host plant.

2. The Dynamics of Plant-Derived Stimuli on Insect Herbivory

Plants "cross-talk" with other plants, with insect herbivores that feed on them and subsequently with insect parasites, predators, or pathogens via external signals, composed of high-molecular-mass nonvolatile cuticular components and low-molecular-mass volatile semiochemicals (Despres et al., 2007). Insects confront a heterogeneous mixture of secondary plant compounds in their natural environment, which impact on their behavior and thus determine host-choice, either encouraging, or conversely preventing or terminating the existing plant-herbivore interaction, thereby ultimately affecting the fitness of the insect in question. Tritrophic interactions are dependent on internalization of plant compounds by the insect, which may be sequestered and recycled for use "as is," or derivatized to metabolites involved in subsequent biotic interactions between the insect and its parasites, predators, or pathogens. The chemical diversity of plant constituents and their effects on insect development within a changing habitat have been reviewed extensively (Baldwin and Preston, 1999; Bennett and Wallsgrove, 1994; Despres et al., 2007; Wink, 2003).

2.1. PLANT AND INSECT SURFACE CUTICULAR HYDROCARBONS–COMPARATIVE CHEMISTRY AND INTERACTIVE PHYSIOLOGY

Arthropods and higher plants share considerable similarity in the composition of their respective cuticles (Hadley, 1991). Nonpolar lipids and high molecular mass polar lipids are dominant surface components on both plant and insect epicuticles (Buckner, 1993; Howard, 1993; Nelson, 1993). The plant epicuticular surface lipids serve as an important interface in trophic interactions by affecting insect herbivory (Eigenbrode and Espelie, 1995; Jenks and Ashworth, 1999). Although germination of entomopathogenic fungi normally occurs on the integument of insects, adventitious entomopathogen persistence and germination on the plant leaf on which it has landed passively can occasionally occur, but is confronted by

leaf surface cuticular composition (Meekes et al., 2000). Entomopathogen fungal development is often aborted on plants, but it should be noted that secondary sporulation by entomopathogenic fungi, whether on plant surfaces or even on arthropod cuticles, is actually a very important event for many of them, perhaps an obligatory developmental process, with the secondary conidium being the primary infective unit. This is the case for most species of *Entomophthora* sensu stricto (Eilenberg et al., 1995). Presumably, direct landing of spores on the cuticle of a potentially susceptible host, rather than on a plant surface, should be in the best interests of entomopathogenic fungi.

2.2. SECONDARY PLANT SUBSTANCES – THEIR CHEMISTRY AND EFFECT ON INSECT BEHAVIOR AND DEVELOPMENT

In some cases, plant-derived lipophilic semiochemicals are secreted onto the plant surface, in others they accumulate within leaf surface trichomes and are released thereafter, in some others they are produced in specialized glands or tissues and subsequently released as a consequence of damage to the plant tissue during herbivore foraging. Volatile secondary metabolites provide host-plant information to insect herbivores from a distance. For example, the flavonoid aglycone quercetin and derivatives accumulate in the glandular trichomes of *Nicotiana attenuata* and are released on to the leaf surface. Quercetin is more abundant on younger leaves and is a feeding attractant to the mirid homopteran *Tupiocoris notatus* (Roda et al., 2003). Much research has focused on describing the diversity of plant chemicals and their effects on insect herbivores (Blande et al., 2007; Kaplan et al., 2008; Staudt and Lhoutellier, 2007; Ton et al., 2007; van Dam and Poppy, 2008; Wei et al., 2007).

Structurally related secondary metabolites are often coincident with related plant taxa, but the response and host distribution of insects of a certain insect taxon is not necessarily coincident with specific plant taxa (Jermy, 1984). This is especially true in the case of "generalist" insects, where host choice is initially affected by orientation to common plant volatiles of broad distribution in plants, and nonchoice, by the levels of secondary metabolites that deter or are detrimental to feeding and digestion. More clearly defined relations occur in cases of "specialist" insects that are, by definition, mono- or oligophagous. This does not mean that specialists always appear in defined taxa with common structurally related secondary metabolites: they may, but in many cases, do not. Such functional capacities dictate host selection.

Examples of specialist insect interrelations with plants, evoked by taxon-specific semiochemicals are:

1. Crucifer-derived volatile isothiocyanate aglycones that attract specialist herbivores of the same plant family (Bartlet et al., 1997; Johnson et al., 2005; Mewis et al., 2002) and similar compounds may attract or otherwise affect specialists on other plant taxa as well as some generalists (Lahtinen et al., 2004;

Seibt et al., 2000; Weissenberg et al., 1998). They are released from nonvolatile glucosinolate storage reserves, the latter also functioning as feeding stimulants to oligophagous herbivorous insects with chewing mouthparts (Griffiths et al., 2001; Hopkins et al., 1997; Renwick et al., 1992).
2. Presumptive sequential evolution of bruchid beetles in congruence with legume seeds, based on an array of seed protease inhibitors (Amirhusin et al., 2007; Modgil and Mehta, 1997), triterpenoid saponins (Modgil and Mehta, 1997), and nonprotein amino acids (Venugopal et al., 2000).

In cases where generalist insect species choose distantly related plants as hosts, interactions may be based on unrelated secondary plant metabolites, deterrent or inhibitory (Bassman, 2004).

2.3. PLANT SECONDARY METABOLITES AND CUTICULAR HYDROCARBONS ARE CO-OPTED BY INSECTS AND AFFECT INSECT–PATHOGEN INTERACTION

Endogenous secondary plant low-molecular-mass compounds and high-molecular-mass surface hydrocarbons, lipids, and derivatives may be ingested by herbivorous insects with chewing mouthparts and either utilized unchanged, or recycled as precursors for biosynthesis of derived endogenous bioactive compounds within the body of the insect recipient or on its surface. This implies that cuticular hydrocarbons of herbivorous insects and/or associated lipophilic compounds may and often do reflect to some extent the composition of the plants they forage on. In some cases, infectivity is affected by the physical structure and location of cuticular hydrocarbons: The infectivity of the entomopathogenic fungus *Pandora neoaphidis* to the pea aphid, *Acyrthosiphon pisum*, is affected by the degree of wax bloom on different pea plant varieties, due to better attachment of fungal conidia on leaf surfaces with reduced wax bloom (Duetting et al., 2003; Steinkraus, 2006).

Consistent with this scenario and next in the sequence of events, plant-derived compounds, internalized by the insect or present on its surface, may affect the behavior and performance of entomopathogenic fungi. The presence of allelochemicals on the insect or leaf cuticle may adversely affect the survival of entomopathogenic fungi, supporting the concept of functional diversity of plant-derived allelochemicals by insect herbivores. On the surface, the pathogenicity of the entomopathogenic fungus *Metarhizium anisopliae* to the chrysomelid mustard beetle *Phaedon cochleariae* was found to be affected by various crucifers-derived stimulatory and inhibitory compounds (Inyang et al., 1999). *Bemisia* whiteflies were significantly less susceptible to infection by *Beauveria bassiana* or *Paecilomyces fumosoroseus* when reared on cotton versus melon (Poprawski and Jones, 2001) while *Trialeurodes vaporariorum* whiteflies were significantly less susceptible to infection when reared on

tomato versus cucumbers (Poprawski et al., 2000). The nature of the compound responsible for protecting whiteflies on cotton is still uncertain. In vitro exposure to gossypol, a major metabolite produced by glandular cells in cotton, did not incriminate this compound (Poprawski and Jones, 2001). Tomatine, an alkaloid present in tomatoes, may be antifungal (Poprawski et al., 2000).

In some cases, plant-derived surface compounds do not even have to be internalized by the insect in order to influence subsequent trophic affects; sufficient that powdery waxes on the plant surface dust the exterior of the insect, or that sticky substances on the plant surface adhere to the insect integument. For example, larvae of the tobacco hornworm, *Manduca sexta*, amass high levels of duvatrienediols on their integument from the surface of the tobacco plant (Espelie and Bernays, 1989).

To some extent, direct interaction between plant metabolites and entomopathogenic fungi is also known to occur. Thus, fungal inhibitors produced by the plant may protect the insect against pathogens (Ramoska and Todd, 1985). Species-specific secondary plant metabolites may be incorporated into the leaf surface and exhibit antimicrobial properties against an array of nonadapted organisms. They may substantially impede conidial germination and attachment by changing the chemical composition and physical characteristics of the leaf surface. For example, the persistence of *Aschersonia aleyrodis*, a fungal pathogen of whitefly, was studied on cucumber, gerbera, and poinsettia. Germination capacity and infectivity of conidia, which remained on the plant leaves for up to 1 month, was low but most of the conidia were shown to be viable when transferred from the leaf to water agar, even after having been on the leaf surface for 1 month. Germination capacity was highest on cucumber, followed by poinsettia and lowest on gerbera (Meekes et al., 2000). The fact that leaves of poinsettia contain cytotoxic triterpenoids (Smith-Kielland et al., 1996) may be relevant to this observation. Germination of blastospores of the entomopathogen *P. fumosoroseus* is affected by exposure to allelochemicals, found to be chemically identical to leaf components present on the insect cuticle (Vega et al., 1997). The insect diet can influence infection of the Colorado potato beetle by *B. bassiana*, rendering the insect more susceptible (Hare and Andreadis, 1983).

Less is known of the mechanisms that have evolved in fungi to overcome those plant-derived chemical defenses, or conversely, to use them in a context advantageous to the insect (Brooks et al., 1996; Eigenbrode and Espelie, 1995; Green et al., 2003).

3. The Evolutionary Ecology of the Entomopathogenic Fungus within the Host-Plant Environment

Insects, including the plant herbivores mentioned herein, are susceptible to a variety of pathogens and parasites. Entomopathogenic fungi have adapted to the

insect niche in a manner generally similar to entomopathogenic viruses, bacteria, nematodes, parasitoid wasps, and other organisms. Based on recent fungal classification (James et al., 2006; Hibbett et al., 2007; Sung et al., 2007), entomopathogenicity appears to have arisen independently and frequently by convergent evolution (Humber, 2008). In entomopathogenic species, an "arms race" leads to a variety of particular adaptations and specialized mechanisms enabling them to invade and infect their hosts (Roy et al., 2006; Shah and Pell, 2003).

Fungal entomopathogens are found worldwide in many of the known insect orders, including Lepidoptera, Diptera, Homoptera, Coleoptera, Isoptera, and others, as well as in other noninsect arthropods (Samson, 1974). These fungi are as taxonomically diverse as their hosts, belonging to various classes, mostly in the divisions Zygomycota, Ascomycota, and Deuteromycota, but also in the Chytridiomycota and Oomycota (Boucias et al., 1988).

Fungi that naturally infect insects can be either facultative or obligate pathogens (Fargues and Remaudiere, 1977). In both categories, they can either be specialists – selective and specific to a restricted host range and analogous in this respect to insect plant-host choice, albeit without direct choice, or generalists – infecting a broad range of host insects, depending on nutritional requirements and other adaptations. Specificity and host range can vary not only between fungal species, but even between different isolates of one species (Shah and Pell, 2003). The possible developmental basis of fungal adaptation is expressed as differences in growth rate, conidial yield, and other variables (Acevedo et al., 2007; Altre and Vandenberg, 2001a, b, 1c; Alves et al., 2002; Antunez et al., 2007; Devi et al., 2003; Nielsen et al., 2005; Talaei-Hassanloui et al., 2007; Wraight et al., 1998), whereas at the physiological and molecular level, there is evidence that certain fungal enzymes and other substances play an important role in specificity and virulence (Fuguet and Vey, 2004; Joslyn and Boucias, 1981; Miller et al., 2004; Murad et al., 2006; Nahar et al., 2004; St Leger et al., 1987; Zayed et al., 2002).

Fungal pathogens are unique amongst entomopathogenic microorganisms, in that in most cases they need not be ingested in order to invade their host (Charnley, 2003; Gillespie et al., 2000). However, oral entry does occur in entomopathogenic *Ascosphaera* (James and Buckner, 2004) or *Culicinomyces* (Scholte et al., 2004), and *Leptolegnia chapmanii* has been shown to be infective either by zoospore germination on mosquito cuticles or by ingestion and germination of ingested encysted zoospores (Lord and Fukuda, 1988; Zattau and McInnis, 1987). Infection per os does occur, but rarely, in entomopathogenic *Fusarium* species (Teetor-Barsch and Roberts, 1983).

In most cases, the hydrophobic entomopathogenic fungal conidia, primarily wind-borne or released in the soil, passively land or contact the outer surface of potential insect hosts, whereby fungal dissemination can be regarded as adventitious. However, in some cases, the fungus may even attenuate the behavior of the infected insect in such a manner that conidial dissemination is facilitated. Under particularly favorable circumstances (*e.g.*, on parasitizing aphids forming compact

colonies), the discharge of conidia from conidiophores creates a high probability of infection of a new host, this probability strengthened by coordination of the period of conidial discharge with a definite time and a definite ecological situation in the host population. Clearly, mobility, insect pest population density, and host-plant choice increase the probability of pathogen–insect interaction (e.g., dispersal of alate forms of aphids; Feng et al., 2007).

The fungal infection process in the insect mostly begins with conidia attaching to the host's cuticle and directly invading through the integument in a process conducted by physical and enzymatic means (Clarkson and Charnley, 1996; Roberts et al., 1992). Docking occurs only if hydrophobicity is attenuated and appropriate chemical stimuli are present. Broadly speaking, interactions between the insect outer surface and entomopathogenic fungi are analogous to the interactions between plant surfaces and fungal plant pathogens (Clarkson and Charnley, 1996; St Leger et al., 1997). Surface substances and structures serve as signals that facilitate conidial docking and in some cases appressorium formation precedes spore germination (Clarkson and Charnley, 1996), whereas hyphal penetration is facilitated primarily by fungal proteases and assisted by chitinases (Clarkson and Charnley, 1996; St Leger et al., 1997).

It is reasonable to assume that physical and metabolic conditions occurring during the molting process in insects, regardless of the status of the immune system, affect the invasion of fungi into the insect and the establishment of infection. Thus, shortly after ecdysis, the newly deposited cuticle has not yet hardened, and this facilitates fungal penetration. It is also likely that a higher percentage of germinating conidia succeed in penetrating nonscelerotized cuticle, contributing to pathogenicity.

Figure 1 describes a typical infection cycle of entomopathogenic fungi: Once penetration of the cuticle and epidermis is complete, the entomopathogenic fungus proliferates within the host hemolymph and tissues, eventually leading to mortality by the depletion of reserves, the disruption of a variety of physiological functions in the host, and the production of metabolites toxic to the host (Dowd, 1999). When such fungal establishment occurs and the fungus exhausts the insects reserves, its hyphae exit the insect's body via the cuticle, sporulation occurs under appropriate levels of humidity, and conidia are released into the environment (Charnley, 2003; Clarkson and Charnley, 1996; Gillespie et al., 2000).

Entomopathogenic fungi have been studied, in both laboratory bioassays and field experiments, as potential agents for biocontrol of various agricultural and forest pests for many years. Fungi have been evaluated against hemipterans, coleopterans, lepidopterans, orthopterans, dipterans (Geetha and Balaraman, 1999; Lacey and Shapiro-Ilan, 2008; Shah and Pell, 2003; Vu et al., 2007), cockroaches (Salehzadeh et al., 2007), heteropterans (Bandani et al., 2006), thysanopterans (Mikunthan and Manjunatha, 2006), and isopterans (Rosengaus et al., 2007). *M. anisopliae* and other fungi have also been evaluated as control agents of mites (Lacey and Shapiro-Ilan, 2008; Meikle et al., 2007; Smith et al., 2000; Zhioua et al., 1997).

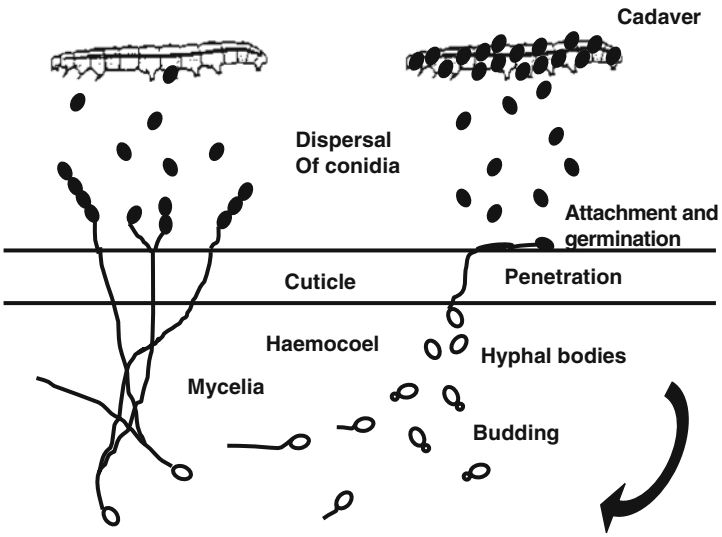

Figure 1. A typical entomopathogenic fungal infection cycle in an insect. Clockwise: Dispersal of conidia from a larval cadaver, their attachment to the cuticle of a new host, breaching the cuticle, proliferation in the form of budding hyphal bodies, germination and growth of mycelium, formation of mycelial mass, exit through the cuticle and formation of new aerial conidia, etc.

In the context of agricultural entomology, entomopathogenic fungi may impact negatively by their effect on economically beneficial insects (e.g., the honey bee *Apis mellifera* and the pollinating bee *Megachile rotundata*) (Higes et al., 2007; James and Buckner, 2004; Qin et al., 1993). Analogous to hyperparasitism, some fungi are infective to bee hive pests such as the varroa mite (Davidson et al., 2003; Meikle et al., 2007) and the beetle *Aethina tumida* (Muerrle et al., 2006) and in this case are regarded as beneficial. "Who is the enemy and who the friend?" depends on the position of the subjective observer: "The enemy of my enemy is my friend."

3.1. INOCULATION, ATTACHMENT, AND GERMINATION

Conidia of many entomopathogenic fungi are passively dispersed from previously infected cadavers. In some cases, conidia are actively discharged under hydrostatic pressure (Shah and Pell, 2003). After detachment from the cadaver of the previous host, conidia can be either air-borne (Hemmati et al., 2001) or carried by insect vectors (Roy et al., 2001). In some entomophthoralean species, conidia were found to be discharged while the host insect was still alive (Shah and Pell, 2003).

Having landed on the cuticle of a potential host, fungal conidia must attach to the surface as a prerequisite to penetration. One problem conidia face after

landing on the insect's cuticle is the movement of their host and its organs during normal behavior such as walking or crawling, which may shake conidia off the surface. Cuticular structures, such as setae or spines on the surface of various insects, can trap conidia and reduce their dislodgement off the cuticle, thus numerically increasing germination (Hajek and Eastburn, 2003). In other insects, such structures may provide protection against fungi, and other protective devices, such as a mucus film over the cuticle, are also known. However, to maximize attachment, fungi have adopted strategies such as rapid germination and penetration of the cuticle, as well as improved attachment efficiency (Altre et al., 1999; Hajek and Eastburn, 2003). The surface of conidia varies from dry and hydrophobic, to sticky and hydrophilic, even within the Deuteromycotina alone (Boucias and Penland, 1991). Hydrophobic conidia attach to the waxy, hydrophobic exterior layer of the insect integument firmly and passively (Boucias et al., 1988). In contrast, hydrophilic conidia presumably require the aid of sticky secretions for that purpose, although a sticky coat is seen in hydrophobic conidia too (Arruda et al., 2005). Nevertheless, it seems that the degree of cuticle surface hydrophobicity plays a role in specificity of entomopathogenic fungi towards their potential hosts (Hajek and Eastburn, 2003). The extent of conidial attachment can also be influenced by grooming behavior in social insects (Traniello et al., 2002). The efficiency of grooming for removal of conidia varies between different fungal species (Yanagawa et al., 2008).

After the initial landing on the surface, fungal conidia need to "dock" onto the cuticle. In many fungi, the germ tube that grows from the conidium produces an appressorium as a means of docking (Arruda et al., 2005; Campos et al., 2005; Clarkson and Charnley, 1996; Kumar et al., 2004; Kumar et al., 1997; Nadeau et al., 1996). The necessity of appressorium formation may depend on cuticle surface topography, which differs between insect hosts and their different body parts (Nadeau et al., 1996) and can thus be a factor in fungus-host specificity. Appressoria in different fungi are known to produce a variety of enzymes, which are involved in cuticle degradation (Arruda et al., 2005; Clarkson and Charnley, 1996; St Leger et al., 1996), probably for the nutritional exploitation of degradation products as well as to prepare the substrate for penetration. Secretion of enzymes by the appressorium, involved in pathogenicity, is known in plant–pathogenic fungal interactions too (Kleemann et al., 2008; van den Ende and Linskens, 1974).

The percentage of conidial germination of the generalist entomopathogens *B. bassiana* and *M. anisopliae* is affected by the nutritional quality of host cuticle components (Crespo et al., 2000; Safavi et al., 2007). Production of enzymes by germinating conidia was found to be affected by nutrition too (Qazi and Khachatourians, 2008). Germination of *M. anisopliae* was found to be suppressed by an aldehyde present in the cuticle of the stinkbug *Nezara viridula* (Sosa-Gomez et al., 1997). Lipids and other components of the silverleaf whitefly, *Bemisia argentifolii*, were found to differentially inhibit germination of *B. bassiana* and *P. fumosoroseus* (James et al., 2003). Secondary plant metabolites were also found to affect germination in *P. fumosoroseus* (Vega et al., 1997) and

B. bassiana (Poprawski and Jones, 2001). By affecting germination, the nutritional quality of the cuticle and inhibitory components in the insect integument, and possibly metabolites originating from the insect's host plant, can play a part in the specificity and virulence of entomopathogenic fungi.

3.2. BREACHING THE CUTICLE

A distinction is observed between those fungi that produce spores containing sufficient internal resources for breaching the cuticular barrier immediately after anchoring, a procedure termed germinative development, and vegetative development, dependent on initial exogenous nutrient sources (Manners, 1966).

In entomopathogenic fungi that develop appressoria, depletion of nutrients on the insect integument surface may be the signal for transition from the saprophytic-like to the pathogenic mode of growth (Clarkson and Charnley, 1996). At this stage, penetration of the cuticle begins with the growth of a penetration peg from the appressorium (Clarkson and Charnley, 1996; Kumar et al., 2004; Nadeau et al., 1996).

In other species of entomopathogenic fungi, the aerial conidia themselves, or secondary conidia, are the infective units, directly growing a germ tube to penetrate the cuticle (Altre and Vandenberg, 2001c; Kumar et al., 1997; Nadeau et al., 1996). Penetration involves both mechanical pressure, leading to separation of cuticular lamellae by the penetrating hyphae, and enzymatic degradation of cuticle components (Altre and Vandenberg, 2001c; Arruda et al., 2005; Campos et al., 2005; Clarkson and Charnley, 1996). For the purpose of breaching the insect cuticle, which is comprised mainly of chitin fibrils embedded in a protein matrix (Andersen, 1979), entomopathogenic fungi produce a variety of degradative proteases (Campos et al., 2005; Gillespie et al., 1998; Joshi et al., 1995; Krieger de Moraes et al., 2003; Samuels and Paterson, 1995; St Leger et al., 1987, 1993, 1996) and chitinases (Bogo et al., 1998; Campos et al., 2005; Krieger de Moraes et al., 2003; Nahar et al., 2004; Screen et al., 2001; St Leger et al., 1996). Some fungal entomopathogens are capable of producing polysaccharidases as well (St Leger et al., 1997), probably to facilitate the saprophytic mode of existence in the absence of an appropriate host. Production of these enzymes is carefully regulated by environmental cues such as pH levels and certain components in the insect cuticle (Bye and Charnley, 2008; St Leger et al., 1998), and could be of significance to host specificity.

3.3. FIRST DEFENCE

While breaching the insect exoskeleton, invading fungi may also encounter not only nutrients, but also molecules that participate in the insect's innate immune response. As a first line of defense, the integument maintains a wound-repair system

that is based primarily on melanization (Lai-Fook, 1966), a process significant for the developmental processes of hardening and darkening of the insect cuticle as well as for the immune system. An interesting study suggested a translocation of the key enzyme in melanization, prophenoloxidase (PPO) from the hemolymph to the cuticle in *B. mori* (Asano and Ashida, 2001). The inactive PPO zymogene was found in the cuticles of other insects (Colonello et al., 2003; Feng and Fu., 2004; Lai et al., 2002; Zufelato et al., 2004), as well as the active enzyme, phenoloxidase (PO) (Barrett, 1987; Hiruma and Riddiford, 1988). The PPO-activating-enzyme (PPAE) was found in the cuticle of *B. mori* (Satoh et al., 1999), and an enzyme likely to be of the same function was found in *M. sexta* cuticle (Aso et al., 1985). Interestingly, in the mealworm beetle, *Tenebrio molitor*, resistance to *M. anisopliae* was found to positively correlate with the degree of cuticular melanization (Barnes and Siva-Jothy, 2000). In another study, the activity of chitinase from *M. anisopliae* was inhibited by solubilized melanin (Nahar et al., 2004), implying a role melanin may play in suppressing fungal infections poised at the stage of cuticle penetration. Another role melanin was found to play in defense against fungi in the cuticle is by encapsulation of the penetrating germ tube by diffuse integumental melanization (Golkar et al., 1993). Not only components of the melanization cascade, but other substances that are usually considered part of the humoral immune response, such as the antimicrobial peptide cecropin, may be produced by cuticular epithelial cells and oppose the invading fungus, as was seen in *B. mori* (Brey et al., 1993).

3.4. THE "TROJAN HORSE" – COLONIZATION AND PROLIFERATION WITHIN THE INSECT

To complete cuticle penetration, invade the insect body cavity and establish infection, entomopathogenic fungi presumably need to possess mechanisms enabling them to evade, or confront, immune responses concurrent with or shortly after breaching the hosts' cuticle. To multiply in large numbers and establish infection, these hyphal bodies specialize in evading the insect immune system, which is constantly on guard against invading foreign objects (Hoffmann, 1995; Kanost et al., 2004; Lavine and Strand, 2002). Many fungi initially proliferate in the form of yeast-like hyphal bodies (Alves et al., 2002; Kawamoto and Aizawa, 1986; Kumar et al., 1997; Pendland et al., 1993; Shah and Pell, 2003), variously termed protoplasts or blastospores in different taxa or developmental stages. Evasion is facilitated by either lack of sugar-rich residues in the hyphal body cell membranes or their masking (Butt et al., 1996; Pendland and Boucias, 1998; Pendland et al., 1993). In both cases, detection by the pattern recognition factors in the insect cells responsible for nonself-recognition is prevented (Hajek and St Leger, 1994; Morrow et al., 1989a, b). Evasion of the insect immune system at early stages of infection allows the fungus to proliferate unimpeded, except for responses that may have been

induced earlier during penetration of the cuticle (Roxstrom-Lindquist et al., 2004). Being essentially undetected, the hyphal bodies spread throughout the insect body and guarantee fungal success within the living host, which, in many cases, does not show any external physical symptoms to its illness prior to mortality.

3.5. THE SECRET LIFE OF THE FUNGUS - BELATED RECOGNITION AND DEFENCE

The different stages of conidia germination may affect the efficacy of certain immune responses. The capability of *Galleria mellonella* hemocytes to phagocytose foreign objects varies throughout the germination stages of *Aspergillus fumigatus* conidia, which affects infection rates too (Renwick et al., 2006). Specific responses to entomopathogenic fungi and their secretions have been observed in insects (De Gregorio et al., 2002b; Lemaitre et al., 1997; Schuhmann et al., 2003; Shin et al., 2006; Vilcinskas et al., 1999).

From the "point of view" of the insect, this occurs only at a later stage of infection, possibly after depletion of most nutritional resources. At that time, the propagated hyphal bodies develop mycelia which, in contrast to the hyphal bodies, are recognized by insect recognition factors involved in the induction of immune responses (Pendland and Boucias, 2000). However, such immune responses may arise at a stage when the fungus has developed within the host to such a degree that precludes the ability of the immune system to cope. Furthermore, entomopathogenic fungi may also actively work to suppress immune responses that might be detrimental to them. Several fungal metabolites from *Aspergillus* and *Penicillium* species have been identified and shown to inhibit phenoloxidase in the cuticle and hemolymph of the noctuids *Spodoptera frugiperda* and *Helicoverpa zea* (Dowd, 1999). A metabolite from a *Tolypocladium* species seems to interfere with the induction of immune responses (Bandani, 2004). Metabolites from some fungi were found to inhibit cellular responses (Vey et al., 2002; Vilcinskas et al., 1999).

There does not seem to be one universal mechanism used by fungal entomopathogens for killing the host. Often, the feeding behavior in insects is reduced in a matter of days after fungal infection (Roditakis et al., 2008; Roy et al., 2006). Blood feeding in the mosquito *A. gambiae* was reduced following *M. anisopliae* infection (Scholte et al., 2006). *Nosema fumiferanae* was seen to indirectly affect feeding behavior in the lepidopteran *Choristoneura fumiferana*, by disturbing establishment of feeding sites (van Frankenhuyzen et al., 2007). A toxin from *Lecanicillium (Verticillium) lecanii* acts as an antifeedant in *B. tabaci* (Wang et al., 2007).

The production and secretion of toxins and mutagenic substances by entomopathogenic fungi was seen in various studies in *N. rileyi* (Onfore et al., 2002), *M. anisopliae* (Krasnoff et al., 2006; Rao et al., 2006; Vey et al., 2002; Wang et al., 2004), *Paecilomyces fumosoroseus* (Asaff et al., 2005), *Paecilomyces tenuipes*

(Nam et al., 2001), *B. bassiana* (Quesada-Moraga and Vey, 2004), the *Hypocrella* genus (Watts et al., 2003), *L. (Verticillium) lecanii* (Wang et al., 2007), and *Aspergillus parasiticus* (Drummond and Pinnock, 1990). However, the exact roles of these toxins in the infection process and pathogenicity are unclear (Clarkson and Charnley, 1996). Fungi may also interfere with crucial developmental processes in their hosts. The overall fecundity of the desert locust *Schistocerca gregaria*, infected with *M. anisopliae* var. *acridum,* is reduced, presumably because of the chronic exploitation of storage reserves by the fungus and their diversion from support of oogenesis to nutrition of the developing fungus. However, if infection occurs in newly fledged females, egg development is temporarily accelerated, possibly by allatal stimulation, as has been observed after mating in *Drosophila melanogaster* (Moshitzky et al., 1996). In an evolutionary context, this could be regarded as a response of the female locust to realize a part of her reproductive potential, before it is too late (Blanford and Thomas, 2001).

Common physiological strategies by different groups of entomopathogens and parasites appear to have evolved independently, and some evidence suggests that the entomopathogenic fungi may extend a host's life span by inhibiting molting, as is more substantially documented where this strategy is exploited by insect parasites and viruses. Such manipulation of the length of the susceptible stages in the host's life can be expected in the repertoire of a parasite or a pathogen to fit the host's processes to its own developmental requirements, and/or to maximize the nutritional potential before moving on to the next host. This phenomenon is known from parasitoid wasps in lepidopteran hosts (Beckage et al., 2002; Grossniklaus-Burgin and Lanzrein, 1990). It was also seen in interactions of viruses with hemipteran (Rozas-Dennis and Cazzaniga, 2000) and lepidopteran hosts (Palli et al., 2000; Shikata et al., 1998; Toister-Achituv and Faktor, 1997). Similarly, *N. rileyi* inhibits the activity of ecdysteroid hormones essential for the molting process in the silkworm *B. mori* (Kiuchi et al., 2003). Interruption of molting is also observed in whiteflies infected with sublethal amounts of *B. bassiana* (Torrado-Leon et al., 2006). Infection with three different fungi disrupts ecdysis in the gypsy moth *Lymantria dispar*, thereby prolonging the duration of larval development (Solter et al., 2002). As in many generalities, the number of examples cited for each broad taxon is insufficient evidence to regard this strategy as universal, but a tendency to support this thesis emerges.

Subsequent to extension of the hosts' life, successful fungal infections eventually lead to the hosts' death. The fungus then exits, and formation of new conidia usually occurs on the exterior of the cadaver, given appropriate conditions of humidity (Shah and Pell, 2003). At this stage, the pathogen depends on efficient transmission to maximize its fitness and on effective persistence. For a summary of "endgame" behavior of pathogen-infected behavior of insect hosts affecting conidial dispersal, see Roy and Pell (2000). Conidia produced postmortem disperse, reach new hosts, and continue the fungal life cycle (Roy et al., 2006), but do not always succeed in doing so.

4. Constitutive and Induced Innate Immunity in Insects

To confront the various infections and diseases they are susceptible to, insects possess a defense system poised to potentially recognize, confront, and eliminate invaders. Unlike vertebrates, insects do not possess an adaptive immune system, and lack the ability to produce specific antibodies (Hultmark, 2003; Marmaras et al., 1996). Their immune system consists of innate defense mechanisms only. This leads to a need, in case of pathogen invasion, for rapid responses and in fact, the innate immune system in insects is intriguingly complex and fit to confront a variety of hazards.

To provoke an immune response, the invading pathogen must be recognized as "nonself" by a front-line defense system, involving proteins that bind to bacterial peptidoglycan or lipopolysaccharide (LPS), or to fungal β-1,3-glucan (Hoffmann et al., 1999; Schmid-Hempel, 2005). Detection of invaders is carried out by specialized plasma membrane proteins of the insect cell designated peptidoglycan recognition proteins (PGRP's) and pattern recognition receptors (Chai et al., 2008; Eleftherianos et al., 2007; Hedengren-Olcott et al., 2004; Hoffmann and Reichhart, 2002; Irving et al., 2005; Wilson et al., 1999), with extracellular domains such as lectins and hemolins. Consequently, an array of immune responses is initiated, consisting of humoral and cellular reactions (Hultmark, 2003; Zhu et al., 2003a).

Humoral reactions of the insect immune system consist of the selective *de novo* synthesis of antibacterial, fungicidal or fungistatic peptides that are secreted by the fat body and hemocytes into the hemolymph. A variety of such peptides were identified in insects of many orders (De Gregorio et al., 2001; Dimopoulos et al., 1997; Glinski and Buczek, 2003; Hoffmann et al., 1996; Hultmark, 2003; Lemaitre et al., 1997; Lowenberger, 2001; Wood and Jacinto, 2007; Yamakawa and Tanaka, 1999; Zhu et al., 2003a). The synthesis of these molecules in *D. melanogaster* is known to be mediated by two distinct pathways regulating gene expression: Imd and Toll. These pathways are known to be activated differentially by various types of pathogens. While Toll responds to Gram-positive bacteria and fungi, Imd was seen to react to Gram-negative bacteria, although another report suggests both Toll and Imd respond to fungi (De Gregorio et al., 2002b; Hoffmann and Reichhart, 2002; Hoffmann et al., 1996; Hultmark, 2003; Levashina et al., 1998). The two pathways are also known to mediate the expression of peptides differentially. In *D. melanogaster,* Toll was found to regulate attacin, defensin, and others, while the regulation of diptericin, drosocin, and more is attributed to Imd. Both were found to mediate the expression of metchnikowin, cecropin, and drosomycin (De Gregorio et al., 2002a; Hoffmann and Reichhart, 2002; Hultmark, 2003; Lemaitre et al., 1996). In the lepidopteran *Bombyx mori*, Toll was found to regulate the production of attacin, cecropin, and lebocin (Tanaka et al., 2005).

Additionally, various studies have shown that the production of the different antimicrobial peptides discriminates between G+ bacteria, G- bacteria, and fungi,

which facilitates rapid, semi-specific responses. In *D. melanogaster*, cecropin and diptericin are induced under challenge by fungi as well as G- bacteria, metchnikowin by G- bacteria, and G+ and by fungi, and drosocin and drosomycin are more selective and induced by G- bacteria or fungi, respectively (Ekengren and Hultmark, 1999; Lemaitre et al., 1997). In *B. mori*, moricin is induced by both G+ and G- bacteria (Furukawa et al., 1999; Hara and Yamakawa, 1995), while attacin, cecropin and lebocin are specific to G+ bacteria (Tanaka et al., 2005). In the wax moth *Galleria mellonella*, the defensin-like gallerimycin acts against fungi (Schuhmann et al., 2003), but another defensin is active against G- bacteria (Lee et al., 2004). In *A. aegypti*, cecropins and defensins act differentially against G+ and G– bacteria, and some reports state that such peptides also act against eukaryotic parasites (Dimopoulos et al., 1997; Lowenberger, 2001). These are only a small sample of many more reports regarding various defense peptides in many insect orders.

Additional molecules that can be considered a part of the humoral defense response are lysozyme (Bogus et al., 2007; Freitak et al., 2007; Roxstrom-Lindquist et al., 2004) and serine protease inhibitors (serpins). Amongst their many known roles, serpins were also seen to directly inhibit fungal proteases (Eguchi et al., 1994; Kanost, 1999). Figure 2 is a schematic presentation of the various immune functions in response to fungal infection in insects.

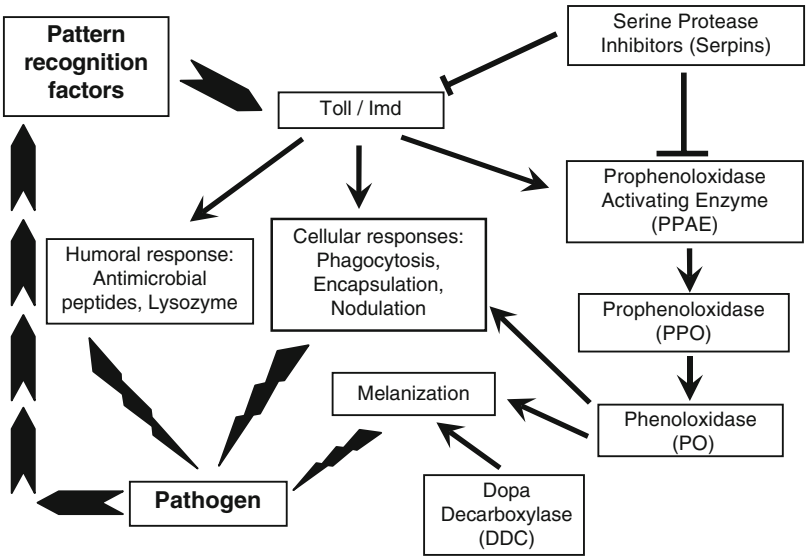

Figure 2. Schematic presentation of major insect anti-fungal defense factors and their possible interrelations.

The cellular defense mechanisms involve mobilization of circulating hemocytes for phagocytosis or encapsulation and nodulation of the foreign objects. Low concentrations of invading microorganisms are predominantly cleared by phagocytosis, whereas at higher concentrations invaders are entrapped by aggregations of cells and certain cellular secretions, known as nodules. Larger foreign bodies are encapsulated by hemocytes, forming layers of flattened cells around the invader (Marmaras et al., 1996).

Hemocytes also participate in production and secretion of defense peptides in conjunction with the fat body, and signal the fat body in case of infection (Wood and Jacinto, 2007). There are a variety of hemocytes, which differ in morphology and in function, as well as in the mode of action in the immune response (Akai and Sato, 1973; Harpaz et al., 1969; Lavine and Strand, 2002; Wood and Jacinto, 2007).

The composition of hemocytes in an insect, in terms of types and total count, was found to change in response to infections in insects. Early stage fungal infection by *N. rileyi* altered the hemocyte type composition and increased the cell count in the noctuid *Mamestra brassicae* (Lee et al., 2005). A parasite in the mosquito *Culex quinquefasciatus* caused alterations in the proportional count of different cell types, with a general increase in early infection and a decrease in a later stage (Brayner et al., 2007). A similar pattern of fluctuation in total count was seen in the locust *S. gregaria* infected by the fungus *M. anisopliae* (Gillespie et al., 2000). Differential hemocyte count following yeast infection was seen in the blowfly *Chrysomya megacephala* (Faraldo et al., 2008). Parasitization by ectoparasitoid wasps led to a decrease in total hemocyte count in the noctuid *Pseudaletia separata* (Suzuki and Tanaka, 2007) and changes in hemocyte composition in *D. melanogaster* were seen in response to components of the long gland (an organ of the reproductive system) of a parasitoid wasp (Labrosse et al., 2005). Cellular responses can play a crucial part in the degree of resistance of insects towards pathogens, and thus in specificity of pathogens to insects. The fungus *Conidiobolus coronatus* induced phagocytosis and encapsulation responses differentially in different insect species, which varied in resistance to the fungus (Bogus et al., 2007).

The molecular basis of insect cellular responses and the pathways involved in them have been studied extensively in recent years. In *D. melanogaster*, the Toll receptor is known to activate hemocytes through the same pathway that leads to the production of the antifungal peptide, drosomycin (Hultmark, 2003). In *Ceratitis capitata*, a Ras/Mitogen-activated protein kinase (MAPK) signal transduction pathway is involved in the attachment of hemocytes, while Integrin is involved in phagocytosis of bacteria (Foukas et al., 1998). Another study from the same group indicated the involvement of an FAK/Src complex in phagocytosis (Metheniti et al., 2001). The activation of insect plasmatocytes for adhesion and spreading in encapsulation and nodulation has been studied as well (Lavine and Strand, 2002), and one important

molecule, a 23 amino acid Plasmatocyte-Spreading Peptide (PSP), was found essential for encapsulation by plasmatocytes in the noctuid *Pseudoplusia includens* (Clark et al., 1997). The involvement of DOPA-decarboxylase-dependent pathways in phagocytosis and nodulation was seen in the medfly *C. capitata* (Sideri et al., 2007). The importance of eicosanoids, essential-fatty-acid derived molecules, in cellular responses has been studied extensively in various insect–pathogen interactions (Bedick et al., 2001; Durmus et al., 2008; Miller et al., 1999; Tunaz, 2006).

Alongside cellular and humoral immune responses, melanization plays an important part in defense reactions. It participates in a process known as melanotic encapsulation, wherein layers of cells adhere to a foreign body and melanin is deposited onto the invader (Vass et al., 1993). Melanin is also utilized in nodulation reactions, as part of the biopolymer matrix secreted by hemocytes to coat the pathogen (Krishnan et al., 2000) and produced by an enzymatic cascade, which utilizes tyrosine as precursor.

By-products of the melanization cascade, such as quinones and reactive oxygen intermediates, may be directly toxic to the fungus (Nappi and Ottaviani, 2000; Zhu et al., 2003b), while melanotic encapsulation traps the fungal units and additionally contributes by excluding water or nutrients. These effects may terminate the fungal infection or only temporarily retard the establishment of the fungus, which in the later case either outgrows the melanin deposition or breaches the melanized capsule or granuloma by mechanical force.

Phenoloxidase (PO), which plays a major role in melanization (Huang et al., 2005; Lee et al., 2000; Zhu et al., 2003b), belongs to a broad family of enzymes that catalyze the oxidation of phenolic compounds (Jaenicke and Decker, 2004). This enzyme family includes tyrosinases and laccases, known to participate in melanization processes in insects (Barrett, 1987). PO is synthesized as the inactive prophenoloxidase zymogen (PPO), which is activated by prophenoloxidase activating enzyme (PPAE), a serine protease, which is part of a serine protease cascade. The melanization cascade in lepidopterans reacts positively to fungal infection, mediated by an increase in hemolymph PO (Bidochka and Hajek, 1998; Zhu et al., 2003b). The same effect was seen in *Plodia interpunctella* parasitized by the wasp *Habrobracon hebetor* (Hartzer et al., 2005), and changes in PO secretion from cells were seen in virus-infected *Helicoverpa armigera* (Kalia et al., 2001). Another study (Gillespie et al., 2000) with locusts showed a decrease in PO activity alongside an increase in PPO concentrations after fungal inoculation. Expression of the activating enzyme PPAE increased in the mosquito *Anopheles dirus* infected with the parasite *Plasmodium yoelii* (Xu et al., 2006). Bacteria elicited a hemocyte-mediated melanization response in the mosquito *Armigeres subalbatus* (Hillyer et al., 2003). Induction of another enzyme related to melanization, DOPA-decarboxylase, was found in a bacteria-challenged coleopteran mealworm, *T. molitor* (Kim et al., 2000).

4.1. SIGNIFICANCE OF NUTRITION AND PLANT SUBSTANCES ON EXPRESSION OF THE INSECT IMMUNE SYTEM

The immune system is affected by the quality of nutrition in the mosquito *Anopheles stephensi*, where sugar intake significantly influenced the melanization immune response (Koella and Sorense, 2002). Larvae of the blood-feeding bug, *Rhodnius prolixus*, that were fed on plasma alone and compared to larvae fed on whole blood, suffered a significant reduction in the ability to produce an antimicrobial peptide and lysozyme activity, as well as a reduction in hemocyte count and nodule formation (Azambuja et al., 1997). In caterpillars of the noctuid moth *Spodoptera littoralis*, challenged with a virus, protein intake greatly influenced melanization as well as lysozyme activity and encapsulation response (Lee et al., 2006).

A few studies found that the immune responses in insects can be particularly affected by metabolites originating from plants. Physalins, seco-steroids from *Physalis angulata*, were seen to depress various aspects of the immune response in the Chagas' disease vector *R. prolixus* (Castro et al., 2008). A reduction in hemocyte count was seen in *Spodoptera litura* treated with sweet flag (*Acorus calamus*) rhizome oil (Sharma et al., 2007).

Differences in plant primary and secondary plant chemicals influence the composition of insects feeding on the different plants, and these differences thereby influence fungal infection indirectly. This may impart relative resistance of insect specialists to certain entomopathogenic fungi and differential resistance to a generalist pathogen challenging a generalist insect host, able to potentially develop on various host plants with different compositions. For example, the host plant can influence the infectivity of the generalist pathogen *B. bassiana* to the specialist Colorado potato beetle, a pest of solanaceous crops (Hare and Andreadis, 1983). Furthermore, inhibitors produced by the plant may also protect the chinch bug *Blissus leucopterus leucopterus* from infection by *B. bassiana* (Ramoska and Todd, 1985).

5. Cooperative or Synergistic Interactions with other Organisms

Entomopathogenic fungi interact with other natural enemies of insect pests including nematodes and arthropods (Furlong and Pell, 2000; Lecuona et al., 2007; Roy et al., 2002). Fungi and other biocontrol organisms may act independently (a cumulative detrimental effect on the host insect) or interact either synergistically or antagonistically (Ferguson and Stiling, 1996). Antagonistic interaction may occur if the entomopathogenic fungus is a generalist and adversely affects predators or parasitoids. At the level of the insect host population, foraging predators or parasitoids may increase mobility of the host, thereby increasing the probability of transmission of the fungus amongst the host population of aphids (Fuentes-Contreras et al., 1998; Roy et al., 1998) or moth larvae (Furlong and Pell, 1996, 2000). The efficacy of this action is of course affected by the insect host population density.

5.1. INTERACTIONS WITH NEMATODES

While interactions between entomopathogenic fungi and most other natural enemies of insect pests have been scarcely studied, their interaction with nematodes has attracted considerable attention. Combination of either of the entomopathogenic nematodes *Heterorhabditis indica* or *Steinernema carpocapsae*, with either of the generalist entomopathogenic fungi *M. anisopliae* or *B. bassiana*, had an additive effect on larval mortality of the weevil *Curculio caryae* (Shapiro et al., 2004), whereas interaction between the entomopathogenic nematode *Heterorhabditis bacteriophora* and the entomopathogenic generalist fungus *B. bassiana*, each independently active against larvae of the noctuid moth *Spodoptera exigua*, demonstrated that a combination of the two pathogens caused higher than cumulative host mortality when used separately (Barbercheck and Kaya, 1991). Synergism between the entomopathogenic fungus *M. anisopliae* and the entomopathogenic nematodes *Heterorhabditis megidis* and *Steinernema glaseri* against third-instar grubs of the Turf June Beetle *Hoplia philanthus* was observed in laboratory and greenhouse experiments (Ansari et al., 2004). The nematode species *S. carpocapsae*, the entomopathogenic fungus *Beauveria brongniartii*, and a combination of both were evaluated against the turf grubs *Ectinohoplia rufipes* and *Exomala orientalis* (Coleoptera: Scarabaeidae) in the field (Choo et al., 2002). In another study, a combination of a highly virulent *H. bacteriophora* nematode isolate with a moderately virulent *M. anisopliae* fungal isolate caused the most rapid host death in the sugar cane borer *Diatraea saccharalis* (Acevedo et al., 2007). In some cases, high efficacy of the entomopathogenic nematode and fungus treatments was attributed to the proximity of the grubs to the soil surface, which allowed for excellent pathogen–host contact and to favorable soil temperatures, sandy soil, post irrigation application, and/or rain. Such results indicate the significance of environmental factors on trophic interactions.

6. Stress

The development and physiology of the fungal entomopathogen is affected by stress either directly, as a consequence of depletion of reserves in the insect by the pathogen and the deterioration of the insect–host humoral composition, or indirectly, via interactions between the environment, the host plant, and the insect herbivore. The delayed molt or metamorphosis induced by the fungus and a consequence of hormonal inbalance induced in the insect prolongs the duration for fungal development, presumably a favorable situation for the pathogen.

In the case of entomopathogens,which are also facultative saprobes, generational development on nonhost diet, such as is the case when cultured on artificial diets, often leads to a reduction in virulence on the insect hosts. Such stress-induced reduction in infectivity is a common although not universal occurrence

in entomopathogenic fungi. It may be the cumulative result of various marginal changes in unconnected characteristics, such as in the degree of adhesion to the insect epicuticular surface, decreased germination and penetration of the cuticle, due to significantly lower levels of a subtilisin-like protease, as was seen in *M. anisopliae* (Wang et al., 2002), and affected by the nutritive composition of the fungal diet (Shah et al., 2005). It may also be the result of compromised evasion of the innate immune response of the insect (Butt et al., 1996). Attenuated cultures of *N. rileyi* fail to produce yeast-like hyphal bodies (Morrow et al., 1989a), the propagative stage of fungal development, which has been shown in *M. anisopliae* to evade induction of the immune response (Wang and St Leger, 2006) and attenuated cultures of *M. anisopliae* do not produce destruxins (Wang et al., 2003).

While attenuation of various functional characteristics has been documented (Morrow et al., 1989a; Zuckerman et al., 1989), the basic molecular mechanisms controlling reduced infectivity and pathogenicity of the fungus are not well known.

7. Epilogue and Future Prospects

Many of the issues discussed in this chapter are important to food production. Numerous insects are pests of agricultural crops. The use of fungal entomopathogens within the context of Integrated Pest Management (IPM) to supplement synthetic insecticides (Ericsson et al., 2007; Furlong and Groden, 2001; Purwar and Sachan, 2006; Santos et al., 2007; Thompson et al., 2007; Tian and Feng, 2006) is an accepted procedure in principle, while less in practice. Entomopathogenic fungi are insufficiently exploited in plant protection, due to economic constraints inherent in producing and maintaining a consistent fungal product. Interactions between different entomopathogenic fungi (Hughes and Boomsma, 2004; Thomas and Read, 2007; Tounou et al., 2008) might enhance pathogenicity within the context of IPM.

Recent studies have addressed the possibility of increasing the virulence of entomopathogenic fungi by means of genetic engineering, including fungal transgenes expressing chitinase (Fan et al., 2007) and scorpion neurotoxin (Wang and St Leger, 2007). The application of fungal metabolites as potential bioinsecticides is also being examined (Binod et al., 2007; Shim et al., 2006). More effective metabolites could be sought as products of genetically modified, virulence-enhanced entomopathogenic fungi. A combined treatment of the moth *Lacanobia oleracea* with *B. bassiana* and a wasp venom proved highly efficient (Dani et al., 2004) and might serve as an indication of another avenue to explore.

It has previously been suggested that the ability of predators to transmit pathogens between infected and uninfected host populations could also be manipulated for IPM (Roy and Pell, 2000) but this approach has not been exploited hitherto. A variant of this approach might be the mass-production and release of fungus-infested or infected males as species-specific vectors for physical pathogen transmission to mating-receptive females in the field.

Unexpectedly, some entomopathogenic fungi are endophytic in plants serving as hosts for insects potentially susceptible to the entomopathogen (Harri et al., 2008; Posada et al., 2007; Posada and Vega, 2005; Vega, 2008; Vega et al., 2008). Whether such interactions provide potential benefit to the physiology, environmental fitness and performance of the host-plant and/or the entomopathogen deserves further study. Symbiotic colonization of plants that might serve as a source of entomopathogen inoculum and potential defence against crop damage deserves attention. This might be considered in cases where oral inoculation by hyphal bodies or mycelia occurs or can be induced. Seedlings, inoculated via irrigation before transplantation, could then harbor a latent entomopathogen endophyte, imparting cryptic resistance to susceptible plant pests.

Having noted the above tentative recommendations for future research, their relevance to insect control in the field should be regarded with a measure of reserve, as succinctly expressed in a slightly different context: "The view of natural enemy ecology that has emerged from laboratory studies, where natural enemies are often isolated from all elements of the biotic community except for their hosts or prey, may be an unreliable guide to field dynamics" (Rosenheim, 1998).

8. Summary

Fungi occupy a unique position in the tritrophic interaction of herbivorous insects with their host plants and entomopathogenic microorganisms. Ingestion of the inoculum is in most cases not a prerequisite for initial infection, but several sequential barriers do challenge the fungus and determine its success, or failure: Conidia must germinate on an appropriate substrate and dock on the outer surface of the insect, the hyphae must breach the cuticle, proliferate within the insect while evading recognition as nonself and immune response as long as possible, and preferentially delay host mortality, postponing the insect's developmental progression and metamorphosis.

Basic aspects of these interactions are self-evident, and yet insufficiently understood to date. They determine fungal virulence, how it is affected by the developmental status of the individual insect host, how the insect coordinates its response and allocates resources common to innate immune response, cuticle hardening, energy expenditure, and ultimate reproduction when challenged with a potential pathogen; next, what determines host specificity in this context. Is there a common strategy of evasion of the entomopathogenic fungus to avoid recognition as nonself by the insect host immune system, or have only some fungal entomopathogens developed possible schemes to avoid host responses, while most entomopathogens elicit strong responses? Does the insect host "over-react" as a precautionary measure, and the entomopathogens use brute force solutions to defeat those responses by growing faster than the host can cope with?

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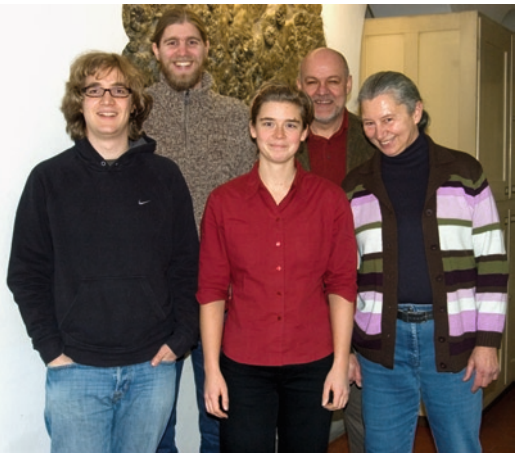

From left to right: Benjamin Läuchli, Frank Gitter, Christine Böhmer, Alexander V. Altenbach, Hanne-Lore Wieczorek

SYMBIOTIC FORAMINIFERA AND STRESS

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1. Foraminifera Outlined

Traditional systematic rankings in micropaleontology are based on the morphology, chemistry, and wall structure of the shell ("test") that most foraminifers develop. Tests may be coiled and substructured in diverse complexity. Test walls are secreted and crystallized in meticulous arrangements, composed of organic matter, agglutinated with foreign matter, mineralized with calcite, aragonite, high-magnesium calcite, or silica. There are several thousand genera characterized by morphotypes (Loeblich and Tappan, 1988). They are unique among protists because they have branching and anastomosing networks of reticulopodia with rapid bidirectional transport of intracellular granules and membrane domains driven by specific tubulin assemblies. Many have life cycles that include a haploid sexually reproducing phase and a diploid asexually reproducing phase. Molecular genetic analysis revealed the early radiation of naked and single-chambered forms, and that the rotaliid and miliolid foraminifera are monophyletic (Pawlowski et al., 2003). Foraminifera (or Granuloreticulosea, see Lee et al., 2001) are considered a stand-alone phylum or First rank group within the eukaryotic Super-group Rhizaria at present (Adl et al., 2005). Classifications are still in transition, as shown by the addition of naked freshwater species, or abyssal giant Rhizaria formerly considered incertae sedis (Adl et al., 2005).

1.1. SYMBIONT-BEARING FORAMINIFERA

The important compilations and textbooks by Lee and Corliss (1985), Hemleben et al. (1989), Lee and Anderson (1991), Hallock (2000), and Lee (2006), provide an extensive source of references on symbiont-bearing foraminifera. The references provided in this chapter are closely focused on stress induced observations, and most recent contributions enlarging this topic.

Modern Foraminifera are hosts for an amazing diversity of phototrophic endosymbionts (dinoflagellates, chlorophytes, rhodophytes, chrysophytes, diatoms, cyanobacteria; see Lee, 2006; Lee and Anderson, 1991). Modern symbiont-bearing benthic forms show morphological test adaptations, such as enlarging and flattening the test, sub-structuring the chambers into chamberlets, and optimizing the cross chamber flow of host cytoplasm. Such adaptations are also obvious for several fossil lineages. Evidence for fossil symbiosis is not incontrovertible, but there are repeated evolutionary patterns since the Palaeozoic (Beavington-Penney, 2004; Lee, 2006; Wade et al., 2008).

Planktonic Foraminifera are not found in the fossil record before the lower Jurassic (BouDagher-Fadel et al., 1997). Their occurrence was concurrent with the global rise of sea levels and the evolution of modern planktonic primary producers (Falkowski et al., 2004; Martin et al., 2008). Their test morphology is consistent with their complex planktonic life styles and their abilities to shelter symbionts, or not (Hemleben et al., 1989). Nevertheless, symbiosis with phototrophs is considered a driving force for their radiation (Lee and Anderson, 1991; Norris, 1996; Wade et al., 2008). Oligotrophic to mesotrophic regions are dominated by symbiont-bearing planktonic taxa, favored by their nutrient recycling and the translucent surface waters. They are outnumbered by asymbiotic planktonic foraminifera only in areas with enhanced primary productivity (Zaric et al., 2005).

In earth history, the onsets of phototrophic symbiosis in benthic and planktonic lineages repeatedly follow oligotrophic limitations. Surface oceans with heavily suppressed nutrient exchange from deeper water masses favored the development of an enclosed nutrient recycling by host–symbiont interactions (Lee and Anderson, 1991).

Non-phototrophic prokaryotes were observed to be free living within the cytoplasm of the very basal benthic foraminiferal clades, as well as in higher ones (Richardson and Rutzler, 1999; Bernhard, 2003; Bernhard et al., 2000, 2006). Such bacterial endosymbionts, or sequestered chloroplasts are most often encountered in hosts facing stressors such as oligotrophy or oxygen depletion (Bernhard and Bowser, 1999, 2008; Bernhard and Sen Gupta, 1999; Bernhard et al., 2006).

2. Phototrophic Symbiosis and Stress

2.1. BENTHIC FORAMINIFERA

Larger benthic foraminifera with symbiotic phototrophs ("LBF") generate a considerable amount of carbonate deposition in reef environments. The annual production ranges from less than 10 gm^{-2} to more than 1 kg m^{-2} (per square meter), amplified mainly by increasing water motion (Fujita et al., 2008). Within the last decades, bleaching affected reefs (Douglas, 2003) and LBF populations on a global scale, accompanied by reproductive dysfunction, malformation, and breakage of the tests. This was first observed in populations of *Amphistegina* spp., and correlated with stressors such as increased water temperatures and irradiance (Williams et al., 1997;

Toler and Hallock, 1998). LBF adapt to a broad range of radiation, either due to the plasticity of their symbionts to changing irradiation (Nobes et al., 2008), or by active movement toward light conditions more appropriate for the growth and reproduction of the holobionts (Lee, 2006). A saturation of the growth rate for *Amphistegina gibbosa* is reached at a photosynthetically active radiation ("PAR") of 6–8 μmol photon m^{-2} s^{-1}, and darkening in color takes place as a photo-protective response when the UVB to PAR ratios exceeded 0.003. Increasing PAR flux and exposure to shorter wavelengths enhances bleaching, and significant growth inhibition occurs above 0.1 W m^{-2} UVB (Williams and Hallock, 2004). Mottled and bleached specimens reveal membrane disintegration, symbiont digestion, and cellular abnormalities such as lysosomes adjacent to symbionts, enlarged vacuoles in the cytoplasm, fewer mitochondria and other organelles, and enhanced granulation of the host cytoplasm (Talge and Hallock, 1995). Such hosts tend to produce malformed ("teratologic") tests, with uneven surfaces, abnormal shapes, poorly defined pore cups, local excess calcification, and degenerated organic matrices. The anomalies are induced by dysfunctional Golgi apparati and endoplasmic reticulae, the sites of glucoprotein and glucosaminglycan synthesis (Toler and Hallock, 1998). The tests lose elasticity, and are more vulnerable to bioerosion and breakage. High light was considered the triggering stressor for the intracellular anomalies, because heat stress alone (32°C or more) was observed to induce symbiont loss, but not the degradation of the host endoplasm (Talge and Hallock, 2003). However, the adaptability on high irradiance may decline with rising water temperatures. *Amphistegina* sampled from a back reef with stable water temperatures near 30°C reacted to high irradiance combined with temperatures of 32°C by darkening, expelling symbionts, and bleaching. The impact of the same stressors declined for other species disposed to larger temperature variations in their natural environment (Strasser et al., 1999).

Combined heat and high-light as stressors do not hinder the stress adaptability of photosystem II, but the quantum yield of the electron transport system (Tsimilli-Michael et al., 1999). ATP levels are higher in specimens subject to high-light stress and partial bleaching, as compared to unaffected specimens (Lee, 2006). Subsequent low-light conditions offer strong thermoprotection and enhanced reversibility from the stress. But in the dark, when host and symbionts turn to respiration, the heat stress generated is most detrimental to the symbiotic system. Due to the fact that under these conditions the photosynthetic capacity is widely reduced (Tsimilli-Michael and Strasser, 2001), the authors conclude that heat stress at night is a major factor for bleaching.

Foraminifera from such heavily stressed populations were considered to show no loss of control during calcification of the test on the ionic level, as Mg/Ca ratios of dissolved tests from stressed and non-stressed populations show no difference (Toler et al., 2001). The dissolution of larger portions of carbonatic tests results in a spatial average of Mg/Ca distributions. Spatial fine structures are mixed in this case. But by applying electron probe microanalysis at 2 μm spot size, high orders of spatial heterogeneity were shown (Raja et al., 2005). The authors assumed that photosynthesis and respiration of symbionts and host were the major cause for the compositional alternations observed.

Reefs heavily affected by nutrient stress and macro-algal dominance offer elevated levels of dissolved N and P for the symbionts. The zooxanthellae outgrow their host to such an extend that the intracellular control of the turnover gets lost (see Lee 2006). Such reefs are settled by specific species of LBF, indicating a more opportunistic behavior and an increased tolerance for amplifying nutrient levels (Renema, 2008). Increased resistance for stressors is achieved by hosting more resistant symbionts, or a multiple symbiotic partnership (Pochon et al., 2007). In contrast, even slightly elevated heavy metal concentrations have a significant negative impact as has been shown when the holobionts have been exposed to copper used as an herbicide (Carnahan et al., 2008). This sensitivity obviously results from the symbionts, as asymbiotic foraminifera are more tolerable, and may even positively correlate with metal pollutants (Carnahan et al., 2008).

Host strategies employed in response to stress are multifold, but guided in first by the variability and imprint of environmental perturbations on the host, not on the host–symbiont interactions. Strategies developed solely for the demands of the symbionts, or their operational optimization, can be deduced from test morphologies (gigantism, test flattening, compartmentation). This is a long-term evolutionary process, evolved in benefit of the host. Short-term stressors not directly perceivable to the host, but critical for the symbiosis, such as heat stress in the dark, or heavy metal poisoning of the symbiont, seemingly are equilibrated less sufficiently. In both cases, a dysfunction of the cell-to-cell signaling system (Lee, 2006) seems reasonable.

2.2. PLANKTONIC FORAMINIFERA

Active control on buoyancy and vertical migration impose smaller and much more lightweight test constructions for planktonic foraminifera, as compared to LBF. Spines are used to support expand the reticulopodial areal space that exposes the algal symbionts to sunlight (Hemleben et al. 1989). Reproductive cycles are rapid, within lunar to semilunar cycles for many species, but environmental perturbations can enlarge or shorten this time span (Hemleben et al., 1989). As the symbionts aid in test calcification, stress caused by symbiont deprivation results in stunted growth and early reproduction (Be et al., 1982). Such progenesis foreshadowed the extinction of several planktonic foraminiferal lineages in the past (Kelly et al., 2001).

Vital effects are substantial for the chemical gradients of the diffuse boundary layer surrounding a living planktic foraminifer with phototrophic symbionts; mainly the carbonate system and the pH may largely differ from bulk sea water (Wolf-Gladrow et al., 1999). During photosynthesis, the algal symbionts preferably remove the lighter ^{12}C, leaving the ambient water enriched in ^{13}C. This leads to enriched incorporation of ^{13}C during test calcification. The fractionation gradient steepens with enhanced photosymbiotic turnover rates, as given by increased test size and number of symbionts, or diminishes with symbiont loss (Erez, 1978; Wade et al., 2008).

The ratio of magnesia and calcium precipitated in the diurnal growth bands of the test of the planktic foraminifer *Orbulina universa* is acutely sensitive to alterations of respiration and photosynthesis of the host and its algal symbionts (Eggins et al., 2004). The authors consider all stressors affecting the vitality of either host or symbiont as influential. These findings bring into question the fundamental premise often made in palaeo-tracer studies, that chemical ratios recovered from foraminiferal tests mirror environmental gradients of the surrounding water body (Eggins et al., 2004; Raja et al., 2005).

The tiny and rare members of the family Guembelitriidae are disaster opportunists in earth history, more resistant to stressors leading to mass extinctions than any other planktic foraminiferal clade (Keller and Pardo, 2004). Facultative symbiosis might be speculated to be reasonable for their extreme resistance to environmental perturbations. But this was never investigated, neither for the fossil taxa, nor for the only modern successor *Gallitella vivans* (Kroon and Nederbragt, 1990).

3. Non-phototrophic Symbionts and Stress Environments

A newly described organic walled ("allogromiid") foraminifer with high numbers of sulfur-oxidizing bacterial endosymbionts thrives under long-term sulfidic conditions in the deep sea (Bernhard et al., 2006). Rod-shaped bacterial endosymbionts, tentatively identified as sulfide oxidizers, were also observed in *Virgulinella fragilis* from sulfidic environments (Bernhard, 2003). In both cases, the metabolism of the symbiotic prokaryont might provide a source of sulfide detoxication urgent for the eukaryotic host which still needs oxygen as a terminal electron acceptor. Chloroplasts sequestered from ingested algae show distinct arrangements in the cytoplasm of *V. fragilis*. As chloroplasts produce hydroxyl radicals, they may offer a source of oxygen for the host by H_2O_2 breakdown, in conjunction with peroxisome proliferation (Bernhard and Bowser, 2008). This "hydroxyl-theory" seems convincing for several reasons. Many foraminiferal species thriving near redox boundary conditions shelter such kleptoplasts (Bernhard and Bowser, 2008). They are often sequestered at water depths, where photosynthetic activities are excluded for energetic reasons. As they may retain functionality for at least one year in the cytoplasm, other purposes have to be considered (Grzymski et al., 2002). Reaching redox boundary conditions, the host is enforced to adapt to heavily toxic stressors, such as free sulfides or sulfide radicals (Grzymski et al., 2002). This might be a functional task for the kleptoplasts as well (Bernhard and Bowser, 2008). The recently discovered autigenic and complete denitrification by Foraminifera at redox boundary conditions (Risgaard-Petersen et al., 2006; Høgslund et al., 2008) proves the extensive self-determination of the foraminiferal intracellular environment. It also confirms an alternative foraminiferal metabolic pathway for oxygen-depleted environments.

Nutritional limitations are a prime threshold for deep-sea environments, most other physical and chemical factors are comparably stable. From this point of view, it seems enigmatic that larger agglutinated taxa may sponsor enormous hydrolytic activities in the deep sea. The values reported for the large, tube-shaped *Hyperammina* sp. surpasses ranges recorded for symbiotic bacterial–macrofaunal interactions by far (Köster et al., 1991; Meyer-Reil and Köster, 1991). Bacterial symbiosis or gardening may be considered here (Richardson and Cedhagen, 2001). But these preliminary findings were never followed by detailed investigations, thus plasticity toward environmental perturbations are unknown.

The observation of chemotrophic bacterial symbionts, functional kleptoplasts and the facultative metabolic pathway provided by complete denitrification expands our overall ideas on foraminiferal ecophysiology. All these symbiotic and physiological adaptations reach into oxygen-depleted and anoxic environments. At present, our knowledge on the chemotrophic symbionts and the number of foraminiferal taxa concerned is preliminary. But it is most fascinating for the evolution of foraminifera that these newly recovered concepts find growing evidence in earth history. During the early evolution of foraminifera, the majority of benthic environmental setups, trophic structures, and available bacterial counterparts for gardening or symbiosis were exposed to hypoxic to sulphidic conditions (Martin et al., 2008). Organizing the intracellular chemistry under anoxic conditions, activating functional kleptoplasts derived from ingested algae, or bacterial gardening and symbiosis with chemotrophs will have been of prime importance for the naked foraminiferal ancestors, and their organic-walled, Proterozoic successors, the allogromids. For the test-constructing successors in the Paleozoic, it is worth to note that hard-shelled foraminifera were found more resistant to prolonged anoxia than allogromiids (Moodley et al., 1997). The early formation of agglutinated (Lower Cambrian) and calcitic (Lower Silurian) tests thus may have had a functional correspondence with anoxia. Toward the Mesozoic and Cenozoic, such necessity will have declined stepwise, in conjunction with the shifting marine oxygen budgets. But the ability to thrive under anoxic and even sulfidic conditions is still carried by the most basal clade (allogromiids), as well as by modern taxa developed in the Cenozoic (*V. fragilis*). Observed adaptations are recorded in conjunction with chemotrophic symbionts, autigenic denitrification, and/or functional kleptoplasts. This may provide a key factor for the evolution of foraminifera, much more influenced by gardening and symbiosis, and reaching much more back in time than hitherto suspected (Richardson and Rutzler, 1999; Bernhard et al., 2006).

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ARBUSCULAR MYCORRHIZAL SYMBIOSIS UNDER STRESS CONDITIONS: BENEFITS AND COSTS

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1. Introduction

Mycorrhization is a highly prevalent association of plants with fungi. Most plant species harbor symbioses with arbuscular mycorrhizal fungi (AMF), which take place within the plant roots. Arbuscular mycorrhizal (AM) symbiosis plays a major role in ecosystems, facilitating nutrient cycling by providing plants with essential nutrients. The AMF are members of the fungal phylum *Glomeromycota* (Schüssler et al., 2001) and form symbiotic associations with most terrestrial vascular flowering plants (Smith and Read, 1997). In addition to increasing nutrient uptake, other key contributions of AMF to plants have been recorded, including improved rooting and plant establishment, improved vegetative growth, and accelerated budding and flowering (Smith and Read, 1997). Moreover, the plant–AMF symbiosis has been shown to promote the plant's ability to withstand numerous abiotic stress conditions. This phenomenon is the subject of the present review.

Stress is composed, according to the literature, of two major processes: the first is a shortage or excess in the availability of a given resource. The second is damage to the biological structures of the organism, originating from damage to the membranes, proteins, or nucleic acids. This type of damage may have a negative effect on metabolism, provoking a response that is intended to limit or reduce the stress effect on the stressed organism (reviewed by Pierce et al., 2005).

Here, we review studies that have demonstrated the effects of AMF on the host plant's responses in general and to stress conditions in particular (Fig. 1). Stress conditions include drought and high soil salinity, conditions of mineral depletion, and growth under low light intensity. In addition, in an attempt to better understand the effects of the fungi on their host plant, we describe some of the physiological and chemical events that take place between the two symbiotic

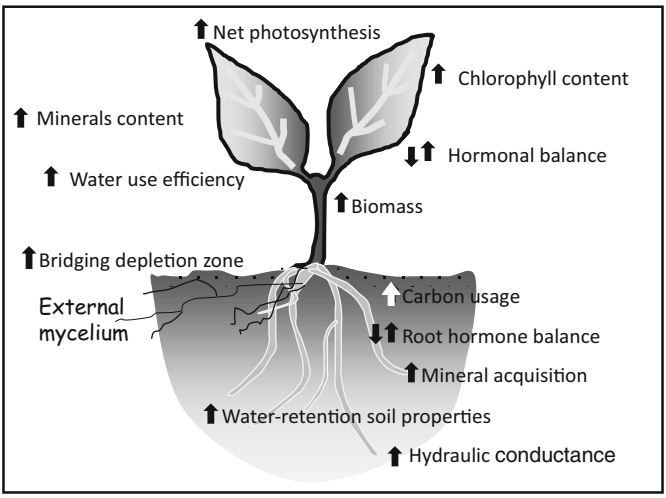

Figure 1. A schematic representation of the physiological and biochemical alterations in the properties of a mycorrhizal plant during symbiosis with AMF. Arrows pointing up represent an increase relative to non-mycorrhizal plants; arrows pointing down represent a decrease relative to non-mycorrhizal plants. Black arrows denote benefits of symbiosis, white arrow, cost of symbiosis.

partners. We suggest some notions as to the mechanisms that govern each of the AM-enhanced plant responses to the abiotic stress conditions. Some possible future applications of the symbiotic association are presented, and the costs and benefits of AM symbiosis under stress conditions are discussed.

The biological, chemical, and molecular characteristics of AMF have been the subject of numerous studies, and these have been discussed in a number of recent reviews (e.g., Gianinazzi-Pearson and Brechenmacher, 2004; Balestrini and Bonfante, 2005; Balestrini and Lanfranco, 2006; Genre and Bonfante, 2005; Harrison, 2005; Hause and Fester, 2005; Karandashov and Bucher, 2005; Paszkowski, 2006; Bucher, 2007; Reinhardt, 2007; Koltai et al., 2010).

2. Arbuscular Mycorrhizal Symbiosis

It has been suggested that the coordinated biological processes that occur in both the fungus and the plant are highly controlled by both symbiotic partners. A reflection of the plant's coordination of the symbiotic process might be the presence of mutants that "resist" colonization by the fungi (David-Schwartz et al., 2001, 2003; Gadkar et al., 2001; Paszkowski et al., 2006; Reddy et al., 2007); the existence of such mutants suggests genetic control, by the host plant, of mycorrhizal establishment.

The AMF–host symbiotic association can be functionally divided into two stages:

The first is a "pre-symbiotic" stage, during which soil-borne propagules, which are resting chlamydospores (i.e., large, thick-walled, multicellular, resting spores), germinate (this happens even if the host root is absent) and grow to a limited extent. In the presence of host root, the hypha grows and starts to branch (Mosse and Hepper, 1975; Gianinazzi-Pearson et al., 1989; Giovannetti et al., 1993a, b, 1994; Buée et al., 2000; Nagahashi and Douds, 2000; Vierheilig and Piché, 2002; Bećard et al., 2004; recently reviewed by Requena et al., 2007). Once the hypha comes into contact with a host root, an appressorium is formed by the fungus as a contact structure with the root epidermis, and a pre-penetration apparatus is formed by the host root in a cell-layer-controlled fashion (Genre et al., 2005; Siciliano et al., 2007). These morphological changes necessitate a reciprocal exchange of signals between the two symbionts, and are necessary in order to proceed to the second stage (reviewed by Paszkowski, 2006).

The second stage is the "symbiotic" stage, during which the growing fungal hyphae penetrate the root epidermis and develop within the root cortex, forming characteristic functional structures called arbuscules. However, in practice, no cell penetration occurs: the fungal arbuscules remain in the apoplastic compartment of the cell, surrounded by an interfacial matrix and a peri-arbuscular membrane that protrudes into the plant cell. These arbuscular constructions are the site of bidirectional exchange between the fungus and the plant: the fungus translocates mineral elements, and in turn absorbs carbon from the host (reviewed by Harrison, 2005). In parallel, an extraradical mycelium is produced by the fungus from which chlamydospores are eventually formed (Smith and Read, 1997). During both the pre-symbiotic and symbiotic stages, growth and differentiation of both the plant root and the fungus are tightly coordinated, and are likely to involve reciprocal recognition and regulation, either in an intimate cell-to-cell fashion or by exchange of diffusible signals between distantly positioned symbiont structures.

3. Effect of Arbuscular Mycorrhizal Symbiosis on Plant Stress Responses

AM symbiosis involves both benefits and costs. On the one hand, the fungi provide the plants with a variety of minerals, primarily those which are less mobile in the soil solution (e.g., phosphorus [Pi], zinc [Zn], iron [Fe]), benefiting the host. On the other, AMF are obligate symbionts, and as such they require fixed carbon from their host; this is the cost of the symbiosis to the host. AMF mediate carbon partitioning in the plant host: they affect the carbon source and whole-plant carbon partitioning. Moreover, the fungi may take up glucose in an amount estimated at between 4 and 20% of the plant's total photosynthetic products (reviewed by Douds et al., 2000).

As the AM symbiosis is known to enhance host resilience under stress conditions, the relative weights of the costs and benefits may change in such situations.

3.1. DROUGHT

Drought significantly inhibits plant growth and development (Kramer and Boyer, 1997). It imposes osmotic stress on the plant; this stress is perceived by the plant and provokes a defensive response (reviewed by Pierce et al., 2005).

A number of studies have shown that AM symbiosis can improve plant resistance to drought (e.g. Subramanian and Charest, 1998; Ruiz-Lozano and Azcón, 2000; Porcel et al., 2003; Bolandnazar et al., 2007): both increased dehydration-avoidance and increased tolerance have been reported. These two measures are discussed in Allen and Boosalis (1983), Davies et al. (1993) and Augé (2001), and are further discussed here in the Summary. In contrast, in other studies, little or no AM enhancement of drought resistance was demonstrated (e.g. Hetrick et al., 1987; Simpson and Daft, 1991).

AMF have been shown to affect plant resilience to drought conditions via several different mechanisms. One is an effect on water movement: AMF were shown to affect the rate of water movement into, within and out of the plant (reviewed by Ruiz-Lozano, 2003), an ability that is mediated by their effect on the plant's osmotic adjustments (i.e., osmoregulation). Osmoregulation is the cell's ability to decrease its osmotic potential by actively accumulating organic compounds, in order to maintain water content, cell turgor and associated cellular processes (Morgan, 1984; Hoekstra et al., 2001). AMF were shown to enhance the plant cell's ability to accumulate organic compounds, such as sugars and amino acids (reviewed by Ruiz-Lozano, 2003), and to increase levels of ions (of magnesium [Mg^{2+}], potassium [K^{+}] and calcium [Ca^{2+}]) and sugars, including soluble sugars and soluble starch. Porcel and Ruiz-Lozano (2004) found that AM roots of soybean accumulate more proline than non-AM roots under drought conditions, while the opposite was observed in shoots; proline is an amino acid known to be accumulated in plants due to drought stress conditions and to act as osmoregulator (Porcel and Ruiz-Lozano, 2004 and references therein). Proline levels, however, were shown to be reduced during drought conditions in citrus, suggesting osmotic adjustment of mycorrhizal plants mainly via adjustment of ion and sugar levels, rather than proline (Wu and Xia, 2006).

Mycorrhizal plants demonstrate increments in leaf stomatal conductance, accompanied by a higher rate of gas exchange (Augé et al., 1987, 1992, 2004, and reviewed in Augé, 1989, 2001; Bethlenfalvay et al., 1987; Ruiz-Lozano et al., 1995a,b; Duan et al., 1996; Goicoechea et al., 1997; Cho et al., 2006). AMF also affect leaf water potential, and leaf osmotic potential as well, albeit to a lesser extent (Allen and Boosalis, 1983; Augé et al., 1986; Cho et al., 2006). These effects may be due to the fungi's ability to enhance water uptake and transport in the plant. Accordingly, enhanced cumulative transpiration was observed in mycorrhizal plant leaves (Querejeta et al., 2007).

Recently, it was shown that AM symbiosis affects leaf hydraulic homeostasis, the latter preventing large and potentially harmful fluctuations in transpiration-induced water potential gradients across the leaf. The results suggested that leaf

hydraulic homeostasis tends to be higher in leaves of mycorrhizal plants. In addition, leaf hydraulic homeostasis was correlated with higher productivity potential. Hence, higher leaf hydraulic homeostasis of mycorrhizal plants may be consistent with their higher rates of gas exchange. These changes are presumed to be necessary to supply the AMF with its carbon needs (Augé et al., 2008), and hence to require sufficient light intensities.

Another mechanism of increased drought tolerance that is facilitated by AMF may be mediated via the effect of AM symbiosis on the soil's water-retention properties (Augé et al., 2001). It has been suggested that fungal exudates, which make up the mycorrhizosphere, affect soil structure (Jastrow and Miller, 1991; Oades and Waters, 1991) and promote soil aggregation (Rillig et al., 2002). This, in turn, increases soil moisture retention (Hamblin, 1985) and reduces changes in soil matric potential upon drought (Augé et al., 2001).

In plants, drought conditions lead to increased oxidative stress, which is associated with degenerative inter- and intracellular reactions. One of the routes to mediating oxidative stress in plants is producing or activating reactive oxygen species, such as superoxide dismutases (SODs; reviewed in Blokhina et al., 2003). Increased plant-originated SOD activity was evident in mycorrhizal plants relative to non-mycorrhizal plants upon drought conditions (Ruiz-Lozano et al., 1996b, 2001).

Increased concentrations of antioxidant enzymes and non-enzymatic antioxidants were found in mycorrhizal citrus plants. These antioxidant compounds were found to protect the plants against oxidative damage, and this was suggested to enhance drought tolerance (Wu et al., 2007).

Lower oxidative damage to lipids in shoots of desiccated AM soybean plants relative to their non-AM counterparts was also demonstrated (Porcel and Ruiz-Lozano, 2004). However, the lower oxidative damage to lipids in AM plants was not correlated with the activity of antioxidant enzymes (Porcel and Ruiz-Lozano, 2004).

Mycorrhization was also found to decrease stress-response levels: mycorrhizal plants were shown to regulate their levels of abscisic acid (ABA; a coordinator of stress responses in plants; Wilkinson and Davies, 2002) better and faster than non-mycorrhizal plants. This was accompanied by a more adequate balance between leaf transpiration and root water movement in mycorrhizal plants than in non-mycorrhizal plants during drought and drought-recovery periods (Aroca et al., 2008).

Hence, in most cases, AMF enhance plant resilience under drought conditions. A recent attempt to utilize AMF technology in commercially grown pepper showed that AMF application may enhance water conservation: under desert conditions, where irrigation with saline water is practiced, a substantial amount of the recommended irrigation regime goes to leaching the salts from the root zone and not to plant water uptake. Reductions in the applied water levels lowered leaching from the root zone, led to increased salt concentration in the soil solution of the active root zone, and, as a result, to a significant reduction in

productivity. However, mycorrhitic pepper plants grew well under these conditions, despite the increased soil salinity, and yielded as much as non-mycorrhitic plants irrigated with the recommended level of water. Hence, mycorrhiza may be included in growth protocols of peppers that are commercially grown under semi-arid conditions, where water resources are limited. Inclusion of the mycorrhiza may lead to reduced water consumption and to alleviation of the effects of abiotic stresses that occur throughout the growing season (Pivonia et al., 2008).

3.2. HIGH SALINITY

Host salt resistance is improved by AMF colonization in a large number of crops, including clover (Ben Khaled et al., 2003), cucumber (Rosendahl and Rosendahl, 1991), guayule (Pfeiffer and Bloss, 1987), lotus (Sannazzaro et al., 2007), maize (Feng et al., 2002), mung bean (Jindal et al., 1993), *Sesbania* sp. (Giri and Mukerji, 2004) and tomato (Al-Karaki, 2000; Al-Karaki et al., 2001). High soil salinity may affect plants by creating both drought conditions and salt toxicity (Munns, 2002). The salinity-resistant phenotype of AMF-inoculated plants was suggested to be a result of reductions in sodium (Na) uptake, together with an associated increase in Pi, nitrogen (N) and Mg absorption and high chlorophyll content, relative to non-mycorrhizal plants (Cho et al., 2006 and references therein). Similarly, mycorrhizal zucchini plants, even under high Pi concentration (which is unfavorable for AMF; Menge et al., 1978; Thomson et al., 1991), exhibited a higher absolute value of yield and shoot biomass than non-mycorrhizal plants, all grown under saline conditions. In addition, higher leaf chlorophyll content, higher relative water content, higher K concentration, and lower Na concentration were recorded in leaf tissues, compared to non-mycorrhizal plants. The Pi content, however, was similar in this study between mycorrhizal and non-mycorrhizal zucchini leaf tissue, grown under both low and high Pi concentrations (Colla et al., 2008).

Another effect of mycorrhiza on plants that was suggested to improve their resilience to salinity stress was improvement of osmoregulation (also discussed above, for drought stress), mediated via the accumulation of polyamines and proline (Sannazzaro et al., 2007). In contrast, mycorrhizal lettuce had higher water and lower proline levels when responding to salt stress, relative to non-mycorrhizal plants (Jahromi et al., 2008). Neither Pi supplementation nor a high level of nutrients alone could mimic AM-related protection against salinity stress in lettuce and alfalfa (Ruiz-Lozano et al., 1996a; Azcón and El-Atrash, 1997).

As with resilience to drought stress, reduced salt-stress injury was suggested as a possible mechanism for AMF enhancement of the plant's resilience to salt stress: reductions in the expression of a stress-marker gene and in ABA levels were recorded in mycorrhizal plant roots as compared to non-inoculated roots, suggesting lower levels of response to salt stress in mycorrhizal plants (Jahromi et al., 2008).

Finally, it might be that the AMF-induced drought response is mediated by a reaction to high salinity. This is due to the fact that under conditions of soil drought, as the soil dries, solutes may concentrate in the soil solution adjacent to the roots, leading to high-salinity conditions near the roots (Stirzaker and Passioura, 1996). Cho et al.'s (2006) results, however, do not support this notion: they show that in some cases, the addition of salt could abolish, rather than enhance, the AMF-induced drought response in plants (e.g., as reflected by stomatal conductance measurements).

3.3. MINERAL DEPLETION

Mineral deficiencies are very common in both natural and agricultural soils. This is because the accessibility of mineral elements, in most soils, is lower than that required by the plant for optimal growth. Hence, mineral depletion is one of the most common abiotic stresses experienced by plants during their growth.

To meet the nutrient demand of soil-grown plants, nutrients must reach the root surface, and this is mainly mediated by movement or transport within the soil solution. In some soils, concentration and mobility of minerals depend upon complex formation between the minerals and organic ligands or between the minerals and solid soil particles. Hence, two factors need to be considered when assessing nutrient supply to the roots. One is the concentration of nutrients in the soil solution. Many factors, such as soil moisture, depth, pH, cation-exchange capacity, redox potential, organic matter content, and microbial activity can affect soil-solution mineral contents (Marschner, 1995). The second factor, in many cases, is the availability of minerals for direct uptake. For example, the availability of major soil minerals, important for plant growth, such as Pi, Ca, aluminum (Al) and Fe phosphates, may be altered by the rhizospheric soil pH (Marschner, 1995).

Although supplementing the soils with chemical fertilizers may rectify the nutrient depletion, to genuinely meet the nutrient demands of soil-grown plants, nutrients must not only be present in sufficient concentrations, but also be available to the root uptake machinery. Moreover, when the roots are active in mineral and water uptake, the mineral concentrations in close proximity to the root surfaces decrease rapidly, leading to the development of a "depletion zone". If the depletion zone is not replenished, conditions of nutritional stress are created.

One of the possible mechanisms by which AMF enhance plant nutrition is by bridging the depletion zone, which they do by providing a greater root surface area. The AMF hyphae associated with the plant root can exploit larger volumes of soil. This greater root surface area may enable the plant to overcome the deficits in mineral nutrients and water typically found in the depletion zone, near the root surface, by exposing a greater surface, outside the depletion zone, for mineral uptake. In addition, the small diameter of the fungal hyphae (averaging 3–4 μm) enables them to penetrate soil pores and cavities and contact soil particles that are

inaccessible to roots and root hairs (the latter having an average diameter of >10 μm). Fungal hyphae normally transport mineral nutrients from further away than non-mycorrhizal roots; the mycorrhiza expands the root absorption zone by several millimeters, to as much as 10 cm (Rausch and Bucher, 2002).

Pi is a major structural element in nucleic acids, phospholipids, and several enzymes and coenzymes. Pi is also involved in energy metabolism and signal-transduction cascades. The most pronounced growth enhancement elicited by AMF is due to the improved supply of several low-mobility minerals from the soil solution, predominantly Pi (Smith and Read, 1997). Moreover, Pi is usually depleted from the rhizosphere, as its absorption rate by roots is higher than its diffusion rate in the soil. This leads to the depletion zone at the root surface and, as a result, to Pi constraints on plant growth (Marschner, 1995).

The external mycorrhizal hyphae can absorb and translocate Pi to the host from beyond the root depletion zone; the uptake rate of Pi per unit root length is two or three times higher in mycorrhizal vs. non-mycorrhizal plants (Smith and Read, 1997). Several factors contribute to the high uptake efficacy of the AMF hyphae in Pi absorbance. One is the small diameter and large surface area presented by the hyphae, the second is the accumulation and storage of polyphosphates in the fungal cell vacuoles (Poirier and Bucher, 2002), and the third is the presence of specific Pi transporters. The efficacy of AMF in providing Pi to the host plants strongly depends on the AMF species and ecotype, and varies among plant species (Smith and Read, 1997).

Pi transporters can be divided into two groups. One group is the plant-originated Pi transporters; these plant Pi transporters may be either constitutively expressed or induced only upon AMF colonization (Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002). The second group is the fungus-originated Pi transporters. Two AMF Pi transporters, exhibiting high affinity towards Pi, have been identified: GvPT and GiPT from *Glomus versiforme* and *Glomus intraradices*, respectively (Harrison and van Buuren 1995; Maldonado-Mendoza et al., 2001; Benedetto et al., 2005). It was suggested that most Pi can be taken up via the mycorrhizal uptake pathway (Smith et al., 2003, 2004); these fungal Pi transporters were shown to be located on the external hyphae, which are responsible for the uptake of rhizospheric Pi and its translocation to the host plant (Brundrett, 2002). Nevertheless, only little is known about the mechanism governing Pi translocation from the fungus to the plant. This translocation may be facilitated by the plant Pi transporters, perhaps those that are induced by the AMF (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001), especially because they have a higher affinity for Pi than the fungal Pi transporters (Harrison et al., 2002), which may lead to Pi translocation from the fungi to the plant cells.

Minerals other than Pi are found at distinctly higher levels in mycorrhizal vs. non-mycorrhizal plants, including N, Zn, copper (Cu), Ca, Mg, K, sulfur (S), and manganese (Mn) (Raghothama, 2000); however, their uptake mechanism is not well understood.

To conclude, AMF support plant mineral nutrition under conditions of mineral depletion. However, under conditions of high levels of minerals, as found in highly fertile soils, fungal spore germination and growth are inhibited. High Pi levels strongly inhibit various stages of the spore's ontogenic cycle and do not result in the establishment of symbiosis (Menge et al., 1978), whereas high levels of organic amendment lead to the production of toxic compounds, such as ammonia, which suppress the soil biota, including AMF (Pfleger and Linderman, 1994).

3.4. LOW LIGHT INTENSITY

Conditions of low light intensity are especially prevalent under the tree canopy in tropical ecosystems, and as such represent a most important growth factor there (Lee et al., 1996). This is because low light intensity may dramatically delay plant growth and development. Nevertheless, only a small number of studies have methodically examined the ability of mycorrhizal symbiosis to enhance plant growth under conditions of low light intensity.

Bereau et al. (2000) exposed *Dicorynia guianensis* Amshoff, a tree species endemic to the Amazonian forest, to different light-intensity regimes. Under medium light intensity (14% and 50% of ambient sunlight), the AMF-inoculated plants exhibited a better growth performance than the non-inoculated ones. However, under low light intensity (1% of ambient sunlight), AMF-inoculated trees exhibited a lower number of leaflets and a higher rate of mortality than non-inoculated trees. In parallel, AMF development was more prominent under medium light intensity than under low light intensity (Bereau et al., 2000).

Five understory shrub species of moist neotropical forests were studied by Kyllo et al. (2003) to determine the effects of AM symbiosis on root hydraulic conductance in both carbon-rich and carbon-limited host plants. AM colonization improved root hydraulic conductance for the more shade-tolerant species (i.e., those adapted to low light intensities) when growing in low light. These results suggested that AMF are an important determinant of the plant's ability to grow under certain environmental conditions, due to effects on both nutrient and water uptake (Kyllo et al., 2003). The AMF's ability to determine plant growth success under low light intensities may also be a result of the carbon needs of the fungi (i.e., the cost of symbiosis to the plant): it could be that only plant species that are adapted to low light intensities can provide the fungi's need for carbon (due perhaps to their greater carbon-assimilation ability).

Together, these results suggest that above a certain threshold of photosynthesis, mycorrhizae may benefit the host's growth under low-light-intensity stress. However, low light conditions limit the plant's photosynthetic ability, especially in species that are not adapted to low light conditions; as mycorrhizae rely on the efflux of photosynthesized products from the plant, their benefit to the host plant, under these conditions, may be questionable.

4. Summary

Under most of the examined stress conditions, mycorrhizal symbiosis appears to increase the host's resilience to adverse abiotic stress. Mycorrhizal plants grow better under conditions of drought, salinity, and mineral depletion. However, under stress conditions that directly limit the carbon source, particularly under low light intensity in species that are not adapted to low light conditions, fungal development may become a burden below a certain threshold of carbon production, inhibiting host growth, rather than promoting it. Under these conditions, the carbon demanded by the fungi becomes a cost that is too high for the stressed plant to pay.

Moreover, various stress conditions may further define the nature of the plant–AMF interaction. Plant growth responses to different mycorrhizal isolates, within an ecosystem, can range from highly mutualistic (i.e., symbiosis) to low mutualistic and even parasitic (Klironomos, 2003). The coordinates of the mycorrhizal association along this mutualistic–parasitic continuum may be defined by the environmental stress conditions. For example, under drought or mineral depletion, when the carbon source is not limited, the presence of AMF may benefit the host, forming a true symbiosis, with both partners gaining mutual benefits from the association. However, under low light conditions, in which carbon sources are limited and hence the cost imposed by the AMF association is greater than its benefit, the association may tend towards parasitism.

The AMF-induced elevation of plant resilience to stress may be a result of either avoidance or tolerance (or both) (Allen and Boosalis, 1983; Davies et al., 1993; Augé, 2001) to stress conditions. Increased tolerance by AMF is probably a result of the activation of preexisting mechanisms in the plant, with mycorrhizal colonization making them more vigorous or rapid, relative to non-mycorrhizal plants.

AMF-facilitated avoidance of stress conditions may be a result of the plant's ability to postpone, to some extent, exposure to extreme conditions. Generally, the classification of stress tolerance (or sensitivity) of a plant species is based on two parameters: a threshold, beyond which a reduction in plant performance is recorded, (Fig. 2) and the slope beyond the threshold, i.e., the rate of decrease in plant performance (e.g. biomass, yield, etc.). The threshold remains the same for mycorrhizal and non-mycorrhizal plants (Fig. 2, point A), regardless of the stress condition induced. However, the AM plant meets this threshold with a higher growth rate. Intensifying the stress conditions, beyond the threshold, leads to a similar reduction in growth rate for both mycorrhizal and non-mycorrhizal plants (similar slopes for +AM and -AM in Fig. 2); however, at each given point (stress level), the relative growth rate of the mycorrhitic plant is significantly higher than that of the non-mycorrhitic plant (Fig. 2). This situation brings the AM plant to a critical point at a higher stress level (Fig. 2, point C) than the non-AM plant (Fig. 2, point B).

The ability of AMF to elevate a plant's resilience to stress may have been one of the factors promoting the coevolution of plants and fungi: AMF have been

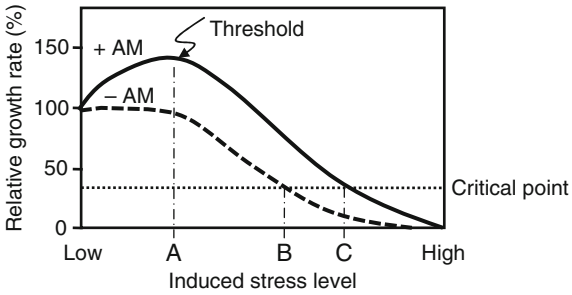

Figure 2. Schematic representation of the relative growth rate of mycorrhizal (+AM) and non-mycorrhizal (-AM) plants under increasing levels of stress. (A) The threshold for non-mycorrhizal and mycorrhizal plants; (B, C) critical points for non-mycorrhizal and mycorrhizal plants, respectively.

dated to the early Devonian Period (417–354 million years ago [MYA]) or even earlier (Remy et al., 1994). During the Silurian-Devonian era, 443–354 MYA, nutrients were also limited, and might have constituted the primary selection pressure for plant growth (reviewed by Pierce et al., 2005). Perhaps stress conditions during these eras enhanced the evolution of mycorrhizal symbiosis. Alternatively, enhancement of resilience to stress conditions may have evolved for both symbiotic partners within the evolving symbiosis.

Interestingly, it may be that, by promoting host stress resilience (by, for example, increasing leaf hydraulic homeostasis; Augé et al., 2008), the fungi increase the host's ability to assimilate carbon. This, in turn, increases the fungi's own supply of carbon. Hence, the need for carbon, rather than the enhancement of host resilience to stress conditions, may be the fungi's ultimate motive for effecting the physiological changes described in the host.

Due to the fungi's ability to promote plant resilience to stress conditions, the mycorrhizae's contribution to the ecosystem may consist of increments in sustainability, especially under prolonged stress conditions. It has been suggested that AMF be considered as dispersal agents (reviewed by Purin and Rillig, 2008), promoting the plant's adaptiveness to a given ecosystem, and therefore increasing ecosystem productivity.

This is especially important for agricultural practices: in view of the predicted changes in the global environment, AMF symbiosis may serve as a force for the enhancement of crops' adaptation to the altered environment, developing a new agricultural cropping environment. For example, it is already evident that AMF implementation in agricultural systems can reduce water use. This is important not only for today's practice of propagating crops in semidesert and desert areas, but also for future agriculture, as it is expected that new and current crop varieties will have to withstand the stress conditions that are developing as a result of the impending global environmental changes.

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MODULATION OF AQUAPORIN GENES BY THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS IN RELATION TO OSMOTIC STRESS TOLERANCE

Aquaporin in AM Plants Under Osmotic Stress

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1. Introduction

Plants are constantly confronted with environmental constraints of both biotic and abiotic origin. Abiotic stresses such as drought, salinity, and extreme temperatures are the most common environmental stress factors experienced by soil plants (Seki et al., 2003). All these stresses share a common osmotic component as they cause a dehydration of plant tissues. Thus, we refer to them as osmotic stresses. The dehydration caused by those stresses is a consequence of the imbalance between the water lost in the leaves and the water taken up by roots (Aroca et al., 2001). Indeed, water deficit caused by osmotic stresses is one of the most common environmental stress factors experienced by soil plants. It interferes with both normal development and growth and has a major adverse effect on plant survival and productivity (Kramer and Boyer, 1997; Bray, 2004). There is broad consensus that climate change continues to occur and that stresses from climatic extremes will continue, and possibly increase, and thus impose significant difficulties on plant and crop growth in many parts of the world. These difficulties will be particularly pronounced in currently semiarid agricultural zones and/or under conditions of irrigation that often exacerbate soil salinization (Araus et al., 2003; Denby and Gehring, 2005).

The negative water potential in drying or saline soils obliges plants to face the problem of acquiring sufficient amount of water (Ouziad et al., 2006), a process in which aquaporins participate (Luu and Maurel, 2005). Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient (Kruse et al., 2006). These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Maurel, 2007). Two major classes of plant aquaporins, located in the plasma membrane (PIPs) or tonoplast (TIPs), respectively, have been identified so far. Another two classes of plant aquaporins are the homologues to the soybean Nodulin-26 aquaporin (NIPs) and the small basic intrinsic proteins (SIPs) (Johanson et al., 2001). The localization and function of SIPs are not clear at the moment

(Luu and Maurel, 2005), although the membrane of endoplasmic reticulum seems to contain SIPs (Ishikawa et al., 2005).

The discovery of aquaporins in plants has caused a significant change in the understanding of plant water relations. In recent years, much effort has been concentrated on investigating the function and regulation of aquaporins. High levels of aquaporin expression have been shown not only in tissues with high water fluxes across membranes, e.g., in fast-growing regions, in shoots and leaves, but also in roots where water uptake occurs (Otto and Kaldenhoff, 2000). Thus aquaporins seem to play a specifically important role in controlling transcellular water transport in plant tissues (Javot and Maurel, 2002; Zhao et al., 2008). However, the relationship that exists between aquaporins and plant responses to water deficit still remains elusive and contradictory (Aharon et al., 2003; Lian et al., 2004). In addition, although many aquaporins are highly selective for water, uptake experiments with *Xenopus laevis* oocytes have clearly shown certain aquaporins to be permeable to small solutes such as glycerol, urea, amino acids, CO_2, and/or NH_3/NH_4, or even small peptides and ions (Uehlein et al., 2003, 2007; Kaldenhoff et al., 2007), raising many questions about the physiological roles of aquaporins.

Most terrestrial plants can establish a symbiotic association with a group of soil fungi called arbuscular mycorrhizal (AM) fungi. The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions (Smith and Read, 1997). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through, and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews see Augé, 2001, 2004; Ruiz-Lozano, 2003). Thus, it is accepted that AM symbiosis can protect host plants against the detrimental effects of water deficit and that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional, and cellular effects (Ruiz-Lozano, 2003). Studies carried out so far have suggested several mechanisms by which the AM symbiosis can alleviate drought stress in host plants. The most important are: direct uptake and transfer of water through the fungal hyphae to the host plant (Hardie, 1985; Ruiz-Lozano and Azcón, 1995; Marulanda et al., 2003), better osmotic adjustment of AM plants (Augé et al., 1992; Ruiz-Lozano et al., 1995; Kubikova et al., 2001), enhancement of plant gas exchange (Augé et al., 1992; Ruiz-Lozano et al., 1995, Goicoechea et al., 1997; Green et al., 1998), changes in soil water retention properties (Augé et al., 2001), and protection against the oxidative damage generated by drought (Ruiz-Lozano et al., 1996, 2001; Porcel et al., 2003; Porcel and Ruiz-Lozano, 2004).

The AM system is an excellent example for the extensive morphological alterations that plant root cells undergo in order to accommodate the presence of symbionts. Since most of the mycorrhiza-induced changes in plant root cells concern cytoplasmic or vacuolar membrane systems, a variation of expression patterns concerning genes that encode membrane-associated proteins such as aquaporins can be

expected (Krajinski et al., 2000). In the following sections, we summarize the current information regarding alteration of aquaporin-encoding genes by the AM symbiosis under a variety of stresses sharing a common osmotic component, and its relation with the enhanced tolerance to water deficit conferred by the AM symbiosis.

2. Expression of Aquaporin Genes in AM Plants Under Drought Stress Conditions

The literature on aquaporins modulation by AM symbiosis has risen significantly in the last decade as reviewed recently by Uehlein et al. (2007). The first report on the modulation of aquaporin genes by AM symbiosis was provided by Roussel et al. (1997) followed by Krajinski et al. (2000), who found mycorrhiza-induced expression of TIP aquaporins in parsley and *Medicado truncatula*, respectively. Krajinski et al. (2000) related the changes in aquaporin gene expression to the changes in plant roots due to fungal colonization. In fact, during AM formation the plant plasma membrane extends to form a novel periarbuscular membrane, which closely surrounds the fungal hyphae resulting in an estimated three- to ten-fold increase in the outer plant cell surface (Bonfante and Perotto, 1995; Gianinazzi-Pearson, 1996). It was proposed that the up-regulation of aquaporins by the AM symbiosis probably optimizes nutrient and water exchange between both symbiotic partners (Krajinski et al., 2000). However, the studies by Roussel et al. (1997) and Krajinski et al. (2000) were carried out under well-watered conditions and they did not test the expression of the aquaporin gene in AM plants under water deficit conditions.

Several aquaporin-encoding genes have been shown to be up-regulated in ectomycorrhizal poplar plants, and this was correlated with an increased water transport capacity of mycorrhizal poplar roots (Marjanovic et al., 2005). PIP and NIP aquaporin genes from *Medicago truncatula* were also shown to be induced by mycorrhization, while other four aquaporin genes analyzed did not change their expression pattern as consequence of mycorrhization (Uehlein et al., 2007). Authors of this work related the mycorrhiza-induced change in expression of the two genes with physiological changes in the plant roots, i.e., the symbiotic exchange processes located at the pariarbuscular membrane (Uehlein et al., 2007). In contrast to the induction of aquaporin gene expression by mycorrhization, Ouziad et al. (2006) showed a decrease in the expression of PIP and TIP aquaporins by mycorrhizal colonization and salt stress in tomato plants.

The effects of reduced expression of the PIP aquaporin-encoding gene *NtAQP1* were investigated in mycorrhized *NtAQP1*-antisense tobacco plants under both, drought stress and well-watered conditions (Porcel et al., 2005). The objectives were to elucidate whether or not the impairment in *NtAQP1* gene expression affected the AM fungal colonization pattern and to find out if such impairment had any effect on the symbiotic efficiency of AM fungi. Reduction of *NtAQP1* expression had no effect on the colonization of the plant root by two

AM fungi, suggesting that either *NtAQP1* function is irrelevant for the process of root colonization or that the impairment in *NtAQP1* gene expression has been compensated by changing the abundance or the activity of other aquaporins (Eckert et al., 1999; Johansson et al., 2000). In contrast, when Porcel et al. (2005) measured the symbiotic efficiency of two AM fungi (in terms of plant biomass production), they observed that under drought stress, mycorrhizal wild-type plants grew faster than mycorrhizal *NtAQP1* antisense plants. This indicates that the symbiotic efficiency of both AM fungi was greater with wild-type than with antisense plants and that the water transport meditated by NtAQP1 seems to be important for the efficiency of the symbiosis under drought stress conditions (Porcel et al., 2005). This may be related to the fact that *NtAQP1* allow CO_2 passage and is involved in plant growth promotion (Uehlein et al., 2003).

The results obtained in tobacco raised the question of what happened with aquaporin genes in AM plants when subjected to water deficit. In fact, mechanisms of osmotic adjustment and modulation of tissue hydraulic conductivity are required to maintain tissue water potential under water deficit conditions. Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurel, 2007). Since aquaporins are regulated both at transcriptional and activity levels (Martre et al., 2002), we have studied whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanism to enhance host plant tolerance to water deficit. To achieve this, genes encoding plasma membrane aquaporins (PIPs) from soybean and lettuce were cloned and their expression pattern studied, in AM and non-AM plants cultivated under well-watered or drought stress conditions (Porcel et al., 2006). The starting hypothesis was that if AM fungi can transfer water to the root of the host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be up-regulated in order to allow a higher rate of transcellular water flow (Javot and Maurel, 2002).

In contrast to the above hypothesis, results obtained showed that the *PIP* genes studied were down-regulated both in soybean (Fig. 1a) and lettuce (Fig. 2) under drought stress and that such down-regulation was even more severe in plants colonized by *Glomus mosseae* than in non-AM plants (Porcel et al., 2006). A similar result was obtained by Ouziad et al. (2006) regarding the expression of PIP and TIP genes in roots of AM tomato plants subjected to salt stress. When the expression of *GmPIP2* gene from soybean was analyzed in a time-course (Fig. 1b), it was clearly visible that AM plants already down-regulated that gene significantly at 5 days after inoculation (dai) and 12 dai, while both non-AM control plants still maintained *GmPIP2* gene expression almost unaltered. At 20 dai, the more intense down-regulation of that gene in AM plants than in both non-AM plants was still clearly visible. Finally, at 35 days all treatments had the same level of *GmPIP2* gene expression.

The effect of the AM symbiosis anticipating the down-regulation of *GmPIP2* gene may have a physiological importance to help AM plants to cope with drought stress. In fact, according to Aharon et al. (2003), the overexpression of a PIP

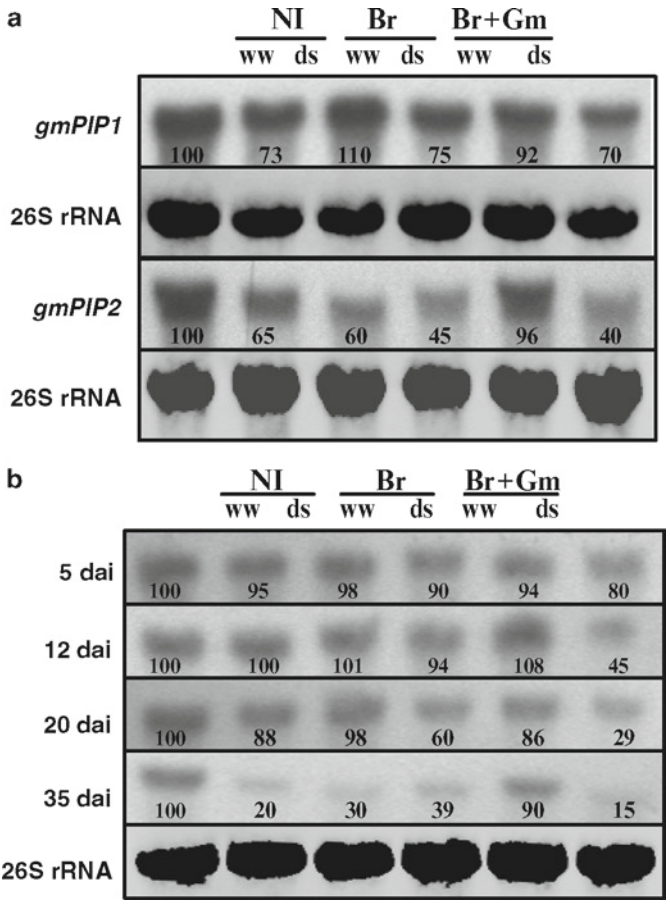

Figure 1. (**a**) Northern blot of total RNA (15 µg) from soybean roots using *gmPIP1* and *gmPIP2* gene probes. (**b**) Northern blot of total RNA (15 µg) from soybean roots harvested 5, 12, 20, or 35 days after inoculation (dai) using *gmPIP2* gene probe. Treatments are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br+Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern. The lower panel shows a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). (Reproduced from Porcel et al., 2006. With kind permission of Springer Science and Business Media.)

aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions, but the overexpression of such *PIP* gene has no beneficial effect under salt stress, and has even negative effect during drought stress, causing fast wilting. A similar result has been obtained more recently by Jang et al. (2007) regarding two different PIP aquaporin genes in *Arabidopsis* and tobacco under dehydration conditions. Hence, the decreased expression of plasma membrane aquaporin genes during drought stress in AM plants can be a regulatory mechanism to limit the water lost

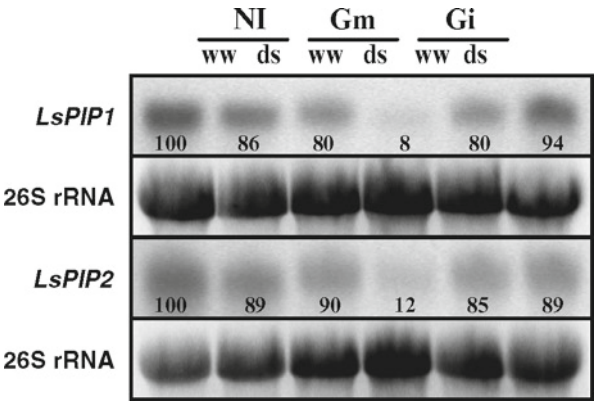

Figure 2. Northern blot of total RNA (15 μg) from lettuce roots, using *lsPIP1* and *lsPIP2* gene probes. Treatments are designed as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern. The lower panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). (Reproduced from Porcel et al., 2006. With kind permission of Springer Science and Business Media.)

from the cells (Barrieu et al., 1999). In support of this hypothesis data on leaf Ψ and relative water content (RWC) showed that AM plants (soybean and lettuce) had higher leaf Ψ and water content than non-AM plants (Porcel et al., 2006).

Data obtained with lettuce plants also colonized by *G. mosseae* point in the same direction (Fig. 2), namely that under drought stress conditions, there is a higher down-regulation of the *PIP* genes studied (and also at the protein level, as revealed by western blot) in AM plants than in non-AM plants. In contrast to *G. mosseae*, plants colonized by *G. intraradices* do not exhibit such down-regulation of *PIP* gene expression or protein accumulation. The expression of *PIP* genes under drought stress in these plants is similar to control non-AM plants.

The exact reason for the different influence of *G. mosseae* and *G. intraradices* on lettuce *PIP* gene expression is not known. However, in a previous study, also with lettuce, we evaluated the ability of six AM fungal species, including *G. mosseae* and *G. intraradices*, to enhance the amount of soil water uptake by these plants (Marulanda et al., 2003). The study demonstrated that there were substantial differences among the six AM fungi used. One of the most efficient fungi stimulating water uptake by plants was *G. intraradices*, while *G. mosseae* showed a reduced ability to improve plant water uptake. This may suggest that the strategy of both fungi to protect the host plant against water deficit is different. *G. intraradices* seems to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that the water movement in these roots must be enhanced and thus, the root water permeability must also increase, maybe by maintaining high

levels of PIP aquaporin gene expression as we observe in this study. Contrarily, *G. mosseae* seems to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant and by that reason down-regulates the expression of *PIP* genes. Such down-regulation of *PIP* genes has been interpreted as a mechanism to decrease membrane water permeability and to allow cellular water conservation (Yamada et al., 1995; Smart et al., 2001). In any case, both strategies seem to protect the host plant in a similar way as lettuce plants had similar RWC and leaf Ψ regardless of the fungus colonizing their roots (Porcel et al., 2006).

3. Expression of Aquaporin Genes in AM Plants Under Salinity or Cold Stresses

To further illustrate the complexity of the response of aquaporin genes to AM fungi we analyzed the responses of mycorrhizal lettuce plants (colonized by the same isolate of *G. intraradices*) to salt stress (Jahromi et al., 2008). Results showed that, in the absence of salinity, the expression of *LsPIP1* and *LsPIP2* genes was inhibited by mycorrhization (Fig. 3), which agrees with the previous findings on these aquaporin genes (Porcel et al., 2006). Under saline conditions, mycorrhizal plants maintained almost unaffected the expression of *LsPIP2* gene, while up-regulating the expression of *LsPIP1* gene, mainly at 100 mM NaCl. This last result is just the opposite of that obtained for the same gene under drought stress

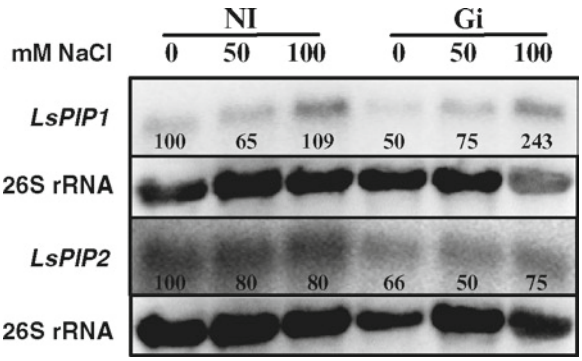

Figure 3. Northern blot of total RNA (15 μg) from lettuce roots using *LsPIP1* and *LsPIP2* gene probes. Treatments are designed as NI, noninoculated control or Gi, plants inoculated with *Glomus intraradices*. Plants were subjected to 0, 50, or 100 mM NaCl. The lower panels show the amount of 26S rRNA loaded for each treatment. Numbers close to each Northern represent the relative gene expression (after normalization to 26S rRNA) as a percentage of the value for control plants cultivated under nonsaline conditions. (Reproduced from Jahromi et al., 2008. With kind permission of Springer Science and Business Media.)

conditions. Hence, these results clearly illustrates that the same aquaporin gene responds differently to each AM fungus analyzed and that the response depends also on the intrinsic characteristics of the osmotic stress applied. This highlights the complex regulation of aquaporin genes in response to the AM symbiosis (Jahromi et al., 2008).

Additional examples of such a complexity come from a study with mycorrhizal *Phaseolus vulgaris* plants (Aroca et al., 2007). In this study the expression of four PIP aquaporin genes from *P. vulgaris* (Aroca et al., 2006) was analyzed in mycorrhizal and nonmycorhizal plants subjected to three different osmotic stresses: drought, cold, or salinity. Three of these *PIP* genes showed differential regulation by AM symbiosis under the specific conditions of each stress applied (Fig. 4). In fact, *PvPIP1;1* was slightly inhibited by *G. intraradices* under drought stress conditions, while nonmycorrhizal plants did not change its expression pattern. Cold stress inhibited its expression similarly in AM and non-AM plants. Finally, salinity raised the gene expression in both groups of plants, but the enhancement was considerably higher in AM plants. The gene *PvPIP1;2* was inhibited by the three stresses in the same way in AM and non-AM plants. In contrast, *PvPIP1;3* showed important differences in AM and non-AM plants according to the stress imposed. This gene was clearly induced in non-AM plants under drought stress but inhibited in AM plants. Under cold stress the behavior was the opposite since it was inhibited in non-AM plants and induced in AM ones. Finally, under salinity it was also induced in both groups of plants, especially in AM ones. The gene *PvPIP2;1* was induced in non-AM plants under drought stress but inhibited in AM plants. The response of this gene to cold stress was not significant for any of the two plant groups and, again, the gene was considerably up-regulated under salinity, especially in AM plants.

The up- or down-regulation by drought stress of mRNAs encoding aquaporins homologues has been described in the roots of many plant species (Javot and Maurel, 2002). There are currently two opposite descriptions of the role of aquaporins in response to dehydration stress (Smart et al., 2001). The first is based on evidence that expression of some aquaporins is induced under dehydration stress (Barrieu et al., 1999; Jang et al., 2004), which is predicted to result in greater membrane water permeability and facilitated water transport. The second is based on the fact that aquaporin expression is down-regulated under dehydration stress, which should result in decreased membrane water permeability and may allow cellular water conservation (Yamada et al., 1995; Smart et al., 2001) during periods of dehydration stress.

The most interesting finding of Aroca et al. (2007) is that each *PIP* gene responded differently to each stress depending on the AM fungal presence. Valot et al. (2005) already found that several plasma membrane proteins were differently regulated by inoculation with *G. intraradices*, some of them were down-regulated and others were induced. Since *G. intraradices* has the capacity of altering root hydraulic properties (Marulanda et al., 2003; Khalvati et al., 2005), it is not strange that the fungus also changes *PIP* gene expression.

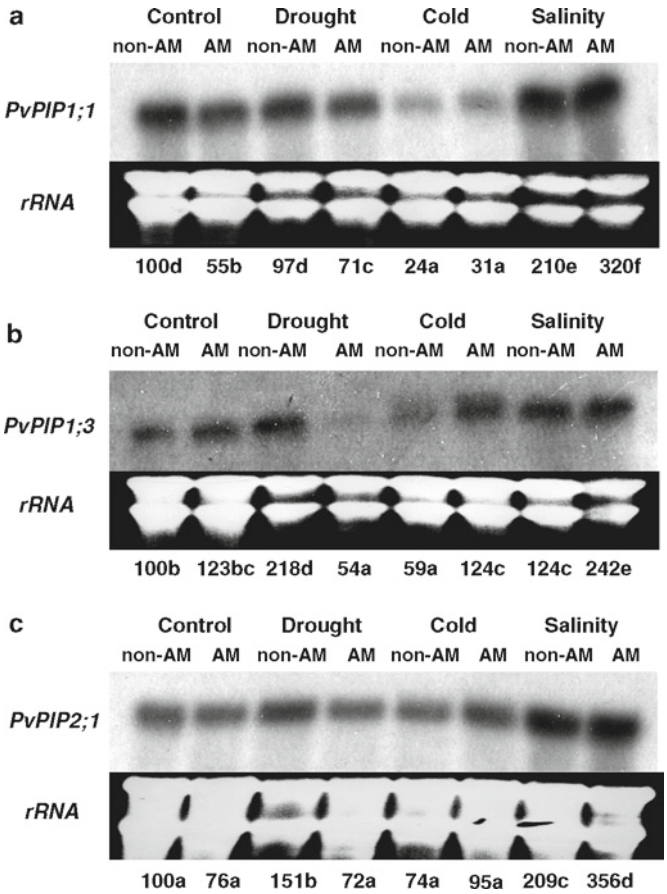

Figure 4. Northern blots analysis using 3′ UTR as probes of *PvPIP1;1* (**a**), *PvPIP1;3* (**b**), and *PvPIP2;1* (**c**) in total RNA of *Phaseolus vulgaris* roots not inoculated (non-AM) or inoculated (AM) with AM fungi *Glomus intararadices*. Plants kept at 23°C and watered at full capacity with tap water were referred as Control. Plants kept at 23°C and subjected to no watering during 4 days were referred as Drought. Plants transferred to 4°C during 2 days and watered at full capacity with tap water were referred as Cold. Plants kept at 23°C and watered each 2 days during 6 days with 10 mL of 0.5 M NaCl solution were referred as Salinity. Quantification of the gene expression was performed by dividing the intensity value of each band by the intensity of corresponding rRNA stained with ethidium bromide. Control value of NI roots was referred as 100. Treatments with different letters are significant ($p < 0.05$) different after ANOVA and Fisher LSD tests. $n = 3$. (Reproduced from Aroca et al., 2007. With permission from New Phytologist.)

4. Expression of Aquaporin Genes in AM Tomato Plants and in an ABA-Deficient Tomato Mutant (Sitiens)

It has been shown that the plant hormone ABA modulates the expression of some *PIP* genes in roots and leaves (Jang et al., 2004; Zhu et al., 2005; Aroca et al., 2006). Thus, we carried out a study with tomato and an ABA-deficient mutant (sitiens)

to analyze the expression of four *PIP* aquaporin genes depending on mycorrhizal presence, exogenous ABA application, and the plant ABA phenotype used (Aroca et al., 2008). In this study, we observed differential expression of some of the genes in AM and non-AM plants after ABA or drought treatments depending on the plant ABA phenotype. For example, the application of exogenous ABA under well-watered conditions enhanced the expression of *SlPIP1-4* gene in root of wild-type plants (both in AM, line 4 versus line 2 and in non-AM plants, line 3 versus line 1) (Fig. 5). In contrast, in *sitiens*, the application of ABA decreased the expression in non-AM roots (line 11 versus line 9) and did not affect the expression of AM roots (line 12 versus line 10). Also, drought duplicated the expression of *SlPIP1-4* in roots of wild-type plants (both AM, line 6 versus line 2 and non-AM plants, line 5 versus line 1), but in *sitiens* plants, drought decreased the expression of the gene in non-AM plants (line 13 versus line 9) and did not change the expression in AM plants (line 14 versus line 10). Similarly, the *SlPIP1-5* gene was induced by drought in roots of wild-type non-AM plants (line 5 versus line 1) and remained unchanged in wild-type AM ones (line 6 versus line 2) (Fig. 5). In contrast, in *sitiens* plants, drought decreased the expression of this gene in roots of both AM (line 14 versus line 10) and non-AM plants (line 13 versus line 9). This suggests that this gene is not only regulated by drought, but also by ABA and needs high levels of ABA to

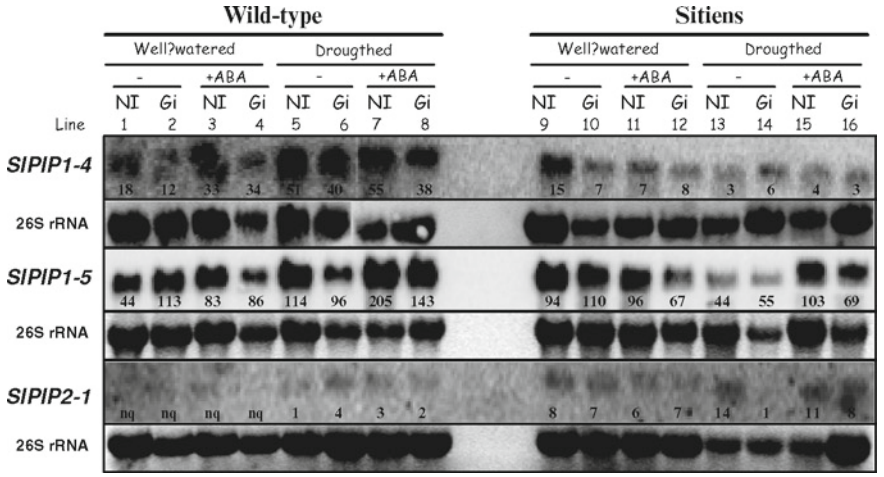

Figure 5. Northern blot of total RNA (15 μg) from tomato roots (wild-type and *sitiens*) using *SlPIP1-4* (Accession AF218774), *SlPIP1-5* (Accession X73848) and *SlPIP2-1* (Accession BI929127) as gene probes. Treatments are designed as NI, noninoculated controls or Gi, plants inoculated with *Glomus intraradices*. Plants were cultivated under well-watered conditions or subjected to drought stress with or without addition of exogenous ABA. The lower panels show the amount of 26S rRNA loaded for each treatment. Numbers close to each northern represent the relative gene expression after normalization to rRNA, nq = not quantifiable. (Reproduced from Aroca et al., 2008. With kind permission of Springer Science and Business Media.)

induce its expression. If the levels of ABA are low (as in *sitiens* plants) its expression is down-regulated by drought. Finally, drought induced the expression of *SlPIP2-1* gene in roots of wild-type plants AM (line 6 versus line 2) and non-AM (line 5 versus line 1) (Fig. 5). In contrast, in *sitiens* plants, drought enhanced the expression only in non-AM roots (line 13 versus line 9) and decreased the expression of this gene in AM roots (line 14 versus line 10).

The reasons for such effects are currently unknown. It may be possible that AM fungal presence can directly regulate gene expression independently of ABA, as has been evidenced for a variety of genes (for reviews, see Gianinazzi-Pearson and Brechenmacher, 2004; Balestrini and Lanfranco, 2006). However, differences in compartmentation of ABA within the cell or tissues or differences in the rate of ABA metabolism (Wilkinson and Davies, 2002; Hartung et al., 2005; Zhang et al., 2006) between AM and non-AM plants can also account for such a differential gene expression. In any case, these results showed that mycorrhization regulated differently the expression of the PIP aquaporin genes analyzed during drought stress and after exogenous ABA application and this effect was dependent on the plant genotype studied. This agrees with results by Lian et al. (2006) who found that *PIP* genes in rice responded in a different way to water stress and ABA, indicating that during water deficit the regulation of *PIP* genes involves both ABA-dependent and ABA-independent signaling pathways. These results suggest that the AM symbiosis exerts a differential control on expression of aquaporin genes, inducing or inhibiting particular genes, and this depends on the endogenous ABA content in the host plant.

5. Conclusion

The results obtained so far on regulation of *PIP* aquaporin gene expression by the AM symbiosis show that the effects of the symbiosis on *PIP* gene expression depends on the own intrinsic properties of the osmotic stress (Table 1). Under drought stress conditions, the AM symbiosis usually decreases or anticipates the decrease of *PIP* gene expression. Under salt stress, the trend is just the opposite since the AM symbiosis enhanced the expression of most of the *PIP* genes analyzed. The regulation of *PIP* gene expression under cold stress is less evident since one of the genes analyzed was down-regulated by the AM symbiosis, another was up-regulated and two genes were not affected by the symbiosis under such conditions. It seems also that the effects of the AM symbiosis on *PIP* gene expression depends on the endogenous levels of ABA in the host plant. In any case, the induction or inhibition of particular aquaporins by AM symbiosis should result in a better regulation of plant water status and contribute to the global plant resistance to the stressful conditions (Yamada et al., 1995; Barrieu et al., 1999; Jang et al., 2004) as evidenced by their better growth and water status under conditions of water deficit. In addition, the results obtained recently by Uehlein et al. (2007) suggest that the role of aquaporins in the AM symbiosis could be more complex

Table 1. Summary of the different effects of the mycorrhizal symbiosis on aquaporin gene expression under nonstressed or under osmotic stress conditions. The consequences on plant water relations (when measured) and the proposed hypothesis are also included.

		OSMOTIC STRESS		
	NO STRESS	**DROUGHT**	**COLD**	**SALINITY**
	Effect Source	**Effect Source**	**Effect Source**	**Effect Source**
Mycorrhizal effects on AQP genes	↑ PcTIP (Roussell et al., 1997) ↑ MtTIP Krajinski et al., 2000 ↑ PttPIP1.1 ↑ PttPIP2.3 (Marjanovic et al., 2005) ↑ PttPIP2.5 ↑ MtPIP2.1 (Uehlein et al., 2007) ↑ MtNIP1	↓ GmPIP1 ↓ GmPIP2 ↓ LsPIP1 (Porcel et al., 2006) ↓ LsPIP2 ↓ PvPIP1.1 = PvPIP1.2 ↓ PvPIP1.3 (Aroca et al., 2007) ↓ PvPIP2.1	↓ PvPIP1.1 = PvPIP1.2 ↑ PvPIP1.3 (Aroca et al., 2007) = PvPIP2.1	↓ LePIP1 ↓ LeTIP (Ouziad et al., 2006) = LePIP2 ↑ LsPIP1 = LsPIP2 (Jahromi et al., 2008) ↑ PvPIP1.1 = PvPIP1.2 (Aroca et al., 2007) ↑ PvPIP1.3 ↑ PvPIP2.1
Consequence	Plant water status not measured (Roussell et al., 1997; Krajinski et al., 2000; Uehlein et al., 2007) ↑ L_0 (Marjanovic et al., 2005)	↑ Ψ_{leaf} (Porcel et al., 2006) ↑ RWC (Porcel et al., 2006; Aroca et al., 2007) ↑ Sap flow rate (Aroca et al., 2007)	= RWC = L_0 (Aroca et al., 2007) = Sap flow rate	Plant water status not measured (Ouziad et al., 2006) ↑ RWC (Aroca et al., 2007; Jahromi et al., 2008) ↑ Sap flow rate (Aroca et al., 2007) L_0 (Aroca et al., 2007)
Proposed hypothesis	The enhanced AQP gene expression ameliorates the exchange of water and nutrients between both symbiotic partners	The down-regulation by the AM symbiosis of plant AQPs allows conservation of water in plant tissues under drought	The AM fungi have little effect on plant water relations under cold stress	The up-regulation of *AQP* genes improves plant water flow and water status under salt stress

than simply regulating plant water status. In fact, they described the induction by the AM symbiosis of specific PIP and NIP aquaporin isoforms exhibiting permeability to water and ammonia, respectively. The authors suggest that these aquaporins could be involved in the symbiotic exchange processes between the fungus and the plant, which opens new perspectives in the study of aquaporins in the AM symbiosis.

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Biodata of **Ann M. Hirsch**, author of ***"How Rhizobia Survive in the Absence of a Legume Host, A Stressful World Indeed"***

Ann M. Hirsch is a Professor in the Department of Molecular, Cell and Developmental Biology at UCLA (Los Angeles, California). Her major area of research is in plant–microbe interactions, an area of study in which she combines her prior training at the University of California-Berkeley (Ph.D.) and Harvard University (postdoctoral) in plant development, molecular biology, and microbiology. Dr. Hirsch was the first person to show that early nodulin gene expression was induced independently of rhizobia by altering the endogenous hormonal levels of legume roots. Also, in collaboration with Dr. Y. Kapulnik, her group demonstrated that signal transduction pathways based on common gene expression patterns were conserved in the nitrogen-fixing symbiosis and in mycorrhizae. Dr. Hirsch's laboratory also unambiguously demonstrated that introducing a lectin gene into a nonhost altered that legume's rhizobial host range. With Dr. M. Valdés, Dr. Hirsch identified a new group of nitrogen-fixing bacteria, non-*Frankia* actinomycetes that also have cellulolytic activity. Her group also developed novel DNA-based systems to authenticate botanical identity and to analyze herbal supplements for contaminants and adulterants. Recently, Dr. Hirsch and coworkers discovered that the core nodulation (*nod*) genes of *Sinorhizobium meliloti*, the nitrogen-fixing endosymbiont of alfalfa, are required for the establishment of mature biofilms. Her expertise in biological nitrogen fixation has led to valuable and ongoing collaborations with scientists in Israel, Mexico, Thailand, Brazil, and Pakistan. She was awarded an NSF Faculty Award for Women and a Research Prize from the Instituto Politecnico Nacional of México in the Programma Institucional de Medio Ambiente y Desarrollo Sustentable. She was named a Fellow of the American Society of Plant Biologists of the Inaugural Class of 2007 and was elected as a corresponding member of the Mexican Academy of Sciences in 2007.

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HOW RHIZOBIA SURVIVE IN THE ABSENCE OF A LEGUME HOST, A STRESSFUL WORLD INDEED

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1. Introduction[1]

Numerous studies have been made of the severe conditions under which *Rhizobium*–legume symbioses persist, including exposure to salt, desiccation, acidic or alkaline pH, temperature extremes, nutrient deficiency, and soil toxicity brought about by heavy metals or hazardous chemicals (Zahran, 1999; Sadowsky, 2005). In addition, the host plants also influence rhizobial survival (Hirsch, 1996). Selection of hosts and their nitrogen-fixing endosymbionts that are tolerant to a broad range of environmental stresses is important for agriculture in marginal lands as well as for the inoculant industry, and has been described in numerous reports. This review asks the question: how do rhizobia survive under stressful conditions in the absence of their symbiotic partner? The issue of rhizobia as a soil saprophyte is rarely discussed, but it is extremely relevant because these bacteria are nonspore formers and do not have a resting stage or form. The most likely bacteria to survive severe environmental stress such as dehydration are those that are dormant or in stationery phase, whereas actively growing bacteria usually die (Davet, 2004).

Our hypothesis is that nonspore formers such as rhizobia survive in the soil environment because they establish biofilms on either biotic or abiotic surfaces (Fujishige et al., 2006, 2008). We wrote: "It was tempting to speculate that biofilm formation is important for the overall fitness of rhizobia in the soil and in rhizosphere microenvironments, thereby contributing to an efficient symbiosis" (Rinaudi et al., 2006). Biofilms are surface-attached communities of bacteria, consisting of either a single or multiple species, and contained within a self-produced extracellular matrix (Costerton et al., 1995; Stanley and Lazazzera, 2004). For reasons of carbon sufficiency and in some cases protection from predation, attachment to biotic surfaces

[1] This review is dedicated to memory of the late John G. Streeter, a pioneer in studying the stress responses of the *Bradyrhizobium japonicum*–soybean symbiosis.

is more likely to enhance survival. In addition, adherence to abiotic surfaces can be advantageous because rhizobia in a biofilm are protected from environmental insults as a consequence of their exopolymeric matrix and lowered metabolic rate. A recent study of the gram-negative, nonspore-producing *Rhizobium* NGR234 attached to dry sand showed a decline in viability after 9 weeks of desiccation stress. Nevertheless, 10^4 viable cells per gram of dry sand were detected months later. In addition, the cells remained symbiotically competent (Gorbushina et al., 2007). In contrast, under the same conditions, all the vegetative cells of a gram-positive, spore-forming species, *Bacillus megaterium*, differentiated into spores within a week (Gorbushina et al., 2007). Thus, in spite of the fact that rhizobia are nonspore formers, they retain viability following severe stress. Estimates have been made indicating that some rhizobial species survive in soil at least 4–5 years without their host, but a few cases demonstrated that rhizobia might survive up to 15 years (Fred et al., 1932)! Nonetheless, the numbers of rhizobia that are present in bulk soil are orders of magnitude less numerous than those found in the rhizosphere (Hirsch, 1996).

Biofilms comprised of synergistic or syntropic consortia of bacteria would provide an additional survival advantage to rhizobia. Because the biofilm state is a means of survival for many bacteria subjected to environmental stress or antimicrobial assaults (Costerton et al., 1999; Hogan and Kolter, 2002), it seems logical to assume that biofilms could be a means that rhizobia use to survive stress. However, surprisingly little is known about rhizobial biofilms and whether genes that are expressed in response to stress are also expressed in biofilms. This review is an attempt to bring some of these literatures together, focusing mainly on desiccation, pH, and nutrient availability. Details about other stresses affecting rhizobia are found in Zahran (1999), Sadowsky (2005), and Vriezen et al. (2007).

2. Rhizobia and Stress

Rhizobia are found in bulk soil, attached to soil particles, but more frequently *Rhizobium* species establish mutualistic or commensal relationships with the roots of both legume hosts and nonlegumes (Foster et al., 1983; Schwieger and Tebbe, 2000). Attaching to roots or living in the rhizosphere offers a competitive advantage for rhizobial survival in part because of root exudation; bulk soil is a desert by comparison. One gram of root is estimated to release 50–100 mg of exudate, enough to support 2×10^{10} bacteria (Morgan et al., 2005). Even nonlegumes can support a substantial number of rhizobia. For example, *Rhizobium leguminosarum* bv. *trifolii* cells were recovered from the internal tissues of rice roots at 10^6 cells per gram of root fresh weight (Yanni et al., 1997). These rhizobia were capable of effectively nodulating berseem clover (*Trifolium alexandrinum* L.) demonstrating that they were symbiotically competent. However, many rhizobial strains isolated from soil are often classified as symbiotically incompetent (Laguerre et al., 1993), leading some to argue that loss of the symbiotic plasmid enhances survival (Squartini, 2001). Nonsymbiotic bradyrhizobia have also been

isolated (Pongsilp et al. 2002); these bacteria do not have plasmid-borne but rather have chromosomally located symbiotic genes. However, nonsymbiotic strains can readily become symbiotic by the acquisition of a symbiotic plasmid or island. For example, the transfer of a "symbiotic island," as shown for a soil-inhabiting $Nod^{-}Fix^{-}$ *Mesorhizobium loti*, allowed the new $Nod^{+}Fix^{+}$ strain to nodulate *Lotus* sp. (Sullivan et al., 1995; Sullivan and Ronson, 1998). In some cases, however, the strains may become poorly effective or even ineffective after lateral transfer of a symbiotic island (Nandasena et al., 2006, 2007). Because most studies evaluate symbiotic competence on the basis of whether rhizobia nodulate a particular legume host, strains defective in a single gene critical for nodulation or for nodule effectiveness, or if tested on the wrong host could be judged as symbiotically incompetent. Thus, quantification of the numbers of symbiotically incompetent strains in soil could be skewed because of the aforementioned limits on nodulation. Also, many earlier studies evaluating the numbers of rhizobia in the soil have been culture dependent rather than culture independent, so the actual numbers of rhizobia and their symbiotic status in bulk soil and the rhizosphere may be underestimated. Indeed, several lines of recent evidence suggest that symbiotic genes are important for rhizobial survival in response to stress (Domínguez-Fererras et al., 2006) and also for biofilm formation (Fujishige et al., 2008).

With some exceptions, the studies on rhizobial responses to stress have been performed in vitro on planktonic cells and not in situ on biofilm cells. Nevertheless, such an approach has facilitated the identification of genes that are upregulated/downregulated or induced in response to stress. Moreover, studies on other bacteria have shown that genes characteristic of various stress responses, e.g., nutrient deprivation, desiccation, etc. are expressed in biofilms (Whiteley et al., 2001).

2.1. DESICCATION

Many rhizobia are salt tolerant (NaCl) or osmotically tolerant and thus, capable of living under severe moisture deficiency (Zahran, 1999; Sadowsky, 2005). Several of the responses of rhizobia to other stresses, such as high temperature tolerance and oxygen radical defense mechanisms, overlap with desiccation resistance. Nonetheless, understanding the details of global gene regulation in response to various stress parameters in rhizobia is fragmentary. So far not much information about the signaling pathways/networks that mediate desiccation tolerance, specifically, or stress resistance, generally, is available for this group.

As free-living rhizobial cells encounter dry conditions, a number of profound changes occur: (i) water activity is reduced; (ii) the osmotic potential rises as salts, which can be toxic to rhizobia, accumulate; (iii) transcription and translation slow down and DNA may become damaged; and (iv) membranes become leaky as they shrink away from the cell wall. To circumvent cellular damage, many bacteria accumulate various carbohydrates or osmoprotectants. For example, trehalose, a disaccharide made up of two glucose molecules

joined together by an α,α-1,1 linkage, is employed by many organisms to protect membranes and proteins from desiccation stress (Streeter and Gomez, 2006). Rhizobia also accumulate trehalose, among other carbohydrates, and also betaine and proline, in response to desiccation. Trehalose and sucrose are the only carbohydrates that are synthesized de novo in response to stress. To synthesize trehalose, bacteria utilize either a single pathway or up to three different pathways, the OtsAB, TreYZ, and TreS pathways. For example, the TreYZ pathway is common to many rhizobia (Streeter and Bhagwat, 1999), whereas the OtsAB and TreYZ pathways are found in *R. leguminosarum* bv. *trifolii* strain NZP561 (McIntyre et al., 2007). All three pathways have been detected in *Bradyrhizobium japonicum* strain USDA110 and *B. elkanii* (Streeter and Gomez, 2006). Trehalose at relatively high concentrations is also present in *B. japonicum* bacteroids residing within nodules, suggesting that these differentiated nitrogen-fixing cells are under stress. Even adding trehalose to *B. japonicum* cells enhances their survival in response to dryness (Streeter, 2003). Saccharides such as trehalose may protect desiccated cells by their ability to form glasses under dry conditions, in this way maintaining the native conformation of proteins and other macromolecules (Ramos et al., 2001). Trehalose levels also increase in *R. leguminosarum* bv. *trifolii* TA1 cells as they encounter osmotic stress (Streeter, 1985; Breedveld et al., 1993). *R. leguminosarum* bv. *trifolii* strain NZP561 accumulates trehalose upon entry into stationary phase (McIntyre et al., 2007), but in this rhizobial strain, trehalose synthesis is constitutive and modified posttranscriptionally rather than induced as in other rhizobia. Mutations in *otsA* or *treY* individually in strain NZP561 did not dramatically affect trehalose accumulation, but double *otsA treY* mutants did not accumulate trehalose and were more sensitive to desiccation. They were also less competitive with regard to occupying nodules than were wild-type strains (McIntyre et al., 2007).

A global approach to study rhizobial stress responses is to do a genome-wide transcriptional analysis. Domínguez-Fererras et al. (2006) employed a DNA microarray to examine gene expression in planktonic, exponentially growing *Sinorhizobium meliloti* cells following increased NaCl or sucrose stress to monitor high salinity and hyperosmotic stress, respectively. Overlapping effects on gene transcription in response to NaCl or sucrose were observed, and a large number of genes were differentially expressed. As expected, genes for trehalose synthesis were upregulated. Although many of the genes whose expression levels changed were unknown, interestingly, a large number of genes on pSymB, one of the two *S. meliloti* megaplasmids, was differentially expressed following osmotic stress. In some case, as for ribosomal proteins and ancillary functions, cognate genes were repressed by both stresses. Genes encoding proteins important for the production of the low molecular weight form of succinoglycan, e.g., *exoHK*, were induced, and many genes known to be involved in stress-responsiveness were also induced. As expected, genes encoding proteins for central metabolism and carbon uptake were downregulated, whereas genes involved in glycogen metabolism were

upregulated. Numerous genes related to chemotaxis and cell motility were downregulated in response to stress. Similarly, many genes known to be induced by oxygen, nitrogen, or carbon starvation, as well as other stresses, were upregulated in *S. meliloti* following increased salt or osmotic stress (Domínguez-Fererras et al., 2006). Furthermore, rhizobial growth in response to salt stress was found to require pSymB, and thus, these authors concluded that this large plasmid is essential for *S. meliloti*'s saprophytic competence.

Cytryn et al. (2007) also undertook a genome-wide transcriptional analysis, but this time to analyze *B. japonicum*'s response to drought. In addition to a large category of hypothetical proteins, genes encoding proteins involved in trehalose synthesis were highly upregulated. Other genes were also upregulated, including many important genes for transcriptional regulation such as *rpoN2*, DNA repair and cell cycle regulation, cation uptake and heat shock, pili assembly proteins and flagellin, transport of sucrose and other molecules, succinylation of osmoregulated periplasmic glucans, energy transfer, and various aspects of metabolism (Cytryn et al., 2007). The upregulation of genes encoding flagellin differs from the results of Domínguez-Fererras et al. (2006) where *S. meliloti* flagellar biosynthesis genes were downregulated after osmotic upshift. However, like *S. meliloti*, a number of genes for succinoglycan (EPSI) biosynthesis, including *exoP* (succinoglycan biosynthesis), *exoM* (UDP-hexose transferase), and *exoN* (UTP-G1P uridylyltransferase) were also strongly upregulated upon the induction of drought stress. A putative LPS synthesis transferase was upregulated in drought-stressed *B. japonicum* (Cytryn et al., 2007).

Interestingly, the transcriptional analysis of the genome of *B. japonicum* subjected to desiccation stress indicated that genes critical for pilus assembly, e.g., *pilA*, *pilA2*, and *ctpA*, are upregulated (Cytryn et al., 2007). Pili, especially type IV pili, are often important for biofilm formation (Shime-Hattori et al., 2006; Jurcisek and Bakaletz, 2007). It is extremely likely that desiccation-stressed *B. japonicum* cells show some of the same patterns of gene expression, as do cells in biofilms. Similarly, *S. meliloti* cells grown under salt and osmotic stress (Domínguez-Fererras et al., 2006) upregulate some of the same genes uncovered in transcriptome arrays of biofilm cells of other bacteria (An and Parsek, 2007).

Biofilms are one of the many ways that bacteria use to protect themselves from desiccation and it is well known that EPS, an important component of the biofilm matrix, protects bacteria from drought stress. Loss-of-function EPS mutants of many bacteria show impaired biofilm formation (Yildiz and Schoolnik, 1999; Danese et al., 2000; Whiteley et al., 2001; Matsukawa and Greenberg, 2004), as do *S. meliloti exoY* loss-of-function mutant cells (Fujishige et al., 2006) and EPS mutants of *M. tianshanense* (Wang et al., 2008). Nevertheless, it is not known whether EPS-deficient mutants that are incapable of biofilm formation are less capable of surviving desiccation stress under field conditions.

Several studies have measured oxygen levels in biofilms using microelectrodes (as well as other gases) (see Stewart and Franklin, 2008). Biofilms often

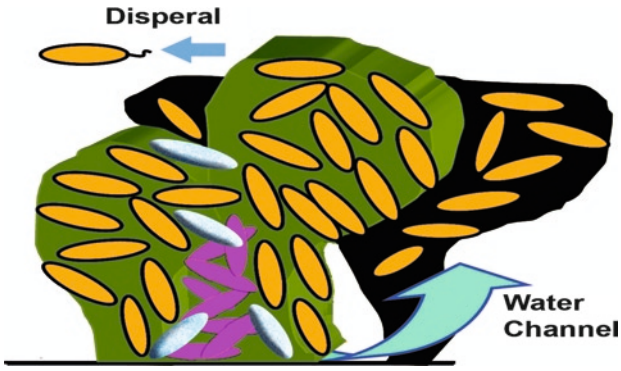

Figure 1. Diagram of a mature biofilm showing water channels and towers composed of cells of different developmental states: yellow, cells with intact membranes; speckled, nutrient-deprived cells; pink, oxygen-deficient cells.

exhibit an oxygen gradient with the lowest levels at the center of the biofilm (Fig. 1). The gradient, however, may not necessarily be established by the presence of a polysaccharide matrix. Instead, the physiological heterogeneity of the biofilm, such that actively respiring cells on the outside reduce the concentration of oxygen on the inside, may be key. For rhizobia, these types of studies have not yet been performed. Our preliminary results on a single species biofilm of *S. meliloti* carrying *gusA* fusions to promoters of genes expressed in reduced oxygen levels demonstrated that the expression of several of these promoters was induced in the biofilm (V. Tovar, P.L. De Hoff, and A.M. Hirsch, 2008, unpublished results).

Moreover, biofilms are often metbolically diverse because they are composed of multiple species. Thus, rhizobia under natural conditions, as members of mixed biofilms, may tolerate or survive stress through the efforts of their neighbors.

Nutrient availability, pH, and concentration of solutes show similar gradients in biofilms as do oxygen and other gases (Stewart and Franklin, 2008). Rhizobial biofilms are likely to show the same gradients, but so far this has not been investigated. *S. meliloti* is a salt-tolerant species, tolerant to salt concentrations ranging from 0.3 to 0.7 M (Zahran, 1999). However, biofilm formation in strain Rm1021 was significantly decreased at 0.15 M NaCl, a concentration that had no effect on growth (Rinaudi et al., 2006). This suggests that the higher concentrations of NaCl may be toxic due to the accumulation of charged ions (Ejlsheikh and Wood, 1989), eliciting either DNA or membrane damage that precludes biofilm formation. *S. meliloti* Rm1021 growth in the presence of 0.3 and 0.6 M sorbitol was adversely affected, but biofilm formation was again more sensitive, showing a decrease at 0.06 M. Although the explanation for this sensitivity is unknown, it may be that older biofilms, which establish profound physiological gradients, become more osmotically tolerant.

2.2. SOIL pH

Many agricultural soils are either so alkaline or acidic as to hinder rhizobial growth and subsequent establishment of a viable nitrogen-fixing symbiosis with a legume host. Even if a legume–rhizobia association is established, the rhizobia may not persist in alkaline or acidic soils once the legume crop is harvested. Soils may exhibit different pHs because of the accumulation of ions, particularly Ca^{2+}, making it difficult to distinguish negative growth effects brought about by Ca^{2+} versus pH (Davet, 2004). Indeed, soil pH is often adjusted by the addition of limestone.

Fungi are thought to be more common in acid soils, whereas bacteria proliferate in neutral or alkaline soils (Davet, 2004). However, some rhizobia are quite tolerant to acidity. *B. japonicum* can survive in acid soils down to a pH of 4.0, whereas *R. leguminosarum* bv. *trifolii* and bv. *viciae* cannot grow in soils below pH 4.7; the lower limit for most *S. meliloti* strains is pH 5.0 (Hirsch, 1996). Low soil pH can also result in increased solubility of certain metal ions, e.g., aluminum, copper, and zinc, to the point where toxic levels are reached. In contrast, alkaline soil conditions can lead to deficiencies as the metals become increasingly unavailable. A number of studies have focused on the effects of heavy metals on rhizobia and the mechanisms of excluding or taking up of metal ions, but these will not be described here.

Rhizobia employ various mechanisms for maintaining intracellular pH including (i) decreased membrane permeability, (ii) internal buffering, (iii) amelioration of external pH, (iv) proton extrusion/uptake, and (v) prevention of metal ion toxicity (Dilworth and Glenn, 1999). But first, a change in pH must be sensed. One likely pH sensor in *S. meliloti* and *S. medicae* is the two component regulatory system, ActS/ActR (Tiwari et al., 1996). These genes, which are homologs of RegBA and PrrBA, are essential for growth in acidic pH. They regulate a number of genes representing a broad range of metabolic process under low pH conditions including carbon fixation and nitrogen assimilation (Fenner et al., 2004). Approximately 20–50 genes are predicted to be involved in acid tolerance (*act*), and some 15–20 of them may be essential (Glenn et al., 1999). Included among these are *actA*, *actP*, *actR*/*S*, *exoH*, and *exoR*. However, the mild acid-sensitive phenotype of an *exoR* mutant may be indirect because the extra EPS produced by the mutant may impose a growth defect that is particularly noticeable in cells grown under acidic conditions. The mutants grow after prolonged incubation on acidic medium (W.G. Reeve, 2008, personal communication).

Not as many genes have been found that are induced under alkaline conditions in part because fewer studies have been made of alkaline tolerance. An *S. meliloti* mutant originally described as Fix^- was found to be sensitive to K^+ and not able to survive at alkaline pH in the presence of this cation (Putnoky et al., 1998). The mutation was determined to be in the *pha* operon, which includes genes that encode proteins resembling subunits of a Na^+/H^+ transporting system. A recent investigation showed that *tfxG*, one of the genes that encodes trifolitoxin, is critical for *S. meliloti* to tolerate alkaline growth conditions (Tang et al., 2007). This gene

is upregulated as the pH is changed from 7.0 to 10.0, and mutants show growth impairment at pH values greater than 9.0.

A strategy to select for acid-tolerant rhizobia among natural genetic populations has led to the identification of a number of strains that grow under these difficult soil conditions. *R. tropici* CIAT899, which nodulates common bean (*Phaseolus vulgaris* L.), is well adapted to nodulate its host, which grows in highly acidic soils. Under laboratory conditions, *R. tropici* CIAT899 grows at a pH as low as 4.0 (Martinez-Romero et al., 1991; Graham et al., 1994), but under natural conditions, the cells survive better at pH values over 4.5 (Graham et al., 1982). Tn*5* mutagenesis identified loci that are involved in acid tolerance in this species. The mutants were acid sensitive and also incapable of forming nitrogen-fixing nodules (Vinuesa et al., 2003). One of the mutations was in a gene orthologous to the *avcB* gene of *Agrobacterium tumefaciens* and was renamed *atvA* by the authors for acid tolerance and virulence. This gene is induced by acid shock (Vinuesa et al., 2003). The first gene in a putative operon, *lpiA*, which encodes an integral membrane protein with 13 transmembrane helices, is also reported to be acid induced.

Strains of *S. meliloti* are among the most acid-sensitive rhizobia. They generally do not grow below pH 5.5 in laboratory media. In contrast, *S. medicae* (formerly *S. meliloti*) WSM419 is one of the more acid-tolerant sinorhizobia (Goss et al., 1990; Glenn and Dilworth, 1994). Several genes required for the growth of this strain at low pH have been identified. Both transcriptional and proteomic analyses were performed to deduce expression pattern differences between *S. medicae* WSM419 grown under neutral and low pH (pH 5.7) conditions (Tiwari et al., 2004; Reeve et al., 2004). Short-term (30 min) exposure to low pH had only a minor effect on protein expression, whereas long-term exposure (5 days) resulted in more than 50 proteins showing changes in expression levels (Reeve et al., 2004). Among these were GroES and DegP, which were upregulated, and an ATP-binding cassette (ABC) transporter as well as several hypothetical proteins that were downregulated. Transcriptional analysis identified *phrR*, which encodes a putative repressor; *lpiA*, a putative membrane protein; *kdpBC*, a potassium importing ATPase; putative ABC transporters, and several hypothetical proteins, among others (Tiwari et al., 2004). In *S. medicae* WSM419, *lpiA* was found to be induced 20-fold in low pH conditions relative to that seen at pH 7.0 (Reeve et al., 2006). Although no effect of a mutation in *lpiA* was observed in cells grown at pH 5.7, *S. medicae lpiA* mutant viability was strongly reduced at pH 4.5. In contrast, a significant number of wild-type *S. medicae* WSM419 cells remained viable at this low pH. Recent evidence suggests that *lpiA* expression results in the synthesis of a lipid, lysyl-phosphatidyglycerol (LPG), in the membranes of *R. tropici* CIAT899 when grown at pH 4.5 in minimal medium (Sohlenkamp et al., 2007). The latter researchers found that mutants with defective *lpiA* are less likely to survive at low pH than wild-type rhizobia after challenge with polymyxin B. The increased LPG in the membrane may alter its surface charge, thereby enhancing survivability.

Little connection between what is known regarding genes important for acid or alkali tolerance and rhizobial biofilm formation has been made so far. Biofilm formation in response to acidic conditions has been pursued only for *S. meliloti* strain Rm1021, the sequenced strain, which is not as acid tolerant as *S. medicae* WSM419 and certain other *S. meliloti* strains. When Rm1021 cells were examined after 24 h of growth at pH 4.0–8.0, optimal growth and biofilm establishment were found to occur at pH 7.0 (Rinaudi et al., 2006). Interestingly, cells grown at pH 7.0 on glass cover slips for 6 days established towers and ridges that were typical of mature *S. meliloti* Rm1021 biofilms (Fujishige et al., 2006). They also fluoresced green after staining with the LIVE/DEAD fluorescent stain, indicating that the cells were viable (Rinaudi et al., 2006). In contrast, Rm1021 cells grown at pH 4.0 and subsequently treated with the LIVE/DEAD fluorescent stain were red, suggesting that the cells were either dead or had leaky membranes. The presence of actively swimming red-staining cells, however, suggested the latter (Rinaudi et al., 2006).

pH has been shown to influence the profile of Nod factors secreted by *R. tropici* CIAT899, which is tolerant of acidic conditions (Morón et al., 2005). At least seven different classes of Nod factor structures were identified, differing in either the reducing or nonreducing end substitutions on the core *N*-acetylglucosamine oligosaccharide. More than 50 different Nod factors were detected at pH 4.5, and greater induction of the *nod* genes also occurred at this pH. This diversity of Nod factor structure may facilitate nodulation of bean at an acidic pH. An earlier study by McKay and Djordjevic, 1993) demonstrated that *R. leguminosarum* bv. *trifolii* Nod factor production is influenced by environmental parameters such as pH, temperature, and nutrient availability.

2.3. NUTRIENT AVAILABILITY

An early study (Wei and Bauer, 1998) demonstrated that C, N, or P starvation resulted in a loss of motility and a transient increase in chemotaxis in *S. meliloti*. Only a subset of cells lost flagella in response to starvation, however. As discussed in the previous section, genes involved in chemotaxis and motility were downregulated in *S. meliloti* after osmotically induced stress (Domínguez-Fererras et al., 2006). They were also downregulated in response to phosphate limitation in *S. meliloti* (Krol and Becker, 2004). Phosphate limitation also resulted in the upregulation of a number of genes, including the *pta-ackA* genes coding for phosphotransacetylase and acetate kinase activity, respectively, heat shock gene *dnaK*, and several others (Summers et al., 1999) including those involved in exopolysaccharide synthesis, both succinoglycan (EPS I) and galactoglycan (EPS II) (Mendrygal and Gonzalez, 2000; Krol and Becker, 2004). Recently, a gene for an inducible catalase, *katA*, found to be upregulated in *S. meliloti* following phosphate stress (Krol and Becker, 2004), is transcribed from a PhoB promoter rather than from an OxyR-dependent promoter as are other catalase genes

(Yuan et al., 2005). A global expression profile of phosphate-limited *S. meliloti* cells earlier showed that a number of genes involved in protection against oxidative stress are also induced (Krol and Becker, 2004).

Some recent data implicate an oligopeptide ABC transporter (Opt) in both stress resistance and symbiosis in *R. etli* (Vos et al., 2007). Rhizobia mutated in various genes of the *opt* operon establish nodules that fixed only about 50% of the nitrogen levels (monitored by acetylene reduction assays) of controls (Vos et al., 2007). When challenged with antibiotics or when grown under hyperosmotic stress, the *opt* mutant cells grew more slowly than the wild-type controls did, and were also more susceptible to certain antibiotics such as ampicillin, but were resistant to others, namely bacitracin. These effects may be a consequence of the lack of uptake of required peptides, for example, glycine- and proline-containing peptides, which may protect the cells from osmotic stress. The symbiotic defect could potentially be a result of a nutritional defect. The nodules are infected normally and the bacteroids are surrounded by peribacteroid membrane (Vos et al., 2007), suggesting that these stages of nodule development are intact. More studies are needed.

Nutrient availability modulates the depth and structure of many bacterial biofilms (Stanley and Lazazzera, 2004). Biofilms of gram-positive bacteria such as *Bacillus subtilis*, which form spores in response to various stress factors, exhibit upregulation of sigma factors that are indicative of sporulation and nutrient starvation (Stanley et al., 2003). Gram-negative bacterial biofilms also exhibit numerous changes in response to nutrient starvation. The center part of a large biofilm may be nutrient starved and the biofilm may be undergoing maximal dispersal of cells to seek new nutrient sources (Fig. 1). Several studies have shown that rhizobia establish biofilms more quickly when grown in minimal media than when cultivated in rich media (Fujishige et al., 2006; Rinaudi et al., 2006; Russo et al., 2006), indicating that changes in physiology brought about by nutrient limitation positively impact biofilm formation. Both N and P limitation resulted in greater biofilm formation in *S. meliloti* (Rinaudi et al., 2006).

3. Symbiosis and Stress

Many of the genes described above influence symbiosis in some way, most likely indirectly by causing nutrient deprivation or membrane damage. Little is known about the global regulation networks that modulate stress responses in rhizobia. A few alternative sigma factors, which are responsible for controlling gene expression in response to stress in a number of other gram-negative bacteria (Ramos et al., 2001), namely *rpoN2* and *rpoE2*, have been identified in rhizobia (Domínguez-Fererras et al., 2006; Sauviac et al., 2007). RpoE2 is described as a major global regulator of *S. meliloti*'s general stress responses. At least 44 genes identified by a transcriptome analysis, many of which encode proteins known to be involved in stress responses such as *katC* and *rpoH2*, are controlled by RpoE2

(Sauviac et al., 2007). Transcriptome and proteome analyses have provided information about many of the downstream players in rhizobial stress responses, but the details of how these genes are regulated is still unknown. For example, *rpoE2* mutants do not differ from wild-type cells in their resistance to various stresses in either exponential or stationary phases of growth (Sauviac et al., 2007).

A gene of unknown function that appears to be a master switch for both symbiosis and environmental stress has been described for *S. meliloti* (Davies and Walker, 2008). It was uncovered using a two-part screening strategy to find mutants that were sensitive to H_2O_2 and at the same time, symbiotically defective with alfalfa. The white, ineffective nodules were found to be completely devoid of bacteroids, suggesting that the symbiosis is blocked early in development, before the release of bacteria from infection threads (Davies and Walker, 2008). Based on sequence analysis, the gene codes for a putative metal-dependent hydrolase, with homologs present in a wide range of alpha-proteobacteria, many of which do not nodulate legumes. Interestingly, the mutant is sensitive to a broad range of environmental pressures, including oxidative stress, agents of DNA damage, and inhibitors of cell wall synthesis, among others, suggesting that the wild-type protein plays a central role in *S. meliloti's* stress response (Davies and Walker, 2008). Some genes neighboring the *opt* operon include two that overlap with pH effects, *lnt* and *phrR*. However, the *S. meliloti* mutant does not show increased sensitivity to acid pH, arguing that the stress effect is independent of pH.

4. Future Directions

One would predict that many of the genes described above as being upregulated by stress are likely to be expressed in biofilms. Based on studies in other bacteria, genome-wide transcriptional analyses show that this is the case (Whiteley et al., 2001). However, transcriptome and proteome analyses are fraught with difficulties due to the nonstandard conditions used to study biofilms and because the cells of the biofilm are highly heterogeneous (An and Parsek, 2007; Stewart and Franklin, 2008). Nevertheless, the various genes identified from the studies already accomplished could provide a tremendous opportunity to learn more about their expression in rhizobial biofilms. For example, fluorescent gene reporters that respond to pH or other stress-elicited cues could be followed in flow cells or by epifluorescence or confocal microscopy. For this, better reporter genes need to be designed; for example, green fluorescent protein (GFP) and its derivatives require oxygen for expression, and as described earlier, many centrally located biofilm cells are oxygen starved. Perhaps reporter genes that are expressed under anaerobic conditions could be developed to circumvent this difficulty. Viability stains such as the LIVE/DEAD stain are also useful, but can give false-negative results. Details of methods that could be used are described fully in Stewart and Franklin (2008).

Another possibility is to develop in situ RNA or protein localization methods, similar to those used in eukaryotic tissue systems. This would require fast freezing of

the biofilm, cryosectioning, and then detecting transcripts with fluorescently labeled probes. The disadvantage of this method is that it is an end-point protocol, resulting in the termination of the biofilm. Technological advances are needed to integrate the knowledge gained from the investigations of planktonic cells to stress and rhizobial biofilms with the hopes of extrapolating this knowledge to the field situation.

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LIFE ON A LEAF: BACTERIAL EPIPHYTES OF A SALT-EXCRETING DESERT TREE

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The surfaces of aboveground parts of plants – the phyllosphere – are normally colonized by a variety of bacteria, yeasts, and fungi (Lindow and Leveau, 2002). Bacteria are the most numerous colonists of leaves, often being found in numbers averaging 10^6–10^7 cells/cm^2 of leaf (Andrews and Harris, 2000; Beattie and Lindow, 1995; Hirano and Upper, 1989). In spite of their worldwide distribution (Morris and Kinkel, 2002), studies of the composition of bacterial communities on leaves have been relatively limited in scope, mostly focusing on potential pathogens of agriculturally relevant plants (Beattie and Lindow, 1994; Dik, et al., 1992; Ercolani, 1991).

In recent years, we have studied a unique phyllosphere niche: the leaf surfaces of the salt-excreting tree, *Tamarix* (Salt Cedar; Eshel; Waisel, 1961). We have shown (Qvit-Raz et al., 2008) that this habitat is home to a specialized microbial community, the life of which is governed by high temperatures, strong radiation, and a very low humidity that dictates a daytime existence in complete desiccation. Damp nights, a very common occurrence in the Israeli Negev desert, allow the microbial population to temporarily proliferate in a hypersaline (~22% – over fivefold higher than seawater) solution, before drying up again after sunrise. This proliferation is made possible by the fact that the dew is not only very saline due to the dissolution of the sodium chloride excreted by the leaves, but is also extremely rich (>4 g/L) in organic compounds, mostly sugars. These compounds may be used by the bacteria not only as carbon and energy sources, but also as desiccation- and osmotic-protectants.

1. Bacterial Life on Leaf Surfaces

Compared to most other bacterial habitats, there has been relatively little examination of phyllosphere microbiology. Epiphytic bacterial populations can vary sharply in size between and within plants of the same species and over short timescales (Hirano and Upper, 1991) as well as over the growing season (Ercolani, 1991; Thompson et al., 1993). These variations in population sizes are caused in great part by the large fluctuations in the physical and nutritional conditions characteristic of the phyllosphere. Additionally, plant species appear to influence the microbial carrying capacity of the leaf since the total number of cultivable bacteria recovered from broad-leaf plants such as cucumber and beans was

significantly greater than that recovered from grasses or waxy broad-leaf plants (Kinkel et al., 2000; O'Brien and Lindow, 1989). Reflective of marked differences in the physicochemical environment of aboveground versus subterranean plant surfaces, the epiphytic flora differ substantially from that of roots. For example, pigmented bacteria, only rarely found in the rhizosphere, dominate leaf surfaces (Stout, 1960a, b), presumably because solar radiation influences the ecology of the phyllosphere (Jacobs and Sundin, 2001; Jacobs et al. 2005; Sundin et al. 1996. Sundin and Murillo, 1999; Sundin and Jacobs, 1999). The differential composition of leaf and root bacterial communities is further evidenced by the failure of common root colonizers such as *Rhizobium* (O'Brien and Lindow, 1989) and *Azospirillum* (Jurkevitch and Shapira, 2000) to become established on leaves.

Studies of the composition of bacterial communities on leaves have been numerous but rather limited in scope, generally focusing on populations of potential plant pathogens. A few exhaustive studies of the variations in the microbial community of leaves over multiple time and space scales have provided important detailed knowledge about the identity and the ecology of bacterial leaf inhabitants (Ercolani, 1991; Thompson et al., 1993). Ercolani (1991) made an extensive inventory of cultivable aerobic bacteria isolated from the surface of olive leaves over six growing seasons and reported distinct bacterial community structures on leaves of the same age at a given time of the growing season over the entire sampling period. Thomson et al. (1969) analyzed 1,236 bacterial strains from immature, mature, and senescent leaves of field-grown sugar beet over a complete growing season. They identified 78 species, and 37 named and 12 unnamed genera of bacteria. Most importantly, like Ercolani (1991), they found distinct patterns of microbial colonization at different times of the year, with bacterial community diversity being lowest during the warmest and driest months of the season and highest during the cooler and rainy months.

The microbial ecology of the phyllosphere has been viewed mainly through the biology of gram-negative bacteria such as *Pseudomonas syringae* and *Erwinia* (*Pantoea*) spp., two of the most ubiquitous bacterial participants of phyllosphere communities. Much attention has also been devoted to ice nucleation active bacteria, including the previously mentioned *P. syringae, Xanthomonas campestris*, and *Erwinia herbicola* along with *P. fluorescens*. Free-living nitrogen fixers (Fürnkranz et al., 2008; Kampfer et al., 2005; Ruinen, 1975) have also been investigated because of their possible contributions to plant productivity (Hirano and Upper, 1989). "Pink-pigmented facultative methylotrophs" (PPFM), capable of utilizing single-carbon compounds (methanol, methyl amine) as carbon and energy sources, though slow growers (Hirano and Upper, 1989) can constitute up to 80–90% of the bacteria recovered from some plants (Corpe and Rheem, 1989; Hirano and Upper, 1995). There have also been reports (Brandl and Mandrell, 2002; Obrien and Lindow, 1989; Ott et al., 2001) of human enterics, *Salmonella enterica, Escherichia coli*, and *Enterococcus* spp. surviving on leaves during dry periods. Some leaf bacteria such as *Enterobacter* sp. may alter leaf surface permeability and affect plant growth (Kampfer et al., 2005).

Molecular approaches have only rarely been applied to the study of phyllosphere microbiology. Not unexpectedly, when this was attempted, Yang et al. (2001) have revealed a higher community complexity in the phyllosphere by culture-independent methods than conventional cultivation-based methods; the majority of 16S rRNA sequences recovered from leaf washings of various plant species were from bacteria not previously described in the phyllosphere, with some sequences representing undescribed species (Yang et al., 2001). Such results support the statement of Lindow and Leveau (2002), that "there are many phyllosphere inhabitants that have never been investigated and which may harbor unique traits enabling them to thrive on leaves." This statement is particularly valid for the *Tamarix* phyllosphere, the subject of the present chapter; the only somewhat similar system previously studied is the salt-secreting desert bush *Atriplex halimus* (Simon et al., 1994), also from the Israeli Negev.

2. *Tamarix*: A Native Tree in Asia, Africa and the Mediterranean, an Invasive Pest in the USA

The genus *Tamarix* (Tamarisk) comprises about 50–60 species of flowering plants in the family Tamaricaceae, native to drier areas of Eurasia and Africa. They are deciduous or evergreen shrubs or small trees growing to 1–15 m in height and forming dense thickets. Taxonomic divisions are most often based on flower size and structure. *Tamarix aphylla* (Feinbrun Dothan, 1972), the species from which the data presented here have been obtained, is an evergreen tree, up to 15 m in height, that often grows on saline soils. Excess salt is excreted by salt glands on the surface of the leaves (Thomson et al., 1969; Waisel, 1961, 1991), which are often covered with salt encrustations (Fig. 1). Salinity and mineral contents of the excreted solution depend upon both the tree species and the composition of the water in the root environment. Reproduction can be either sexual or by adventitious roots or submerged stems.

Tamarix was apparently introduced in the USA as an ornamental shady shrub in the 1800s, and was also employed to set up wind breaks or to stabilize eroding stream beds. It was first reported outside of cultivation in the 1870s, and today it is the most widespread tree in riparian areas throughout the American Southwest. It is believed to exert several negative effects on its environment, including increased soil salinity, water consumption, wildfire occurrences, and frequency and intensity of flooding (Brock, 1994).

Two points that emerge from a survey of the scientific *Tamarix* literature are of particular relevance: its association with different classes of insects, and the occurrence of sugary substances on the leaves, partially at least due to insect secretions (Grieve Facciola, 1990). In fact, *Tamarix*-associated honeydew was mentioned as one of the possible origins of biblical "manna." Chemical analysis of the "manna" from *Tamarix* and other unidentified desert plants (Leibowitz, 1944) revealed the predominance of sucrose, glucose, fructose, and trehalose.

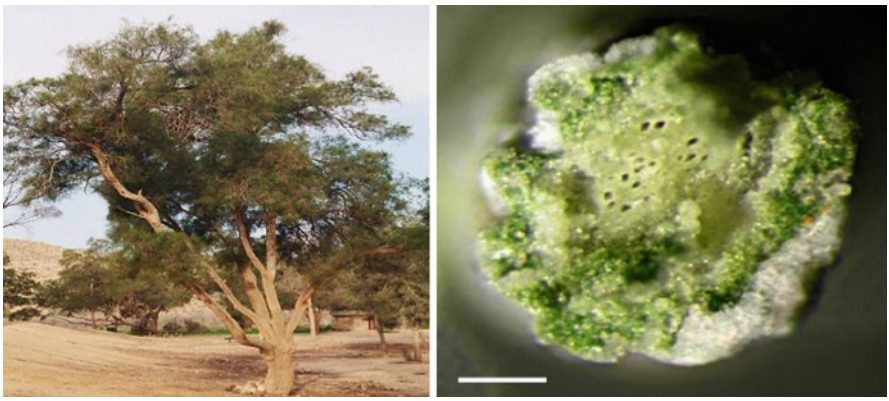

Figure 1. *Tamarix aphylla* tree (*left*; height ~4 m) and a close-up of a salt-encrusted leaf cross section (*right*; bar 1 mm).

3. Stress Factors on a *Tamarix* Leaf and Microbial Adaptations to Desiccation

A bacterium inhabiting the *Tamarix* leaf surface should be adapted for growth, or at least for survival, in the face of a complex set of environmental stress factors. In arid regions, such an organism would exist primarily on dry leaves, and concentrate most of its growth and metabolic activities into short but frequent periods (Southern Israel is characterized by ~200 nights of dew/year) when the leaf is wet. Unlike epiphytes of "regular" plants, on a *Tamarix* leaf these active hours might be spent in a highly saline solution that becomes more and more concentrated as water evaporates after sunrise. Furthermore, the leaf surface of *T. aphylla*, probably due to high carbonate concentrations, is, as described below, also alkaline. Thus, successful colonists of the *Tamarix* leaf surface should be resistant to diurnal desiccation/rewetting cycles as well as to saline and alkaline conditions.

Table 1 summarizes the concentrations of some of the main chemical components of *T. aphylla* dew. The bulk of the dissolved minerals is made up of sodium and chloride, with significant contributions of potassium, calcium, and magnesium. Sulfate is present in surprisingly high concentrations, and the total dissolved salt concentration is ~22%, i.e., approximately fivefold higher than seawater. Most notable is the extremely high concentration of organic solutes, with a total organic carbon concentration of over 3 gC/mL. A significant fraction of the organic carbon is made up of sugars such as trehalose (not shown). Another major organic component of *T. aphylla* dew is glycerol. This is demonstrated in Fig. 2, which displays an NMR spectrum of a dew sample compared to a glycerol reference. Another significant chemical characteristic of *T. aphylla* dew is the high pH, probably a result of the high concentrations of inorganic carbon (Waisel, 1961, 1991). While direct pH determination yielded an average value of 8.5, the pH of a tenfold diluted solution was one unit higher, indicating the buffer

Table 1. Main chemical constituents of early morning *Tamarix* dew (Qvit-Raz et al., 2008). Averages of 12 individual samples collected from three *Tamarix aphylla* trees are presented. No significant statistical differences were observed between individual trees, or between different samples collected from the same tree.

Constituent	Average ± standard deviation (mg/L)
Sodium	38,800 ± 15,700
Potassium	5,960 ± 2,550
Calcium	870 ± 230
Magnesium	8,090 ± 2,510
Chloride	68,760 ± 15,600
Nitrate	920 ± 620
Phosphate	220 ± 160
Sulfate	23,400 ± 7,300
Organic carbon (mgC/mL)	3,280 ± 1,400
Inorganic carbon (mgC/mL)	320 ± 110

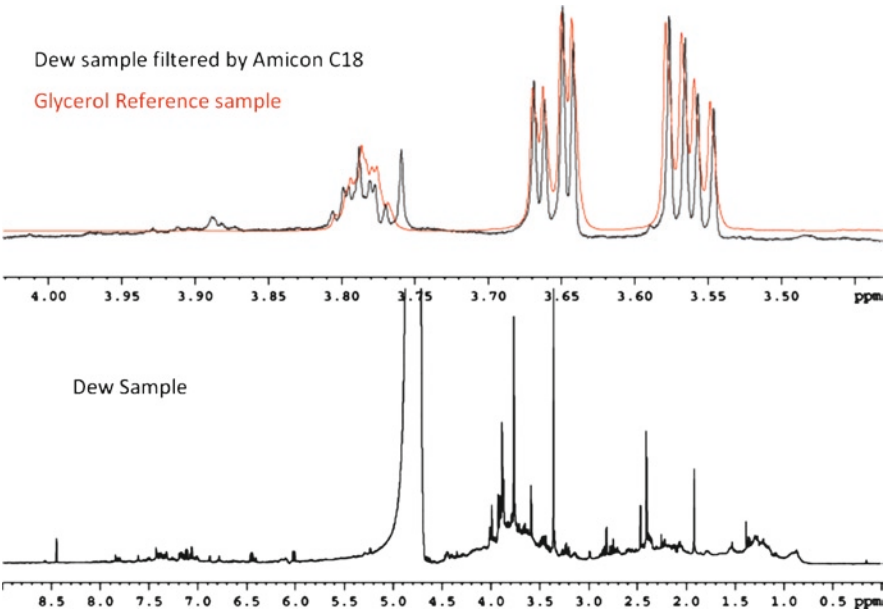

Figure 2. NMR spectra of a *Tamarix* dew sample and a glycerol reference.

capacity of the dew as well as pointing out the limitations of direct measurements of proton activity in a highly saline solution

While the biochemical and molecular mechanisms permitting life at high salinities have been relatively extensively studied (Madigan and Oren, 1999), this

is somewhat less true for adaptations to high pH and even less so for the mechanisms conferring revival after drying, clearly essential for life on leaves. The removal of intracellular water causes drastic changes in inter- and intramolecular interactions (Wolkers et al., 2002), and may damage/denature proteins, membranes, and other cellular components. A common strategy for combating many of these effects is the accumulation of high concentrations of sugars, such as sucrose or, more often, trehalose. There are records of desiccated preservation of vegetative (i.e. not spores) prokaryotic cells for decades and centuries (Kennedy et al., 1994). While at present there are no indications of the strategies employed by *Tamarix* epiphytes, it is tempting to hypothesize that the high sugar concentrations on the leaves may provide osmoprotection as well as aid in desiccation resistance.

4. Phyllosphere Bacterial Populations

Direct isolation of leaf bacteria yielded a large number of colonies (approximately 10^6–10^7 colony forming units per gram of dry leaves) that grew on solid media of varying salinities. As typical of other leaf systems (Lindow and Brandl, 2003), most of the isolates were strongly pigmented. Composition of the bacterial populations was analyzed by characterizing the phylogenetic affiliation of 16S rRNA gene sequences obtained from amplified PCR products from three sources: (a) DNA extracts of these isolates; (b) total DNA extracts obtained from leaf wash samples and separated by DGGE analysis (Muyzer et al., 1993); (c) total DNA extracts obtained from leaf wash samples and segregated using *E. coli* clone libraries.

The phylogenetic affiliation of over 200 16S rRNA gene sequences obtained by all three methods was determined using BLAST analysis based on the Genbank database. The collection of sequences could be grouped into 52 different genera (or less-defined deposited sequences) mostly belonging to five phylogenetic groups; two of these, Actinobacteria and Firmicutes, are gram-positive, and three are gram-negative: Bacteroidetes, Alpha-, and Gammaproteobacteria (Qvit-Raz et al., 2008). Only two sequences seemed to be related to other phyla: one to Betaproteobacteria and the other to the *Deinococcus-Thermus* group. The suitability of the latter to life under extreme desiccation is obvious, although this leaf inhabitant is probably phylogenetically remote from its closest sequenced *Deinococcus* relative. One group that has been conspicuously absent from all our inventory lists of the *Tamarix* phyllosphere deserves a special mention: no archaeal strain was yet isolated from the leaves, nor have we succeeded in amplifying archaeal 16S rRNA genes from DNA extracted from the leaf surfaces using several sets of archaeal-targeted PCR primers. This may be surprising in view of the harsh conditions prevailing on the leaves, but in line with the observation that archaea are not a normal constituent of the phyllosphere.

Analysis of the distribution of genus-level data referred to above according to the methodological approach by which they have been identified revealed a

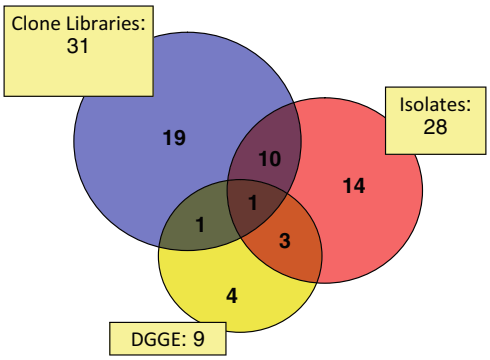

Figure 3. Numbers of identified (closest relatives) genera of *T. aphylla* phyllosphere bacteria, separated according to the methodology by which the data were obtained; red, isolates growing on solid media; yellow, 16S rRNA gene sequences from DGGE gels; blue, 16S rRNA gene sequences from clone libraries. (From Qvit-Raz et al., 2008. With permission.)

significant overlap (Fig. 3). Although each of the methodologies appears to be characterized by a certain bias (Curtis et al., 2002), the overlapping of the circles in Fig. 2 indicates that together they describe a relatively compact grouping of a rather limited diversity. The single genus identified by all three approaches was *Halomonas*, which was also one of the most abundant sequences in our collection. This well-studied genus of mostly moderately halophilic species (Euzéby, 2006) was previously isolated from a variety of saline environments (Garcia et al., 2005; Llamas et al., 2006; Mata et al., 2002). The ability of members of this genus to metabolize aromatic molecules (Garcia et al., 2005) may be of significance for life on a tree surface, the exudates of which may contain such compounds.

To investigate the phylogenetic affiliation of the *Tamarix* bacterial inhabitants, detailed phylogenetic trees were constructed for each of the five phyla that contain representatives of the phyllosphere populations (Qvit-Raz et al., 2008). A study of these trees reveals that in each case, the *Tamarix* sequences group into very specific branches, displaying what appears to be a very limited local diversity. To illustrate this point, Fig. 4 presents an overview of the bacterial domain and its main subdivisions, highlighting the limited areas where the *Tamarix* sequences congregate. For example, all Actinobacteria sequences found on *Tamarix* congregate in only three families (Brevibacteriaceae, Dermabacteraceae and Micrococcaceae), grouped closely together among the 43 families in the order Actinomycetales; this order, in turn, is the only one out of the six in this phylum to which we could associate *Tamarix* sequences. Interestingly, the nearest neighbors in practically all cases are bacterial species isolated from other saline habitats, indicating several independent common halophilic origins.

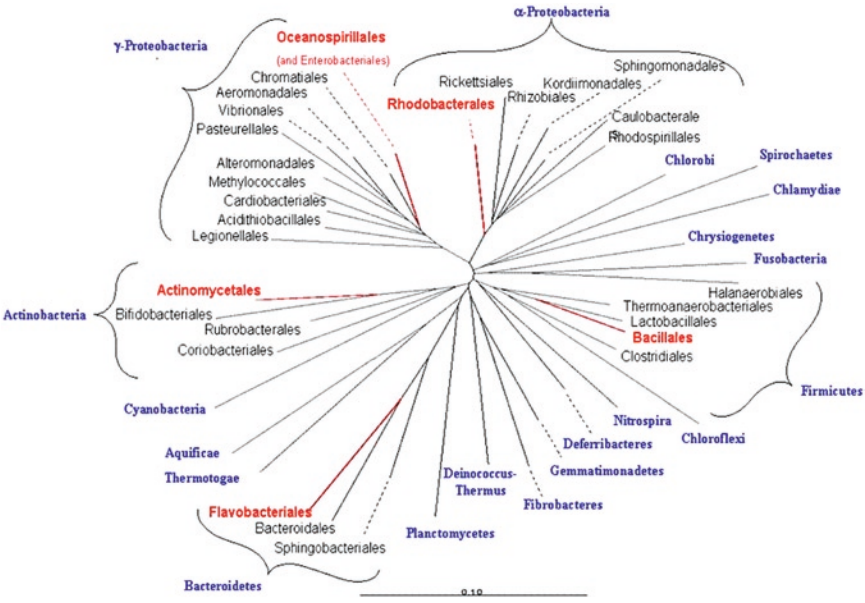

Figure 4. Bacteria phylogenetic tree (constructed using ARB), highlighting the branches where *Tamarix* sequences congregate. Phyla are marked in blue; the five phyla harboring *Tamarix* representatives were expanded to the order level, and the orders inhabited by these representatives were marked in red. (From Qvit-Raz et al., 2008. With permission.)

5. Estimating Microbial Diversity

A question increasingly encountered in the microbial biodiversity literature addresses the number of different microorganisms expected to be found in a specific environment; in practical terms, this often translates to the extent to which a set of samples reflects the true biodiversity of a microbial community (Hughes et al., 2001). In marine microbial communities, estimates range from hundreds to thousands of operational taxonomic units (OTUs) per milliliters in open water (Schloss and Handelsman, 2005) and sediments (Kemp and Aller, 2004; Ravenschlag et al., 1999), respectively. Sogin et al. (2006), by moving beyond the 16S rRNA genes into a hypervariable region of rRNA, revealed a much greater diversity in ocean waters, most of it occupied by representatives of the "rare biosphere," composed of low abundance operational taxonomic units. Microbial biodiversity estimates in plant environments have been carried out to a much more limited extent. Recent reports describe a high diversity in rhizosphere environments (Edwards et al., 2006), with no attempt to estimate the total number of OTUs. In fact, very little use has been made to date of nonculture-dependent

Table 2. Phylum-specific composition of the microbial population of *Tamarix aphylla* phyllosphere.

	Fraction (%) of population according to:	
Phylum	**16S rRNA gene sequencing**	**FISH analysis**
Alphaproteobacteria	7.3	18 ± 1
Gammaproteobacteria	18.2	15 ± 3
Firmicutes	41.5	20 ± 4
Bacteroidetes	7.8	10 ± 4
Actinobacteria	19.5	11 ± 4
Others	5.6	
Unrecognized		26 ± 13

methods for the study of phyllosphere bacterial populations and of their diversity. Yang et al. (2001) identified a limited number of strains in agricultural plants and did not attempt to calculate diversity but justifiably concluded that phyllosphere communities are more complex than previously thought. Lambais et al. (2006) have surveyed bacterial diversity in the leaf canopy of a tropical Atlantic forest of Brazil. The authors estimate the number of distinct OTUs in the different trees to be around 100–400.

Our diversity estimates of phyllosphere bacterial populations were based on five independent *E. coli* 16S rRNA gene clone libraries, each containing ca. 50–60 samples of amplified 16S rRNA genes (Qvit-Raz et al., 2008). The genes were restricted by three endonucleases and visually grouped according to their amplified ribosomal DNA restriction analysis (ARDRA) patterns, which were then defined as our operational taxonomic unit (OTU). The use of several richness estimators, including nonparametric Chao-1and parametric Jackknife 1 indices, indicated that the population is composed of approximately 70–80 OTUs, out of which our samples appear to cover over 60%. In an attempt to gain a different look at the composition of the phyllosphere population, specific fluorescence in situ hybridization (FISH; Amman et al., 2001) probes were designed to microscopically detect individual cells belonging to each of the five taxonomic groups mentioned above. These probes were applied to bacterial suspensions washed off the leaves, and representatives of the five groups were individually counted by direct epifluorescent microscopic observation. Table 2 displays the results, along with those obtained from 16S rRNA gene sequencing.

While for the three gram-negative groups the different approaches yielded very similar results, this was not the case for the Firmicutes and the Actinobacteria, the percentage of which was much higher according to the molecular analysis. The size of the unrecognized fraction (out of DAPI-positive cells) that did not hybridize with any of the FISH probes was significant, indicating that the contribution of the group currently referred to as "others" may in fact be much higher than currently indicated.

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PHYSIOLOGICAL RESPONSES TO STRESS IN THE VIBRIONACEAE

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1. The Vibrionaceae

1.1. A GENERAL DESCRIPTION

The family Vibrionaceae (Domain Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria) is comprised mostly of motile gram-negative chemoorganotrophs, possessing at least one polar flagellum (Farmer III and Janda, 2005; Thompson and Swings, 2006). Vibrios are facultative anaerobes, having both respiratory and fermentative metabolisms, and the mol% G + C of the DNA is 38–51% (Farmer III and Janda, 2005). Cells are usually 1 μm in width and 2–3 μm in length, and most are oxidase positive. The vast majority of vibrios require Na^+ for growth and survival, usually 0.5–3% NaCl for optimum growth. Additionally, most species are susceptible to the vibriostatic agent 0/129 (Thompson and Swings, 2006). In recent years, a two-chromosome configuration, one large and the other small (both circular), has been discovered to be a universal feature for all members of the Vibrionaceae (Iida and Kurokawa, 2006). The Vibrionaceae are ubiquitously distributed throughout aquatic habitats, freshwater and marine waters (Madigan and Martinko, 2006), including rivers, estuaries, lakes, coastal and pelagic oceanic waters, the deep sea, and saltern ponds (Urakawa and Rivera, 2006). Although as many as eight genera have been assigned to the *Vibrionaceae*, the two most specious are *Vibrio* and *Photobacterium* (Thompson and Swings, 2006). A third genus, *Salinivibrio* is worthy of mention due to its unusual ability to grow in a wide range of salinity (0–20% NaCl; Ventosa, 2005) and temperature (5–50°C; Bartlett, 2006) (refer to Table 1).

Numerous species are pathogenic and cause disease in aquatic animals and humans (Farmer III et al., 2005), *Vibrio cholerae* being the most notorious example as the causative agent of cholera (Colwell, 2006). *V. vulnificus* and *V. parahaemolyticus* can also cause severe illness in humans as a result of consuming contaminated seafood (Hulsmann et al., 2003; Wong and Wang, 2004). Furthermore, every year *V. harveyi* (Owens and Busico-Salcedo, 2006), *V. anguillarum* (Miyamoto and Eguchi, 1997; Crosa et al., 2006), and *V. parahaemolyticus* (Austin, 2006) cause substantial economic losses to the aquaculture industry worldwide.

Table 1. Some members of the Vibrionaceae (genera *Photobacterium*, *Vibrio*, and *Salinivibrio*) for which published literature exists on stress physiology.

Stress	Microorganisms	References
Temperature	*P. profundum, V. anguillarum, V. coralliilyticus, V. diabolicus, V. fischeri, V. logei, V. parahaemolyticus, V. salmoncida, V. shiloi, V. vulnificus, V. wodanis*	Amaro et al. (1995), Bartlett (2006), Bordas et al. (1996), Bryan et al. (1999), Datta & Bhadra (2003), Hilton et al. (2006), Huels et al. (2003), Hulsmann et al. (2003), Larsen et al. (2004), Lin et al. (2004), Marco-Noales et al. (1999), McGovern & Oliver (1995), Nishiguchi (2000), Rosenberg et al. (2007), Urakawa & Rivera (2006)
pH	*V. parahaemolyticus, V. vulnificus*	Hulsmann et al. (2003) Kim et al. (2005; 2006), Park et al. (2004), Rhee et al. (2002; 2006), Wong & Wang (2004)
Starvation	*V. anguillarum, V. angustum, V. cholerae, V. parahaemolyticus, V. vulnificus*	Hulsmann et al. (2003), Larsen et al. (2004), McDougald & Kjelleberg (2006), Smith and Oliver (2006)
DNA damage	*V. harveyi, V. vulnificus*	Park et al. (2004), Zielke et al. (2003)
Oxidation	*V. fischeri, V. fluvialis, V. harveyi, V. parahaemolyticus, V. shiloi, V. vulnificus*	Ahn et al. (2005), Banin et al. (2003), Bose et al., (2007), Hulsmann et al. (2003), Kim et al. (2005; 2006), McDougald & Kjelleberg (2006), Park et al. (2004), Ruby & McFall-Ngai (1999), Szpilewska et al. (2003), Vattanaviboon & Mongkolsuk (2001)
Osmolarity	*P. profundum, V. alginolyticus V. anguillarum, V. cholerae, V. fischeri, V. parahaemolyticus, V. vulnificus, S. costicola*	Bartlett (2006), Hulsmann et al. (2003), Lee & Choi (2006), Park et al. (2004), Ventosa (2005), Xu et al. (2004; 2005)

Study of the Vibrionaceae also has applications in ecoysystem health and conservation biology, especially in the light of increasing contemporary concerns about human-induced global climate change. It is already clear that temperature is an abiotic factor that is critical for numerous vibrio symbioses (as discussed below), and it is possible that anthropogenic increases in the prevailing ocean temperature could have profound effects on ecosystems mediated partly through alterations in these symbioses. For example, *V. shiloi* is a pathogen of corals that causes coral bleaching at warmer ocean temperatures such as those expected to prevail in the future (Banin et al., 2003). For these reasons, the Vibrionaceae has galvanized tremendous basic and applied research. Increasing interest in recent years in the utilization of the genes responsible for light production from the bioluminescent bacteria *V. fischeri* for developing bioreporter monitoring and biosensor technologies illustrates this (Ripp et al., 2006).

1.2. SYMBIOSES WITHIN VIBRIONACEAE

Vibrio species not only occur as free-living members of the bacterioplankton but also regularly form symbioses – relationships between two or more organisms that encompass parasitisms, mutualisms, and commensalisms – with other aquatic organisms, including fish, invertebrates, algae, and other microorganisms (Nishiguchi and Nair, 2003; Meibom et al., 2005). Within marine animals, *Vibrio* species are commonly found in the digestive tract and on their surfaces, including skin and chitinous exoskeletons (Urakawa and Rivera, 2006). Host-associated vibrios are provided with a microenvironment rich in nutrients and organic molecules compared to the surrounding seawater (Urakawa and Rivera, 2006). Hence, the vibrio population within or on the host is often several orders of magnitude higher than in the oceanic water column (10^2 cells/ml). *V. cholerae* reach a population level as high as 10^4–10^6 cells/copepod, while *V. halioticoli* can reach a population size at 10^6–10^9 cells/g of fresh gut in abalones (*Haliotis discus hannai*; Sawabe et al., 1995). Although some vibrios are pathogenic toward their hosts, numerous *Vibrio* species are part of the normal microflora of animals living in the ocean, such as oysters (Olafsen et al., 1993), blue crabs (Davis and Sizemore, 1982), sharks (Grimes et al., 1985), and hydroids (Stabili et al., 2006). The metabolic, physiological, and genetic traits permitting the Vibrionaceae to attach, colonize, proliferate in, and circumvent the defense mechanisms of their hosts to cause disease are undoubtedly homologous to those responsible for the establishment of mutualisms. These traits have a common and ancient evolutionary origin, giving rise to many different independent lineages (Nishiguchi and Nair, 2003).

In some instances, vibrios are intimate symbionts providing such an essential role that their hosts would be unable to survive in nature without them (Douglas, 2002). These roles include protection from pathogens, enhanced metabolic function, elevated environmental tolerance, or nutrient acquisition. The symbiosis between *V. halioticoli* and abalones (*Haliotis*) is one such example. In this case, the bacterial partner serves in alginate degradation, a brown algal polysaccharide the abalone consumes while grazing, and provides the gastropod host with an important energy source (Sawabe, 2006). Another example is *V. fischeri*, which is a bioluminescent symbiont of sepiolid squids and monocentrid fishes, and benefits these animals through a behavior termed counterillumination, allowing the hosts to conceal themselves from potential predators or prey (Jones and Nishiguchi, 2004). Considering host interactions partaken by vibrio bacteria encompass the entire symbiosis continuum, from pathogen to indispensable microbial mutualist (Nishiguchi, 2001; Nishiguchi and Jones, 2004), a paradigm shift is emerging where some *Vibrio* species are considered beneficial and may have potential in the development of probiotics for commercially important aquaculture animals (Verschuere et al., 2000).

2. Stress Regulation

2.1. GENERAL DESCRIPTION

Despite the fact that biologists uniformly recognize some environments as stressful, attempts to unequivocally define or quantify stress are difficult (Lenski and Bennett, 1993). The *Oxford Dictionary of Ecology* defines stress as, "A physiological condition produced by excessive pressures that are detrimental to an organism" (Allaby, 2005), while the *Dictionary of Ecology, Evolution, and Systematics* states a stress is "…Any environmental factor that restricts growth and reproduction of an organism or population or causes a potentially adverse change in an organism or biological system; any factor acting to disturb the equilibrium of a system" (Lincoln et al., 1998). For many evolutionary biologists and ecologists, a more satisfying definition is one treating stress as any environmental factor (biotic or abiotic) reducing fitness (Lenski and Bennett, 1993).

"Stress," broadly considered, must also include any biotic or abiotic factors that fluctuate, and thus require organisms to adapt to them physiologically in order to survive. Most bacteria encounter such stressful changes in the environment, including the Vibrionaceae. They grow and survive in a multitude of habitats while possessing various lifestyles: aquatic sediments, fresh and brackish waters, oceans, symbionts of host organisms, saprophytes on detritus, and as free-living cells (Nishiguchi and Jones, 2004; Urakawa and Rivera, 2006; Dunlap et al., 2007). These different environments and lifestyles should not be viewed as static and permanent but rather as transient and cyclical (Urakawa and Rivera, 2006; Dunlap et al., 2007), where microbes migrate between each habitat while encountering stressful conditions (McDougald and Kjelleberg, 2006). These different habitats vary in a myriad of abiotic and biotic factors; consequently, the Vibrionaceae have evolved diverse physiological responses to stress and variable environments.

Previous research has shown fluctuating environments and stressors (e.g., oxygen and reactive forms, extreme salinities/temperatures) have important influences in symbiosis (Xu et al., 2004). For instance, a temperature downshift from 26°C to 18°C caused dramatic changes in the microbiota of the gastrointestinal tract in red hybrid tilapia, with tremendous proliferation of *Vibrio* spp. and a concomitant decrease in *Flavobacterium* (LeaMaster et al., 1997). Vibrio bacteria have also been shown to be distributed differentially both within host species located in different habitats, as well as in various seasons throughout the water column (Jones et al., 2006, 2007). The effect of fluctuating environments on the growth of non-host associated vibrios has been investigated less, but there are still some intriguing recent findings. For example, saline stress has been shown to affect the quality of organic carbon produced by vibrios living in simple, microbial loop foodwebs. This phenomenon affects the quality of carbon available to other trophic levels (Odic et al., 2007).

The purpose of this review is to discuss the physiological responses of non-cholera vibrios to stress, especially to stressors likely encountered during

symbiosis or during transitions from one host or lifestyle to another. We will also draw connections, wherever possible, among work that addresses vibrios from evolutionary, ecological, and molecular physiological points of view. We refer readers interested in *V. cholerae* to another recent review (Prouty and Klose, 2006).

2.2. TEMPERATURE

Vibrios encounter a broad range of temperatures, from those prevailing in marine habitats, to the higher temperatures tolerated by vibrios that can infect humans. Temperature is a significant determinant in shaping ecological associations of vibrios with countless host organisms, including eels (Amaro et al., 1995; Marco-Noales et al., 1999), squid (Nishiguchi, 2000, Jones et al., 2006), sea bream (Bordas et al., 1996), oysters (Kaspar and Tamplin, 1993), and coral (Rosenberg et al., 2007). For example, *V. shiloi* and *V. coralliilyticus*, both pathogens of coral, produce virulence factors implicated in bleaching and killing their hosts. In both cases, the production of these virulence factors is strongly regulated by temperature. At winter temperatures (16–20°C), virulence factors are not produced, while summer temperatures (25–30°C) induce virulence factor production (Rosenberg et al., 2007).

Temperature is a critical abiotic factor affecting other pathogenic symbioses, too. For example, chemotaxis is important for virulence of the fish pathogen *V. anguillarum*, and it is strongly affected by temperature. *V. anguillarum* is most robustly chemotactic at 25°C, and the chemotactic response diminishes in both cooler (5°C, 15°C) and warmer (37°C) conditions (Larsen et al., 2004). The stationary phase-associated sigma factor encoded by *rpoS* is required for *V. vulnificus* to survive heat shock (Hulsmann et al., 2003). An important virulence factor in *V. vulnificus* is capsular polysaccharide (CPS); CPS production appears to be controlled by a phase variation mechanism that can be detected by examining colony phenotype. Encapsulated cells make opaque colonies, while *cps*− cells make translucent colonies. Conversion from CPS+ to *cps*− (from opaque to translucent) is affected by temperature, as increasing the temperature from 23°C to 37°C increased switching for several different isolates (Hilton et al., 2006).

Since Vibrionaceae are aquatic microorganisms residing mostly within oceans, which are the largest cold environment on earth (Urakawa and Rivera, 2006) making up 71% of the earth's surface (Atlas and Bartha, 1998), some members of this group have been extensively selected to thrive in cold temperatures (Bartlett, 2006). Thus, although clinical and human pathogenic Vibrionaceae are mesophilic and capable of growth at ≥37°C, some members of this bacterial family's ancient lineage have adapted to low temperatures. Examples include *Photobacterium profundum*, *V. logei*, *V. wodanis*, and *V. salmoncida*. *Photobacterium* spp. have been more frequently observed to be the more prevalent member of the Vibrionaceae in the cold deep-sea, whereas the genus *Vibrio* is more common in cold ocean surfaces. These species are capable of growth at ≤5°C. Vibrios such as *V. diabolicus*, isolated from a deep-sea hydrothermal vent annelid *Alvinella*

pompejana, are heat tolerant, but no evidence exists that any member of the Vibrionaceae are thermophilic (Urakawa and Rivera, 2006).

Cold shock responses have been studied in *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* (McGovern and Oliver, 1995; Bryan et al., 1999; Datta and Bhadra, 2003; Huels et al., 2003; Lin et al., 2004). Within the later two species, cold shock response increases survival at lower temperatures by a translation-dependent process. Research in this area is particularly important when considering applications of low temperature usage for food storage of shellfish (Bryan et al., 1999). As is generally true of stress responses in microorganisms, cold shock response involves changes in gene expression. Expression of cold shock proteins (CSPs) reach maximal levels during acclimation and includes the upregulation of several proteins, including small homologous peptides 65–70 residues long in the CspA family (Ermolenko and Makhatadze, 2002). Most studies of the CspA family have been studied in greater detail in bacteria such as *E. coli* and *B. subtilis*. Proteins in this family have five antiparallel β strands that form a β barrel, creating a characteristic cold-shock protein domain well conserved throughout all three domains of life. CSPs often bind single-stranded mRNA and DNA, and are believed to assist bacteria in coping with unstable secondary structures at lower temperatures during ribosomal translation, mRNA degradation, termination of transcription, and perhaps nucleoid condensation, thereupon giving CSPs the function of nucleic acid chaperones. Additionally, there may also be a suppression of protein synthesis to prevent miscoding of polypeptides until the cold shock response is initiated (Ermolenko and Makhatadze, 2002).

To maintain functional membrane fluidity with decreasing temperature, vibrios are known to increase the unsaturation of fatty acids comprising their cell membranes. Adaptation to a fully psychrophilic lifestyle regularly, but not always, involves a decrease in enthalpy-driven interactions for the catalytic activity of enzymes, increasing the number of functional conformations permitted for enzyme-substrate complexes (Bartlett, 2006). For instance, the amino acid residues of psychrophilic enzymes within the cytosol display additional hydrophilic associations with the solvent, while simultaneously lessening internal hydrophobic interactions. This yields enzymes that are less condensed relative to mesophilic counterparts, which can result by increasing the α-helix and decreasing the β-sheet character of the secondary structure.

2.3. pH STRESS

pH stress is ecologically and evolutionarily significant because symbiotic vibrios include some gastrointestinal pathogens that must somehow survive the acidic challenge encountered in the digestive environment. Recently, molecular mechanisms of survival in the face of pH stress have been studied most intensively

in the species *V. vulnificus*. This microorganism is an opportunistic pathogen of humans, acquired by ingesting contaminated seafood. There appears to be multiple overlapping signal transduction networks that together sense and respond to acid challenge. For example, the alternative sigma factor encoded by the *rpoS* gene is required for *V. vulnificus* to survive acid stress (pH < 5) in both stationary and exponential phases of microbial growth (Hulsmann et al., 2003; Park et al., 2004). Other regulatory proteins such as CadC (accessory protein for the Cd^{2+} efflux ATPase CadA), SoxR (superoxide response regulator), and Fur (ferric uptake regulator protein) are also needed for survival of acid stress. The CadC regulator in *V. vulnificus* induces *cadAB* expression, leading to the production of CadA (lysine decarboxylase) and CadB (lysine-cadaverine antiporter). Lysine decarboxylation is one step toward the production of cadaverine, which accumulates in the extracellular space during an acid stress response. The AphB transcription factor also enhances expression of *cadAB* under stressful acidic conditions, by directly activating a promoter that drives *cadC* production (Rhee et al., 2002, 2006).

The physiological response that leads to survival of acid stress is connected to the response that protects *V. vulnificus* from superoxide stress. The SoxR regulator, already known to regulate genes important for surviving oxidative damaging agents, also induces the *cadAB* operon (Kim et al., 2006). This induction does not require the CadC regulator, and cells lacking CadA are more sensitive to oxidizing agents than wild-type cells. Furthermore, simply increasing the amount of *cadAB* expression by supplying these genes on a multicopy plasmid is sufficient to reduce the induction of a superoxide dismutase that the cells would normally produce upon challenge with the oxidizing agent, methyl viologen. Together, these results suggest that extracellular cadaverine not only neutralizes the local environment surrounding cells in acidic medium but also scavenges superoxide radicals (Kim et al., 2006).

Yet another connection between survival of acid and oxidative stresses in *V. vulnificus* was revealed with the discovery that cells grown to exponential phase and then subsequently exposed to acidic (pH = 5.0) conditions induce the expression of *sodA*, a locus encoding a manganese-containing superoxide dismutase (MnSOD; Kim et al., 2005). MnSOD is positively regulated by SoxR and negatively regulated by Fur, but is not transcriptionally regulated by *rpoS*. Regulation by SoxR is likely indirect, while regulation by Fur is direct via binding to the *sodA* promoter. An explanation for the induction of MnSOD upon low pH is that shocked cells accumulate superoxides. In fact, use of a scavenger to prevent intracellular superoxide accumulation eliminated pH-dependent induction of MnSOD. Thus, it appears that MnSOD is not induced directly by acidic conditions per se, but rather by oxidizing agents that are themselves produced in response to acid shock. Deletion of any SOD in *V. vulnificus* (FeSOD, encoded by *sodB*; CuZnSOD, encoded by *sodC*, or MnSOD) led to decreased survival of exponential cells exposed to acid stress (pH = 5.0). Therefore, acid resistance in

V. vulnificus involves not only stress responses to acidity itself but also counteractions to superoxides that accumulate intracellularly upon acid stress (Kim et al., 2005). Additional investigations of pH stress in vibrios other than *V. vulnificus* are beginning to expand this area of research (Wong and Wang, 2004).

2.4. NUTRITIONAL STRESS

Ninety-five percent of the open ocean is oligotrophic, averaging a scant 50 g of carbon fixed per square meter per year by primary productivity (Atlas and Bartha, 1998). Host organisms, however, are nutrient rich. As vibrios experience transient free-living and host-associated life cycles, these microbes thus encounter feast or famine conditions in which they are either host-associated (feast) or living in the water column or sand (famine). They must, therefore, undergo long intervals with little or no growth and metabolic dormancy in their free-living state, followed by brief periods of rapid growth during symbiosis (McDougald and Kjelleberg, 2006). Given this natural history, it is no surprise that many *Vibrio* species possess extraordinarily quick generation times during periods of high nutrient availability, enabling them to out-compete and outgrow other microbial species (Eilers et al., 2000; Giovannoni and Rappe, 2000).

They also appear to have been selected for effective starvation response mechanisms, and the molecular basis of these responses has been studied in some detail (Urakawa and Rivera, 2006). Researchers found that incubation of *V. vulnificus* in chambers suspended in natural estuarine waters led to in situ expression of both *rpoS* and *katG* (catalase peroxidase), regardless of different prevailing temperatures and salinity conditions found in the summer and winter (Smith and Oliver, 2006). Perhaps, these genes were expressed specifically to adapt to nutritional stress during the in situ incubation; regardless of the time of year, the dissolved organic carbon was only 2.83 mg/L. This in situ work is consistent with previous findings, which demonstrated that *rpoS* mutants were less able to survive starvation conditions initially, compared with wild-type counterparts, but after 14 days exhibited survival identical to wild-type counterparts (Hulsmann et al., 2003).

A study of *V. anguillarum*, which are fish pathogens, demonstrated a linkage between nutritional stress and virulence. Chemotaxis is an essential activity during infection, and starving (through incubation in phosphate-buffered saline), and *V. anguillarum* cells remained as virulent as exponential-phase cells after 2 days, and were still chemotactic post 8 days starvation using LD_{50} (Larsen et al., 2004).

2.5. DNA DAMAGE

Like all cells, *Vibrios* must have mechanisms for repairing DNA damage caused by common environmental assaults such as exposure to UV irradiation. In *V. vulnificus*, *rpoS* mutants are much more sensitive to UV irradiation than their

wild-type counterparts in exponential phase (Park et al., 2004). In *V. harveyi*, the small GTP-binding protein CgtA is required for survival upon exposure to ultraviolet light. Its role in the repair of damaged DNA is likely indirect, as CgtA stimulates *recA* gene expression (Zielke et al., 2003). The coevolution of DNA-interacting proteins and genome dialects, intergenome differences as a result of horizontal gene transfer, has recently been attributed to stress (Paz et al., 2005). Evolution of bioluminescence as a mechanism to aid DNA repair via the activation of light-dependent photolyase has also been proposed (Czyz et al., 2003).

2.6. OXIDATIVE STRESS

Vibrio species encounter oxidative stress during colonization of animal hosts, even during mutualistic associations such as the symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Ruby and McFall-Ngai, 1999). Thus, the question of the mechanisms by which certain *Vibrio* species survive oxidative stress has been under intense investigation. Several groups have investigated the role of the *V. vulnificus* sigma factor encoded by *rpoS* in survival following a challenge with the oxidizing agent H_2O_2. In one circumstance (strain C7184o), an *rpoS* mutant was much more sensitive to H_2O_2 than its wild-type counterpart during stationary phase (Hulsmann et al., 2003). Another case using a different pathogenic isolate of *V. vulnificus* (ATCC 29307), the *rpoS* mutant was more sensitive than wild-type to H_2O_2 challenge during exponential phase, but not during stationary phase (Park et al., 2004). However, the (ATCC 29307) *rpoS* mutant had reduced catalase activity in both exponential and stationary phases, despite the fact that differential survival upon challenge with H_2O_2 was only observed in exponential phase. Therefore, different roles of *rpoS* during oxidative challenge between these two *V. vulnificus* isolates might indicate that adaptation to oxidative stress has taken different pathways (i.e., convergent evolution) in distinct *V. vulnificus* isolates.

The SoxRS regulon and superoxide dismutases (SODs) have also been implicated in physiological responses needed to survive oxidative stress. In many cases, the production of SODs is linked to survival of multiple stressors. For example, in *V. vulnificus*, extracellular acid stress provokes the accumulation of intracellular superoxides and so further provokes a superoxide response (Kim et al., 2005, 2006). An extracellular SOD is an important virulence factor in the coral pathogen *V. shiloi*; its production is induced by high temperature, indicating a connection between survival of oxidative stress and temperature stress (Banin et al., 2003). In *V. harveyi*, a pathogen of the farmed black tiger prawn, exposure to the superoxide-generating drug menadione induces expression of both the OxyR and SoxRS regulons. *V. harveyi* also seem to exhibit physiological adaptation to oxidative stress, as exposure to sublethal doses of menadione protects *V. harveyi* cells from subsequent exposure to otherwise lethal concentrations of H_2O_2. Growing *V. harveyi* cells in high-salinity medium prior to exposure to

menadione also led to increased protection to this oxidizing agent, suggesting a coupling between osmotic stress physiology and oxidative stress responses in this organism (Vattanaviboon and Mongkolsuk, 2001).

V. harveyi, like most vibrios, are bioluminescent. The enzyme luciferase directly catalyzes the production of photons and is encoded by the *luxAB* genes. The LuxD protein, encoded in the same operon, is an acetyltransferase that produces fatty acid substrates for the luminescence reaction. Mutants with null mutations in *luxA* or *luxB*, but not mutants with a null mutation in *luxD*, are hypersensitive to several oxidative stressors such as H_2O_2, cumene hydroperoxide, *t*-butyl hydroperoxide, and ferrous ions. Curiously, this hypersensitivity was found over a narrow range of concentrations of these agents, occurring neither above nor below this range. Nevertheless, *luxA* and *luxB* mutants were rescued by supplied antioxidants in the growth medium. This suggests that bioluminescence may have evolved as a response to oxidative stress (Barros and Bechara, 1998; Szpilewska et al., 2003).

As in *V. harveyi*, bioluminescence in *V. fischeri* consumes reducing power. The physiology suggests a possible relationship between redox homeostasis, responses to oxidative stress, and bioluminescence. Recent evidence demonstrates that the ArcAB system in *V. fischeri* represses expression of the *luxICDABEG* operon. Possible inactivation of the ArcA repressor by oxidative stress, experienced during the early stages of host colonization, likely derepresses *luxICDABEG* expression when *V. fischeri* colonize *E. scolopes*. This hypothesis, may explain why some strains of symbiotically competent *V. fischeri* such as ES114 are not visibly luminescent in vitro, yet are visibly luminous during symbiosis (Bose et al., 2007).

Additional connections between oxidative stress physiology and host colonization may be present due to the need to survive stressors produced by the host. For example, in halophilic *V. fluvialis*, an opportunistic pathogen that causes gastroenteritis in humans, requires the *hupO* gene for surviving exposure to H_2O_2 during exponential phase (Ahn et al., 2005). HupO is a virulence factor that binds to hemin and likely affects intracellular accumulation of hemin-associated iron during infection. The mechanism of *hupO*-associated H_2O_2 resistance is independent of catalase activity; therefore, the molecular details of how iron deficiency is connected to oxidative stress through HupO remain to be determined.

A relatively unexplored but related topic is the question of survival in the face of nitrosative stress, which symbionts also encounter when colonizing a host. It is increasingly clear that *V. fischeri* encounter nitric oxide during host colonization, as the host tissues lining the spaces where *V. fischeri* must traverse to colonize juvenile squids contain cells that produce NO and nitric oxide synthase (NOS; Davidson et al., 2004). NO production is normally considered to be a defensive strategy to prevent harmful bacterial infections, so it is striking that in this case, NO seems to function as part of the normal process of host–symbiont colonization that leads to a highly specialized and mutually beneficial symbiosis.

2.7. OSMOTIC STRESS

Vibrios live in environments that vary in salinity, and therefore experience high (hyperosmolar) and low (hypoosmolar) osmotic stress. During hypoosmolarity, the obstacles to cellular homeostasis are maintaining appropriate cytoplasmic concentrations of metabolites and ions, preventing cell lysis, and preserving ionic strength and pH (Bartlett, 2006). During hypoosmotic shock, some vibrios may increase putrescine content to compensate for decreased K^+ that are necessary to stabilize the phosphate backbones of nucleic acids. Hyperosmolarity, however, promotes dehydration and shriveling of cells. Microorganisms must be able to import or synthesize counterbalancing solutes that are compatible with metabolic and physiological functions. K^+ uptake is frequently stimulated to compensate for the increased external osmolarity. However, negative counter-ions (e.g., glutamate) must also be concurrently imported into the cell or synthesized de novo to sustain the same intracellular net charge (Sleator and Hill, 2001). Alternatively, cells can forgo K^+ uptake and import or synthesize neutral compatible solutes, as they carry no charge. Ectoine is such an example and its biosynthesis may be unique to the genus *Vibrio* (Bartlett, 2006). *V. fischeri* is also known to possess the ability to synthesize the disaccharide trehalose, which is also a neutral compatible solute for high osmolar stress. Incorporating polyunsaturated fatty acids in the cell membrane may also alleviate vibrios of excess toxic Na^+ by allowing their departure through the more fluid membrane (Valentine and Valentine, 2004).

As mentioned previously, *V. vulnificus* is an opportunistic human pathogen that can survive a range of osmolar conditions, from high-salt (or sugar) environments used to curb colonization of shellfish intended for human consumption, to those typical in marine environments and lower osmolarites as encountered in some compartments of the human body. For example, *rpoS* mutant *V. vulnificus* (C7184o) in stationary phase were much more sensitive to hyperosmolarity stress than their wild-type counterparts (Hulsmann et al., 2003). In contrast, *rpoS* mutant *V. vulnificus* (ATCC 29307) was no more sensitive to hyperosmolarity stress than its wild-type counterpart (Park et al., 2004). Perhaps, these isolate-specific observations indicate that the *rpoS* regulon is not identical across all *V. vulnificus* isolates, evidence that a strain-specific genomic context is present for gene expression. Also in *V. vulnificus*, loss-of-function mutations in the *putAP* operon cause hypersensitivity to high osmolarity (Kim et al., 2006). The operon encodes a proline dehydrogenase and a proline permease; proline dehydrogenase is part of a pathway that converts proline into glutamate, a well-known osmoprotectant. Two promoters, separated by six base pairs, control production of two transcripts from this operon. One is monocistronic and encodes only *putA*, while the second encodes both *putA* and *putP*. Expression of mRNA that hybridizes to a *putA* probe declines in stationary phase, while both *putA* and *putAP* transcripts are induced by proline and negatively regulated by glutamate (Kim et al., 2006). In contrast, high osmolarity induced higher *putA* mRNA levels but did not affect

levels of bicistronic *putAP* mRNA, suggesting that only one of the two promoters is responsive to osmotic conditions. Additionally, both *putA* and *putAB* transcripts were dramatically reduced in a *crp*− mutant, suggesting a possible connection between survival of acid stress and nutritional status (as surveyed by intracellular cAMP levels; Lee and Choi, 2006). *Crp* mutants in other bacteria, such as *E. coli*, can have pleiotropic effects, so this relationship between nutritional status and survival of saline stress remains tentative in vibrio bacteria.

Osmotic stress has also been observed to have effects in other vibrios. For example, in the fish pathogen *V. anguillarum*, chemotactic responses to serine are decreased by high osmolarity (≥1.8% NaCl) relative to optimal osmolarity conditions (0.8% NaCl; Larsen et al., 2004). Proteomic analyses have been completed of *V. alginolyticus* and *V. parahaemolyticus* at different NaCl concentrations to examine resultant changes in gene expression through these physiological shifts (Xu et al., 2004, 2005). Since marine pathogens constantly face changes in osmolarity as they shift between marine waters and their native hosts, proteins such as outer membranes are selected to accommodate such changes. Outer membrane proteins OmpW, OmpV, and OmpTolC were discovered to be responsive osmotic stress proteins in *V. alginolyticus* (Xu et al., 2005). OmpV was expressed at low NaCl concentrations, but not at higher concentrations. Conversely, OmpW and OmpTolC displayed reverse changes, being expressed at high NaCl concentrations and downregulated at low NaCl levels. Interestingly, differential expression of outer membrane proteins has been suggested by several researchers to play significant roles in symbiosis, including immunogenicity and virulence (Xu et al., 2005; Jones and Nishiguchi, 2006). Not only were OmpW and OmpV identified in *V. parahaemolyticus* osmoregulation, but elongation factor TU and polar flagellin were implicated as well (Xu et al., 2004). Elongation factor TU and polar flagellin were respectively downregulated and upregulated at higher salinities, while OmpW and OmpV showed analogous patterns of expression, as in *V. alginolyticus*.

3. Experimental Evolution and the Viable but Non-Culturable State

3.1. EXPERIMENTAL EVOLUTION WITH VIBRIOS

In recent years, experimental evolution with microorganisms has emerged as an exciting new subdiscipline of evolutionary biology addressing diverse issues (Lenski et al., 1991; Bennet, 2002; Lenski, 2002), including microbial adaptation to variable environments and stress (Lenski and Bennett, 1993, 1997, 1999). The elegance of this scientific approach is the ability investigators have to control the selective regimen of the experimental conditions, to observe evolution and adaptation on a human time scale due to short generation times of microorganisms, and the ability to compare evolving lineages from different evolutionary time points directly to a specifically known ancestor through

the usage of a –80°C "frozen fossil record." This cryogenically preserved "fossil record" allows identification of changes in gene expression responsible for adaptation and loci subject to selection through subsequent genetic analysis (Riehle et al., 2003). Although experimental evolution studies in the past were largely initiated with *E. coli* as the major study microorganism, the list of other microbial species used in parallel studies has expanded in recent years. To date, the usage of Vibrionaceae in experimental evolution has principally been absent; however, such work is currently underway in our laboratory, as we are in the process of conducting serial passage experiments with *V. fischeri* derived from the sepiolid squid *Euprymna scolopes* (Hawaiian species) and evolving them through the novel host congener *E. tasmanica* (Australian species; Nishiguchi et al., 1998; Nishiguchi, 2002). Moreover, we are expanding such experimental evolution projects to address the ability of *V. fischeri* to adapt to abiotic factors at extreme limits of permissible growth based on previous studies that have shown introgression of various *V. fischeri* haplotypes to different habitats (Jones et al., 2006), as well as seasonal changes that cause changes in the viable *V. fischeri* baceterioplankton community (Jones et al., 2007).

3.2. THE VIABLE BUT NON-CULTURABLE STATE

A substantial literature now exists and continues to develop surrounding the viable but nonculturable (VBNC) state, whereby microorganisms normally culturable do not grow in liquid or agar media because of their entry into a dormancy where cells are still metabolically active and presumed to enhance resistance and survival to stress, a phenomenon first widely reported in the Vibrionaceae but is now believed to exist in other prokaryotes (Roszak and Colwell, 1987; Colwell, 2000). Nevertheless, the existence of VBNC cells has been contested and continues to be challenged (Bogosian and Bourneuf, 2001; Wong and Wang, 2004; McDougald and Kjelleberg, 2006). Past research on cells ostensibly in the VBNC state have included the identification of molecules and mechanisms (e.g., temperature upshift) that apparently resuscitate VBNC cells, enabling them to regrow in microbiological culture media once again. Nonetheless, skepticism persists because of the possibility that any observed regrowth is the result of injured cells having recovered their healthy state, and not the result of resuscitating cells from a genuine VBNC condition (Bogosian and Bourneuf, 2001). Skeptics point out, that as yet no genes have been identified, through null mutations or knockouts, that may be responsible for vibrios entering a developmental program or pathway leading to a physiologically differentiated VBNC state. Convincing evidence would perhaps require loss-of-function experiments followed by complementation or over-expression gain-of-function studies (Bogosian and Bourneuf, 2001; McDougald and Kjelleberg, 2006). Proponents of the VBNC state remain convinced of its validity, perhaps not least because of the state's power to explain

some ecological observations related to isolation of vibrio colony-forming units during different times of the year. Continued work into this area will surely lead to intriguing research, along with lively debate, for years to come.

4. Conclusion

Finally, extended examinations into the genetic traits and physiological responses characteristic of Vibrionaceae – quorum sensing, biofilm formation, two-chromosome architecture, induction of recombination machinery in the utilization of integrons, and horizontal gene transfer – is essential to more completely understand their roles in regulating cellular homeostasis against stress (Boucher and Stokes, 2006; Iida and Kurokawa, 2006; Rowe-Magnus et al., 2006). There has not been much recent work specifically on stress and its effects on any of these phenomena, providing fertile ground for additional physiological investigations. Future work in areas such as experimental evolution, community ecology, and population structure of vibrios in the environment, as well as specific trade-offs between symbiotic and free-living lifestyles should provide key insights into the adaptive radiation and speciation of this extensive group of bacteria.

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THE STRESSED LIFE OF MICROBES IN PLANTS

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1. Introduction

The microbes most frequently found in plants belong to eubacteria, cyanobacteria, and fungi. Some of these microbes induce the formation of visible nodules on roots, but many others do not reveal their presence by external symptoms on the host. Most of them provide the host with nitrogen and obtain protection against predators and competing organisms in the soil. In other cases, the symbiont promotes the production of plant growth substances and receives protection against soil-competing organisms. However, it is difficult to define all the types of plant–microbe associations as the relationships between the associated partners are multiple and various.

A focus concerning the plant–microbe association is the response of the host to the various steps of the association, from the adhesion to the localization of the microbe in the host cytoplasm or tissues or in the apoplast. Other questions concern the microbe behavior during the steps from outside until localization and cooperation with the host. Among these problems, those less known are the final stages of the microbes in the host and their survival in the soil.

In order to answer the last question, we compared the behavior of bacteria and cyanobacteria in different association systems such as Cycad-*Nostoc*; *Azolla-Anabaena*; Legume-rhizobia; and tomato-*Azospirillum*. There are associations in which the symbionts are intercellular in coralloid roots or in leaf cavities, or intracellular in special structures as root nodules or only in cortex or other organs and tissues, without visible modifications in the plant. Due to this, we mainly pinpoint the ways in which microbes undergo and overcome the stress conditions when inside the host. More specifically, we look at a group of proteins and other compounds, such as Superoxide dismutase (SOD), Poly-β-hydroxybutyrate (PHB), trehalose, and melanin, found in the associated microbes. We also review the formation of spore-like cysts and akinetes to interpret the stress symptoms that predict the final fate of the microbes in the plant.

In all the above-cited systems, bacteria or cyanobacteria adhere to the plant roots after being attracted by plant-excreted molecules and penetrate the host via passive ways, as through broken hairs in *Azospirillum*, or modified hair wall and the resulting formation of thread infection in rhizobia. During these steps,

cyanobacteria and bacteria often undergo changes in their shape, and dimensions, as well as in their metabolism. Similarly, other changes characterizing situations of microbial stress occur in the microbes once they are inside the host (Grilli Caiola, 1992; Grilli Caiola et al., 2004).

Stress symptoms in microbes have been reported in cultures grown in different nutrient concentrations, mainly in nitrogen-fixing microorganism; moreover, carbon compound availability, pH or salt and water variation induce stress (Gerson et al., 1978; Tung and Watanabe, 1983; Rai and Rai, 2000).

Trehalose (alpha-D-glucopyranosyl-(1-1)alpha-glucopyranoside) has been discovered in many bacteria and cyanobacteria, in which it often builds high tolerance levels to different abiotic stresses (Benaroudj et al., 2001; Garg et al., 2002; Wingler, 2002). Trehalose and its hydrolyzing enzyme trehalase appear to be less frequent in plants but common in plant–symbiotic microbes (Mellor, 1992). In symbiotic organs, trehalose seems to prevent phagolysosome fusion in host cells, could act as a reserve or storage form of reduced carbon, or may help in thermotolerance, in resistance to water stress, e.g., desiccation, or in the stabilization of biological structures. It occurs in *Nostoc* associated with cycads, in the *Nostoc-Gunnera* symbiosis, in Rhizobiaceae that live in legume root nodules (Mellor, 1992; Muller et al., 1995; Aeschbacher et al., 1999).

PHB is a polymer related metabolically to lipids. It is a highly reduced polymer made up exclusively of D-β-hydroxybutyric acid units in ester linkage. Its tertiary structure is a compact right-handed coil with a twofold screw axis and a pitch of 0.60 nm. PHB appears as small or large roundish-shaped electron transparent granules surrounded by a single membrane, which appears as a dense line approximately 3 nm in thickness under a transmission electron microscope. It occurs only rarely in free-living cyanobacteria, whereas it is common in other free-living and symbiotic bacteria. It is considered a reserve material in bacteria involved in the maintenance of nitrogen fixation when the photosynthesis is restricted (Gerson et al., 1978; Trainer and Charles, 2006). In *Azospirillum brasilense*, the synthesis and utilization of PHB as a carbon and energy source under stress conditions apparently favors the establishment of this bacterium and its survival in competitive environments (Kadouri et al., 2003).

Melanin is a dark pigment produced by some free-living and/or plant-associated bacteria. It is a polymer occurring inside the cytoplasm as small electron-dense granules. It is formed often in nitrogen-fixing bacteria as well as in aging vegetative cells in culture and symbiosis. Melanin production is the result of the oxidative polymerization of phenolic compounds by the polyphenol oxidases:tyrosinase that has monophenol monooxygenase (EC 1.18.14.1) and *o*-diphenol:oxygenoxidoreductase (EC 1.10.3.1) activities, and by laccase (EC 1.10.3.2) that has *p*-diphenol:oxygen-oxidoreductase activity. Tyrosinase has been reported in *Rhizobium* and *Synorhizobium*. Laccase has been found in *Azospirillum* and *Rhizobium*, and the beneficial effects of coinoculation of both bacteria have been reported in legumes.

Superoxide dismutases (SODs: EC 1.15.1.1) are metalloproteins that rapidly convert superoxide O_2 to hydrogen peroxide (H_2O_2) and molecular oxygen in all

aerobic organisms(Fridovich, 1995). They prevent damage caused to cellular membranes by oxygen species (ROS), and act as a primary defense during oxidative stresses to which organisms are exposed. Due to their superoxide detoxifying capacities, SODs are considered a hallmark of plant defense responses to pathogens. Moreover, convincing evidence has demonstrated a positive correlation between levels of FeSOD and nitrogenase activity of cyanobionts, supporting the hypothesis that FeSOD protects nitrogenases against ROS damage (Canini et al., 1992).

Spores are formed by free-living bacteria and less frequently in associations. No spores or cysts have been reported in rhizobia in root nodules legume. Cysts (spore-like) have been described in *Azospirillum* free-living in the soil as well as when inside the roots of tomato (Sadavisan and Neyra, 1987; Grilli Caiola et al., 2004). A cyst derives from a vegetative cell, which after losing motility, develops a thickened outer coat, assumes an enlarged spherical form, and accumulates abundant PHB granules. The encystation is accompanied by a production in the cytoplasm of a dark brown pigmentation due to melanin.

Akinetes of *Anabaena* occur in *Azolla* sporocarps, but rarely in the leaves (Grilli et al., 1992), and *Nostoc*, too, rarely forms akinetes inside the cycad coralloid roots (Grilli Caiola and De Vecchi, 1980; Grilli Caiola, 1992, 2002). Akinetes derive from vegetative cells whose differentiation is controlled by a critical level of nitrogen and carbohydrate. They contain high amounts of cyanophycin and glycogen, both facilitating long-term survival under difficult environmental conditions.

Of course, the behavior of stressed microorganisms depends on their own ability to react to the strong influence of the host. In the following, we present a brief summary of associations in which the microbes are localized inside a newly formed structure of their symbiotic partners.

2. Cycad-*Nostoc*

Cycad coralloid roots house cyanobacteria belonging to *Nostoc* or *Anabaena*. These are localized in the cyanobacterial zone, the intercellular spaces of the zone formed by radially elongated cells (Grilli Caiola, 2002). Mainly vegetative cyanobacterial cells are developed in the apical part of coralloid roots, whereas the number of heterocysts increases from the median parts of the coralloid toward the basal ones (Fig. 1a), where most of the heterocysts degenerate.

The association allows the host to access nitrogen even when it is poorly available in the soil. On the other hand, cyanobionts benefit from the nutrient availability and the more stable conditions inside the coralloids. Coralloid roots form independently from the presence of the symbiont. They represent apogeotropic roots with a short life. Because they are usually annually developed, the advantage for the symbiont is limited to one or a few years after the coralloid degenerates, and the cycad utilizes the product of the reduced nitrogen, e.g., in the form of glutamine or citrulline.

In this system, the cyanobiont accumulates trehalose (Lindblad and Bergman, 1989) and cyanophycin at first. The latter is detectable as large granules inside the nucleoplasm or in the symbiont's thylakoidal apparatus. Both compounds disappear in the vegetative cells present in the oldest parts of the coralloid, suggesting sharp changes of the metabolic conditions inside the host. Together with these metabolic changes in the oldest part of the coralloid, many vegetative cells and, above all, heterocysts undergo degenerative processes, and probably autolysis. Only a few of the small vegetative cells survive and probably return alive to the soil (Fig. 1b). Akinetes are very rare in cyanobacteria–cycad association, and no bacteria or other microbes usually accompany cyanobionts.

Cyanobacterial relation to the host is regulated in a manner that suggests a dominance of the cycad over the partner. In fact, high oxygen pressure in the heterocysts inhibits the nitrogenase activity and the utilization of derived nitrogen compounds, inducing the degenerative process in these specialized cells (Canini and Grilli, 1993).

The synthesis and regulation of SOD appears to be a possibility to defend heterocysts against the superoxide anion originating from the high respiration necessary to provide the energy for N_2 reduction. A pattern of FeSOD labeling has been revealed in the cycad cyanobiont: FeSOD particle densities in heterocysts are in line with nitrogenase activity. FeSOD was never localized in degenerate, non-nitrogen-fixing heterocysts. No melanin or PHB has been observed in cyanobacteria associated with cycads.

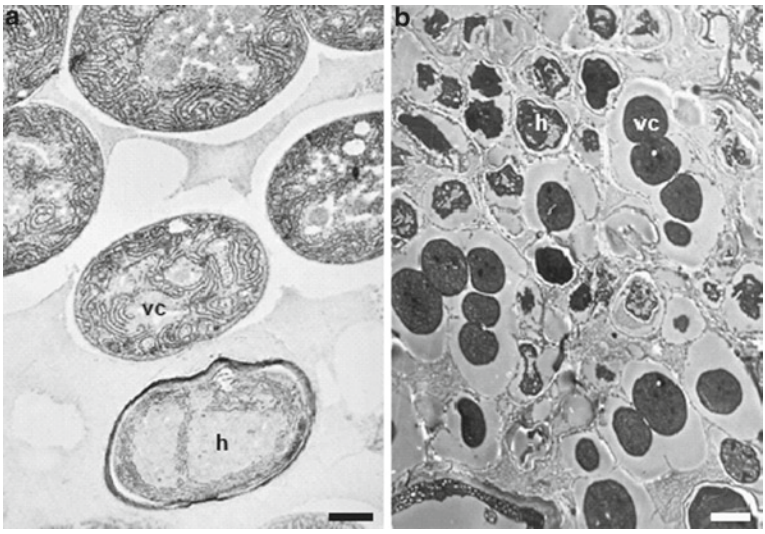

Figure 1. Cyanobacteria in *Cycas revoluta* coralloid root at TEM. (**a**) Healthy vegetative cell (vc) and heterocyst (h) in the median part of coralloid. Bar = 1 μm. (**b**) Basal part of coralloid with healthy cyanobacterial vegetative cells (vc) and degenerated heterocysts (h). Bar =1.2 μm.

In cycad–cyanobacteria association, the cyanobacteria cell morphology and metabolism is different from the free-living stages. The reduction of size and the relative composition of cell types from the apical parts to the basal ones of the coralloid, lead to the conclusion that the conditions of the cyanobacteria inside the coralloid are rather stressful, and for a majority of the cells, survival is difficult (Grilli Caiola, 2002).

3. *Azolla-Anabaena*-Bacteria

In previous papers (Grilli Caiola, 1992; Canini and Grilli Caiola, 1995; Grilli Caiola and Forni, 1999), the life of the prokaryotes, *Anabaena* and bacteria, in the leaf cavities of *Azolla* has been examined. *Azolla* is a genus of small aquatic fern. The species are distributed in almost every continent as spontaneous or cultured plants much appreciated for their water and soil enrichment in nitrogen due to nitrogen fixation occurring in their leaf cavities. The leaves are overlapping, each with a dorsal floating chlorophyllous lobe and a ventral submerged lobe. Their floating lobe has a cavity (Fig. 2a, b) containing many simple hairs, one primary branched hair, one secondary branched hair, the cyanobacterium *Anabaena azollae*, and bacteria. Recent studies have pointed out the existence of different cyanobionts strains among *Azolla* species, and diversity within a single *Azolla* species (Papaefthimiou et al., 2008). Based on the latter, cyanobiont seems to have more genotypic affinity to the genus *Anabaena* than to other Nostocaceae.

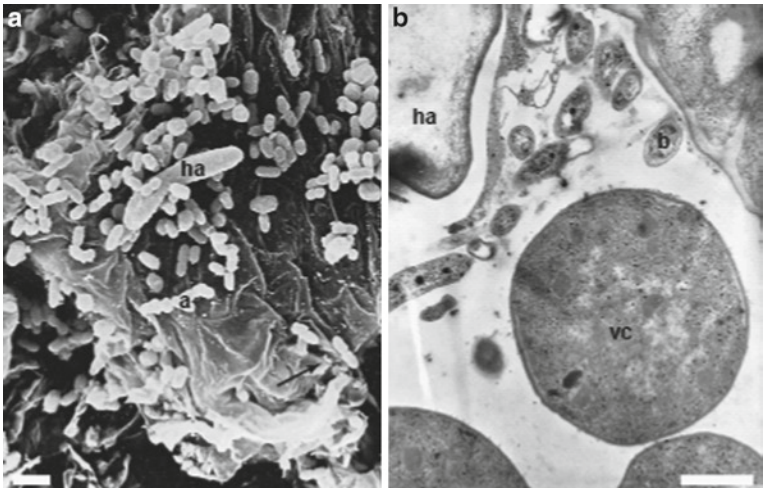

Figure 2. *Azolla* leaf cavity. (**a**) Leaf cavity of *Azolla Mexicana* with a simple hair (ha), *Anabaena* (**a**) *Azolla mexicana* and bacteria (*arrow*). SEM. Bar = 6 μm. (**b**) Vegetative cells (vc) of *Anabaena azollae*, bacteria (**b**), and apex of an hair (ha) in a leaf cavity of *Azolla caroliniana.* TEM. Bar = 2 μm.

The Cyanobiont is hosted inside *Azolla* during all its life cycle (Canini and Grilli Caiola, 1995). In fact, the *Azolla* sporocarps present beneath the indusium *Anabaena* akinetes and bacteria. When the megaspore germinates it give rise a gametophyte which after sexual reproduction originates a new sporophyte. On this latter, small colonies of *Anabaena* vegetative cells derived by akinete germination occur. Such colonies are capable of maintaining the symbiont in the host so that *Azolla* and *Anabaena* are transmitted together from *Azolla* sporophyte via gametophyte to a new sporophyte.

The *Anabaena* heterocysts fix nitrogen. A coordinated work inside the leaf cavities results in the reduction of nitrogen molecules and the uptake of nitrogen compound by *Azolla*. Bacteria accompanying *Anabaena* cooperate in the reduction of oxygen concentration inside the leaf cavities to enhance nitrogenase activity (Grilli Caiola and Forni, 1999).

However, by comparing young and old leaves of *Azolla*, we can conclude that cyanobionts and bacteriobionts could not have an easy life in the host. In fact, *Azolla* acts on its "own behalf." In addition, whenever the host's life becomes too stressful, the symbiont can be eliminated.

Symptoms from suffering of the cyanobacterial symbiont can be recognized in the old leaves where reduced number of vegetative cells, degeneration of heterocysts, the appearance of akinetes under the indusium of micro- and megasporocarps are observed (Grilli Caiola et al., 1992). No trehalose has been found in *Anabaena* vegetative cells (Newton and Herman, 1979). Isolated *A. azollae* can grow on glucose and fructose, and synthesize glycogen; its mixotrophic growth results in an increased growth rate, higher heterocysts frequency, and nitrogen fixation.

The SOD activity increases in the symbiont from the median leaves to the basal ones. The accumulation of cyanophycin and the reduction of dimension in the vegetative cells of the old leaves are the aspects that can be related to a stressful condition of the *Anabaena* and its endeavor to survive the hostile conditions when the vegetative cycle of the host is approaching its end. A typical pattern of FeSOD distribution was evidenced in *Anabaena* living in *Azolla* cavities: higher particle densities of FeSOD in heterocysts than in vegetative cells; the FeSOD labeling trend overlaps that of nitrogenase activity of leaf cavity heterocysts. Moreover, low or degenerated, non-nitrogen fixing heterocysts exhibited low or zero FeSOD labeling (Canini and Grilli Caiola, 1995).

4. Legume–Rhizobia Association

In 1988, the first centenary of the discovery of symbiotic nitrogen fixation in leguminous plants by Hellriegel and Wilfarrh and of the isolation of the rhizobia by the Dutch bacteriologist M.W. Beijerinck, was held in Cologne (Quispel, 1988). Updated reviews by Hirsch (1992), van Rhijn and Vanderleyden (1995), Michiels and Vanderleyden (1994), and Sprent (2007) on the rhizobia associated with the

legumes have well documented the morphology and the molecular basis of the establishment and functioning of the nitrogen-fixing root nodule.

Nodule origins on legume roots by association with soil bacteria of the genera *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium*, and *Azorhizobium.* Rhizobia are rod flagellate Alphaproteobacteria free-living in the soil (see also Hirsch, this volume). Most of the natural and cultivated leguminous plants attract rhizobia by means of root exudates containing molecules specific for each species. However, a legume plant can host one or more rhizobia strains. Bacteria penetrate the host through root hairs, which undergo a curling process thereafter. Hairs cooperate in forming a thread infection in which bacteria are present. After infection, the cells of root cortex tissues begin divisions, thus leading to a nodule emerging on the root. The root nodule shape varies from roundish to elongated and on the root, it can be isolated or in groups, this in relation to the presence or not of a nodule meristematic zone. In the spherical shape, the growth of nodule is determinate and the nodule has no meristematic zone. In indeterminate nodules, a meristem allows growth and lengthening for a long period of time and the shape of nodule becomes elongated. In a transversal section, a nodule shows a medullar zone in which the host cells contain numerous bacteria whose morphology depends on the steps and age of nodule. In fact, once the thread infection has reached a cell of the nodule, the bacteria are released into the cytoplasm embedded in a mucilage envelope. Here they divide (Fig. 3a) and contain electron

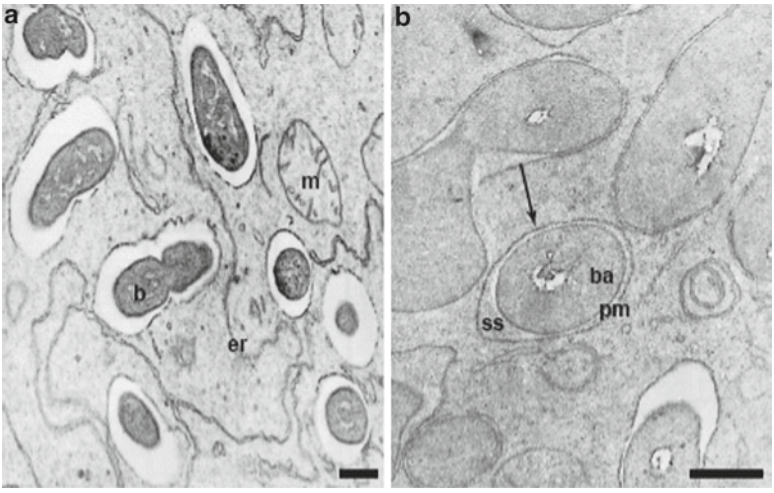

Figure 3. Rhizobia in legume root nodules at TEM. **(a)** Initial stage of infected cell of pea (*Pisum sativum*) nodule with dividing rhizobia (b). Host cell shows abundant endoplasmic reticulum (er) and mitochondria (m). Bar = 0.15 μm. **(b)** Symbiosomes of indeterminate nodule of broad bean (*Vicia faba*). Bacteroids (ba) are elongated, singly enclosed in the peribacteroid membrane (pm and arrow), and in a reduced peribacteroid space (ss). Bar = 0.15 μm.

transparent inclusions of poly-β-hydroxybutyrate, often resembling vacuoles due to their electron transparency.

Inside the host, the rhizobia undergo deep changes in shape and metabolism. Frequently, bacteria in this stage also have electron-dense granulations of dark material comparable to melanin. At a subsequent stage however, when they are released from the infection thread into the host cell cytoplasm, rhizobia enlarge and form variously branched and unbranched bodies known generally as bacteroids. Similar forms are produced when young rhizobia are subjected to certain environmental stresses (Jordan and Coultier, 1965). Bacteroid is a modified form of rhizobium inside the nodule cell. It contains nitrogenase and occurs in the symbiosome, the structural unit for the nitrogen fixation in association with the host plant. A symbiosome consists of the bacteroid, the peribacteroid space and the peribacteroid membrane (Fig. 3b), where the leghemoglobin is synthesized to regulate the entry of oxygen inside the symbiosome. The organization of the symbiosome is different in the various legumes as it results from the comparison of nodules (Fig. 4a–c) of bean (*Phaseolus vulgaris*) to pea (*Pisum sativum*), cowpea (*Vigna sinensis*), broad bean (*Vicia faba*), and lupine (*Lupinus albus*), or *Robinia pseudoacacia* (Grilli, 1963, 1964). However, not all the nodules are active in nitrogen fixation (ineffective nodules). The life of rhizobia in nodule is related and limited to the vegetative period of the host, from flowering to fruit maturation, if the legume has annual cycle. In the oldest nodule or in the oldest medullar zone, bacteroids undergo degeneration processes concluding with the lysis of nodule and the contained bacteria (Grilli, 1963, 1964).

During the invasion phase, inside the thread infection and in the host cell release, many dividing rhizobia contain numerous granulations of PHB, whereas melanin granules have been detected in the bacteroid, mainly in the final stage of nodules. These granules are sometimes accompanied by the presence of dark brown pigmentation of the old part of the nodule. PHB seems to indicate that inside the nodule, there is abundant carbohydrate compound available for the symbionts, compared to the amount present in the soil. PHB are numerous in the bacteria of pea, bean, and particularly in bacteroids of bean and in those of lupine and pea in the final stage.

Melanin, on the other hand, represents a product of phenol metabolisms related to age and stress conditions for bacteria inside the nodule. They are very abundant in the bacteroids of bean and lupine. It is unclear whether melanin production by *Rhizobium* plays any role in the symbiotic process (Cubo et al., 1988). Probably, it is involved in the detoxification of the phenolic compound in nodules and roots of senescent bean plants.

During the elongation of the thread, bacteria are exposed to an oxidative burst due to the host plant with releasing of hydrogen peroxide and superoxide (ROS). For proper symbiotic development, bacteria encode a set of enzymes to defend against ROS, including SODs and catalases. Several SOD isoenzymes have been detected. *Sinorhizobium meliloti*, for example, encodes an SOD, SodB that can use either Fe^{2+} or Mn^{2+} with a strong role for manganese to carry its protective physiological role (Davies and Walker, 2007). Several pieces of evidence suggest

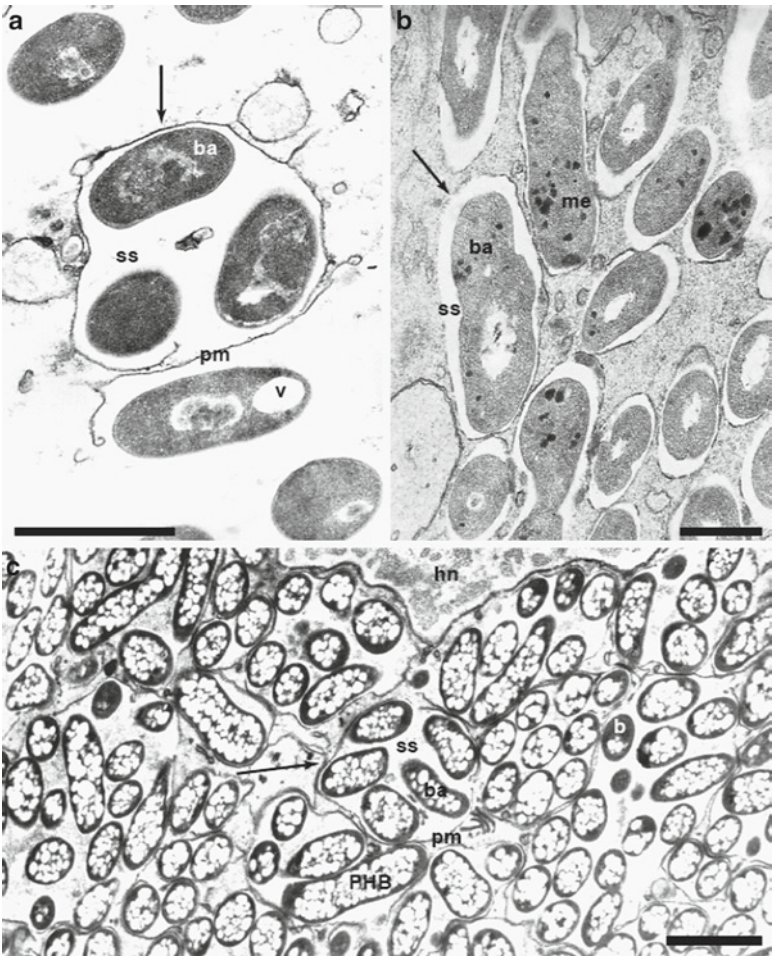

Figure 4. (**a**) Symbiosomes in a cow pea (*Vigna sinensis*) determinate nodule. Several bacteroids (ba), are enclosed in a peribacteroid membrane (pm). The large peribacteroid space (ss) and the vesiculated host cell cytoplasm indicate the senescent condition of the nodule. Among the symbiosomes some free vacuolated (v) bacteria occur. Bar = 0.5μm. (**b**) Symbiosomes in indeterminate nodule of lupine (*Lupinus albus*). The elongated bacteroids (ba) contain dark granules of melanin (me) and are individually enclosed in a peribacteroid membrane (*arrow*) with a reduced peribacteroid space (ss). Bar = 1μm. (**c**) Symbiosomes in a determinate nodule of bean (*Phaseolus vulgaris*). Several bacteroids (ba) are enclosed in a peribacteroid membrane (pm and *arrow*) and in a rather large peribacteroid space (ss). Bacteroids contain numerous PHB granules (PHB). Bar = 1 μm.

an active role of SOD for the development of effective and efficient symbioses (Tavares et al., 2007). However, a model by which ROS and antioxidants interacting with hormones should orchestrate the nodule senescence has been proposed by Puppo et al. (2005).

5. Endophytes in Higher Plants: Tomato-*Azospirillum*

The tissues of healthy plants were originally considered to be sterile by Pasteur (1876) and subsequent authors such as Fernbach (1888). However, since then a number of investigators have reported instances in which bacteria were found in various parts of healthy plants such as the storage organs (Hollis, 1951; Tonzig and Bracci Orsenigo, 1955), fruits (Samish and Dimant, 1959; Samish et al., 1961), ovules and seeds (Mundt and Hinkle, 1976), root xylem, or many different organs (Schanderl, 1939). After Dobereiner (1961) isolated bacteria from sweet cane roots, a new viewpoint arose about the significance of microbes in the plants. Endophytes was the term generally used to indicate the microbes present in different organs and tissues of plants without disease symptoms. Although it is commonly used, this term has been criticized by some authors and the significance of the presence of many microbes inside the host plant is still unknown. An increasing number of bacteria belonging to different taxa such as *Acetobacter*, *Herbaspirillum*, *Pseudomonas*, *Azoarcus*, have been found associated to spontaneous and cultivated monocotyledonous and dicotyledonous plants (e.g., Reinhold-Hurek and Hurek, 1998). In some cases, they prove to be associated to the external root surface; in others, they are found inside the plant as small colonies spread in different tissues or organs from which they have been isolated in higher amounts (Chi et al., 2005).

Rhizobacteria are bacteria living in the soil in a zone termed rizoplane where roots also grow and, due to their capacity to invade the roots, many of them are considered endophytes. Bacteria and fungi inhabit this space in large number and in different relation to plant roots. Many fungi are able to envelop roots and penetrate the plant's cortical tissues, thus giving rise to the well-known symbiosis mycorrizae. Rhizobia and Actynomycetes can also penetrate the roots and invade the inner tissues of the plant, causing the formation of structures as tubercules as they occur in legumes and *Alnus*. Rhizobacteria adhere to the plant roots without inducing apparent modification in the host. Their relationship with the host can be limited to adhering to the external surface or penetrating the cortex root. Root penetration has been reported in many plants, whereas the diffusion inside the tissues of the host is until now limited to cortex and, in a few instances, to xylem tissue, thus reaching the foliar system. Ascending migration of endophytic rhizobia from roots to leaves inside rice plants has been recently reported (Chi et al., 2005). Colonization of internal plant tissues is thought to be largely intercellular and, more rarely, intracellular in living plant cytoplasm.

Among the rhizobacteria, *Azospirillum* is the most studied because of its effect on producing indolacetic acid (IAA) and stimulating optimal growth of the host. In some conditions, it is also capable of nitrogen fixation. Apparently, *Azospirillum* seems to help the penetration in obtaining a more convenient environment regarding the nutrient availability and protection from the competitors in the soil. However, the symbiotic condition is not free from some complications as it results from a study carried out on a system set up between tomato (*Lycopersicum esculentum* Mill.) and *Azospirillum brasilense* Cd (Grilli Caiola et al., 2004).

Azospirillum brasilense is capable of a vegetative phase with motile flagellate cells, but in aged culture, it produces brown colonies forming cysts. Thus, it is a good model for following the bacteria variation when outside in the soil compared to when it lives inside the plant.

Information was obtained through experiments: (a) on tomato seeds inoculated with *Azospirillum brasilense* Cd grown on agarized medium with and without combined nitrogen; (b) on 30-day old tomato plantlets inoculated with *Azospirillum brasilense* grown on modified Okon agarized medium supplied or not with combined nitrogen and (c) on comparing young *Azospirillum* cultures in exponential growth phase and old brown culture in the late stationary phase. Useful results were obtained through an analysis of the previous material at OM, SEM, and TEM.

Comparison of *Azospirillum* in young and old brown cultures and also inside tomato roots has provided important information about the relation between rhizobacteria and the host plant. Young *Azospirillum* cells show a single polar flagellum and several lateral ones (Fig. 5a) that are only synthesized in solid growth medium. Old cultures show aflagellate cells containing large electron-dense forms enclosed within a thick capsule, housing two or more smaller cells that are then released in the medium.

In the brown mature cultures, *Azospirillum* has very electron-dense colonies, with smaller cells and a thicker envelope than observed in younger cultures. Such structures have been identified as the cysts previously reported in *A. brasilense*.

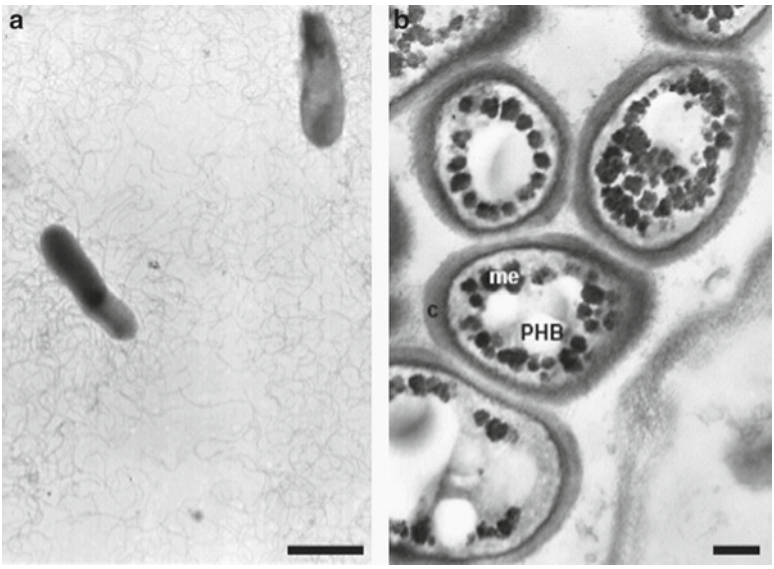

Figure 5. (**a**) Periflagellate *Azospirillum brasilense* Cd in young culture. SEM. Bar = 1.1 μm. (**b**) *Azospirillum brasilense* inside tomato root. Bacteria have a thick coat (c), numerous melanin (me) and PHB granules. TEM. Bar = 0.6 μm.

The brown color of the cultures was attributed to the melanin also present in other bacteria (Cubo et al., 1988; Castro-Sowinski et al., 2002). The bacteria penetrate the host plant via the root hairs and epidermis cells and localize between the host cells, aligned in rows or aggregated into large colonies in the intercellular spaces. Some bacteria occur within cells that appear to be lysed and dead. The morphology and activity of the bacteria inside the tomato roots change with respect to those observed in culture. They do not divide, have a very thick capsule, numerous large PHB (Fig. 5b) and glycogen granules, similarly to cysts observed in aged brown cultures. Cysts and pigmentation have been observed in *A. brasilense* in aging cultures under carbon and nitrogen limitation (Sadavisan and Neyra, 1987). These cysts differ from those observed in cultures rich in melanin, which are frequently divided into smaller cells and lack glycogen.

However, some other aspects of these cysts suggest that they may instead be active forms, with high levels of oxidative metabolisms, as revealed by the large amounts of SOD present and the accumulation of reserves (PHB, glycogen) (Grilli Caiola et al., 2004).

6. Concluding Remarks

The analyzed associations suggest reconsidering the concept of symbiosis. De Bary defined symbiosis as "unlike organisms living together," among other examples, by observing the presence of "algae" in the leaves of *Azolla*. The systems reported above are formed of a cormophyte with one dominant microorganism of a microbial community. The fates of these associations are different, but some common aspects can be recognized. With the exception of the *Azolla-Anabaena*-bacteria association, where the partners live and are transmitted together, in the other examples reported here, there is a defined duration of association. Usually, the host survives, whereas most microbionts die and only a few bacteroids (McDermott et al., 1987) and perhaps vegetative cells of cyanobionts (Grilli, 1992, 2002) survive. Thus, the host prevails over the partner and the association results in a temporary combination for the host's benefit. In this situation, microbes show, more or less pronounced symptoms of suffering until cells lyse and its content is utilized by the host after a first phase, during which it seems to take advantage of the host. The symptoms of such a suffering state are the absence of the microbial cell divisions, the changes in morphology and the production of compounds for a defense against the host metabolism. In addition, the influence of the host on the microbiont can also affect the microbial genotype, some of which are modified, as in the rhizobia so that the surviving forms sometimes show different genetic composition compared to the infecting ones (Simms et al., 2006; Sprent, 2007). This stressed condition becomes evident in the production of storage compound such as PHB, glycogen, or specialized resting cells such as akinetes in cyanobacteria and cysts in bacteria. All these aspects, although not exclusive to microbes in association with plants, occur during all or most of the symbiont life cycle in the host.

Concerning the causes of the stress in microbiont associated to plants, they can be identified in biotic and abiotic origin. Nitrogen deprivation induces heterocyst differentiation and nitrogen fixation both in cyanobacteria as well in bacteroids. In addition, host affects symbiont metabolism, inducing the synthesis of new metabolites. Moreover, high temperature, high salinity, phosphorus deprivation can affect both host as well as symbiont (Tung and Watanabe, 1983; Rai et al., 2006).

In the light of new research results, it is tempting to revise the concept of symbiosis. Recently, Sapp (2004) introduced the concept "symbiome," which defines the organism as a "functional field that includes microbial communities." Carrapico (2002) interpreted the *Azolla-Anabaena*-bacteria symbiosis as a "natural microcosm."

When considering the fate of microbes in plants, a question arises: "Why are most microbes beneficial to their plant hosts, rather than parasitic?" Concerning rhizobia, Denison and Kiers (2004) suggest that multiple strains per plant and root-to-root transmission favor rhizobia, which invest in their own reproduction, rather than symbiotic N_2-fixation. Legumes seems to select for mutualistic strains by controlling nodule O_2 supply and reducing reproduction of rhizobia, which fixes less N_2. A mechanism to suppress non-mutualistic strains could be by sanctions against undifferentiated or less active rhizobia in the nodule (Denison, 2000; West et al., 2001). In this context, an approach based on bargaining theory has been presented as a model for negotiation of benefits (Akçay and Roughgarden, 2007) and as mechanism to prevent exploitation between partners with conflicting interest (Simms et al., 2006).

Despite the numerous studies devoted to researching the deep relations between host and partners and the resulting beneficial effects for the plant, the association of plants with microbes remains an intriguing world to explore.

7. Summary

Many associations of plants with microbes are considered to be mutually beneficial for both partners. The authors of this paper suggest that associations of plants with oxyphototrophic or heterotrophic bacteria are beneficial mainly for plants, the microbes being eliminated at the end of symbiosis. To prove this, different plant–microbe associations such as cycad-cyanobacteria, *Azolla-Anabaena*, legume-rhizobia, and tomato-*Azospirillum* have been examined. All the systems considered show elimination of most microbes in course of time. In fact, only a few individual symbionts may survive in the soil or inside the host as resting form.

The life of the symbiont appears to be more comfortable inside the host than in the free-living stage outside, especially during the first steps of the association. However, afterward, symptoms of stress such as a decrease in growth and division, appearance of trehalose, PHB, melanin, SOD, resting cells like akinetes, and spore-like cysts occur in the microbe. All these mechanisms are involved in response to stress conditions.

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SYMBIOTIC PLANT–MICROBE INTERACTIONS: STRESS PROTECTION, PLANT GROWTH PROMOTION, AND BIOCONTROL BY *STENOTROPHOMONAS*

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1. Introduction

The genus *Stenotrophomonas* is phylogenetically placed in the γ-subclass of *Proteobacteria* (Moore et al., 1997). The genus was first described with the type species *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993), previously called *Pseudomonas maltophilia* (Hugh and Ryschenko, 1961) and later changed to *Xanthomonas maltophilia* (Swings et al., 1983). Actually, the genus comprises eight validly described species: *S. maltophilia*, *S. nitritireducens* (Finkmann et al., 2000), *S. rhizophila* (Wolf et al., 2002), *S. acidaminophila* (Assih et al., 2002), *S. koreensis* (Yang et al., 2006), *S. terrae*, *S. humi* (Heylen et al., 2007), and *S. chelatiphaga* (Kaparullina et al., 2009). However, pheno- and genotypic studies revealed much more differentiation at species level (Ryan et al., 2009). Only two species, *S. maltophilia* and *S. rhizophila* (Wolf et al., 2002), show a strong association with plant hosts. In comparison with *S. maltophilia*, the defining phenotypic characteristics of *S. rhizophila* are: growth at 4°C and no growth at 40°C; the utilization of xylose as a carbon source; higher osmotic tolerance (<5% NaCl [w/v]); and the absence of lipase and β-glucosidase production (Wolf et al., 2002). Both species produce osmoprotective substances (Roder et al., 2005). These are compounds compatible at very high internal concentrations with cellular functions, e.g., DNA replication, DNA–protein interactions, and cellular metabolism; they regulate the osmotic balance and are effective stabilizers of enzymes (Welsh, 2000). A molecular protocol was developed for the differentiation of the two *Stenotrophomonas* species. It targets specifically the *ggpS* gene responsible for glucosylglycerol (GG) synthesis because this marker occurs only in *S. rhizophila*

strains and was absent from all *S. maltophilia* isolates (Ribbeck-Busch et al., 2005). As a further genetic marker the *smeD* gene was used, which is part of the operon coding for the multi-drug efflux pump SmeDEF only occurring in *S. maltophilia* (Alonso and Martinez, 2000).

The great heterogeneity in physiological parameters among *S. maltophilia* strains has already been shown by Van den Mooter and Swings (1990), and also by Palleroni and Bradbury (1993) in the type description of *S. maltophilia*. Heterogeneity has been confirmed by genotypic studies (Gerner-Smidt et al., 1995; Chatelut et al., 1995; Nesme et al., 1995; Hauben et al., 1999; Berg et al., 1999).

2. Occurrence of *Stenotrophomonas* on/in Plants

S. maltophilia and *S. rhizophila* are typical plant-associated microorganisms. Their main reservoir is the rhizosphere of plants (Juhnke and Des Jardins, 1989; Berg et al., 1996). The rhizosphere is defined as the layer of soil influenced by root metabolism. In comparison to root-free soil, the rhizosphere forms a nutrient-rich niche for microorganisms as a result of exudation of organic compounds (Sørensen, 1997). Additionally, this microenvironment is described as "microbial hot-spot" where diverse interactions between organisms, beneficial as well as pathogenic, take place (Whipps, 2001). *S. maltophilia* is reported to be associated with a long list of plant species, which includes all branches of plant phylogeny. Examples are potato *Solanum tuberosum* L. (Garbeva et al., 2001), oilseed rape *Brassica napus* L. (Berg et al., 1996), rice *Oryza sativa* (Sun et al., 2008), sweet flag *Acorus calmus* L. (Marecik et al., 2008), tropical orchids like *Paphiopedilum appletonianum* and *Pholidota articulata* (Tsavkelova et al., 2007), coffee *Coffea arabica* L. (Vega et al., 2005), grape-vine *Vitis vinifera* (Prieto et al., 2007), poplar *Populus* spp. L. (Taghavi et al., 2009), and marram grass *Ammophila arenaria* (L.) Link (De Boer et al., 2001). In many studies, *Stenotrophomonas* was reported as dominant member of the plant-associated bacterial community. The proportion of *S. maltophilia* in the cultivable fraction of bacteria was estimated up to 38% for *Graminaceae* (Juhnke and Des Jardins, 1989) and up to 48% for *Brassicaceae* (Berg et al., 1996). Using cultivation-independent methods, the dominance of *Stenotrophomonas* was confirmed, e.g., for rice plants (Sun et al., 2008). They were also found on bryophytes, which are the phylogenetically oldest land plants (Opelt et al., 2007). Although the rhizosphere seems to be a preferred habitat, strains of *S. maltophilia* are also found in other microenvironments such as phyllospheres (Krimm et al., 2005; Schreiber et al., 2005). Recently, *Stenotrophomonas* strains were isolated from extraordinary plant-associated habitats such as root nodules of herbaceous legumes (Kan et al., 2007) or the plant pathogenic fungus *Fusarium oxysporum* (Minerdi et al., 2008) and arbuscular mycorhizal fungal spores (Bharadwaj et al., 2008). Interestingly, they are also a member of the lichen symbiosis, where they fulfill the function of nitrogen fixation (Liba et al., 2006). It has been assumed that many *S. maltophilia* strains associated with the rhizosphere are also capable to fix inorganic nitrogen promoting growth of the host plants. *S. maltophilia* has not only been isolated from typical terrestrial and

aquatic environments (Minkwitz and Berg, 2001) but also from the extreme environment of a soda lake (Denton and Kerr, 1998). Altogether, *S. maltophilia* was found in a wide variety of environments and geographical regions, and occupies diverse ecological niches including hospitals and medical equipments (Denton and Kerr, 1998).

The second species, *S. rhizophila*, was first described in 2002; since that time, it was also reported from diverse plant species and microenvironments. In contrast to *S. maltophilia*, strains of this species were exclusively found on plants. Possibly, in the older studies on plant-associated bacteria *S. rhizophila* strains were wrongly identified as *S. maltophilia*. In an extensive study, clinical and environmental *Stenotrophomonas* strains from several countries were distinguished according to the protocol of Ribbeck-Busch et al. (2005): none of the clinical strains (>100) was identified as *S. rhizophila* (Berg, 2005, unpublished). Another important criterion, which points to the intimate interaction of *Stenotrophomonas* with plants, is their endophytic occurrence. Both *Stenotrophomonas* species are able to live in the endosphere of plants (Garbeva et al., 2001; Krechel et al., 2002; Vega et al., 2005; Hallmann and Berg, 2006; Sun et al., 2008; Taghavi et al., 2009).

3. Plant–Microbe Interaction

Bacteria showing beneficial interaction with plants comprise the group of plant growth-promoting bacteria (PGPB), which influence plant growth by producing phytohormones or by enhancing the availability of nutrients. Moreover, such strains may induce systemic resistance in plants and may act as antagonistic bacteria for pathogens (Kloepper, 1992; Whipps, 2001). Antagonists are naturally occurring organisms with traits, which enable them to interfere with pathogen growth, survival, infection, or plant attack (Chernin and Chet, 2002). Mechanisms responsible for antagonistic activity include: (i) inhibition of pathogens by antibiotics, toxins, and biosurfactants (antibiosis), (ii) competition for colonization sites and nutrients, (iii) competition for minerals, e.g., for iron through production of siderophores or efficient siderophore-uptake systems, (iv) degradation of pathogenicity factors of the pathogen such as toxins, and (v) parasitism that may involve production of extracellular cell wall-degrading enzymes such as chitinases and β-1,3 glucanase (Bloemberg and Lugtenberg, 2001; Whipps, 2001; Raaijmakers et al., 2008). Furthermore, the importance to recognize and adhere to plant roots for all plant-associated bacteria is corroborated by many studies.

An early step in the establishment of a plant–bacterium interaction is attachment of cells to plant roots, in which, for example, fimbriae and cell-surface proteins are involved (Lugtenberg et al., 2001). For the colonization of plant roots, flagella, O-antigen of lipopolysaccharides (LPS), the growth rate, and the ability to grow on root exudates are important (Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001). Other factors that contribute to rhizosphere fitness include the ability to use seed and root exudates as carbon sources or, more in

general, ecological and nutritional versatility. Which of these factors are already described for *Stenotrophomonas*? An overview about the possible mode of interaction of *Stenotrophomonas* cells with their plant hosts is given in Fig. 1.

Antagonistic mechanisms are reported, especially for *S. maltophilia*. In vitro, the majority of *Stenotrophomonas* isolates show an antagonistic activity toward plant pathogenic fungi (Minkwitz and Berg, 2001). New antifungal antibiotics with strain-specific occurrence were discovered: maltophilin (Jacobi et al., 1996) and xanthobaccins (Nakayama et al., 1999b). A gene encoding a potential pathogenicity factor – the zonula occludens toxin – could be detected, but only in several clinical *Stenotrophomonas* strains (Hagemann et al., 2006). It was earlier described that *S. maltophilia* produces not only antibiotics but also plant-associated as well as clinical strains, which are also highly resistant to several antibiotics (Berg et al., 1999; Alonso and Martinez, 1997). The rhizosphere is an environment, where a lot of bacteria possess antibiotic resistances and preferential horizontal gene transfer occurs to submit the encoding gene cluster (Berg et al., 2005).

These resistances can protect microorganisms against antibiotics produced by other microbial colonizers or against toxic metabolites produced by the plant hosts themselves. Some antibiotic resistance determinants have been carefully analyzed, including aminoglycosides inactivating enzymes (Lambert et al., 1999; Li et al., 2003), β-lactamases (Kataoka et al., 2003), and multidrug (MDR) efflux pumps like SmeABC (Li et al., 2002) or SmeDEF (Alonso and Martinez, 2000, 2001; Vila and Martínez, 2008). The first complete genome sequence of the clinical

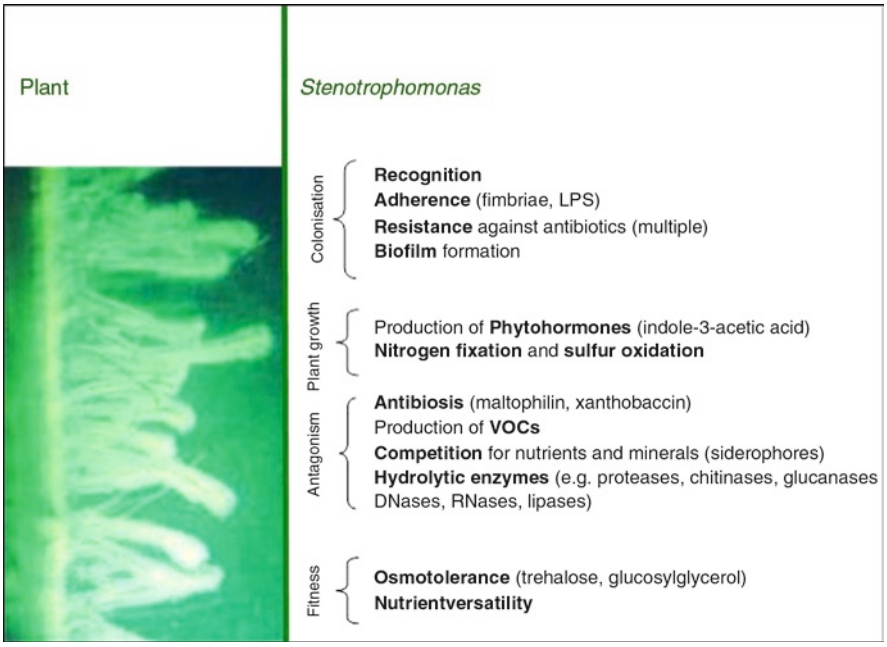

Figure 1. Mode of interaction of *Stenotrophomonas* with plant hosts.

isolate *S. maltophilia* K279a confirms the remarkable capacity for drug and heavy metal resistance (Crossman et al., 2008). In addition to a number of genes conferring resistance to antimicrobial drugs of different classes via alternative mechanisms, nine resistance-nodulation-division (RND)-type putative antimicrobial efflux systems are present. *Stenotrophomonas* strains are also known for their extraordinary high hydrolytic potential. They produce diverse proteases, chitinases, glucanases, DNases, RNases, lipases, and laccases (Debette, 1991; Berg et al., 1996; Dunne et al., 2000; Galai et al., 2008). However, the precise roles of these enzymes in the antagonistic activity of *Stenotrophomonas* remain to be elucidated. Furthermore, *Stenotrophomonas* cells synthesize siderophores, possess siderophore-uptake systems, and are able to take up or scavenge efficiently siderophores from other microorganisms (Jurkevitch et al., 1992). Besides the excretion of soluble antibiotics and enzymes also volatile organic compounds (VOCs) produced by bacteria like *Stenotrophomonas* can inhibit growth of pathogenic fungi (Wheatley, 2002). Furthermore, VOCs may serve as inter- and intraorganismic communication signals in general (Stotzky and Schenk, 1976; Wheatley, 2002). Recently, it has been shown that the VOCs of *S. maltophilia* and *S. rhizophila* inhibit mycelial growth of the soil-borne pathogen *Rhizoctonia solani* to more than 90% in dual culture tests. Out of a vast diversity of VOCs produced by *S. rhizophila*, two, namely dodecanal and β-phenylethanol, could be identified by GC-MS (Kai et al., 2007). Beta-phenylethanol is a typical floral fragrance compound, which exerts its antimicrobial effects by inhibition of macromolecule synthesis and by altering the permeability of the plasma membrane and sugar and amino acid transport processes (Ingram and Buttke, 1984).

In addition, synthesis of compatible solutes by bacteria contributes to survival under changing osmotic conditions, which occur in the rhizosphere (Miller and Wood, 1996). Hiltner (1904) described the phenomenon, designated as the "rhizosphere effect," that in rhizospheres, in comparison to bulk soil, the biomass and activity of microorganisms is enhanced as a result of the exudation of compounds. A long list of diverse substances, such as organic acids, sugars, amino acids, vitamins, and polymeric carbohydrates, is known to be released by plant roots (Bais et al., 2006). However, the nutrient content is not constant and the different amounts of nutrients lead to changing osmolarities in the rhizosphere (Miller and Wood, 1996). The two type strains of *Stenotrophomonas* species, *S. maltophilia* strain DSM 50170 and *S. rhizophila* strain DSM 14405 produce different compatible solutes. *S. maltophilia* accumulated trehalose as the only osmolyte, whereas *S. rhizophila* was shown to produce glucosylglycerol (GG) in addition to trehalose. As expected, the different spectrum and amounts of compatible solutes in these two strains led to differences in terms of their salt tolerance. The human-associated *S. maltophilia* was able to grow in media containing up to 3% NaCl (w/v). In contrast, *S. rhizophila* can be propagated in salinities up to 5% NaCl (w/v). The genes for trehalose and GG synthesis can be used to differentiate the two species (Ribbeck-Busch et al., 2005). Moreover, the molecular basis for GG synthesis was revealed in *S. rhizophila*. This strain possesses a new type of GG-synthesis enzyme, which combines the two-step biosynthesis

employing GG-phosphate synthase and GG-phosphate phosphatase on a single enzyme (Hagemann et al., 2008).

It is common in many bacteria that genes encoding factors involved in interaction are regulated in a cell density-dependent manner. Many plant-associated bacteria produce *N*-acyl homoserine lactones as cell–cell signaling molecules (Berg et al., 2002). In contrast, *S. maltophilia* uses a signaling system mediated by a diffusible signal molecule (DSF) (Fouhy et al., 2007). The latter authors described that DSF activity controls resistance to several antibiotics, aggregative and biofilm behavior, and virulence in a nematode model.

Interestingly, using a genomic approach to analyze endophytic bacteria of poplar and their plant–microbe interaction, Taghavi and coauthors (2009) found none of the well-known genes known for positive interaction (e.g., phytohormone biosynthesis, ethylene degradation) in an endophytic strain of *S. maltophilia*. Additional new insights into the interaction of *Stenotrophomonas* with eukaryotes resulted from the genome sequencing of the clinical *S. maltophilia* K279a strain (Crossman et al., 2008). Many genes encoding for drug resistance, secretion systems of type I, II (*sec*), IV, and V (autotransporter), as well as the twin arginine secretion systems genes are present in the K279a genome. Furthermore, this genome harbors genes for extracellular enzymes such as nonhemolytic phospholipase C, enzymes of the phospholipase D family, DNase, gelatinase, hemolysin, lipases, proteinase K, and proteases. The genome comparison confirmed also the high similarity of the human pathogen *S. maltophilia* to the phytopathogen *X. campestris*. However, in contrast to the closely related genus *Xanthomonas*, no phytopathogenic capacity of *Stenotrophomonas* is known (Palleroni and Bradbury, 1993).

4. Plant Growth Promotion and Biocontrol by *Stenotrophomonas* Treatment

Stenotrophomonas species have an important ecological role in the element cycle in nature (Ikemoto et al., 1980). The biotechnological importance of *S. maltophilia* is partly due to their potential plant growth-promoting effects and application in biological control of fungal diseases in plants. An overview about the use of *Stenotrophomas* strains in biocontrol and plant growth promotion is given in Table 1.

The influence of *Stenotrophomonas* treatment on in vitro plant growth is shown in Fig. 2.

Plant growth promotion was also observed in the highly salinated soils of Uzbekistan, where statistically significant promoting effects of strain DSM 14405 treatments were observed for wheat, tomato, lettuce, sweet pepper, melon, celery, and carrot (Egamberdiyeva, 2008, unpublished results). These treatments resulted in higher germination rates as well as in longer shoots and roots. For example, in tomato the germination rate was 180% and the growth of the shoot was 120% and root was 142% in comparison to the values found for the untreated control. Furthermore, up to 100 mM NaCl in the soil, there is no influence of salt on the survival of *S. rhizophila* in

Table 1. Examples of applications of *Stenotrophomonas maltophilia* and *S. rhizophila* in biocontrol and plant growth promotion.

No.	Strain	Pathosystem: pathogen and/or plant species	Reference
1.	*S. maltophilia*	Soil-borne pathogens	Elad et al. (1987)
2.	*S. maltophilia*	Rhizoctonia damping-off in bark compost media	Kwok et al. (1987)
3.	*S. maltophilia* 3089	*Verticillium dahliae* – oilseed rape	Berg et al. (1994)
4.	Chitinolytic *S. maltophilia*	Summer patch disease of turf grass	Kobayashi et al. (1995)
5.	*Stenotrophomonas* sp. strain SB-K88	Sugar beet damping-off disease	Nakayama et al. (1999)
6.	*S. maltophilia* mutant with overproduction of an extracellular serine protease	Biological control of *Pythium ultimum*	Dunne et al. (2000)
7.	*S. maltophilia* PD3533	Potato – *Ralstonia solanacearum* race 3 biovar 2	Messiha et al. (2007)
8.	Endophytic *S. maltophilia* R551-3	Poplar	Taghavi et al. (2009)

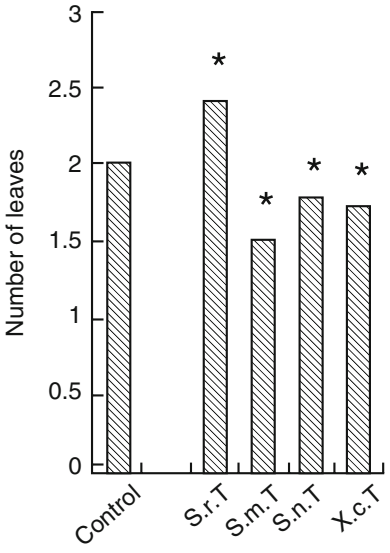

Figure 2. Influence of the treatment of strawberry seedlings with *Stenotrophomonas rhizophila* DSM 14405^T (S.r.T), *S. maltophilia* DSM 50170^T (S.m.T), *S. nitritireducens* DSM 12575^T (S.n.T), and *Xanthomonas campestris* DSM 3586^T on the number of leaves. Control: untreated seedlings. Significant differences are indicated by asterisks. (Adapted from Suckstorff and Berg, 2003.)

the rhizosphere of cucumber and tomato (Egamberdiyeva, unpublished results). In comparison to literature (see Table 1), the plant-promoting effect of *Stenotrophomonas* treatment is much higher under salt stress conditions than in nonsaline soil. Also under in vitro conditions, salt stress has an important influence on the antagonistic activity of *Stenotrophomonas*. Salt stress significantly enhanced the antagonistic activity against phytopathogenic fungi such as *Rhizoctonia solani* and *Verticillium dahliae* (Berg, unpublished); the molecular mechanisms behind this phenomenon have to be analyzed.

5. The Ambivalent Role of *Stenotrophomonas* as Opportunistic Pathogen

In the last two decades, *S. maltophilia* has also become important as a nosocomial multidrug-resistant pathogen associated with significant case to fatality ratios in certain patient populations, particularly in those who are severely debilitated or immunosuppressed (for a review see Denton and Kerr, 1998). *S. maltophilia* is the third most common nosocomial nonfermenting gram-negative bacterium (Sader and Jones, 2005). A study of intensive care patients in the USA found that 4.3% of almost 75,000 Gram-negative infections studied were caused by *S. maltophilia* (Lockhart et al., 2007). *S. maltophilia* is also involved in polymicrobial infections as may occur in wounds, abscesses, and the cystic fibrosis lung (Ryan et al., 2008; Sibley et al., 2008).

6. Possible Applications in Biotechnology

Due to their diverse capabilities, *Stenotrophomonas* is an interesting candidate for many biotechnological applications. Strains are used to biologically control plant diseases or enhance plant growth (see Table 1). The high hydrolytic potential is used to breakdown natural and man-made pollutants applied in bioremediation and phytoremediation strategies (Barac et al., 2004). Furthermore, *Stenotrophomonas* is used for the production of biomolecules of economic value, e.g., enzymes and compatible solutes (Roder et al., 2005).

Opportunistic pathogens cannot be used for field trials and other direct applications. However, *S. rhizophila* is not known as a human pathogen. Strains of *S. rhizophila*, which very often live endophytically, are promising candidates for biocontrol and stress protection on plants.

7. Summary

The genus *Stenotrophomonas* comprises at least ten species of which the most predominant ones on plants are *S. maltophilia* and *S. rhizophila*. Strains of both species are able to colonize more or less in all the microenvironments of plants but

have a preference for the rhizosphere. They show beneficial interactions with their plant hosts, since they are able to promote plant growth as well as plant health. The mechanisms involved in this interaction are discussed. *Stenotrophomonas* strains are able to live under salt stress due to the production of compatible solutes. This observation explains their use in salinated soils, where they may protect plants against diseases and possibly also against salt stress. Strains of *S. maltophilia* are known for their ambivalent behavior. On the one hand, they exhibit an extraordinary range of capabilities that includes detrimental effects as opportunistic multidrug-resistant human pathogens, whereas on the other hand, they can exert beneficial effects such as biocontrol of plant diseases. In this chapter, the importance of symbiotic bacteria in the genus *Stenotrophomonas* for plants as well as benefits and problems is discussed.

8. Outlook

Further insights into the diversity in adaptation and metabolic capabilities will be provided by comparative genomic analysis in the future. The genome sequence of the clinical isolate *S. maltophilia* K279a has recently been published (Crossman et al., 2008). Additionally, the genome sequences of the endophytic strain *S. maltophilia* R551-3 (http://genome.jgi-psf.org/finished_microbes/stema/stema.home.html) and of a marine *S. maltophilia* SKA14 (https://research.venterinstitute.org/moore) will be finished soon. The genome sequences of isolates of different origin will lead to a better understanding of the mechanisms of adaptation and evolution of this fascinating group of organisms, ranging from understanding of medical consequences to environmental applications.

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Dr. Regina S. Redman for the past ten years, I have been associated with the University of Washington as a Research Scientist, and in addition, for the past several years, the Senior Research Scientific Consultant and CEO of a private company (Adaptive Symbiotic Technologies). Both positions involve the study and application of microbial symbiotic technologies to native and agricultural plants. My educational background in science includes an undergraduate B.S. in Genetics at the University of California, Berkley; graduate Ph.D. studies in Genetics/Plant Pathology at the University of California, Riverside; followed by a six year Post-Doctoral study at the University of Washington, Seattle. Collectively, my educational and professional studies for more than 20 years has centered-around unraveling the genetic, molecular, and biochemical basis of plant-fungal symbiotic interactions, and in more recent years, application of this technology to agricultural systems to improve stress tolerance and overall health and yields to crop plants. In 2009, I was elected as the treasurer of the International Symbiosis Society, and have in previous years served as Chair (5 years), and am presently, the Secretary (3 years) for a USDA supported meeting/workshop "Biochemistry and genetics of plant-fungal interactions".

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ADAPTATION AND SURVIVAL OF PLANTS IN HIGH STRESS HABITATS VIA FUNGAL ENDOPHYTE CONFERRED STRESS TOLERANCE

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1. Plants in High Stress Habitats

From the Arctic to the Antarctic, plants thrive in diverse habitats that impose different levels of adaptive pressures depending on the type and degree of biotic and abiotic stresses inherent to each habitat (Stevens, 1989). At any particular location, the abundance and distribution of individual plant species vary tremendously and is theorized to be based on the ability to tolerate a wide range of edaphic conditions and habitat-specific stresses (Pianka, 1966). The ability of individual plant species to thrive in diverse habitats is commonly referred to as phenotypic plasticity and is thought to involve adaptations based on changes in the plant genome (Givnish, 2002; Pan et al., 2006; Robe and Griffiths, 2000; Schurr et al., 2006). Habitats that impose high levels of abiotic stress are typically colonized with fewer plant species compared to habitats imposing low levels of stress. Moreover, high stress habitats have decreased levels of plant abundance compared to low stress habitats even though these habitats may occur in close proximity to one another (Perelman et al., 2007). This is particularly interesting because all plants are known to perceive, transmit signals, and respond to abiotic stresses such as drought, heat, and salinity (Bartels and Sunkar, 2005; Bohnert et al., 1995). Although there has been extensive research performed to determine the genetic, molecular, and physiological bases of how plants respond to and tolerate stress, the nature of plant adaptation to high stress habitats remains unresolved (Leone et al., 2003; Maggio et al., 2003; Tuberosa et al., 2003). However, recent evidence indicates that a ubiquitous aspect of plant biology (fungal symbiosis) is involved in the adaptation and survival of at least some plants in high stress habitats (Rodriguez et al., 2008).

In this chapter, we discuss recent information demonstrating that fungal endophytes can adapt to abiotic stress in a habitat-specific manner and confer stress tolerance to plants. We hypothesize that the intergenomic epigenetic communication responsible for endophyte-conferred stress tolerance allows some plants to make quantum evolutionary leaps necessary for their establishment and survival in high stress habitats.

2. Fungal Symbionts

The fossil record indicates that fungal endophytes have been intimately associated with plants for at least 400 million years (Krings et al., 2007). First described in the 1800s (De Bary, 1879), plant–fungal symbioses are now thought to be ubiquitous in all habitats (Petrini, 1996) and can have profound impacts on plant health, adaptation, and survival. Collectively, fungal symbionts have been shown to confer several fitness benefits to plants including increased root and shoot biomass, increased yield, and tolerance to abiotic stresses such as heat, salt, metal, and drought (Arnold et al., 2003; Bacon and Hill, 1996; Clay and Holah, 1999; Márquez et al., 2007; Redman et al., 2001, 2002; Sahay and Varma, 1999; Waller et al., 2005). Symbiotic fungi can be differentiated into two functional groups (endophytic and mycorrhizal fungi) based on plant colonization patterns, transmission, and ecological functions (Brundrett, 2006). In addition, fungal endophytes can be separated into four classes based on plant host range, colonization pattern, transmission, and conferred fitness benefits (Table 1).

While the primary focus of this chapter is on the ability of class 2 endophytes to confer stress tolerance, it is important to note that multiple classes of fungal endophytes and mycorrhizal fungi commonly coexist in individual plants. Fungal endophytes represent a diverse group of species belonging to the dikara representing both filamentous and single-celled fungi, the majority of which are in the Ascomycota (Arnold and Lutzoni, 2007; Carroll, 1988; Girlanda et al., 2006; Schardl and Leuchtmann, 2005; Van Bael et al., 2005).

The outcome of plant–fungal symbiotic interactions is defined by the fitness benefits realized by each partner (Lewis, 1985). Host fitness benefits can be positive (mutualism), neutral (commensalism and neutralism), or negative (parasitism, competition, and amensalism) depending on the lifestyle expressed by the fungal endophyte. The communication that dictates the outcome of symbiotic associations remains an enigma but subtle differences in communication can alter the symbiotic lifestyle expressed. For example, fungal species within a genus and different isolates

Table 1. Fungal endophytes.

	Clavicipitaceous	**Non-Clavicipitaceous**		
Criteria	**Class 1**	**Class 2**	**Class 3**	**Class 4**
Host range	Restricted	Unrestricted	Unrestricted	Unrestricted
Colonization	Shoot	Shoot and root	Shoot	Root
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits	NHA[a]	NHA and HA[b]	NHA	NHA

[a]Nonhabitat-adapted benefits are common among endophytes regardless of the habitat of origin
[b]Habitat-adapted benefits result from habitat-specific selective pressures such as pH, temperature, and salinity

of a species have been shown to express different symbiotic lifestyles ranging from mutualism to parasitism. This variation in lifestyle expression within a genus or species may occur due to host physiology or edaphic conditions (Francis and Read, 1995; Graham et al., 1996; Graham and Eissenstat, 1998) and is defined as the Symbiotic Continuum (Johnson et al., 1997; Schardl and Leuchtmann, 2005; Schulz and Boyle, 2005). Variation in lifestyle expression was most dramatically demonstrated by a host range study of pathogenic *Colletotrichum* species (Redman et al., 2001). Individual *Colletotrichum* isolates could express either pathogenic or nonpathogenic symbiotic lifestyles depending on the host genotype they colonized. More importantly, several isolates expressed mutualistic lifestyles conferring fitness benefits to plants such as disease resistance, growth enhancement, and drought tolerance. These results exemplify the symbiotic continuum that occurs between fungi and plants. In one geographic location individual endophytes may be expressing pathogenic lifestyles in some plants species and mutualisms in others. While this has not yet been investigated in natural ecosystems, the potential impacts of lifestyle switching endophytes on plant community structure may be profound.

3. Plant Stress Tolerance

3.1. HEAT TOLERANCE IN GEOTHERMAL HABITATS

All plants are known to initiate complex biosynthetic responses to elevated temperatures; these include the synthesis of heat shock proteins (HSPs) and antioxidant systems, and adjustments in osmotic potential and membrane lipids (Iba, 2002). However, few plants are capable of thriving in geothermal soils that impose temperature and drought stress. For example, in Yellowstone National Park (YNP) there are only nine plant species that thrive in, and appear restricted to, geothermal soils even though these soils represent a wide diversity of microhabitats (Stout and Al-Niemi, 2002). The most thermotolerant plant species in YNP is *Dichanthelium lanuginosum* (panic grass), which grows in the geothermal soils that reach root zone temperatures as high as 57°C (Stout and Al-Niemi, 2002). Geothermal soils of YNP have significant annual temperature fluctuations that are influenced by moisture. Winter snows melt on contact with geothermal soils to decrease surface temperatures to around 20°C and a lack of rainfall in summer results in dry, hot soils that reach root zone temperatures up to 57°C. Therefore, panic grass is exposed to high temperatures and drought conditions annually. The adaptive mechanisms responsible for the establishment of plants in geothermal soils is not known but, thought to be based on changes in the plant nuclear genome.

Analysis of 100 panic grass samples from YNP geothermal soils revealed that the plants were symbiotic with the fungal endophyte *Curvularia protuberata* (Redman et al., 2002). All plants were colonized with the fungus, which could be

isolated from several surface-sterilized plant tissues (roots, leaves, seed coats), but was not isolated from seeds. Therefore, by removing seed coats and briefly surface-sterilizing seeds, it was possible to generate nonsymbiotic plants that were free of the endophyte. Comparative studies with symbiotic and nonsymbiotic plants indicated that *C. protuberata* confers thermotolerance to panic grass and that this plant–fungal symbiosis is responsible for survival of both species in geothermal soils. When grown nonsymbiotically, the maximum growth temperature of panic grass and *C. protuberata* is 40°C and 38°C, respectively. However, when these organisms are grown symbiotically they are able to tolerate 10-day root temperature regimes of 65°C for 10 h followed by 37°C for 14 h under laboratory conditions (Redman et al., 2002). Similar results were observed with symbiotic and nonsymbiotic panic grass in geothermal soils of YNP. Soil was removed from four locations that ranged in temperature from 30°C to 45°C, pasteurized to kill resident fungi, replaced into the same holes, and planted with panic grass seedlings. Twelve months after transplanting, symbiotic plants had greater biomass than nonsymbiotic plants at all temperatures (Redman et al., 2002). The biomass differences between these plants increased with temperature and nonsymbiotic plants were unable to survive soil temperature of 45°C. For example, at 30°C, 35°C, and 40°C, the total biomass of nonsymbiotic versus symbiotic plants were 16.2/22.75 g, 21.65/28.4 g, and 8.8/22.2 g, respectively. These were the first experiments to unequivocally demonstrate that a plant–endophyte association was a mutualism since survival of both partners in their native habitat was only achievable via symbiosis. Prior to this, plant–endophyte symbioses were inferred to be mutualistic based on the fact that there were observable benefits to the host such as increased biomass, drought tolerance, or resistance to herbivores, but benefits to endophytes could not be visualized and were surmised to constitute nutrients required for fungal growth that were provided by the plant.

To determine if the ability of *C. protuberata* to confer heat tolerance was a habitat-specific adaptation, the host range and symbiotic lifestyle expression of isolates from panic grass (isolate Cp4666D) and from the nonthermal adapted grass *Deschampsia flexuosa* (isolate CpMH206) were evaluated in genetically unrelated monocot and eudicot plants. One-week-old monocot (corn, panic grass) and eudicot (watermelon, tomato) seedlings were inoculated with both isolates, incubated for 2 weeks and plants surface sterilized to assess fungal colonization (Redman et al., 2001). With the exception of corn seedlings, all plants were asymptomatically colonized by both *C. protuberata* isolates in root and stem tissues (Rodriguez et al., 2008). Exposure of symbiotic and nonsymbiotic plants to a soil temperature of 50°C for 7 days revealed that Cp4666D conferred heat tolerance to tomato, watermelon (Fig. 1), and panic grass seedlings, but CpMH206 did not confer heat tolerance to any plant (Rodriguez et al., 2008). On-symbiotic and CpMH206-colonized plant leaves curled and died after 2 days of thermal stress, whereas Cp4666D-colonized plants did not show any negative effects of the stress throughout the experiment. Cp4666D was reisolated from surviving roots and stems of all symbiotic plants treated at 50°C, indicating that the fungus

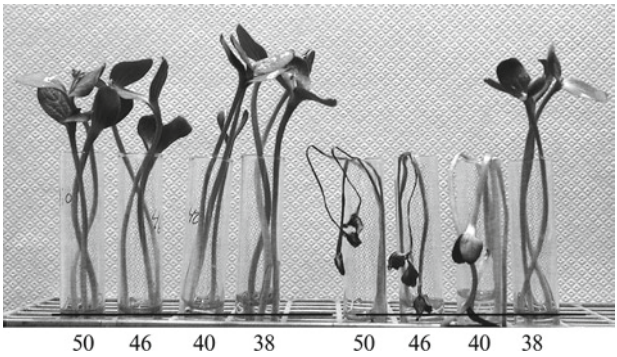

Figure 1. Nonsymbiotic (NS) and symbiotic (S, with Cp4666D) watermelon seedlings exposed to root temperatures of 38°C, 40°C, 46°C, and 50°C, respectively. Nonsymbiotic plants wilted after 2 days and the image was taken after plants were exposed to root temperatures for 7 days. (Methods from Rodriguez et al., 2008.)

was also protected from heat stress. The lack of thermotolerance provided by CpMH206 suggests that symbiotically conferred thermotolerance is a result of habitat-specific adaptation by Cp4666D, a phenomenon defined as "Habitat-Adapted Symbiosis" (Rodriguez et al., 2008). Colonization studies with panic grass supported this conclusion. Ten plants were colonized with Cp4666D or CpMH206 for several weeks, seedlings were homogenized, and homogenates plated on fungal growth medium to determine fungal colony forming units (cfu)/gram of plant wet weight. The average cfu/g for Cp4666D (1,212 cfu/g) and CpMH206 (1,297 cfu/g) indicated that plants were colonized similarly by the endophytes. Therefore, stress tolerance is not a direct result of fungal biomass, but must involve specific communication between stress-adapted endophytes and plant hosts. Moreover, the symbiotic communication responsible for heat tolerance in a monocot host (panic grass) appears to be the same in at least some eudicot hosts (tomato and watermelon). This suggests that the genetic and biochemical bases of symbiotically conferred stress tolerance is conserved and evolved prior to the divergence of monocots and eudicots (estimated at 175–230 mya (Yang et al., 1999; Zhou and Kleinhofs, 1996)).

3.2. SALT TOLERANCE IN COASTAL HABITATS

Plants in coastal habitats can be exposed to salt stress either by salt spray from waves or root inundation during high tides and storms depending on their distance from seawater. While most beach plants are not true halophytes (Kearney, 1904) they can tolerate higher levels of salt than plants from nonsaline habitats (Barbour and DeJong, 1977). Regardless, the biodiversity of plants decreases in the salt inundation zone and it is thought that the ability to tolerate salt stress is a function

of plant genomic adaptations. However, recent experiments with coastal plants indicate that a fungal endophyte is responsible for the adaptation and survival of at least some plants to high salt stress habitats (Rodriguez et al., 2008).

Shaw Island is part of the San Juan Island archipelago in Puget Sound off the coast of Washington state. At the University of Washington's Cedar Rocks Biological Preserve on Shaw Island (CR-SJI), beach substrates range in composition from very coarse sand to small/large pebble and the habitat is dominated by dunegrass (*Leymus mollis*). In this habitat, dunegrass grows in areas that are inundated with salt water during high tides and experiences dry conditions during summer months. Dunegrass plants (N = 100) from the beach habitat were analyzed and found to be symbiotic with one fungal species (*Fusarium culmorum*), which was isolated from rhizomes, roots, stem, leaves, and seed coats (Rodriguez et al., 2008). Using surface sterilization techniques (Redman et al., 2001), nonsymbiotic dunegrass plants were generated and some were recolonized with an isolate of *F. culmorum* (FcRed1) to assess colonization and symbiotically conferred fitness benefits. Based on previous studies that demonstrated Habitat-Adapted Symbiosis in geothermal soils, it was hypothesized that FcRed1 may confer salt tolerance to host plants. Therefore, nonsymbiotic and symbiotic (FcRed1 colonized) dunegrass plants were grown in sand exposed to NaCl at a concentration range of 0–500 mM. At 500 mM NaCl exposure, all FcRed1-colonized plants remained healthy, while the nonsymbiotic plants wilted and died above 100 mM NaCl indicating that FcRed1 confers salt tolerance to the host plant. To ensure that FcRed1-conferred salt tolerance was ecologically significant 20 symbiotic and 20 nonsymbiotic plants were planted into a beach habitat on Shaw Island known to harbor FcRed1 colonized plants. After 3 months of growth the plants were harvested for biomass assessment and fungal colonization. All of the FcRed1-colonized plants survived and achieved a total average biomass of 19.16 g (±5.95 g) while only 40% of the nonsymbiotic plants survived achieving an average biomass of 17.58 g (±9.23 g). While the difference in survival rates between symbiotic and nonsymbiotic plants was significant, it was expected that none of the nonsymbiotic plants would survive the beach habitat. However, microbiological analysis revealed that all of the surviving plants were colonized with FcRed1. Additional studies revealed that a very small population of FcRed1 was present in the beach substrate and it was concluded that survival and final biomass of initially nonsymbiotic plants was dependent on the timing of in situ colonization by FcRed1 (Rodriguez et al., 2008).

To determine if symbiotically conferred salt tolerance involved habitat-specific adaptation of FcRed1, a study was performed to determine if an isolate of *F. culmorum* (Fc18) from a noncoastal habitat (an inland agricultural field in the Netherlands) would confer salt tolerance. Both FcRed1 and Fc18 asymptomatically colonized dunegrass and several genetically distant species (tomato, watermelon, and rice) to equivalent levels (Rodriguez et al., 2008). The ability of these isolates to confer salt tolerance was tested in dunegrass, rice, and tomato. In all three plant species FcRed1 conferred salt tolerance to 300–500 mM NaCl,

while none of the Fc18-colonized or nonsymbiotic plants survived above 100 mM NaCl. This remarkable observation suggests that Habitat-Adapted Symbiosis may be a common phenomenon and plays a significant role in the establishment and survival of plants in high stress habitats. The fact that one fungal endophyte can confer salt tolerance to both monocot and eudicot hosts supports the hypothesis that the communication responsible for symbiotically conferred stress tolerance is conserved and evolved prior to the divergence of these plant groups.

3.3. DROUGHT TOLERANCE

There are numerous reports describing drought tolerance conferred to plants via fungal symbionts (Clay and Schardl, 2002; Schulz, 2006). Although the mechanism of symbiotically conferred drought tolerance is not known, it is thought to involve osmotic adjustments and/or altered stomatal activity (Malinowski and Belesky, 2000). However, the ability of fungal endophytes to confer drought tolerance has been studied in very few plant species and, until recently, it was not known if symbiotically conferred drought tolerance required habitat-specific adaptations. A recent study indicated that regardless of the habitat from which endophytes are derived, they all appear to confer some level of drought tolerance to plant hosts (Rodriguez et al., 2008). For example, geothermal- and nongeothermal-adapted isolates of *C. protuberata* (Cp4666D and CpMH206, respectively), or salt- and nonsalt-adapted isolates of *F. culmorum* (FcRed1 andFc18, respectively)–all confer similar levels of drought tolerance to native hosts and unrelated crop plants such as tomato and rice (Rodriguez et al., 2008). An example of endophyte-conferred drought tolerance is shown in Fig. 2, where FcRed1 allows wheat plants to retain turgor long after they are depleted of water.

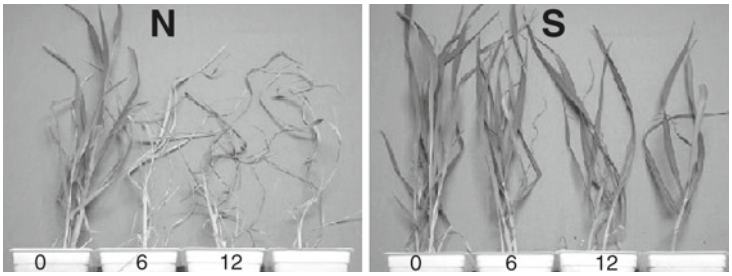

Figure 2. Nonsymbiotic (NS) and symbiotic (S) wheat plants colonized with FcRed1 were grown in sand for 2 weeks with adequate watering. Watering was then stopped and plants left to dry for the number of days indicated below each plant. After 6 days of desiccation, NS plants wilted while the S plants remained healthy after 18 days of desiccation. $N = 30$ plants/treatment. (Methods are from Rodriguez et al., 2008.)

Some interesting results were reported for plant pathogenic fungi (*Colletotrichum* sp.), which can express mutualistic lifestyles depending on the plant host genotype they colonize (Redman et al., 2001). When these virulent pathogens are expressing nonpathogenic lifestyles they confer drought tolerance to a number of host plants including cucurbits, tomato, and pepper (Redman et al., 2001; Rodriguez et al., 2004).

As we observed with temperature and salt tolerance, the symbiotic communication required for drought tolerance is conserved between monocots and eudicots. However, in contrast to heat and salt tolerance, endophyte-conferred drought tolerance does not appear to require habitat-specific adaptations.

4. Stress Tolerance Mechanisms

Decades of physiological studies indicate that plants perceive abiotic stress through receptors and subsequently use secondary messengers such as reactive oxygen species (ROS), Ca^{2+}, and inositol phosphates to transduce stress-related signals within the cells. The messengers act to upregulate stress-responsive genes and this results in the adaptive responses by the plant necessary for stress tolerance and survival. The reaction to stress at the cellular level is coordinated into a whole-plant response by the change in the level of hormones such as abscisic acid (ABA), ethylene, and jasmonates (Christmann et al., 2006). Plants exposed to salinity, drought, and heat stress have physiological responses that are either stress specific or common to all three stresses as described below.

Soils with high salinity have a low water potential, which makes it hard for roots to absorb both water and nutrients. In addition, NaCl competes with other ions, resulting in a decrease in cellular levels of Ca^{2+}, K^+, Mg^{2+}, and NO_3^- (Niu et al., 1995) that can induce nutrient deficiencies. Increases in cellular Na^+ and Cl^- ions result in a disruption of ionic and osmotic equilibrium, and inhibition of enzymes essential for cellular metabolism. These cellular effects cause an overall reduction in plant growth through the inhibition of both cell division and expansion, and a reduction in photosynthesis. Drought conditions also cause a water deficit in plants. The desiccation of plant cells causes an increase in the electrolyte concentration (Bahrun et al., 2002) to levels that are toxic to enzyme function resulting in a breakdown of cellular metabolism. Plant membranes become porous and this loss of membrane integrity leads to a disruption of mitochondria and chloroplast function (Flexas and Medrano, 2002). Collectively, the decrease in membrane integrity and loss of organelle function causes a decrease in plant growth, particularly of the leaf tissue (Salah and Tardieu, 1997) and senescence of older leaves (Rivero et al., 2007). Heat stress is deleterious to photosynthesis; in particular the thylakoid and PSII complexes are disrupted (Kim and Portis, 2005; Tang et al., 2007; Vani et al., 2001). Carbon metabolism is impeded as rubisco activase and the associated proteins become

inhibited (Salvucci and Crafts-Brandner, 2004). HSPs are found to increase in concentration, these act as chaperones protecting the integrity of essential proteins (Kotak et al., 2007).

Physiological responses that are common to salt, drought, and heat stress are based on the fact that all three stresses affect water relations and result in the generation of ROS. To prevent the loss of intracellular water the plant lowers the water potential inside the cell by producing osmolytes. Osmolytes are molecules that can be accumulated to high concentrations without disturbing the cells metabolism and include sugars and sugar alcohols (raffinose, sucrose, trehalose, mannitol, and sorbitol), amino acids (proline) (Yoshiba et al., 1997), and amines (glycine betaine) (Waditee et al., 2005). Not only do osmolytes lower the water potential of the cell, but they act to stabilize the structure of membranes and enzymes and scavenge ROS. Reactive oxygen species are normally produced at a very low concentration in plants; however, when plant metabolism is disrupted by abiotic stress ROS concentrations significantly increase resulting in cellular damage through chemical oxidation, de-esterification of membrane lipids, protein denaturation, and mutation of nucleic acids (Apel and Hirt, 2004). The ROS themselves act as secondary messengers (Neill et al., 2002) leading to hormone-mediated responses such as the further closure of leaf stomata, production of heat shock protein (HSP) to chaperone proteins and initiate an increase in the level of antioxidant scavengers such as catalase, guaiacol peroxidase, and ascorbate peroxidase (Kotak et al., 2007). Plants limit the leaf dehydration and subsequent osmotic imbalance of the cells by closing the leaf stomates. This can occur through direct evaporation from the guard cells or by ABA promoting K^+ efflux and subsequent loss of turgor pressure and stomatal closure. The reduction in transpiration however simultaneously decreases the influx of CO_2 decreasing photosynthesis and upsetting the delicate balance of ROS production and antioxidant quenching.

Contemporary understanding of plant physiological responses to abiotic stresses does not include potential influences of fungal endophytes on plant responses. For example, when exposed to heat, salt, or drought stress, nonsymbiotic panic grass and tomato plants increase osmolyte concentrations (Rodriguez et al., 2008). However, plants that are symbiotic with an endophyte that confers tolerance to each stress (heat and drought tolerance by Cp4666D; salt and drought tolerance by FcRed1; and only drought tolerance by CpMH206 & Fc18) either did not increase in osmolyte levels or had lower osmolyte concentration compared to nonstressed controls (Márquez et al., 2007; Rodriguez et al., 2008). Therefore, increased osmolyte concentrations are not the method of heat, salt, or drought tolerance in symbiotic plants.

Although the mechanisms associated with endophyte-conferred stress tolerance are not yet known, there are three physiological aspects of symbiotic plants that provide insight into this phenomenon: symbiotic plants consume less water, have greater biomass, and produce less ROS compared to nonsymbiotic plants. A concomitant decrease in water consumption and increase in biomass suggests

that symbiotic plants have greater water use efficiency than nonsymbiotic plants, which may be especially useful under restricted water conditions where it would be advantageous for the plants to require less water to survive and fulfill their life cycle. The herbicide Paraquat can be used to test tissue sensitivity to ROS (Vaughn and Duke, 1983). The involvement of ROS in endophyte-conferred stress tolerance was determined with the herbicide Paraquat, which is reduced by photosystem I and oxidized by molecular oxygen forming superoxide molecules, which cause photobleaching. In the absence of stress, leaf tissues of symbiotic and nonsymbiotic plants (panic grass, dunegrass, and tomato) exposed to Paraquat were not susceptible to photobleaching showing that no ROS was produced. However, when the symbiotic and nonsymbiotic plants were exposed to stress (panic grass with heat stress, tomato with heat and salt stress, and dunegrass with salt stress) leaf tissues of nonsymbiotic plants bleached white and symbiotic plants remained green. This suggests that the symbiotic plants had a greater capacity to either scavenge or prevent the production of ROS.

5. Plant Evolution and Adaptation to Stress

Two significant events that occurred over geologic time required adaptive responses by plants: the oxygenation of the atmosphere and the movement of plants from an aquatic to terrestrial habitats. Earth's atmosphere began to slowly accumulate oxygen when photosynthetic bacteria emerged approximately 3,500 mya (Schopf, 1993). Geologic models suggest that atmospheric oxygen reached levels necessary for aerobic metabolism between 500 and 1,000 mya (Canfield and Teske, 1996; Kah et al., 2004). Concomitant with this atmospheric change was a tremendous increase in the biological diversity of aerobic organisms, which eventually resulted in the evolution of terrestrial plant life approximately 400 mya (Cleal and Thomas, 1999). Aerobic respiration and photosynthesis generate metabolic energy (ATP) as a result of transferring electrons from electron donors (inorganic or organic compounds) through an electron transport chain to terminal electron acceptors (most commonly molecular oxygen [O_2]). Transfer of electrons to the terminal acceptor results in the reduction of O_2 to H_2O. In plant photosynthesis the electron donor is H_2O, which gets oxidized to produce O_2 and the terminal electron acceptor is CO_2, which gets reduced to organic compounds such as carbohydrates. A by-product of aerobic respiration and photosynthesis is the production of high-energy forms of oxygen designated as reactive oxygen species (ROS; Apel and Hirt, 2004). There are several different ROSs that can be formed and all of them cause oxidative damage to proteins, DNA, and lipids. Under normal physiological conditions (metabolically balanced) ROSs are scavenged by different antioxidation chemicals or enzymes. However, exposure to abiotic stress results in metabolic imbalances causing an increased production of ROS, which can result in cellular damage and death. The ability of fungal endophytes to protect plants against detrimental effects of ROS may represent an ancient aspect of these symbiotic

associations, one that likely predates the divergence of monocots and eudicots (est. 200 mya) and may reflect the occurrence of these associations very early in plant evolution.

The establishment of plants on land was likely a necessary precursor for the evolution of land animals, which required shelter, food, and developed habitats. However, plants had several physical problems to overcome in transitioning between aquatic and terrestrial habitats (Cleal and Thomas, 1999). One of the greatest problems likely involved water availability and the ability to transport sufficient amounts of water from belowground tissues to prevent the desiccation of aerial tissues. Eventually, plants developed elegant root and vascular systems to efficiently absorb and transport water and nutrients. However, water availability has been something plants have had to deal with throughout their evolutionary history. Fungal endophytes appear to increase the efficiency of water usage in plants, which may provide an important mechanism for avoiding intermittent water availability. This may explain why the class 2 fungal endophytes tested to date confer some levels of drought tolerance to plants.

6. Summary

Symbiotic associations between plants and fungal endophytes provide a clear example of how biological cooperation results in stress tolerance. These associations are ancient and may have been critical for the establishment of plants on land. Moreover, the ability of some plants to establish and survive in high stress habitats appears to be dependent on fungal endophytes. In the coming decades researchers will determine if Habitat-Adapted Symbiosis is required for all plants to establish in high stress habitats and if similar phenomena occur in low to moderate stress habitats. Regardless of the ubiquity of Habitat-Adapted Symbiosis, concepts in plant biology such as biogeography, ecology, evolution, and phenotypic plasticity need to be expanded to include contributions by biological cooperators.

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GRASS ENDOPHYTE-MEDIATED PLANT STRESS TOLERANCE: ALKALOIDS AND THEIR FUNCTIONS

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1. Introduction

The Family Clavicipitaceae (Hypocreales, Ascomycota) includes saprotrophic and symbiotic species associated with insects and fungi (*Cordyceps* spp.) or grasses, rushes and sedges (*Balansia* spp., *Epichloë* spp., *Claviceps* spp.) (Bacon and White, 2000). Symbiotic interactions are a notable feature of the Clavicipitaceae and they range in a continuum from antagonism to mutualism (Schardl et al., 2004). The plant biotrophic forms within this family can be characterized based on the nature of the association with hosts, being epibiotic during part or the entire life cycle or strictly endophytic with hyphae growing intercellular in the aboveground plant parts such as leaves, stems, and culms of the host.

2. Origin and Evolution of Ergot Alkaloids

Secondary metabolites are bioactive compounds nonessential for growth or reproduction produced in large amounts in diverse groups of organisms (Wicklow, 1988). Species in the Clavicipitaceae are a rich source of secondary metabolites. Insect pathogenic species of genus *Cordyceps* and *Hypocrella* produce numerous secondary compounds with biological activity with possible applications in medicine and insect control (Isaka et al., 2005). In the grass endophytes, infection of the grass host is associated with production of an array of compounds, ergot alkaloids, and nonalkaloid secondary metabolites. In the past 3 decades the focus of research has been centered on the associations between *Neotyphodium* endophytes and cool-season forage grasses due to their economic impacts related to detrimental effects of endophyte-infected grasses on livestock.

Ergot alkaloid production is not unique to grass endophytes. Vascular plants in the family Convulvulaceae (e.g., *Ipomoea violaceae* and *Turbina corymbosa*) also produce ergot alkaloids (Tudzynski et al., 2001). Several other genera

in Hypocreaceae and Clavicipitaceae (Hypocreales) such as *Hypomyces*, *Dussiella*, *Hypodermium*, and *Hypocrella* have been reported to produce ergot alkaloids in vitro (Glenn and Bacon, 1997; Rehacek and Sajdl, 1990; Torres et al., 2008). Apparently independently derived in the phylogenetically distant order Eurotiales (Alexopoulos et al., 1996), several *Penicillium* species and *Aspergillus fumigatus* also produce ergot alkaloids (Panaccione, 2005; Wicklow, 1988).

3. The Proposal of Functionality for Ergot Alkaloids

Typically ergot alkaloids are produced in a pathway in which the end product and intermediary products accumulate in large quantities (Panaccione, 2005). This suggests that ergot alkaloid diversity has been selected for functional reasons. The functions of many secondary metabolites are uncertain and frequently a matter of speculation. In *Aspergillus* and *Penicillium*, the fungi concentrate ergot alkaloids in sclerotia in a comparable way to *Claviceps* spp. Since the sclerotium is important in terms of survival, metabolic effort is expended to defend and preserve it. Sclerotia of *A. flavus* are avoided by the dried-fruit beetle (*Carpophilus hemipterus*); this species consumes other fungal parts but avoids the sclerotia (Gloer, 1995).

Ergot alkaloid production in grass–endophytes is hypothesized to be associated with host defense (Clay, 1988; Clay and Schardl, 2002). However, it is not clear that this is the case in insect necrotrophs/plant biotrophs or the epibiotic species, such as *Hypocrella* and *Claviceps*. In these species mycelium is restricted to the surface of plants, replacing the scale insect or in the case of a flower, replacing the ovary. In the epibiotic species where the fungal organism spends the entire life cycle as an external symbiont (*Hyperdermium* spp., *Hypocrella* spp., *Dussiella* spp., *Claviceps* spp., etc.) it is unlikely that there is a transfer or diffusion of compounds from fungal stromata to the plant. In this case alkaloids could act as a "self-defense mechanism" of the stromata to avoid herbivores and/or prevent colonization by other organisms, without any apparent benefit for the plant host that is supplying nutrients. It may be reasoned that with the acquisition of the endophytic habit as in genera *Balansia* and *Epichloë* fungal defensive metabolites developed a "host defensive" function. This host defensive feature seems to stem from at least two characteristics of the endosymbionts, including the internal systemic location of the endophytes in leaves, stems, and seeds and a degree of water affinity and diffusible nature of their defensive metabolites.

4. The Evidence for Defensive Mutualism in the Clavicipitaceae Endophyte–Grass Association

Mutualism has been the prevailing conceptual framework under which the evolution and ecology of grass endophytes have been interpreted. In this view, grass endophytes are considered as plant mutualists primarily because they deter herbivore feeding,

and the primary driving selective force behind the endophyte–plant mutualism is considered a defense against herbivores (Clay, 1988, 1990, 1997).

Endophyte-infected grasses contain a variety of secondary metabolites that are not found in noninfected plants. These compounds, mainly alkaloids, are considered the primary mechanism for antiherbivore and antimicrobial activity (Bacon et al., 1986; Schardl and Philips, 1997; Bush et al., 1997). Several factors influence the types of alkaloids produced and their concentrations. Some factors include the strain of endophyte, plant part, age, growing season, and fertilization status. In general terms alkaloid production and concentration correlates positively with hyphal density and the survival value of the plant part, with meristems, leaf sheaths, and seeds exhibiting high concentrations of alkaloids (Clay, 1990). Alkaloids with known specific biological activities are lolines, peramines, lolitrems, and the ergot alkaloids. Lolines, ergot alkaloids, and lolitrems tend to be limited to leaf sheaths, meristematic zones, inflorescences, and seeds while peramine and lolines are also present in the blade (Keogh et al., 1996; Lane et al., 2000). This broader distribution of loline and peramine is likely due to their greater solubility in the aqueous apoplast. The ergot alkaloids and lolitrems are less soluble and are not likely translocated to great distances through plant tissues.

Approximately 65% of the endophytes are present in the asexual form and they produce some degree of toxicity in herbivores through a range of different kinds of alkaloids (Porter, 1994). The persistence of the alkaloids in a highly evolved relationship is well adapted and likely advantageous for both host plant and endophyte and as demonstrated to having defensive effects (Clay, 1990).

The response of insects to endophyte infection depends in part on the plant part on which they feed and in some cases is age related (Popay and Bonos, 2005) with larvae and first instars showing major reductions of weight gain and developmental rate in fall armyworm (Popay and Rowan, 1994). Other Lepidopteran insects such a black cutworm (*Agrotis ipsilon*), common cutworm (*A. infusa*), and Orthopterans are less affected by consumption of infected grasses (Williamson and Potter, 1997). When consuming the base of the plant, predominately leaf sheaths and basal meristems, insects appear to be more impacted by the toxins. This appears to be the case in feeding by the Argentine stem weevil larvae (*Listronotus bonaerensis*) on perennial ryegrass (Popay and Bonos, 2005), mealybug (*Balanococcus poae*) feeding in tall fescue (Pennel et al., 2005), bluegrass billbug (*Sphenophorus parvulus*) in *Poa pratensis* (Richmond et al., 2000), and black bettle (*A. ipsilon*) adults, which feed on the base of tillers in tall fescue (Popay and Bonos, 2005). Aphids and leafhoppers exhibit diverse responses to endophyte-infected plants (Wilkinson et al., 2000; Popay and Bonos, 2005).

Even though fungal growth does not extend in the root, some alkaloids are reported in root system (i.e., loline alkaloids) and several root-feeding invertebrates, such as nematodes, have been reported to be affected by endophytes, the mechanism remains unknown (Elmi et al., 2000; Bacon and White, 2003).

4.1. THE DEFENSIVE MUTUALISM CONTROVERSY: A DEBATE REGARDING FUNCTION

The defensive mutualism concept of the role of symbiotic endophytic in Clavicipitaceae has been generally accepted in the scientific community (Clay, 1988; Schardl et al., 2004). This hypothesis holds that plants are defended from herbivory by animals and insect herbivores through the production of secondary metabolites. The defensive mutualism explanation for endophyte distribution came under scrutiny recently due to observations that many endophytes do not appear to impart defensive benefits to host plants. Faeth (2002) has argued that a strong anti-insect effect in endophyte-infected grasses appears to be the exception, not the rule. In arizona fescue (*Festuca arizonica*) plants are often infected by endophytes that do not appear to confer insect or animal hervibory deterrence benefits to hosts (Saikkonen et al., 1999). Faeth (2002) has also argued that herbivory is a weak selective force on grasses and unlikely to provide significant selective pressure to drive the evolution and spread of endophyte associations. Grasses are adapted to animal herbivory by developing basal meristems that protected growing parts of plants (Stebbins, 1981). The endophytes grow in these protected parts of plants and are therefore protected in grass rhizomes and meristems and are not susceptible to extinction by herbivory. It would therefore follow that antiherbivore selection pressure is negligible.

It may be incorrect that the primary function of fungal-produced secondary metabolites relates to defense of the host. Two primary needs that must be filled by the fungal symbionts are acquisition of nutrients and water. Water relations are important for both partners in the grass–endophyte symbiosis. In Clavicipitaceae, water is also a determinant step in stroma development (White et al., 1993). One of the most notable attributes of symbiotic associations involving grass endophytes is enhancement of their hardiness to adverse conditions and resistance to abiotic factors, improving overall fitness and persistence of the infected plants (Redman et al., 2002; Rodriguez and Redman, 2008). Directly or indirectly, the array of secondary metabolites produced by endophytes in the plant apoplast is a factor and could function to control or manipulate host plant metabolism. It seems logical that Clavicipitaceae fungi would evolve compounds that would facilitate nutrient acquisition or water from host plants.

4.2. NUTRIENTS FIRST AND DEFENSE SECOND

Biotrophic interactions can have a marked effect on the source–sink balance within host plants (Hall and Williams, 2000) where the hyphae growing in the apoplast could act as an additional sink in the host. Amino acid metabolism is also altered with increases in the concentration of free amino acids (Smith and Smith, 1990). Biotrophic fungi typically have mechanisms to extract nutrients nondestructively from host plants. In powdery mildews (Erysiphales) and rusts and smuts

(Heterobasidiomycetes) the fungal symbionts produce haustoria that function by interfacing internally with host cells to absorb nutrients across a matrix using proton-pumping mechanisms (Griffin, 1994). In these internal interfaces of haustoria, plant membranes lack proton pumping ATPases and, absorption of nutrients is unidirectional from plant to fungus (Buchanan et al., 2000). In general, endophytic hyphae must compete with plant host cells for nutrients in the apoplast. This greatly reduces the efficiency of nutrient acquisition and hence the need for haustoria where nutrient absorption by the fungus is favored (Volaire, 2002). None of the Clavicipitaceae produces haustoria. We hypothesize that they have evolved alternate mechanisms to nonaggressively acquire nutrients from plant tissues or reduce the competition from the plant plasma membrane nutrient transport proteins.

The endophytes growing intercellularlly in plant tissues (see Fig. 1) are presumed to passively obtain nutrients that leak from host parenchyma cells into the apoplast where hyphae are located. Hyphae are generally adherent to host cell walls (see Fig. 1b and c) and when fully developed assumes a sinuous growth pattern (Fig. 1a). The sinuous growth pattern of hyphae may have the effect of maximizing the surface area of interface with host tissues and nutrient leakage

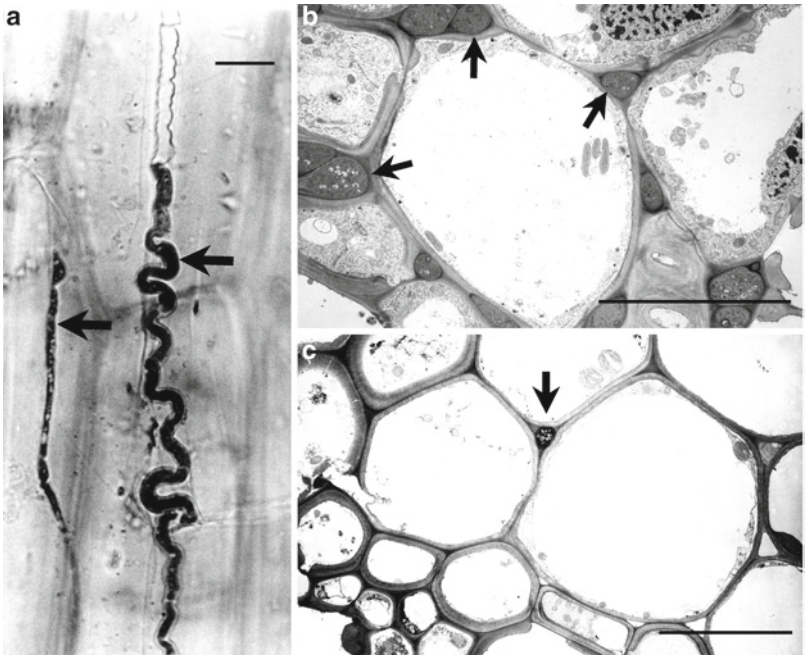

Figure 1. Intercellular mycelium of endophytes in Clavicipitaceae. (**a**) Hyphae of *Neotyphodium coenophialum* growing intercellularly in the tall fescue leaf sheath. Bar = 5 μm. (**b, c**) Longitudinal section through the primordial inflorescence of *Agrostis hiemalis* infected by *Epichloë amarillans* (electronic micrograph). Bar = 5 μm.

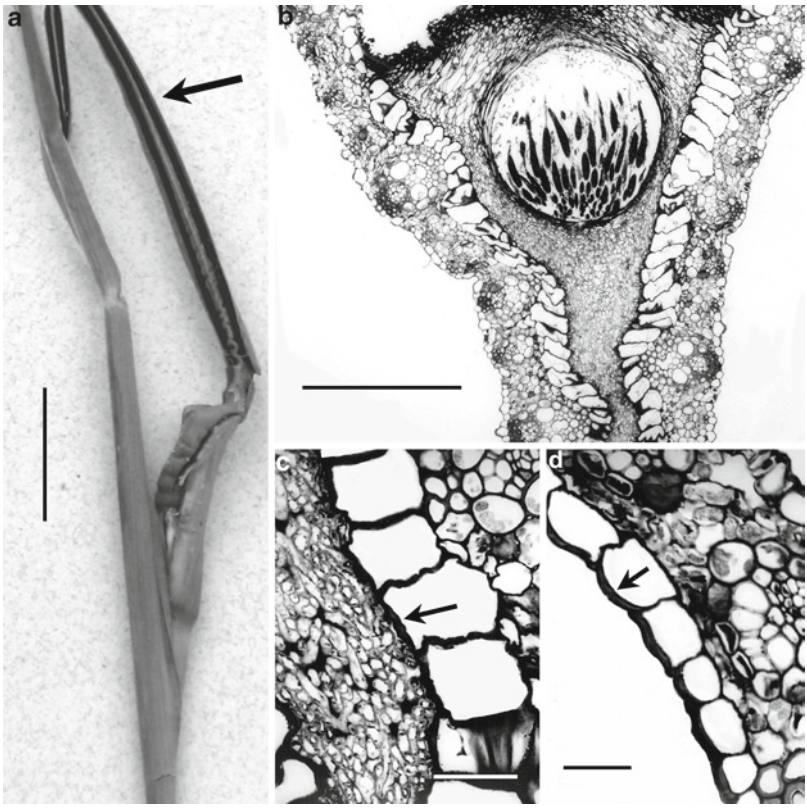

Figure 2. *Myriogenospora atramentosa.* (**a**) Perithecial stroma on leaf. Bar = 2 cm. (**b**) Cross section of stroma on leaf of *Paspalum dilatatum*, showing perithecial cavity. Bar = 300 μm. (**c, d**) Section through epidermis. (**c**) Colonized by epibiont (*arrow* shows outer epidermis wall). Bar = 30 μm. (**d**) Not colonized (*arrow* shows outer epidermis wall). Bar = 25 μm.

intercept. In the epiphytic *Myriogenospora* sp. (Fig. 2a) the fungus grows on developing leaves and modifies development of the epidermis layer causing hypertrophy and failure to produce a waxy cuticle (see Fig. 2b–d). This enables the fungus to remove barriers to nutrient flow from plant to fungus (Smith et al., 1985).

5. Membrane Depolarization Hypothesis for Function of Ergot Alkaloids

Endophytes in Clavicipitaceae may utilize ergot alkaloids as modifiers of the host cell interface in order to increase leakage in the zone of absorptive hyphae. Moubarak et al. (2003) demonstrated that the ergot alkaloids, ergovaline, ergotamine, and ergonovine were inhibitory to ATPases. While this work was

conducted to elucidate toxic effects on herbivores consuming infected plants, it also suggests one mechanism whereby clavicipitaceous fungi may obtain nutrients from hosts. In the membrane depolarization mechanism, the ergot alkaloids function by binding plasma membrane proteins and result in depolarization of the plant plasma membrane. Polarization of the plant cell plasma membranes occurs by plasma membrane H^+ pumping ATPases (Buchanan et al., 2000). Proton pumping is the source of the chemiosmotic H^+ gradient that drives solute uptake from the apoplast. In this chemiosmotic mechanism, the exterior of the membrane becomes positively charged with respect to the interior of the membrane. It is this electrical polarization (typically 100–200 mV) of electrical potential across the plasma membrane that provides the power to import sugars and other solutes into the plant cell (Palmgren and Harper, 1999).

The chemical properties of ergot alkaloids are relate to this function. Ergot alkaloids are considered to be good hydrophobic ligands (with hydrophobic and hydrophilic parts) that would bind to receptor proteins on cells and become partially embedded in the cell membrane (Shappell, 2003). The membrane target of ergot alkaloids has been demonstrated for ergot alkaloids in eukaryotic systems for kidney cells (Moubarak et al., 2003) and membrane receptors, likely proteins, in bovine brain cells membranes (Allgren et al., 1985).

According to this model once embedded at the protein receptors on membranes the hydrophilic component of the alkaloid remains exposed on the exterior surface of the plant plasma membrane. When H^+ protons are pumped to the exterior surface of the plant plasma membrane and the exterior membrane becomes acidic the alkaloid protonates thus remove the proton and reduce the electron potential across the plant cell membrane.

This membrane depolarization or its effects have been demonstrated in several studies including the alkaloid pergolide (Hong et al., 2005) where voltage-gated potassium channels were inhibited. Several ergot alkaloids were shown to cause transient depolarization of snail nerve membranes (Miyamoto et al., 1980). Moubarak, Johnson, and Rosenkrans (2003) have demonstrated apparent binding of several ergot alkaloids to receptors on several ATPase enzymes with resultant inhibition of those systems. The outcome of membrane depolarization is that solute uptake by the plant cells is transiently slowed. This permits the fungus to uptake the apoplastic compounds before absorption by the plant cell membrane. The limited solubility of ergot alkaloids likely limits membrane depolarization to a localized region of the membrane directly proximal to the fungal hyphae.

Hyphae of *Epichloë*/*Neotyphodium* endophytes grow closely to plant cell walls, with the proposed zone of alkaloid influence likely not extending much beyond these hyphae. It is interesting that many clavicipitaceous fungi including *Claviceps*, *Epichloë*, and *Balansia* tend to grow on plant meristematic tissues. These are the tissues where H^+ pumping plasma membranes ATPases are most abundant (Palmgren and Harper, 1999) and where responsible solute uptake from the apoplast is most critical. The effect of ergot alkaloids as membrane depolarizers

would be expected to be most pronounced in these meristematic tissues. Shappel (2003) demonstrated that ergovaline inhibited growth of dividing intestinal caco-2 cells and that mature cells were little affected. Shappell (2003) hypothesized that inhibition was the result of inhibition of electron transport or membrane polarization. Dividing plant tissues (inflorescences and leaves) embedded in the stromal mycelium of *Epichloë* also become arrested in size and fail to continue development and differentiation.

According to the depolarization mechanism model for ergot alkaloid function arrested plant tissue development, as is seen in plant tissues embedded in fungal stromata of *Epichloë*, could be explained by disruption of the electron gradients across the plant plasma membranes and inability of plant cells to acquire nutrients from the apoplastic tissues to complete development and differentiation. Interestingly, a similar mechanism has been proposed for another indolic alkaloid hypaphorine (Martin et al., 2001) produced by an ectomycorrhizal fungus *Pisolithus tinctorius*. Hypaphorine is secreted by mycelium on differentiating root tissues. This alkaloid causes transient depolarization of the differentiating plant cell membranes with the resultant inhibition of root hair development. Ergot alkaloids, also indolic alkaloids, may function similarly.

6. Effects on Apoplastic Osmolytes

The mechanism of nutrient acquisition may involve secretion of alkaloids that affect chemiosmotic functioning of transmembrane symporter proteins in absorption of solutes. This removes competition of the plant cell plasma membrane for apoplastic nutrients. Studies on apoplast infiltrate further support this hypothesis in demonstration of significantly higher glucose and fructuose amounts in apoplast of endophyte-infected grass plants (Richardson et al., 1992).

The fungus further increases nutrients by secreting osmolytes, including loline alkaloids, mannitol, and trehalose (Richardson et al., 1992; Secks et al., 2004), that are osmotically active but cannot be absorbed and degraded by the plant plasma membranes. The increased osmotic potential in the apoplast reduces the osmotic difference between cytoplasm and apoplast and reduces the movement of water and smaller molecules into the more osmotically active cells.

7. Alkaloids as Hormone-Like Compounds: Indole Acetic Acid

Plant growth regulators produced by fungi have been associated with growth and morphological alterations in symbiotic associations (Hall and Williams, 2000; Reboutier et al., 2002; Martin et al., 2001). Several grass endophytes (*Neotyphodium* spp. and *Balansia* spp.) have been reported to produce indole acetic acid (IAA) in vitro (De Battista et al., 1990; Porter et al., 1985). Nevertheless, levels of free IAA in the whole plant are unaffected by the endophyte presence in

tall fescue (0.29–0.31 μg/g dry weight). Probably due to a complex mechanism of IAA synthesis, catabolism and distribution in the plant and localized or transient changes are difficult to detect (Schardl et al., 2004).

Endophyte produced compounds with auxin functions (i.e., IAA) in addition to the auxin produced naturally by the plant, which may have an effect on plant metabolism. In plants, auxin is synthesized in meristems and young organs, transported through the plant by a transport system and ultimately produce specific cellular responses. Approximately 90% of the plant IAA in the plant is in conjugated forms, with only minute amounts of free IAA. IAA has been found to be conjugated to simple sugars, cyclitols, high-molecular-weight polysaccharides, or the carbohydrate component of glycoproteins via an ester or anhydride linkage, by amide linkage to single amino acids, peptides, or proteins. Conjugates are thought to be involved in a variety of hormonally related processes such as transport of IAA within the plant, the storage and subsequent reuse of IAA, protection of IAA from enzymatic destruction, and as components of a homeostatic mechanism for the control of IAA levels. Another important aspect of IAA plant metabolism is that IAA biosynthesis occurs via two separate pathways: tryptophane-dependent (TD) and tryptophan-independent (TI). Wounding alters in a transient way the route of IAA biosynthesis, causing a change from synthesis via the TI pathway to synthesis via the TD pathway. Also, both pathways can be used at different times of development, with the TD pathway predominating during early embryogenesis and seed germination, and the TI utilized during late embryogenesis and vegetative growth (Cohen and Slovin, 1999; Normanly, 1997; Cooke et al., 2002).

Bacteria colonizing plant surfaces are also know to produce IAA. Production of IAA is related to the hyperplasia produced in plants in most plant pathogenic bacteria, while its role in other pathogenic and nonpathogenic plant-associated bacteria is still unclear (Lindow and Brandl, 2003).

7.1. AUXIN MODE OF ACTION AT CELL MEMBRANE AND CELL WALL

One of the most studied auxin action mechanisms is in guard cells where auxins stimulate stomata opening through a turgor-driven process that relies on the accumulation of K^+ salts and sugars in the cytoplasm. It seems that an indirect mechanism of activation exists. Plasma membranes in guard cells have recognition sites for auxin that respond by ion channel opening and membrane depolarization. According to the acid growth theory, a membrane proton pump (H^+-ATPase) is activated by phosphorilazion/desphosphorilation of autoinhibitory domains in the C-terminus portion of the protein. Binding of a protein to the phosphorilated C-terminal region leads to the displacement of the autoinhibitory domain and thus activation of the H^+-ATPase. Activation of H^+-ATPase (proton pumping) results in hyper polarization of the cell membrane potential and in turn activation of

voltage-dependent K^+ ion channels. The proton gradient will provide the driving force for the uptake of Cl^- and/or sugars via H^+-based symporters in the cell membrane (Becker and Hedrich, 2002). Activation of the proton pump in the cell membrane results in acidification of the apoplast (Becker et al., 2002) that produces cell wall extension.

7.2. FUNGAL ALKALOIDS AND IAA CONJUGATES

"Norleucine," a structural component of the ergot alklaoid, ergokryptinine, has been shown to conjugate auxins (Nigovic et al., 1992). Ergokryptinine is produced by numerous species of biotrophic Clavicipitaceae, including *Claviceps purpurea*. Ergot alkaloids and other norleucine-containing alkaloids could function by forming auxin conjugates and in consequence interfering in auxin plant metabolism.

Production of fungal alkaloids that bind auxin might be a part of a mechanism of fungal adaptation to the symbiosis. This mechanism might be important to avoid repair of leaky membranes by the plant host. This would enable fungi to continue to obtain nutrients from host tissues and continue the nutrient flow. In a species like *Claviceps* the developing sclerotia depend on the continuous flow of nutrients from host phloem to sclerotial mycelium. Plants normally respond to wounds or nutrient leakages by producing auxin and other plant hormones at the site of damage and stimulation of wound repair process (Cohen and Slovin, 1999). Removal of plant auxins may affect the capacity of the plant to repair the leaky plant tissues proximal to the developing sclerotia.

Modulations of ionic currents across the cell membrane are among the earliest events in plant–microbe interactions. It has been reported the production of an indole alkaloid (hypaphorine) during the establishment of ectomycorrizal symbiosis where the mycelium accumulates and exudes the alkaloid that acts as IAA antagonist. Hypaphorine plays a role as competitor of auxin activity in the regulation of symbiosis-associated differentiation (Ditengou and Lapeyrie, 2000; Reboutier et al., 2002). More than 1,200 indole alkaloids have been described from fungi and plants. The akaloid hypaphorine counteracts IAA. It is likely that many other indole alkaloids also can compete with auxin-binding proteins based on structural similarities (Jambois et al. 2005).

7.3. FUNGAL ALKALOIDS AND OSMOTIC ADJUSTMENT

Osmotic adjustment and stomata regulation are key factors in plant response to water stress. Both functions appear to be under the effect of the endophyte (Elmi and West, 1995) through the production of secondary metabolites that can be stored in the hyphae or secreted in the apoplast. Elmi and West (1995) suggested accumulation of osmotically active metabolites as a mechanism of osmotic adjustment in endophyte-infected plants.

Osmotic adjustment helps the plant acclimate to dry or saline soils. Chemically diverse, highly soluble organic compounds that do not interfere with the plant metabolism can perform as osmolytes. The amino acid proline is accumulated and its concentration is maintained by a combination of synthesis and catabolism. Other compatible compounds such as monomeric sugars can be released from polymeric forms (starch) in response to stress. Some compatible compounds may perform additional functions. Compounds such as sorbitol, mannitol, myo-inositol, and proline may all function to scavenge hydroxil radicals in vitro and thus show protective antioxidant activity (Buchanan et al., 2000).

In endophyte-infected grasses, the accumulation of several compatible compounds has been reported. Richardson et al. (1992) reported apoplast accumulation of glucose and fructose in infected plants of tall fescue. Accumulation of fungal metabolites such as mannitol, arabitol (Richardson et al., 1992), and trehalose (Secks et al., 2004) has also been reported. Mannitol is a common polyol of fungal origin that is highly correlated with fungal biomass (Rasmussen et al., 2008) and its seems to have different biochemical role in different species, such as osmoprotectant and high temperature stress protection (Rasmussen et al., 2008). Trehalose accumulates in a wide range of drought-tolerant plants, fungi, and bacteria during water deficit. It has been proposed as an osmoregulator, storage or membrane and protein protectant (Crowe et al., 1984). Lyons et al. (1990) reported increased amino acid concentration in infected tall fescue compared to noninfected.

In contrast, in drought stress experiments involving *Festuca* spp. and *Lolium* spp. grasses proline accumulation was not detected as a mechanism of osmotic adjustment (Malinowski and Beleski, 2000). In Addition, Elberson and West (1996) reported lower proline concentration in tall fescue genotypes under drought stress when compared with noninfected genotypes. A possible explanation for a lower concentration of proline in endophyte-infected *Lolium* is that the amino acid proline is a precursor of alkaloids synthesis.

Reactive oxygen species (ROS) like superoxide and hydrogen peroxide are involved in defense response and in maintaining grass–fungal endophyte symbiosis, as it was demonstrated by Tanakaa et al. (2006) for *Epichloë festucae*, and its plant host, *Lolium perenne*.

8. Conclusions

Experimental evidence for the physiological roles of secondary metabolites in enhancing the symbiotic associations is still incomplete. In past research investigators have been unable to separate the many effects on plants of fungal metabolites like the ergot alkaloids. In addition, most work have been conducted using animal tissues and animal models. Because of this an understanding of effects on animals is fairly well developed, but little is known about effects on plants. Additional research should be conducted to elucidate the impacts of ergot alkaloids and other metabolites on the plant plasma membrane and other plant cell parts.

The consistent and widespread production of ergot alkaloids by clavicipitaceous plant biotrophs proves that they may have adaptive value for the fungi. The broad diversity of their forms further suggests that they have multiple cellular targets and perhaps multiple functions. The widely accepted view of these fungus-produced metabolites is that they are defensive in nature and function primarily to deter herbivores from consuming the fungus or its host plant. A great deal of evidence supports defensive effects of fungus-produced alkaloids for host grasses. Host defense may be an important function of ergot alkaloids and similar compounds. However, the primary and immediate needs of the fungus would seem to relate to procurement of nutrients by the fungus from its plant host.

In this chapter, an outline is presented of several ways in which ergot alkaloids and other fungus-produced metabolites may function to impact the fungus–host interface. Several studies demonstrate effects of ergot alkaloids and other indole alkaloids on ATPases and membrane polarization. Alkaloids such as indole acetic acid have been shown to be produced by several clavicipitaceous endophytes. Experimental studies exploring ergot alkaloid effects in plants are now needed to support the hypothesis that the fungal secondary metabolites function to modify the plant–fungus interface and increase the nutrient supply to the fungal symbionts.

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ENDOCYTOSIS IN PLANT – FUNGAL INTERACTIONS

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1. Introduction

Plants are continuously exposed to pathogenic microorganisms in their environment, and possess many mechanisms aimed at mounting an effective defense against these pathogens (Jones and Dangl, 2006; Yang et al., 1997). These defense responses include the strengthening of mechanical barriers, oxidative burst, "*de novo*" production of antimicrobial compounds such as pathogenesis-related (PR) proteins and phytoalexins, and the induction of the hypersensitive response (HR) mechanism, where the tissue surrounding the infection site dies and confines pathogen growth (Hammond-Kosack and Jones, 1996).

The host plant recognizes foreign molecules associated with microorganisms. Some recognition events conform to the model in which a host receptor interacts directly with a molecule of the microbe. These include the interaction between microbe-associated molecular patterns (MAMPs) and MAMP receptors (Nurnberger et al., 2004). They also include the interaction between some effectors and their cognate resistance (R) proteins. Elicitors (MAMPs) that trigger plant defense responses have been isolated from a variety of phytopathogenic and nonpathogenic microorganisms (Ebel and Cosio, 1994; Felix et al., 1999; Fuchs et al., 1989; Ricci et al., 1993) The ability of the plant to recognize and defend itself upon MAMP perception has recently been studied extensively in the context of endocytosis, in particular in connection with bacterial proteins (Martin et al., 2003; Robatzek et al., 2007). Here we present an analysis relating primarily to fungal MAMPs.

1.1. VERTICILLIUM GLYCOPROTEINS

Fungi of the genus *Verticillium* are pathogens responsible for vascular wilt disease in over 200 plant species (Fradin and Thomma, 2006). A few elicitors present in *Verticillium* species have been previously documented, among them a 65 kDa heat-stable glycoprotein (Davis et al., 1998). A locus responsible for resistance against *Verticillium*, termed *Ve*, has been isolated from tomato and was found to confer resistance to strains of *V. dahliae* and *V. alboatrum*. The *Ve* locus contains two genes: *Ve1* and *Ve2*, which encode cell surface leucine-rich repeat (LRR) receptor-like protein (LRR-RLPs; Kawchuk et al., 2001).

1.2. CRYPTOGEIN FROM *PHYTOPHTHORA*

Cryptogein and Capsicein are proteinaceous elicitors isolated from the oomycete *Phytophthora* (water mold) and are capable of eliciting defense responses in tobacco; Cryptogein is 50 times more potent than Capsicein (Ricci et al., 1989).

Cryptogein induces hypersensitive response (HR) and systemic acquired resistance (SAR) in tobacco plants (Lebrun-Garcia et al., 1999). The tobacco response to cryptogein also includes production of active oxygen species, cytosol acidification, membrane depolarization, and MAP kinase activation (Lebrun-Garcia et al., 1999).

Cryptogein was found to bind tobacco plasma membranes in a saturable, specific, and reversible manner, in concentrations required for in vivo activity. The putative Cryptogein receptor may also be glycosylated (Wendehenne et al., 1995).

1.3. *CLADOSPORIUM FULVUM* AVR PROTEINS

C. fulvum causes leaf mold disease on sensitive cultivars of Tomato. Tomato *Cf* genes confer resistance to *C. fulvum* through recognition of fungal Avr proteins. Many tomato *Cf* genes have been cloned. The encoded proteins are type I transmembrane glycoproteins containing extracellular leucine-rich repeats (LRRs), a membrane spanning region and a short cytoplasmic domain (Rivas and Thomas, 2005).

Many host responses have been described as characterizing the interaction between Cf and corresponding Avr proteins, including deposition of callose, production of glucanases and chitinases, production of phytoalexins and pathogenesis-related (PR) proteins, as well as production of active oxygen species, stimulation of protein kinases, and hypersensitive response (HR; Joosten et al., 2000; Joosten and de Wit, 1999). However, no physical interaction was detected between Cf4 or Cf9 and their corresponding Avr proteins, though the possibility was examined in many different experimental systems (Luderer et al., 2001; Rivas and Thomas, 2005). In fact, though Avr4 and Avr9 are the presumed ligands of Cf4 and Cf9, respectively, the molecular mechanism underlying Avr protein perception has not been established.

Interestingly, and possibly due in part to the lack of direct physical interaction between the Cf and Avr proteins, though the tomato Cf receptors Cf4 and Cf9 were reported to contain the conserved endocytosis signal Yxxϕ within the short cytoplasmic domain (Jones et al., 1994; Thomas et al., 1997), endocytosis of the corresponding Avr proteins has not been reported. However, vesicular transport and signaling are no doubt involved in the response to *C. fulvum* Avr proteins, as evidenced by the specific phosphorylation of a syntaxin (SNARE complex protein) early in the Cf9/Avr9 pathway (Heese et al., 2005).

1.4. ETHYLENE-INDUCING XYLANASE FROM *TRICHODERMA*

The fungal protein ethylene-inducing xylanase (EIX) (Dean et al., 1989), is a well-known protein elicitor of defense response reactions in tobacco (*Nicotiana*

tabacum) and tomato (*Solanum lycopersicum*) plants (Avni et al., 1994; Bailey et al., 1990). EIX induces ethylene biosynthesis, electrolyte leakage, expression of PR proteins, and HR in specific plant species and/or varieties (Bailey et al., 1990; 1992; Elbaz et al., 2002; Ron et al., 2000). EIX was shown to specifically bind to the plasma membrane of both tomato and tobacco responding cultivars (Hanania and Avni, 1997). The response to EIX in tobacco and tomato cultivars is controlled by a leucine-rich-repeat receptor-like-protein (LRR-RLP) encoded by a single dominant locus, termed LeEix (Ron and Avni, 2004).

2. Endocytosis in Plants

In the past years, the roles of regulated endocytosis in plant development and plant immunity are emerging (Robatzek, 2007). A variety of membranal receptors, mostly leucine-rich repeat (LRR) receptors, have been identified and are involved in many processes, including cell differentiation and defense signaling. Ligand-dependent (Robatzek et al., 2006) and ligand-independent, constitutive receptor internalization have been documented (Gifford et al., 2005; Shah et al., 2002). As is the case for cell surface receptors in mammalian cells, autophosphorylation of the cytosolic domain of plant LRR-RLK receptors induced by ligand binding has also been demonstrated (Shah et al., 2002). Plant receptors can also undergo recycling back to the PM after internalization (Albrecht et al., 2008). However, despite many recent advances in the field, plant endocytic compartments are not well characterized and the term endosome is often employed generally for compartments containing endocytosed material. A broad range of molecular markers have been developed and, together with lipid marker dyes are used to analyze plasma membrane vesicular recycling and endocytosis, as well as to identify and characterize the corresponding endomembrane compartments in plant cells (Gross et al., 2005; Lam et al., 2007b; Muller et al., 2007; Samaj et al., 2004, 2005).

Styrl dyes such as FM-4-64 have been used to study localization of vesicles, which are putative endosomes (Bolte et al., 2004; Grebe et al., 2003; Lam et al., 2007a; Ueda et al., 2001). Structural studies indicated that the partially coated reticulum (PCR) is analogous to the early/recycling endosomes of mammalians (Galway et al., 1993). Two distinct classes of early endosomes were identified in *Arabidopsis*. One comprises the endosomes in which Ara6 resides, and the other the endosomes to which Ara6 is not targeted (Ueda et al., 2001). Early endosomes have also recently been characterized as SCAMP1 containing tubular-vesicular structures possessing clathrin coats and residing in the vicinity of the trans-golgi (Lam et al., 2007b). Molecule sorting occurs in the early endosomes, from which they are either recycled back to the plasma membrane, transported to the golgi apparatus, or to multivesicular bodies (MVBs, also known as late endosomes; Battey et al., 1999; Jurgens, 2004). The trans-golgi network (TGN) was also found to be involved in early endocytic pathways in Arabidopsis (Dettmer et al., 2006; Lam et al., 2007b). Prevacuolar compartments (PVCs) have been identified as

MVBs in tobacco BY2 cells (Tse et al., 2004). From the MVBs, the endocytosed material is targeted to the vacuole for degradation. Recent studies conducted in plant systems have further elucidated possible functionalities of plant endocytic compartments and the flow of endocytosed material throughout plant cells (Geldner and Robatzek, 2008; Lam et al., 2007a; Muller et al., 2007; Silady et al., 2008; Teh and Moore, 2007).

Clathrin-coated vesicles are most probably a major means of internalization in plant cells. Studies conducted recently have demonstrated that clathrin-dependent internalization occurs in plants (Dhonukshe et al., 2007; Lam et al., 2007b; Leborgne-Castel et al., 2008; Perez-Gomez and Moore, 2007; Tahara et al., 2007). Components that interact with the clathrin-coated vesicles and adaptor proteins such as dynamins and proteins that contain an SH3 domain occur in plants and are involved in endocytosis and vesicle trafficking (Kang et al., 2003; Lam et al., 2001).

3. Ligand-Induced Endocytosis of Fungal Elicitors

Fungal elicitors have been shown to enter plant cells in several instances. In some cases, a specific plant receptor, which recognizes the fungal elicitor, has been identified and isolated. Thus far, many of the plant receptors identified, which recognize fungal elicitors have been shown to be leucine-rich-repeat receptor-like proteins (LRR-RLPs), which contain an extracellular LRR and lack a kinase domain. LRR motifs are often found in proteins involved in specific protein–protein interactions. In the case of some R proteins, the LRR domains are believed to determine the specificity of Avr ligand binding (Hammond-Kosack and Jones, 1997; Thomas et al., 1997). Additionally, some of these receptors contain a Yxxϕ motif for clathrin-mediated endocytosis.

One of the first systems, which demonstrated that endocytosis does indeed occur in turgid plant cells employed the *Verticillium* elicitor. Although this work dates to 1989 it is still current today, and it was the first to indicate the possibility of receptor-mediated endocytosis in plant cells. This work indicated that there probably exists a specific receptor for the *Verticilium* elicitor (Horn et al., 1989). Subsequently, two *Ve* receptors were isolated, and found to be LRR-RLPs containing the Yxxϕ endocytosis motif (Kawchuk et al., 2001) as detailed above.

The *Verticillium* elicitor was shown to enter the cell by an endocytic process in soybean cell cultures (Horn et al., 1989). The rate of elicitor uptake as well as its sensitivity to temperature conform with an endocytic process. A preparation of the *Verticillium* elicitor, which is a glycoprotein as described above, was found to associate with the cell surface and subsequently induce the formation of H_2O_2 within 5 min. Internalization of the labeled elicitor was competitively inhibited by unlabeled elicitor and, 5–7 h after application, a large portion of the elicitor was delivered to the cell vacuole, probably for degradation (Horn et al., 1989).

The Cryptogein elicitor was recently shown to induce endocytosis in correlation with its defense response activation (Leborgne-Castel et al., 2008). Endocytosis

of the lipophylic dye FM-4-64, which is commonly accepted as a marker for clathrin-mediated endocytosis in plant, was found to be stimulated in response to the addition of Cryptogein to a tobacco cell suspension. However, endocytosis of FM-4-64 was not induced in response to a control ligand, which does not trigger defense response signaling. Additionally, cryptogein was found to induce a transitory stimulation of clathrin-coated pits within 15 min of its addition. Both these phenomena were blocked in the presence of tyrphostin A23, which can inhibit receptor-mediated endocytosis. The study presented in Leborgne-Castel et al. (2008) is one of the first to link clathrin-coated pits and vesicles with the endocytosis of a plant defense response elicitor and, given the evidence presented, most probably occurs via a specific receptor, which may contain a clathrin-mediated endocytosis motif.

Hanania et al. (1999) showed that after binding the plant membrane EIX is transported into the cytoplasm. Mutation in the endocytosis motif of LeEix2 resulted in abolishment of induction in HR in response to EIX, suggesting that endocytosis plays a key role in mediating the signal generated by EIX that leads to HR induction (Ron and Avni, 2004).

In a recent work (M. Bar and A. Avni, 2010, unpublished results), we have shown that EIX triggers internalization of the LeEix2 receptor on endosomes, which are dependent on an intact cytoskeleton. Ten to 15 min after EIX application the GFP-tagged LeEix2 receptor can be seen throughout the cell on vesicles. These vesicles were also FYVE positive indicating that they are endosomes. In untreated leaves, GFP-tagged LeEix2 did not appear colocalized with the FYVE marker (data not shown). The FYVE domain has been reported to localize to endosomes in mammalian cells (Stenmark et al., 1996) as well as plant cells (Heras and Drobak, 2002; Jensen et al., 2001; Voigt et al., 2005). The FYVE-positive LeEix2 vesicles were also highly motile, as is characteristic of endosomes. In untreated leaves the FYVE vesicles have similar motility, while the GFP-LeEix2 is localized to the plasma membrane.

4. Defense Receptors and the Involvement of the Endocytic Mechanism in Plant Defense Response Signaling

Leucine-rich-repeat receptor kinase (LRR-RLKs) and LRR-RLPs have been implicated in signaling as well as defense responses in plants (Becraft, 2002; Torii, 2004). The most intensively studied LRR-RLK in the context of plant defense responses is FLS2, which recognizes bacterial flagellin and the flagellin-derived peptide flg22 (Felix et al., 1999; Gomez-Gomez and Boller, 2000; Gomez-Gomez et al., 1999). FLS2 is responsible for flagellin recognition, leading to a response, which includes generation of ROS, MAP kinase activation, ethylene production, and induction of gene transcription (Asai et al., 2002; Felix et al., 1999; Zipfel et al., 2004). The perception of flagellin by FLS2 was shown to be essential for the plant defense response, as FLS2 mutations compromised the ability of the plant

to mount an efficient defense against bacterial pathogens (Robatzek et al., 2006; Zipfel et al., 2004). Interestingly, the kinase activity of RLKs such as FLS2 may be required for receptor internalization and is probably required for receptor signaling (Robatzek et al., 2006).

As detailed above, LRR-RLPs have been implicated in response to pathogens. The tomato *Cf* genes, which mediate resistance to *C. fulvum* encode LRR-RLPs, the LRR domain of which was shown to be important for avirulence (Avr) gene recognition (Takken et al., 1999; van der Hoorn et al., 2005). Genetic compatibility of a Cf protein and its Avr counterpart typically leads to defense responses including oxidative bust, ion fluxes, MAP kinase activation, and induction of HR (May et al., 1996; Piedras et al., 1998; Romeis et al., 1999) that inhibits *C. fulvum* proliferation. Additional LRR-RLPs include the tomato *Ve*-resistant proteins (Kawchuk et al., 2001) and the *LeEix* proteins, as mentioned above (Ron and Avni, 2004). The tomato Ve2-, Cf9-, Cf4-, and LeEix-resistant proteins detailed herein (Jones et al., 1994; Kawchuk et al., 2001; Ron and Avni, 2004; Takken et al., 1998) contain the conserved endocytosis signal Yxxϕ within the short cytoplasmic domain. Mutating this signal in LeEix2 abolishes both endocytosis (M. Bar and A. Avni, 2010, unpublished results) and receptor signaling (Ron and Avni, 2004).

4.1. THE ENDOCYTIC MECHANISM INVOLVED IN PLANT DEFENSE RESPONSES TRIGGERED BY FUNGAL ELICITORS: EIX AS A MODEL

A schematic proposed model incorporating our works relating to the LeEix receptor (Bar et al., 2008; Hanania et al., 1999; Ron and Avni, 2004; Rotblat et al., 2002) is presented in Fig. 1. Upon EIX application, EIX binds the LeEix2 receptor on the outside of the plasma membrane (Hanania and Avni, 1997; Ron and Avni, 2004). This binding was shown not to require additional plant proteins (Ron and Avni, 2004). The ligand–receptor complex probably signals for the binding of an endocytic protein complex to the Yxxϕ motif present within the cytoplasmic tail of LeEix2. One protein in such a complex could be AP-2, which has been shown to bind the Yxxϕ motif of transferrin receptor and participates in transferrin internalization in *Arabidopsis* protoplasts (Ortiz-Zapater et al., 2006). Binding of AP-2, usually via the Yxxϕ motif, has also been shown to be a crucial step in the internalization of several mammalian receptors (Traub, 2003).

Interestingly, EHD proteins in mammalians were shown to bind adaptor proteins, as well as additional proteins of the clathrin-coated vesicle complex (Rotem-Yehudar et al., 2001). This may also be the case in plant cells. It is possible that AP-2 resides in a complex with the cytoplasmic tail of LeEix2 and EHD2, as well as additional proteins.

Binding of EIX allows for entry of LeEix2 into the cell, in an actin- and microtubule-dependent manner. EHD2 has been shown to be linked to the actin cytoskeleton in mammalian cells (Braun et al., 2004; Guilherme et al., 2004),

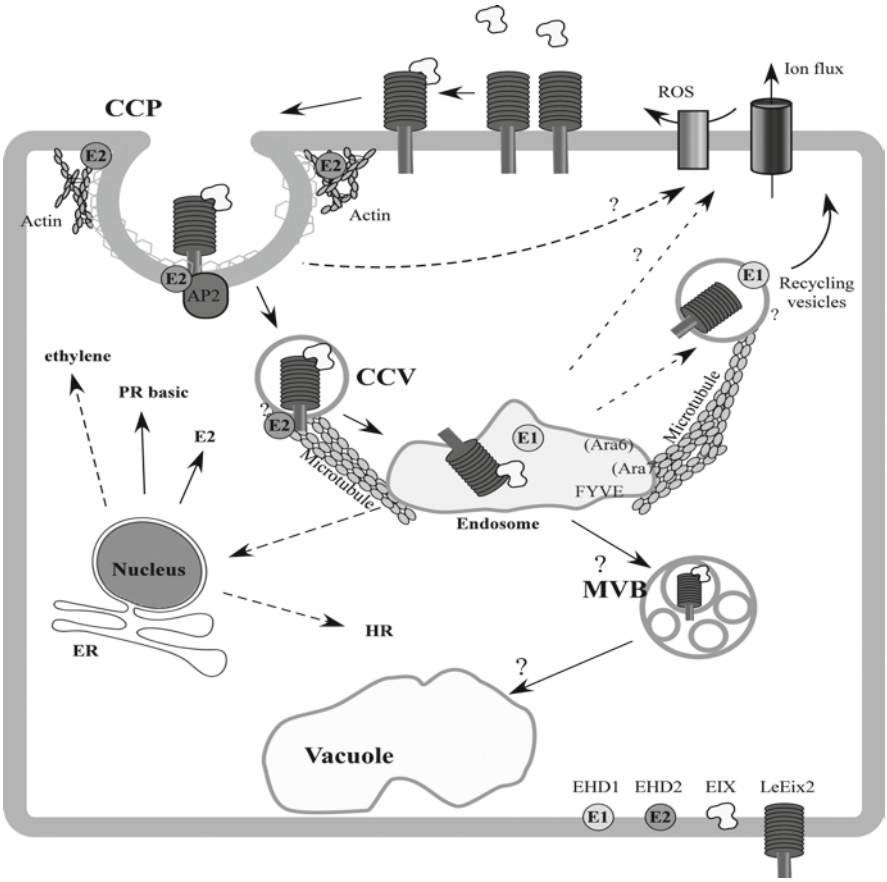

Figure 1. Schematic representation of LeEix localization and putative signaling pathway. Localization of known markers indicated. E1 = AtEHD1, E2 = AtEHD2, CCP = clathrin-coated pit; CCV = clathrin-coated vesicle.

and we have preliminary evidence that this is the case in plants as well (M. Bar et al., 2010, unpublished results).

LeEix2 is internalized on FYVE-positive endosomes, which may also contain EHD1 (Bar et al., 2008); LeEix2 may be recycled back to the plasma membrane on recycling vesicles (which can also contain EHD1 [Rapaport et al., 2006]), as internalization experiments of LeEix2 in the presence of cycloheximide were not significantly different than those conducted without cycloheximide, though LeEix2 did remain on FYVE endosomes for longer periods of time in the presence of cycloheximide. LeEix2 is probably recycled to the plasma membrane via vesicles, a process, which does not obligatorily require protein synthesis but may be amplified by the synthesis of certain proteins involved. LeEix2 may also be degraded via the multivesicular bodies/vacuole pathway, at least in part.

The internalization of LeEix2 is required for induction of defense responses, including ion flux, ROS production, ethylene, and PR protein synthesis (Bailey et al., 1990, 1992; Laxalt et al., 2007). EIX application also triggers NtEHD2 expression, upon which NtEHD2 acts to inhibit the defense response in the short term. Longer exposure to MAMPs leads to a "full-blown" defense response including HR, free of the inhibitory influence of EHD2, suggesting that a control mechanism based on the interplay of different proteins may be at work.

5. Conclusions

Plants are continuously exposed to pathogenic microorganisms in their environment, and possess many mechanisms aimed at mounting an effective defense against these pathogens. In many cases, the host plant recognizes foreign molecules associated with the microorganism, termed microbe-associated molecular patterns (MAMPs). Elicitors (MAMPs) that trigger plant defense responses have been isolated from a variety of phytopathogenic and nonpathogenic microorganisms (Ebel and Cosio, 1994; Felix et al., 1999; Fuchs et al., 1989; Ricci et al., 1993).

Leucine-rich-repeat receptor-like-proteins (LRR-RLP) have been reported to be involved in plants' ability to sense and respond to several microbial pathogens. The transmembranal receptor-like proteins studied include receptor-like kinases (RLKs) such as FLS2 and EFR and receptor-like proteins (RLPs, lacking a kinase domain) such as the LeEix proteins and the Cf proteins. In the cases of plant defense against fungal pathogens, the plant resistance receptors identified thus far are predominantly receptor-like proteins.

LeEix2 mediates the recognition and response to ethylene-inducing xylanase (EIX) elicitor. LeEix2 contains the endocytosis motif Yxxϕ. Mutating the Yxxϕ motif in LeEix2 abolishes EIX-mediated hypersensitive response, suggesting that endocytosis plays a key role in the signal transduction pathway. Endocytosis has also been demonstrated to be involved in the signaling of additional "anti-fungal" RLPs such as Ve1. We have previously shown that EIX triggers internalization of the LeEix receptor and that plant EHD2 is an important factor in the internalization and downstream signaling of EIX/LeEix and RLPs of the Cf family.

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DIE HARD: LICHENS

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1. Introduction

The symbiotic association of a heterotrophic fungal and an autotrophic algal (and/or cyanobacterial) partner produces a unique phenotype, the lichen thallus (Grube and Hawksworth, 2007). With their thallus structures, lichens can establish long-living individuals under almost all climatic conditions of our planet. Lichens cover about 8% of the land surface on Earth (Ahmadjian, 1995). Many lichens have adapted to extreme environmental conditions, which are adverse to most other organisms, and which do not support long-term survival of lichen symbionts alone. Some species are able to grow at the latitudinal and altitudinal limits of life in Antarctica or the Himalayas, while other species are present in hot deserts (Kappen, 1974). On the other hand, lichen thalli degrade rapidly if their preferences regarding their habitat are not matched due to ecological or climate changes. In the mid-nineteenth century, it was noticed that air pollution negatively influenced the growth of lichens in Paris (Nylander, 1866), and similar observations were made in other European cities at that time. Their particularly high sensitivity to sulfur dioxide and other pollutants generally causes rapid death of lichens. Much scientific interest and funds were, therefore, invested on exploring lichens as bioindicators. On the other hand, natural aging and senescence of lichens have been little studied.

Lichens do not have a clearly age-related death rate. In fact, lichen thalli can become extremely old under the optimal ecological conditions. The approximate age of lichen thalli can be determined by lichenometry (Beschel, 1950), a method that has been applied to date glacier retreat: assuming a constant rate of growth, the diameter of an individual relates to its age. Growth of lichens in cool habitats is not fast, but the sizes of some individual thalli suggest that they are of very old age, in the range of several thousands of years (Denton and Karlén, 1973). However, with these high estimates, we must also consider that conspecific thalli may fuse when they grow adjacent to each another, or that old thalli may fragment and the patches can regenerate.

Stress certainly influences aging of lichen symbioses greatly. Stress as any external influence with harmful effects for an organism can be of both abiotic and biotic nature. Occasionally, biotic factors can also contribute to abiotic

stress, e.g., when vegetation of higher plants influences the microclimatic conditions of the lichen habitats. In addition, abiotic stress may make them more vulnerable to biotic stressors. In any case, stress results in specific phenotypic responses, although these responses are likely different from those in other organisms with higher internal organization. Processes such as systemic acquired resistance or hypersensitivity response are poorly understood in lichens, although related phenomena such as oxidative bursts are known (Beckett and Minibayeva, 2003). Thus, it is likely that the lack of such a type of sophisticated response may cause lichens to be particularly sensitive, leading to rapid senescence. However, growing as a symbiotic system, many lichens are surprisingly robust and with an extended ageing phase (Fig. 1).

Transverse sections display ageing across functional layers of a lichen thallus. Old algal cells usually disintegrate from the algal layer and are either released downwards into the medulla layer or pushed upwards to the upper cortex. The latter phenomenon is often observed in crustose lichens, where remnants of algal cell walls form, together with fungal material, a pseudocortex. Thallus growth of many species proceeds with pseudomeristematic zones. These develop into differentiated thalline parts with decreased cell-turnover rates. These parts contain increased proportions of algal cells with arrested cell cycles that exceed the size for autospore formation and, later on, increasing numbers of dead photobionts, while fungal hyphae retain their integrity for longer periods, but fungal plectenchyma tends to become more brittly by age.

Figure 1. Decay and biotic stress in lichens: *Lobaria pulmonaria*. Arrows: o, old parts, partly overgrown by other organisms; f, fungal infections; g; invertebrate grazing damage; y, young and growing parts (Photograph by Peter Bilovitz).

Some lichens exhibit interesting examples of indeterminate growth. Crustose lichens generally expand radially on the substrate surfaces, until there are limited in their further growth by neighbouring lichens or other constraints. Central parts of these lichen individuals may age, and may then be overgrown by other lichens, detach from the substrate, rejuvenate by new outgrowths, or may be re-colonised by the same individual from the periphery. Some foliose lichens, e.g., in the genus *Peltigera* disintegrate in the centre when they grow larger, but perpetually grow at the periphery as long as the habitat is permissive. There can also be repeated waves of growth in some species. The rock-inhabiting *Arctoparmelia centrifuga*, a foliose lichen with leaf-like thallus branches, grows in a centrifugal manner with several rings of active growth (Fig. 2). As the thalli degrade in some distance from the growing edge, space is provided for the inner rings to expand. In soil-covering reindeer lichens (e.g., *Cladina* spp., *Cetraria* spp. *Alectoria* spp. in similar habitats), the branching terminal parts are perpetually growing, while the distal bases senesce. Nutrient translocation occurs from these decaying parts towards the growing tip region (Ellis et al., 2005). All these species display terminal or marginal growth. In other lichens, this behaviour is not so regular and obvious, and senescing of parts can be restricted to some parts of a thallus. Growth in other species also includes, to a certain extent, regular or irregular "intercalary" patterns, with corresponding gradients of photosynthetic activity (Larson, 1983). Growth can also be patchy, as seen in pustulate species in Umbilicariaceae. As lichens are extremely slow-growing organisms, some patience is needed to study the dynamics of thallus

Figure 2. *Arctoparmelia centrifuga*. The lichen grows perpetually at the periphery while it is decaying at the inner side. This leads to concentric rings of lichen growth (Image by Einar Timdal; http://www.nhm.uio.no/botanisk/lav/Photo_Gallery/index.html).

turnover. However, reports suggest that dramatic changes in thallus size and appearance may sometimes can be observed after 2 years (McCarthy, 1989).

The reasons for the regular senescing in older thallus parts are not always clear, but nutrient re-allocation to actively growing parts could play an important role. In reindeer lichens, the gradient of low light and higher humidity towards the thallus bases may add to the nutrient re-allocation effect. Perhaps other organisms are involved in shaping the intrathalline age structure. Generally, senescing parts of lichens can host a range of other microorganisms, in particular, fungi and bacteria. The increasing number of colonizers of older thallus parts can be clearly observed in the microscope, but only few studies have focused on these thallus parts in particular, so far. Aptroot and Alstrup (1999) isolated hyphomycetes from senescing parts of *Cladonia rangiformis*. Preliminary observations show that these parts of reindeer lichens have an altered composition of associated bacteria compared to actively growing parts, with more *Actinobacteria* and *Gammaproteobacteria* (Cardinale et al., in preparation). Large, ageing thalli, e.g. of *Peltigera*, can also disintegrate, perhaps due to mechanical stress, from the substrate organisms (e.g., mosses), to form several independent thalli. The older parts of many lichens may, however, still be vital enough to regenerate in central parts, giving rise to complex thalli of advanced age with many rejuvenating areas in the thallus centre.

2. Biotic Stresses

Generally, long-living lichen thalli create a habitat for other fungi that have specialized on lichens, with variable degrees of host and symbiont specificity. These fungi are known as lichenicolous fungi, which often produce characteristic structures on their hosts. The biological strategies of these fungi range from commensalic to pathogenic. The latter imposes various degrees of biotic stress to the hosting lichen.

Approximately, 1,000 known specific lichen-parasitic fungi can be distinguished according to morphological characters (Lawrey and Diederich, 2003). Some lichenicolous fungi are specialised to parasitise the algal partners, others aggressively degrade fungal plectenchyma, but in many cases, the biologically affected partner of the host symbioses is not so clear, especially with mild pathogens or commensals. A general pattern, however, seems to be that parasites with rather aggressive behaviour, such as *Marchandiomyces lichenicola* or *Athelia arachnoidea*, infest more or less unspecifically on a wider range of host species in variable lineages. On the other hand, species that are apparently confined to a host species often seem to be less aggressive and do not cause damage. They might be acting as commensals in the host lichens. Some of these specialised lichenicolous fungi apparently only occur when the host thalli can reach appropriate ages for infection. This leads to the interesting observation that infections with rare or highly specific fungi are frequently detected in the optimal habitats of the host lichens, in areas with low or no air pollution.

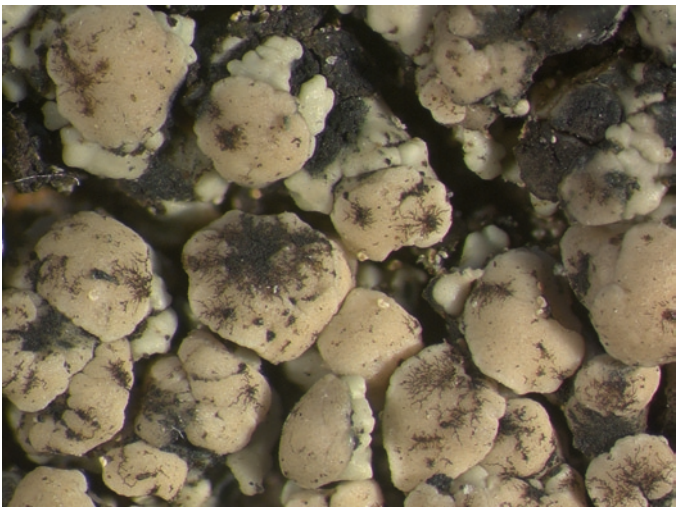

Figure 3. Black fungi infecting fruit bodies of *Lecanora polytropa* (Photograph by Lucia Muggia).

Apart from the phenotypically known lichenicolous fungi, there seem to be many other fungi associated with lichen thalli (Fig. 3). Fungi that do not develop fruiting structures or any other characteristic signs have been hardly considered in microscopic studies, but it is known that fungi can grow out from lichen thalli that are kept under excessive humidity. The isolation of culturable fungi has revealed a high diversity of surface and internal lichen-associated fungi (Arnold et al., 2009; Harutyunyan et al., 2008; Petrini et al., 1990; Prillinger et al., 1997). This diversity may be significantly higher, if as yet unculturable, lichen-specific fungi are considered. A direct quantitative assessment of associated fungi in the host lichen has not yet been achieved, but preliminary results using single-strand conformation polymorphism analyses (SSCP) have revealed up to 14 different fungi in a small piece of a single thallus (Muggia and Grube, 2010). Such results can be confirmed by careful microscopic studies, whereby several other fungi can be shown in different parts of a mature lichen thallus (either by their colour, specific staining, or growth patterns). The number of associated fungi apparently increases with thallus age. An overaged and melanising thallus fragment of *Peltigera* sp. is often completely infected by dark-pigmented foreign fungi, which seem to slowly degrade their morbid host.

Interestingly, clear detrimental effects exerted by bacterial infections are usually not apparent. This may be due to the different physiological requirements of bacteria to achieve rapid degradation of lichen thalli. The usual capacity to reduce metabolic activity in dry stage and recurrent dryness lichen

thalli may perhaps prevent rapid bacterial destruction. However, it is interesting to note that some lichens are hardly degraded by other organisms even under ideal circumstances (e.g., constant high humidity and temperature). Certain tropical species of *Sticta* are not affected by bacterial degradation on the ground for considerable time after falling from the tree branches (personal observation). They are not even eaten by snails, suggesting that compounds could be present to prevent biotic degradation.

Damage by invertebrates can be a threat to lichen thalli, especially in warmer latitudes. Traces of predation by snails have been frequently recognised even in crustose lichens, but unless the whole lichen is eaten, which is rarely the case, lichen thalli can also recover from grazing damage (Fröberg et al., 2006). Nonetheless, snail grazing can lower the population size by impeding the growth of juvenile lichens (Asplund and Gauslaa, 2008). The recovery from grazing certainly differs among species. Successful recovery is particularly the case with bark-inhabiting crustose lichens, which usually lose only the uppermost thallus layers, and which apparently regenerate over the entire affected area by mycelium emerging from the lower bark layers. The same may be true of crustose rock-inhabiting lichens. Regenerating forms are recognised by some morphological alterations, which have sometimes erroneously interpreted as characters of separate species. Regeneration after invertebrate feeding on fruit bodies is usually recognisable as numerous smaller fruit bodies emerging where a formerly coherent hymenium had been eaten.

Several studies indicate that invertebrate grazing is limited by the presence of secondary metabolites (Asplund and Gauslaa, 2008; Nimis and Skert, 2006; Benesperi and Tretiach, 2004). On the other hand, some lichen symbionts can survive the enteric passage of invertebrates, which then even contributes to their dispersal (Meier et al., 2002; Fröberg et al., 2001).

3. Abiotic Stress

Seemingly hostile conditions for other organisms are not necessarily extreme for lichens. Lichens can persist under the coldest, hottest, driest and highest conditions on Earth. This includes the Antarctic McMurdo Dry Valleys (Onofri et al., 2007), deserts, and high elevations in the Himalayas. Owing to their physiology, lichens are particularly well suited to survive under these conditions and can photosynthesise at temperatures far below 0°C (Schroeter and Scheidegger, 1995). Green algae in lichens do not depend on liquid water, but can take up water in the form of vapour. As the preferred habitats suggest, most lichens are highly desiccation tolerant, and can withstand drying up to 5% water content, if dried slowly (Beckett et al., 2008; Kranner et al., 2008). Lichens can also regenerate after many years of anhydrobiosis, and can be cultured even after 36 years (*Cladonia*, E. Stocker, personal communication, 2008), when DNA of these thalli becomes difficult to amplify. Lichens are also able to achieve fully active metabolism after a

very short time, while reactive oxygen species (ROS) are efficiently scavenged upon water uptake (Kranner, 2002; Kranner et al., 2003), apparently by mutually beneficial effect (Kranner et al., 2005). Especially in the dry stage, lichens can endure rather unusual treatments without loss of their capacity to revive, e.g., acetone rinsing (Solhaug and Gauslaa, 2001), or exposition in the outer space (Sancho et al., 2007).

However, in varying degrees, lichens are sensitive against certain air pollutants or any alterations of the species-specific microclimate. Their sensitivity to sulfur dioxide (SO_2) has been especially investigated for a long time, and is the basis of the use of lichens as bioindicators. Lichens exposed to a range of SO_2 concentrations display ultrastructural alterations (Eversman and Sigal, 1987; Holopainen and Kärenlampi, 1984, 1985, Holopainen and Kauppi, 1989; Plakunova and Plakunova, 1987; Sharma et al., 1982). Initial injury includes swelling of the mitochondria, stretching of the chloroplast envelopes, and deformation of pyrenoglobuli, while thylakoid stretching and degeneration of pyrenoids, chloroplast stroma, nucleus, and mitochondria in the algal partner are observed with prolonged exposure to SO_2. The fungus seems to be affected at higher concentrations of SO_2, and displays swelling of mitochondria and vesiculation of the mesosome-like organelles. At the biochemical level, reduction of protein and lipid synthesis was observed (Bychek-Guschina et al., 1999; Malhotra and Khan, 1983). A major effect of SO_2 on cells is acidification, which influences enzymatic reactions. The effect of acidification can be different among species, and recent work indicates that the buffering capacity of secondary metabolites produced by lichens may play an important role in the tolerance of SO_2 air pollution. Hauck and Jürgens (2008) showed that usnic acid makes lichens vulnerable to acidity, which could also explain the scarcity of usnic acid-producing lichens in areas of higher pollution levels. Lichen compounds could help in coping with free radicals that are formed by pollutants (Beckett et al., 2008). However, they are extracellular deposits and their role in intracellular radical scavenging has not been confirmed. Apart from SO_2, strongly oxidising air pollutants, as well as organopollutants, including pesticides can affect lichen biology (Bartók, 1999). Sensitivity to these pollutants alters the composition of lichen communities; after exposure, more sensitive species degrade more or less rapidly, while more tolerant species may persist. Macroscopically, degradation includes discolourations, mostly bleaching due to damage of the photobionts and thus reduction of secondary metabolite production. Lichen thalli also become more brittle under these conditions and finally disintegrate.

Apart from the sensitivity to common air pollutants, the great majority of lichens cannot tolerate oversaturation with water or submergence. In these cases, molds tend to overgrow the thalli, or algae evade from the thallus. Generally, the tolerance of lichens under hydrated conditions towards other stress factors is significantly low. In fact, heat is then tolerated only to temperatures up to c. 35–43°C (Beckett et al., 2008).

4. Adaptive Mechanisms to Stresses

Stress tolerance is a matter of protection and repair in lichens, and has been correlated to the ability to counteract the stress-increased production of ROS. Control of ROS is pivotal for long-term survival under recurrent stress conditions. These phenomena have recently been reviewed in detail by Kranner et al. (2008) and Beckett et al. (2008). Prevention of ROS formation and removal of ROS by enzymatic and non-enzymatic systems efficiently protect lichens. Further effects of ROS can include alteration of fatty acid composition, as a form of oxidative damage (Bychek and Bychek, 1996).

Interestingly, abiotic stress has also been associated with the formation of osmolytes (Honegger et al., 1993). Osmolytes comprise the typical polyols of lichens, which are used as transport sugars from the algal to the fungal partner. Otherwise, desiccation results in drastic shrinkage of algal and fungal protoplasts. Honegger et al. (1996) showed that under these conditions, protoplasts remain in contact with the cell walls, while gas bubbles are formed in the cell walls, which seem to prevent harmful deformation of fungal cells walls and disintegration of the symbiotic coherence. Upon rehydration, these gas bubbles rapidly shrink and disappear.

The intricate association of fungi and algae in the lichen thallus has apparently led to a mutualistic stress response. Exposed to high light in the lichen thallus, the photobiont has lower chlorophyll contents than free-living algae (which can only survive in dim light). This adaptation to high light partly avoids the transfer of energy from excited chlorophyll molecules onto triplet oxygen (ground state oxygen), thus forming the highly reactive singlet oxygen. On the other hand, the concentrations of photosynthetic pigments involved in non-photochemical quenching, and of the lipid-soluble antioxidant α-tocopherol, are higher. Also, the concentration of the water-soluble antioxidant glutathione (GSH) is significantly higher in the symbiotic assemblage than the sum taken from the isolated partners (Kranner et al., 2005). Hence, the antioxidant and photoprotective protection from ROS attack are much more effective in the symbiotic stage that in the separated partners. The latter suffered from oxidative stress due to the inefficient photoprotection in the algae and the slow GSH-antioxidant system of the fungus. Such mutualistic stress responses likely contributed to the evolutionary success of the lichen symbiosis (comprising almost 19,000 species, Feuerer and Hawksworth, 2007). The efficient coordinated and integrative stress responses may also contribute to the longevity of lichen thalli, perhaps at the cost of high metabolic turnover and growth rate.

Thallus longevity also requires certain morphological adaptations that ensure structural integrity, especially in lichens that are exposed to mechanical stresses such as wind. The filamentous and pendulous thalli of beard lichens (*Usnea* spp.) that are exposed to wind in their habitats demonstrate a unique construction principle with a flexible central cord of fungal hyphae that is extremely resistant to traction forces. Contrarily, the erect thallus of *Cladonia* spp. comprises hollow cylinders, which support stability and rigidity of the construction under humid

conditions. These principles recall technical solutions in architecture. The intercellular conglutinate matrices are typical for lichens and bind together the hyphae into plectenchymata of characteristic texture that plays an important role for the stability of long-living lichen thalli. Interestingly, such biological composites can also memorise shape: if hydrated thalli of reindeer lichens are dried under moderate compression, they restore the original shape after rehydration.

Lichens visibly react to stresses by phenotypic changes by modification of their stratified layers. Under high light, production of pigmented secondary "sun screen" products is increased, either of crystallised secondary metabolites or of dark amorphous wall pigments. Also, the thickness of the upper cortex increases with altitude in crustose lichens (e.g., *Lecanora polytropa*). Under extreme conditions, rock-inhabiting lichens can also abandon the epilithic growth and hide underneath the surface as endolithic forms (e.g., *Lecidea* species in Antarctica). Morphological convergences in different lichen lineages suggest certain anatomical adaptations to salt stress, e.g., reduction of intercellular spaces due to more tightly packed fungal hyphae (Poelt and Romauch, 1977).

Adaptation to certain stress factors can also involve a change in the ratio between the symbiotic partners according to a recently outlined "community adaptation hypothesis" (Friedman and Sun, 2005; Sun and Friedman, 2005). In lichens, this hypothesis is confirmed by the relative abundance of photobiont vs. mycobiont in *Cladonia* from Northern Finland and Southern Finland. Southern samples needed more photobionts to balance the higher respiratory needs of the mycobionts, compared to the requirements under colder temperatures of Northern Finland. This hypothesis assumes that lichens from different locations have the same photobionts. However, this is not always the case. For example, certain widespread and ecologically adaptive lichens such as *Lecanora rupicola* can associate with a wide variety of photobionts of the genus *Trebouxia* (Blaha et al., 2006). Some of them are only found in particular habitats, and seem to be species that are better adapted to local conditions. Their ability to select the locally better adapted alga, among others, increases the ecological success of this and other lichen species. This concept of habitat-driven selection can presumably be extended to the functionally active bacterial community in lichens, which may be involved in N-fixation or nutrient mobilisation (Grube et al., 2009). The possibility to switch the algal partner is not necessarily restricted to the formation of new thalli, as observations of intrathalline variation of the algal strains suggest (e.g., Piercey-Normore, 2006). In many lichens, thallus parts can regenerate, and new outgrowths from older parts of the established thalli are regularly observed (Fig. 4). Initial phenotypic observations and molecular analyses suggest that different algae can be present in regenerated lobes in the thallus centre of *Protoparmeliopsis muralis* (Grube, unpublished), which is known to host different algae in the same habitat (Guzow-Krzeminska, 2006). In these cases, the thallus centre could represent a kind of "symbiotic arena," where new partnerships could be tested for fitness under the prevailing conditions. It is thus possible that fitter combinations could in the long run outcompete the original partnerships by overgrowing during the indeterminate life of an individual mycobiont.

Figure 4. *Protoparmeliopsis muralis*. Secondary lobes formed in the thallus centre, left from apothecia-rich areas.

5. Conclusion

Lichens developed various strategies to cope with abiotic and biotic stresses. These adaptations help to avoid the break-down of the symbiotic relationship under seemingly hostile conditions. When lichens suffer from serious stress, some parts may degrade, but regeneration and restoring of thallus parts is still possible. Even after many years of cryptobiosis in the dark, cells can survive. Lichens can readily adapt to a wider range of habitat conditions by mechanisms of community adaptation, including photobiont switches or producer/consumer ratio balancing. However, deviation from the optimal humidity regime may then also include a compositional shift in the community of lichen-associated microbes.

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Biodata of **Elfie Stocker-Wörgötter**, author of ***"Stress and Developmental Strategies in Lichens"***

Associate Professor Elfie Stocker-Wörgötter, received her Ph.D. degree in Botany and Biochemistry from the University of Salzburg, Austria, 1985. There, she started as a postdoctoral assistant researching experimental and developmental aspects of the lichen symbiosis. Currently, she and her co-workers at the University of Salzburg conduct research on the "transcription of PKS genes in lichens" and on "population studies of selected lichen fungi, studying the genetic diversity of their photobionts by using microsatellite markers."

For the period of 1996–1999, she was awarded with an APART (Austrian Programme of Research and Technology) stipendium of the Austrian Academy of Science. During this time, she worked at the research laboratory of the Nippon Paint Company (Osaka, Japan), Duke University (USA), the Smithonian Institution (USA). In 2001, she was invited to work as a Research Professor at the Department of Chemistry (ANU Australian National University, Canberra, Australia) following several projects dealing with the chemistry of lichens and "lichen substances."

In the following years, she was involved in several research projects, sponsored by the European Community and the Austrian Science Foundation. In 2006, she received the Harvey Pofcher Award and a research fellowship from Harvard University and Farlow Herbarium (Cambridge, USA). Since many years, her research interests also focus on forming building processes and dynamics during thallus morphogenesis.

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STRESS AND DEVELOPMENTAL STRATEGIES IN LICHENS

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1. Introduction: by Environmental Signals

In the very beginning, the lichen was simply noticed as a very enigmatic, but uniform plant with an unusual appearance and structure.

In the end of the nineteenth and early twentieth century, by discovering "symbiosis" as a common strategy of life, lichens were recognized to have a double nature and found to be composed of "a fungus and algae/cyanobacteria" (e.g., Schwendener, 1869).

"As the result of my researches, the lichens are not simple plants, not individuals in the ordinary sense of the word; they are, rather, colonies, which consist of hundreds of thousands of individuals, of which, however, one alone plays the master, while the rest, forever imprisoned, prepare the nutriment for themselves and their master. This master is a fungus of the division Ascomycota, a parasite, which is accustomed to live upon others' work. Its slaves are green algae, which it has sought out, or indeed caught hold of, and compelled into its service. It surrounds them, as a spider its prey, with a fibrous net of narrow meshes, which is gradually converted into an impenetrable covering, but while the spider sucks its prey and leaves it dead, the fungus incites the algae found in its net to more rapid activity, even to more vigorous increase…." —from Simon Schwendener's book on lichens published in German in Basel, Switzerland, 1869. Although the choice of words by Schwendener is quite unusual for our modern thinking and understanding of parasitic and symbiotic relationships, it describes essential characteristics of the lichen in a very impressive way.

The following, ongoing controversy among scientists in the late nineteenth century, if the lichen should be considered as a whole or composite plant, had quite surprising consequences that has driven lichen research in a completely new and unexpected direction.

One of the most positive effects of the violent argumentations between "whole-ists" and "symbiolog-ists" was that the double nature of the lichen had to be proven experimentally. For this reason, first trials were initiated to separate the lichen into its two or three components—fungus and algae/cyanobacteria and afterward to resynthesize the symbiotic partners to form the composite organism again. In the beginning, such synthesis experiments, with a few exceptions

(Stahl, 1877; Bonnier, 1887, 1889), were not very successful; but finally, the finding that the algal symbionts did not differ essentially from their free-living forms (e.g., Ahmadjian, 1980), when cultured, contributed to the acceptance of lichens as unusual and fascinating symbiotic life forms.

From the historical point of view, culture experiments with lichens and their symbionts can be considered as the birth of "experimental lichenology."

By the end of the twentieth century and despite manifold experimental approaches, it was still a quite challenging task to understand all essential characteristics of the lichen's "symbiotic life style" and to extract the major properties that would fully reveal all the typical features that make up the lichen.

In agreement with most available data originating mainly from ultrastructural studies, finally the lichen had been recognized as a true symbiotic organism representing an "ideal mutualistic relationship" (e.g., Honegger. 2001). In contrast to the "mutualists," Ahmadjian (1993 and afterward), one of the most prominent authorities in experimental lichenology, defined the relationship of the partners within the lichen as a "controlled parasitism," following the former opinion of Schwendener, but giving more detailed explanations.

In the case of controlled parasitism, the benefit would be more located on the side of the fungus, which gets sugars and sugar alcohols out of the photosynthesis of the algal colonies than on the side of the algae/cyanobacteria, which seem to be "enslaved by the fungus," by being exploited—releasing essential nutrients to the fungus and being strongly handicapped in their own capacities of sexual reproduction.

However, the nutritional benefit of the fungus in the lichen symbiosis considers only two aspects, the close, physical co-existence of both partners and the physiological relationship, whereas essential structural and morphogenetic changes of the fungal mycelia adopting algal cells into a new structure, the thallus, have not been sufficiently considered.

The transition of the mycelia into the lichenized state, the formation of a lichen thallus, housing the algae in a completely organized way, by distributing them in defined layers or sometimes even hosting them in superstructures like fungal networks building up coral-like bodies can be seen as an important innovation in the fungal life style. Referring to the studies of William Sanders (Sanders, 2001a, b, 2006; Sanders and Ascaso, 1995), during evolution, lichen fungi could have advanced from pathogenity, parasitism to a far less aggressive style of life-like symbiosis taking advantage of the photosynthesis of the algae. By changing the type of contact between the partners from haustoria to intraparietal and appressorial hyphae, the algal/cyanobacterial cells could survive within the partnership and were not damaged or killed by the fungal hyphae. In a further step, they could even benefit by being integrated into a common structure that would protect them and make them fit to endure longer periods of drought, high intensities of UV irradiation, etc.

After initially, the "new, thalline," probably more simple symbiotic organism than we deal with and know nowadays (Hallbauer et al., 1977), had been established,

the morphogenetic features of the thallus could further evolve and become improved by time, creating an unimaginable diversity of growth forms. Compared with the single bionts living as unprotected single colonies, the more progressive "symbiotic associations" were certainly able to overcome the challenges for survival in a better way. As a two or even three partner system and "composite structure"; they were able to respond more sensibly to the various environmental signals in complex ecosystems than algae and fungi growing without evident partnerships. There must have occurred a transfer of essential genes, as most of the lichenized ascomycetes are not known as free-living life forms.

If both partners could "behave as one organism" they were able to break up their former limitations of distribution for trying out various habitats and ecological niches and by doing this, would regain more fitness to withstand any harsh conditions, in which, even other life forms and later on, higher plants were not able to compete.

The different lichen thalli and growth forms, we investigate nowadays, including crustose, squamulose, foliose, fruticose thalli, representing themselves as outcomes of probably several parallel on-going evolutions, could also be seen as the products of perfect adaptations and also eventually as co-evolutive processes that have stabilized the symbiotic relationships between the partners as a result of long-term interactions that became fixed and stabilized over time. By studying fungal algal relationships in more detail (Piercey-Normore and DePriest, 2001; Piercey-Normore, 2004, 2005, 2006), a lack of co-evolution was detected in lichens at higher taxonomic levels, due to a high specifity of the lichen fungus and a low specifity of algae at the population level. If a co-evolution actually had taken place, in the more strict sense as expected, it could have occurred at the population scale through adaptations of a more variable algal partner to changing environmental conditions. For this reason, we have to specify the level and scale for which a possible co-evolution between the symbiotic partners in the lichen is discussed. Probably, and most likely, we have to invent a new, more detailed terminology to describe the fungal–algal relationships in the lichens in a more adequate way than solely with co-evolution and co-adaptation.

Such morphogenetic modifications required also surprising, metabolic exchanges producing specific lichen metabolites as the offspring of a surprising chemical evolution; like depsides and depsidones that are unique molecules (e.g., Elix et al., 1984; Elix and Stocker-Wörgötter, 2008) produced by the lichen fungi, after taking up specific polyols and sugars provided by the "symbiotic" algae and cyanobacteria, the photo- and cyanobionts.

One of the major forces, which may have driven lichen evolution, fitness, adaptation, and also chemical evolution, may be summarized as responses of the lichen thalli to the various stress factors and environmental signals. We have only begun to understand these responses in the complex and rapidly changing environments of lichens (Kranner et al., 2005).

Competition alone, according to the former Darwinian hypothesis, obviously cannot be the only motor of survival in complex ecosystems. Nowadays, we could

follow a new trend and apparently more modern concept representing a reasonable and evident rule, stress situations may be better overcome by cooperation than by combat and competition.

Symbiosis and symbiogenesis, referring to Sagan and Margulis (1986), depended on various strategies of cooperation and finally on networking.

The lichen symbiosis may be the best example that cooperation, interaction (metabolic exchanges, mutual/obligate dependence between two or more organisms and finally also networking) could have succeeded over combat in functioning and often very complex ecosystems.

Under this aspect, a more modern view of the lichen symbiosis would define it as a micro-ecosystem, composed of fungus and algae as the major partners living in close association with further symbiotic and asymbiotic, procaryotic and also eucaryotic microorganisms like bacteria and epilichenic, as well as endolichenic fungi. All these random inhabitants of the lichen thalli with partially or completely unknown "symbiotic properties" could be, referring to the rule that there is no or seldom "waste" in nature, decisive for the survival of the lichen thallus in more or less "extreme environments," where diverse kinds of adaptiveness and "stress tolerance" play a very dominant role.

2. "Case Studies" and Experimental Approaches for Understanding Developmental Strategies in Lichens

Considering the "ecological definition" of a lichen, it seems obvious that studies of lichen life cycles and ontogenetic processes have always challenged experimental lichenologists to work in a field where multiple environmental factors and many "unlike" organisms interact in a complex way that until recently, has discouraged researchers to study morphogenetic processes and cell differentiation, in detail.

The morphogenesis of lichen thalli is one of the last, relatively unexplored areas in lichenology. In summary, nearly nothing is known about factors, which enable or direct an amorphous mass of fungal hyphae/mycelia and algae to form the highly differentiated, stable thallus that represents itself as a new "plant."

As in all topics in science, where many partners and parameters have to be studied entangled in a complex interplay, we have to work with model systems to understand, on one hand, general patterns of growth and development, and, on the other hand, how one individual or a small population of lichens behaves in a particular environment.

Despite the fact that lichens have evolved a high diversity of growth forms depending on the habitats they originate from, there are certainly basic, general modes of thallus formation, we have to explore first. But then, apart from those, lichen fungi have invented such a variety of peculiar morphogenetic capacities, which finally forces experimental lichenologists to do "case studies"; how one particular lichen, representing an interesting growth form "type," could be studied over a longer

period of time by simulating "conditions" that would favor its growth in a highly artificial environment, we are certainly creating in laboratory experiments.

Traditionally, lichen morphoglogy has been studied by researchers following three major subjects:

1. Ultrastructural approach: In this case, the lichen phenotype is studied as the final step of thallus development. Then, by looking at different stages and modes of cell differentiation at the ultrastructural level; and by trying to understand from the obtained data sets how the thallus could have formed, details of lichen life cycles can be reconstructed (Honegger, 1986, 1992, 1993; Hammer, 1996, 2000).
2. Ecological approach: In this case, developmental stages of lichens are traced and observed directly in the natural environments, in situ (e.g., Jahns, 1987; Schuster et al., 1985). By investigation of anatomical and structural details, life cycles are reconstructed from data sets and observations obtained outdoors, and afterward also from microscopical studies done in a laboratory. As most lichens are growing and developing very slowly, such studies require long-term experiments in the field and for this reason, only a limited number of studies focussing on lichen development has been done in the field.
3. Microbiological approach: In this case, the formation of a lichen can be studied from the very beginning, from sexual or vegetative reproduction units, like spores and soredia, isidia, and other propagation units (thallus fragments, etc.).

As a pre-step of the latter approach, the mycobionts and photobionts have to be isolated and cultured under sterile conditions. In a further step, followed by extensive trials there have to be found the right culture conditions for studying lichen symbionts and lichens under axenic and artificial conditions. Only in a more advanced step, it may be possible to reconstitute a lichen tallus and then, if this was successful, the complete life cycle of a lichen could be reconstituted under determined and controlled laboratory conditions. In this case, the steps of developmental processes are directly derived from in-vitro experiments; however, for correct conclusions and interpretations, comparisons with results from field studies are absolutely necessary to avoid misinterpretations of data sets. So, the lichenologist, studying ontogenetic processes has to combine field work and laboratory studies.

Although being mainly involved in one of the major disciplines mentioned above, lichen morphologists have always been ready to adopt methods and results from the other disciplines and by time, the previously only ultrastructural, ecological, and microbiological studies have become inter- and multidisciplinary approaches using more and more also molecular genetic methods to understand developmental processes occurring during thallus morphogenesis in a completely novel context.

In summary, we have to be aware that the lichen symbiosis is different than other kinds of symbioses, because the lichen takes on a new body shape that neither the fungus nor the alga has achieved independently, before. Without the alga, the fungus would not develop the organized tissue layers present in the lichen.

The alga is able "somehow" to "turn or switch on" the fungal genes that control morphogenesis.

Surprisingly, the genes that are supposed to be involved in regulating morphogenesis in lichen fungi have not been studied, yet. As known from other fungi (e.g., Vinces et al., 2006), especially in yeasts, morphogenetic transitions occur in response to a variety of environmental signals. Important regulators, e.g., for *Candida albicans*, can change its morphology from the yeast form into the filamentous form due to various environmental cues; these opportunities are thought to be essential for the fungus to switch from a commensal organism to a pathogen and vice versa. It would be a further challenge for a new generation of lichenologists to work on morphogenesis regulator genes in lichens.

Considering the slow growth of lichen fungi, researchers have invested many time-consuming efforts in improvements, optimization of nutrient media and culture conditions for lichen fungi and algae, trying to increase their growth rates and also to study their morphogenetic capacities during growth and resynthesis with their appropriate algal partners.

A true pioneer for testing out nutrient media and favorable culturing methods was Thomas (1939), who was the first to culture different mycobionts under various physiological conditions and temperature regimes. Thomas also recognized that environmental stress factors like alternative drying and watering the cultures, moreover temperature increases and drops by considering the different origins of the lichens regarding climatic conditions are influencing lichen growth and development. In an additional experiment, Thomas succeeded to resynthesize *Cladonia pyxidata* from its isolated and cultured bionts.

Later on, in the seventies of the twentieth century, Ahmadjian (1973) contributed an important overall approach and manual to improve culture methods for lichen fungi (mycobionts) and algae/cyanobacteria (photobionts, cyanobionts), and a first instruction how to re-constitute a lichen from its partners was presented by adapting and introducing novel microbiological methods for symbiotic organisms like lichens and their bionts.

Together with Ahmadjian, the Culbersons (e.g., Ahmadjian and Reynolds, 1961; Ahmadjian, 1973, 1980; Culberson et al., 1992; Culberson and Armaleo, 1992), we were among the pioneers (e.g., Stocker-Wörgötter and Türk 1987, 1988, 1989; Stocker-Wörgötter and Hager, 2008) to study lichen symbiosis and development based on mycobiont isolations from spores; and also the symbiotic algae by doing single-cell isolations using micropipettes and later on micromanipulators.

There are only few studies, which trace ontogenetic processes of thallus formation in laboratory experiments (e.g., Ahmadjian, 1993; Bubrick and Galun, 1985; Stocker-Wörgötter and Elix, 2006). For this reason, I will highlight some of our studies we have done recently and also in the past 10 years.

From a permanently growing culture collection of 250–300 mycobionts and 50–100 photobionts, we have selected several lichen symbionts, originating from morphologically exceptional phenotypes of lichens from the tropical, temperate,

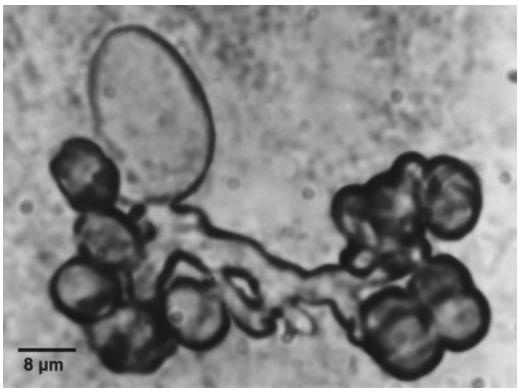

Figure 1. Germinated spore and contact with the algal symbionts in a resynthesis experiment, forming appressoria-like cell to cell contacts between hyphae and division stages of unicellular green algae.

and also arctic climatic regions for doing "case studies" and "resynthesis" experiments (e.g., with *Verrucaria macrostoma* growing on calcareous walls, Fig. 1).

By exploring the inner tropical regions of North East Brazil (Paraiba State), we studied representatives of the *Cladonia verticillaris*-complex (e.g., Stocker-Wörgötter, 1998), first in the field and later on, under laboratory conditions. In our opinion, species like, e.g., *Cladonia imperialis*, C*ladonia calycantha*, *Cladonia calycanthoides*, *C. crinita*, *C. verticillaris* sensu strictu, etc.) of the *Cladonia verticillaris*-complex are morphologically among the most spectacular lichens of South America (Fig. 3; Ahti and Marcelli, 1995). *Cladonia verticillaris* grows in the inner tropics of Brazil. It very rarely forms fruiting bodies (apothecia) during the dry season (typical climate of a savannah), as can be seen in Fig. 4a.

Figure 4b shows germinating spores (*C. verticillaris* in a laboratory experiment, grown on Lilly and Barnett-Agar (4% dextrose) under simulated tropical conditions (27–30°C, 12:12 h light dark regime). Germination depends also on exposure to "natural" day light and cultures were placed near an eastern-orientated window for 3 days; after that the mycelia were grown together with other mycobionts isolated from tropical lichens in an electronically adjusted culture chamber.

As most taxa of the *Cladonia verticillaris* complex do not produce fruiting bodies easily, they mainly reproduce asexually by conidio- or pycnospores (formed in pycnidia, Fig. 2) or vegetatively by thallus fragments that are probably also distributed by ants and mammals grazing on the sandy soils of the Cerrado (NE Brazil), where *Cladonia verticillaris* and also *C. clathrata* grow in pillow-like cushions.

The isolated mycobiont, grown in standard nutrient media like Lilly and Barnett 4% dextrose agar and Malt yeast 4% dextrose agar, forms compact, relatively undifferentiated mycelia (Fig. 5a).

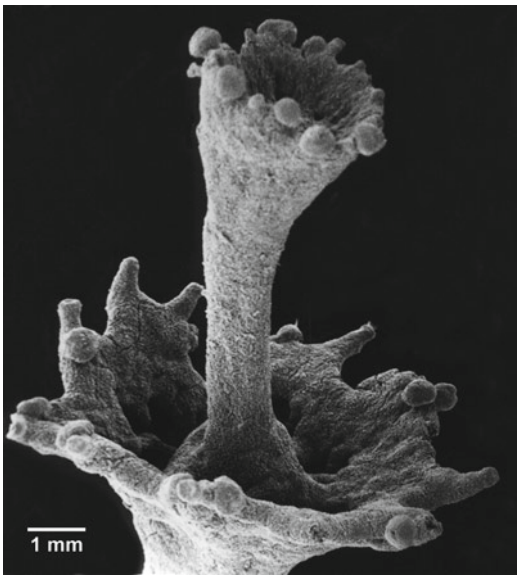

Figure 2. SEM photograph of *Cladonia clathrata*, showing the typical, centrally proliferating scyphus with pycnidia on the margins of the "verticillate" podetia.

Cladonia imperialis is one of the most spectacular and probably the tallest known terricolous lichen worldwide, and also the most exceptional among tropical and subtropical Cladoniaceae in Brazil. In the natural environment, it forms very robust and tall podetia with tiers, achieving an astonishingly high growth rate, forming podetia with a height of 15–35 cm. It grows in open areas of subtropical, cloudy mountain forests in the south of Brazil, where *Araucaria angustifolia* is the dominant tree.

Under stable culture conditions, it forms brownish pigmented and very robust mycelia (Fig. 5b), however, on Sabouraud 4% dextrose agar the aposymbiotically grown lichen fungus is able to produce podetia. Morphogenesis and podetia formation is induced (Fig. 5c, 5d) when the mycobiont cultures are exposed to higher light intensities of about 400 µE $m^{-2}s^{-2}$, a light dark regime of 12:12 h, and higher temperatures of 27:24°C during the day and night cycles. The tallest podetia, in culture, with a size of 1–2 cm (in absence of algal cells) are formed, when the agar cultures have completely dried out (during a period of several months). In the case of *Cladonia imperialis*, it was found that stress by desiccation and treatment with higher temperatures has a very positive effect on podetia morphogenesis, and also induction of the production of the typical lichen substances, like the depsidones protocetraic, fumarprotocetraric, and confumarprotocetraic acids (Fig. 6).

In another experiment, we tried a resynthesis of *Cladonia calycanthoides* from its components fungus and algae. Axenically grown, hyphal segments are

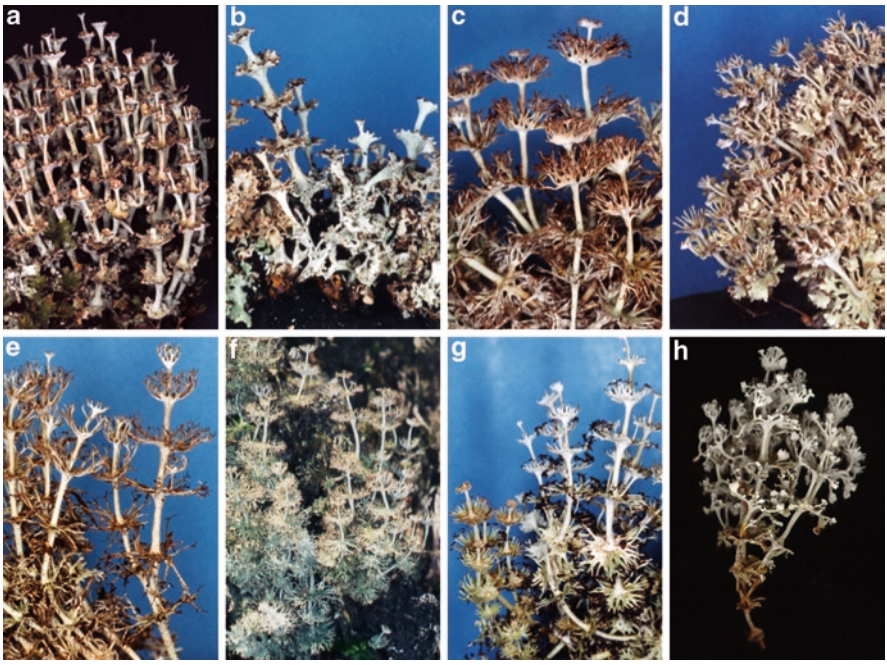

Figure 3. A selection of representatives, species of the *Cladonia verticillaris* complex. **a**. *Cladonia andesita* **b**. *Cladonia calycantha* **c**. *Cladonia crinita* **d**. *Cladonia calycanthoides* **e**. *Cladonia flagellaris* **f**. *Cladonia imperialis* **g**. *Cladonia penicillata* **h**. *Cladonia verticillaris sensu strictu* (picture from Internet: **c**. Apol: matisse.chem.uniroma1.it/apol/cladonia.jpg).

mixed with axenically grown *Asterochloris-isolates* on multiple sterilized sand substrates to avoid any further contaminations. Thallus primordia with a sorediate structure (the majority of the *Asterochloris* cells are contacted by interparietal hyphae) formed after a time span of 3 months.

After an inoculation period of 6 months, the substrate is covered by well-developed, corticated squamules. Interestingly, the podetia grow up from the margin of the squamules. Like with *Cladonia imperialis*, the early, juvenile podetia are club-shaped and only fungal. Gradually, the algae, released from the squamules "colonize" the fungal podetia. It takes about 1 year, until the scyphi are corticated with a well-arranged algal layer, typical for the adult lichen. A further year is necessary to develop the second scyphus, which grows up from the middle of the central cavity of the first. A two-storeyed verticillate podetium is formed within 2 years. If the speed of growth, in the natural environment, is as low as under laboratory conditions (simulating essential stress factors that may be also relevant in nature), a relative high age of multistoreyed and very tall thalli like that of *Cladonia imperialis* has to be predicted. Five to 25 scyphi (arranged like in a storeyed tower or pagode) are typical for thalli of *C. imperialis* and often ten tiers are formed by *C. calycanthoides*, indicating that the mature

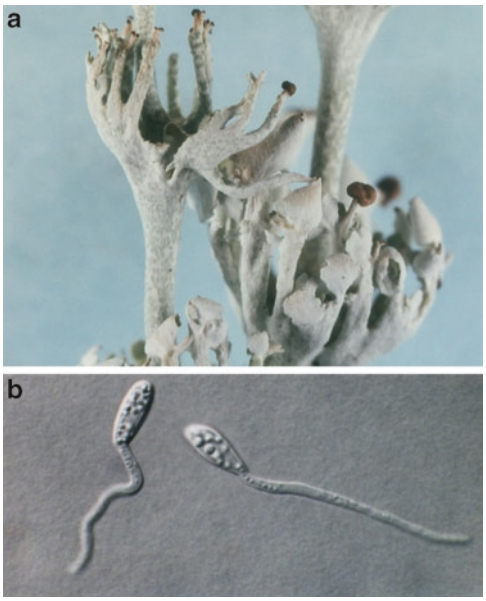

Figure 4. (**a**) and (**b**) Sexual reproduction of *C. verticillaris* forming apothecia and germinated spores (c. 10–12 μm).

lichens are about 5–20 years old. The number of tiers could probably also give a rough estimation of persistence of individual thalli and populations in the natural environment.

The production of polyketides (depsidones) within all the tested mycobionts of the *Cladonia verticillaris* complex is coupled with changes of light and moisture and also with morphogenetic processes like the induction of podetia production.

3. Are "Lichen Substances" Stress Metabolites?

There is no detailed knowledge about lichen metabolites and their exact distributions in growing thalli from the natural environment; e.g., when they are formed and deposited on the outer surface of the hyphae. In adult thalli, the complete pattern of secondary metabolites can be detected; however, variations can occur in different habitats, and in such cases, one or several metabolites are absent, depending probably on the available nutrient supplies and environmental factors.

Lichens are potential slow growers in their natural habitats, and for this reason, the formation of particular secondary compounds has not been studied in connection with a certain developmental stage.

More data about the induction of polymalonyl ("polyketide") and shikimate pathways have been obtained from laboratory studies of lichens and lichen fungi in culture (e.g., Ahmadjian and Reynolds, 1961; Ahmadjian, 1993; Hamada et al., 1996;

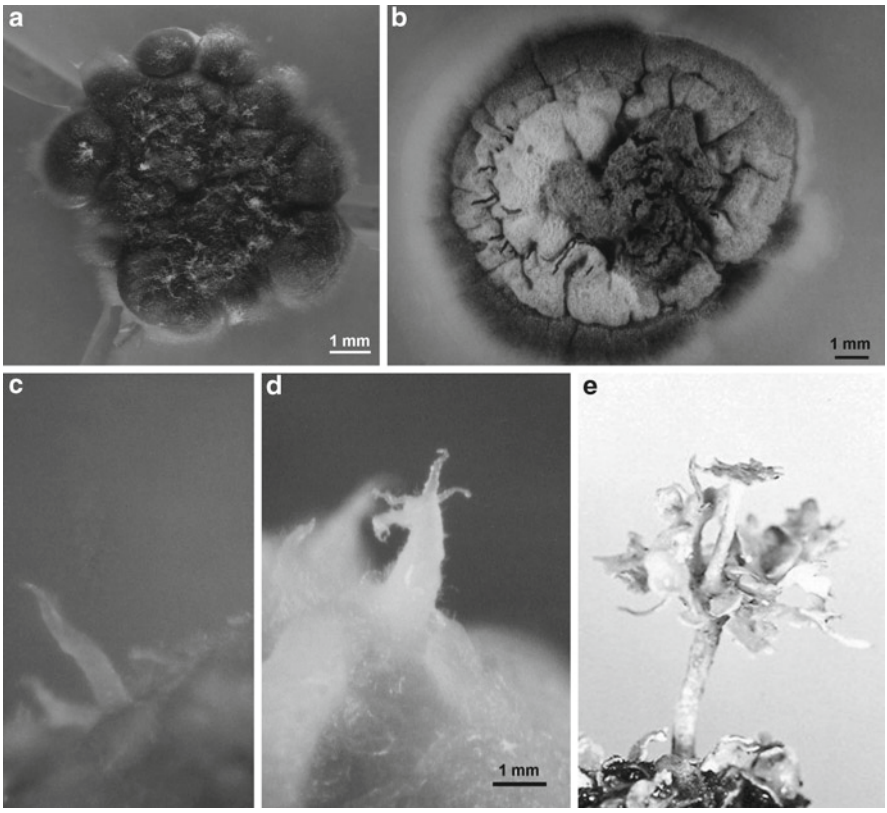

Figure 5. (**a**) cultured mycobiont of *Cladonia verticillaris*, grown from spores, on L & B (4% dextrose) agar. (**b**) Cultured mycobiont of *Cladonia imperialis* on malt yeast medium (4% dextrose) forming compact, undifferentiated structures. (**c–d**) Cultured mycobiont of *Cladonia imperialis* forming podetia in absence of algae. The morphogenesis of the "secondary" mycelium structures starting the reproductive phase; occurs synchronically with the production of the typical secondary metabolites, the "fumarprotocetaric chemosyndrome," present in almost all representatives of the *Cladonia verticillaris* complex. (**e**) Re-synthesis stage of *Cladonia calycanthoides*, forming the first, proliferating tier, 2 years old.

Hamada and Ueno, 1987; Kinoshita, 1993; Kinoshita et al., 1993; Yamamoto et al., 1985, 1995; Yoshimura et al., 1994; Stenroos et al., 2003; Stocker-Wörgötter, 2001, 2002a,b, 2005, 2008; Stocker Wörgötter and Elix, 2002, 2004; Stocker-Wörgötter et al., 2004; Brunauer et al., 2007; Hager et al., 2008).

Several investigations have shown that the chemosyndromic variation from one taxon to another seems to be genetically determined (e.g., Culberson et al., 1992; Stocker-Wörgötter et al., 2004); but that the induction of a specific and typical biosynthetic pathway leading to a particular product or related compounds (e.g., a depside, a depsidone, a dibenzofurane, a shikimic acid derivative, etc.) is also influenced by physiological and environmental factors.

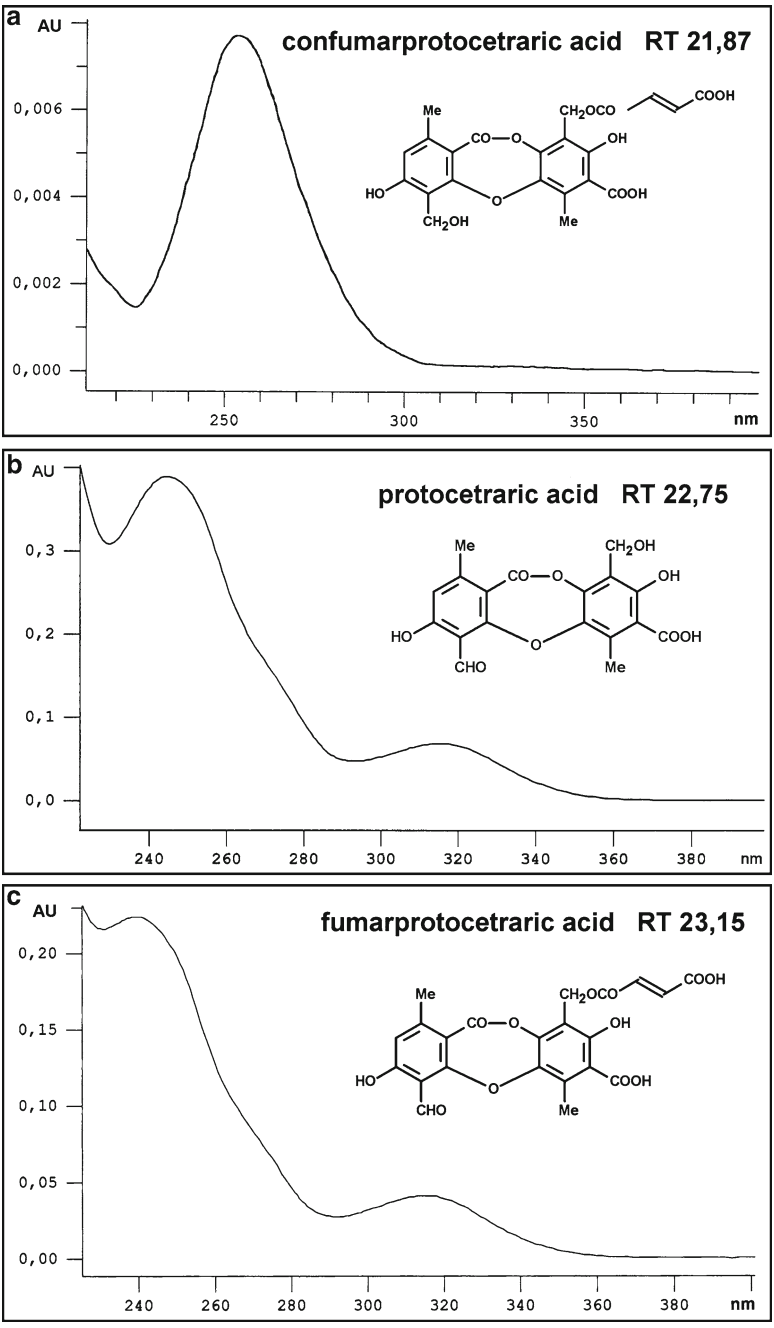

Figure 6. HPLC-UV spectra. Typical and uniform chemosyndrome – depsidones – produced by the different taxa and also cultured mycobionts (mycelia forming podetia on Sabouraud agar) of the *Cladonia verticillaris* complex. Undifferentiated mycobionts that are grown under less "stressy" and stable conditions are found to produce fatty acids, instead of lichen substances.

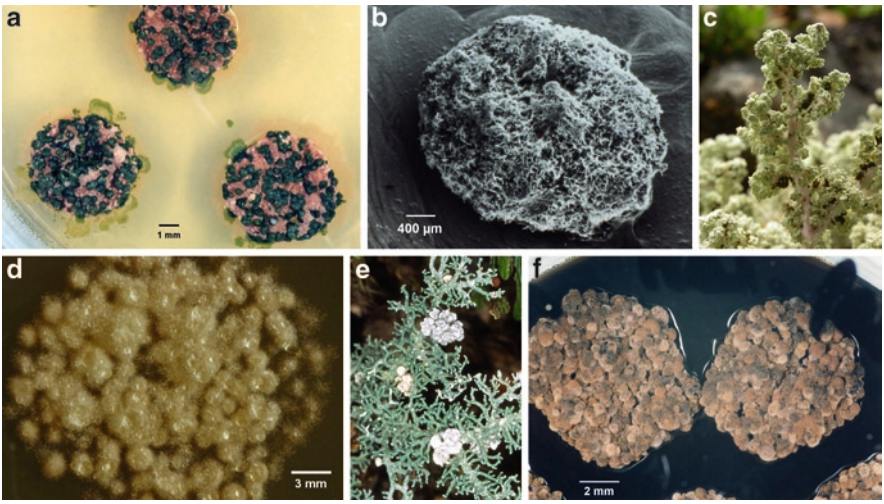

Figure 7. (**a**) Mycobiont inoculated with *Trebouxia*-photobionts does not trigger secondary metabolite production. (**b**) Mycobiont, grown in liquid medium does not produce any lichen metabolites. (**c**) Thallus of *Stereocaulon paschale* with cephalodia, representing a three partner photosymbiodeme. (**d**) Mycobiont of *Stereocaulon paschale*, under stable conditions, forming drops with fatty acids. (**e**): Thallus of *Stereocaulon ramulosum* with cephalodia, growing in the tropics and subtropics. (**f**): Mycobiont of *S. ramulosum* forming typical lichen polyketides (depsides), after exposure to periods of dryness and varied culture conditions.

Interestingly, the presence of the symbiotic partner (microalgae and cyanobacteria) do not have essential impacts on the formation of secondary metabolites in mycobiont cultures (Fig. 7a); however, extensive test series showed that the algal transfer carbohydrates (polyols, glucose) do have an important influence on the production of secondary metabolites; especially, mannitol that is formed by the fungus itself, e.g., after chemically transforming algal polyols and establishing a fungal mannitol pool.

Mannitol was shown, e.g., to influence the anthraquinone production in cultures of *Xanthoria elegans* (unpublished results). When the carbohydrates are dissolved in liquid media, high growth rates of the mycobionts (Fig. 7b) can be obtained, but no typical lichen metabolites like depsides and depsidones, are produced. In other experiments with mycobionts isolated from different epiphytic lichens (e.g., *Pseudevernia furfuracea*, *Hypogymnia physodes*, *Lobaria mediterranea*, *Physcia stellaris*) and grown on solid media, it was demonstrated that sucrose can serve as an essential carbon source for the growth and production of secondary compounds. The disaccharide sucrose is the transport form of sugars in higher plants and mosses. Many foliose epiphytic lichens are known to produce "big" thalli and much biomass; the mycobionts may also use sucrose (composed of glucose and fructose) from higher plants and mosses as an additional nutrient supply beside their own photosynthesis carbohydrates provided by the symbiotic algae/cyanobacteria. Growth of "epiphytic" lichen fungi, in media containing sucrose, can be effectively increased. One of the best media to grow mycobionts

from epiphytic lichens is Murashige Skoog medium, containing the polyol mannitol and the disaccharide sucrose as carbon sources.

Introducing and exploring new media compositions and growing the mycobionts under marked changes of micro-environmental parameters (variable adjustments of the culture chambers instead of stable conditions) has contributed to a detailed knowledge about conditions that initiate the production of secondary compounds in mycobionts.

Mycobiont cultures, isolated, e.g., from *Stereocaulon paschale* (Fig. 7c) that were grown under stable culture conditions were found to produce fatty acids (Fig. 7d) and triglycerides instead of polyketides (Molina et al., 2003; Adler et al., 2004). Moreover, production of lichen metabolites as a response to diverse types of stress has been demonstrated as essential and very successful.

The possibility that high osmotic stress caused by high percentages of sugar contents in the nutrient media would trigger lichen fungi to produce secondary metabolites has not been verified. Contrarily, it was shown that the availability of particular carbon sources like ribitol, mannitol, sorbitol, and sucrose in lower amounts is very effective.

Effects of environmental stress on lichen mycobionts is documented by culture experiments on mycobionts isolated from two species of the genus *Stereocaulon*, *Stereocaulon paschale* (Fig. 7c, 7d), and *Stereocaulon ramulosum* (Fig. 7e, 7f), representing photosymbiodemes composed of the lichen fungus, green photobionts, and cyanobacteria (cephalodia). Species of the genus *Stereocaulon* have a cosmopolitan distribution. Some of the taxa grow in arctic regions (like *St. paschale*), others are also growing in the tropical and subtropical lowlands of the southern and northern hemisphere (like *St. ramulosum*).

The mycobiont of *Stereocaulon paschale* is grown under "standard" culture conditions (20°C, 14:10 h light dark cycles) and also under varied conditions with 10°C/4°C temperature shift and 14:10 day-night regimes; every 6 weeks, the mycobionts are exposed for 1 week to −23° in a freezer in complete darkness. The mycobionts under stable conditions do not produce any secondary metabolites, but the mycelia are covered by fat drops as can be seen in Fig. 7d. Only the cultures, exposed to the varied conditions and the cold temperature treatments, produced the typical set of compounds, atranorin, lobaric, and protocetaric acids.

Studying *Stereocaulon ramulosum* from different lowland and alpine sites, it became obvious that the samples exhibit considerable chemosydromic variations on different continents and locations (Fig. 8).

Mycobiont isolates from the Australian and South American chemotype, again, are grown under stable (at 27°C) and varied conditions (day–night regime of 12:12 h and a temperature change of 29°:15°C), simulating climatic conditions in a temperate rain forest where the voucher specimens came from. Only the mycobionts grown under varied conditions, exposed to additional desiccation for 4 weeks every 6 months, produced the complete chemosyndrome in culture (Fig. 9, UV spectra of HPLC analysis). It was repeatedly shown that periods of dryness are necessary to induce polyketide production in mycobionts from rainforest

Chemotype	Country	Chemistry
I	Argentina Australia	methyl olivetolcarboxylate, divaricatic acid stenosporic acid, perlatolic acid
II	Chile	atranorin, stenosporic acid, perlatolic acid
III	New Zealand	anziaic acid, atranorin, methyl haematommate, perlatolic acid
IV	Jamaica	atranorin, perlatolic acid, anziaic acid
Va	Brazil	atranorin, perlatolic acid
Vb	Brazil	atranorin, norstictic acid

Figure 8. Chemosyndromic variations in *Sterecaulon ramulosum* from different lowland and alpine sites.

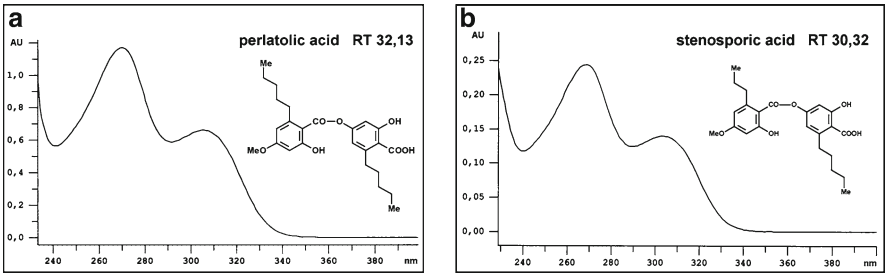

Figure 9. UV spectra of perlatolic (**a**) and stenosporic (**b**) acids: major and satellite compound of the Australian chemotype of *Stereocaulon ramulosum*.

habitats, although these results are in contrast to our former hypothesis that lichen fungi from rainforests would be adapted to very moist conditions with daily and heavy rainfalls. Obviously, the physiological stress caused by complete desiccation (even for a short time) of substrate and slow dehydration of the mycelia is important to influence cell differentiation and secondary metabolite production. Sequential loss of carbohydrates in the nutrient media slows down the growth rates, promotes cell differentiation and morphogenetic processes, which have been often observed to go along with the biosynthesis of secondary metabolites.

Another epiphytic lichen that we have studied intensively is *Cryptothecia rubrocincta* (former *Chiodecton sanguineum*), growing in tropical and also subtropical/temperate rainforests of the Americas and also in South Brazil (Fig. 10a.). It arises attention by producing an eye-catching, red pigment (naphthaquinone), called chiodectonic acid, and for this reason, it was also named the "Christmas lichen." Taxonomically, it belongs to the order Arthoniales and hosts trentepohlioid algae as the photobiont. The isolation of the lichen fungus is quite difficult, because the Brazilian specimens are found to contain several endolichenic fungi (Fig. 10c), which were occasionally isolated together with the mycobiont (e.g., Hawksworth, 1982).

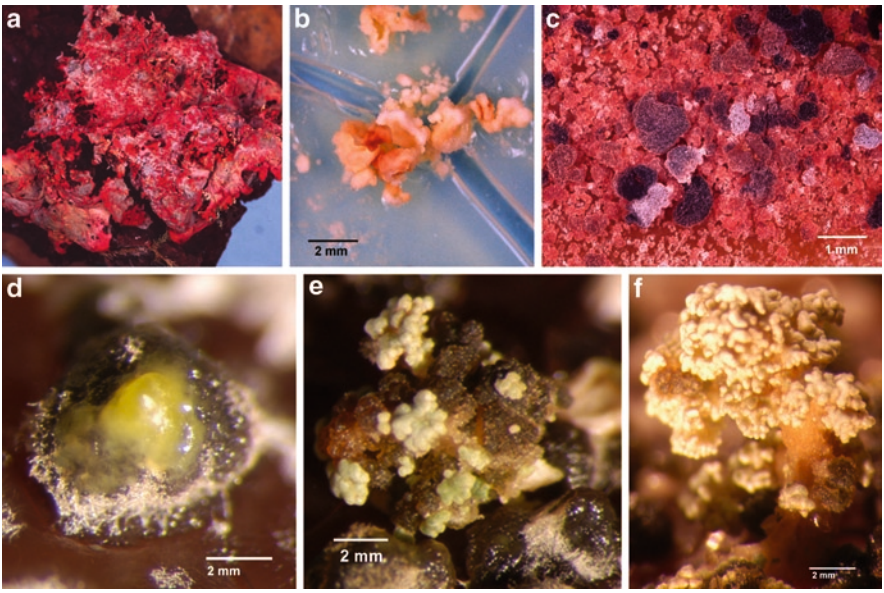

Figure 10. (**a**) The tropical epiphytic lichen *Cryptothecia rubrocincta* forms eye catching spots on various trees; (**b**) Cultured mycobiont of *Cryptothecia rubrocincta* producing chiodectonic and confluentic acids, induced by UV light and a longer period of desiccation; (**c**) Cultured mycobiont growing together with endolichenic fungi, show increased growth rates, and by exposure to UV irradiation and drought, produces a high quantity of chiodectonic acid and also confluentic acid. **d**. Very early stage of resynthesis; triple symbiosis of *Stereocaulon paschale*. The cyanobacteria are invaded by fungal hyphae. (**e**) Resynthesized thallus squamules growing on colonies of cyanobacteria, 6 months. **f**. *Stereocaulon-paschale*: cultivated young and intact thallus with cephalodia (dark areas), after an incubation time of 46 months, on sterile soil substrate.

The axenically grown mycobiont (Fig. 10c) produces the naphthaquinone chiodectonic acid only after a period of drought for 8 weeks and exposure to UV light every 4 days during 3 months of treatment. In this case, the growth of the fungus occurs very slowly and only small quantities of mycelium biomass are produced as can be seen in Fig. 10b. If the fungus grows together with two different types of endolichenic fungi (black and greyish black mycelia), the growth rate of the fungus is very much increased and 10 g of mycelium (dry weight) produce about 30 mg of chiodectonic acid and also lower amounts of the depside confluentic acid, which is also present in the lichens from nature (Fig. 11).

Another highlight was to try a resynthesis of the photosymbiodeme *Stereocaulon paschale*. A multiple, sterilized soil substrate (prepared in petridishes) is first inoculated with cyanobionts (a mixture of axenically cultured *Nostoc and Stigonema* colonies), later on (after 2 weeks) with photobiont cells, isolated from the lichen, as can be seen in Fig. 10d. Fully developed thallus squamules (Fig. 10e) are formed after 6–7 months, depending on the moisture contents of the soil substrates. Every month, the cultures are transferred to a

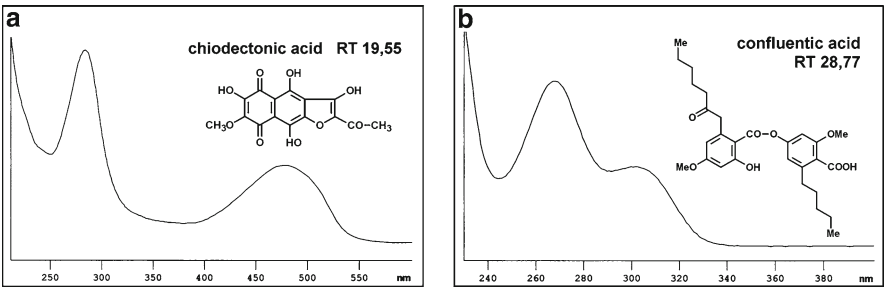

Figure 11. HPLC analyses: UV spectra of chiodectonic (**a**) and confluentic (**b**) acids, produced by the mycobiont growing together with two different types of endolichenic fungi.

refrigerator (+ 4°C) for 1 week to slow down algal growth. The gentle cold temperature treatment is necessary to obtain "healthy, green squamules," as can be seen in Fig. 10e. In the control, which was kept at a stable temperature of 15°C, an undifferentiated mass of cyanobacteria and green algae completely overgrew the lichen fungus and no differentiated thalli were formed.

Young, resynthesized thalli, bearing cephalodia are present after an incubation time of 46 months (3 years and 10 months), indicating that fully developed lichens can be actually obtained under laboratory conditions (Fig. 10f). The "tall" thalli of *Stereocaulon paschale* (with a height of 3–5 cm), we know from nature, compared with our lab experiments, seem to be quite old, even though their growth rate might be increased due to the nitrogen fixing activity of the cyanobacteria hosted in the cephalodia of the lichen.

4. Acknowledgments

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GREEN ALGAE AND FUNGI IN LICHENS:
Symbionts – But Friends or Foes?

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1. Introduction

This chapter may be similar to Don Quixote's quest in attempting the impossible, but it aims simply to attack the very common misconception that lichens are a classic example of mutualistic symbiosis between an alga and a fungus. That classic concept as a generalization is wrong both in terms of the assumed mutualism and in terms of the assumed bilateral partnership (see e.g., Ahmadjian and Jacobs, 1981). Although the classic concept is wrong on both fronts, the notion that lichens are fascinating, sometimes challenging, examples of symbiosis with parallels to the exquisite endosymbiosis of eukaryotic cells is certainly valid and lichens are intriguing organisms that some people fear are doubly in danger of losing scientific attention as organismal biology continues to ebb at research and educational institutions around the world. With declining focus on both mycology and phycology, lichenology would be expected to share in a decline in research attention. That decline in attention would be especially ironic in this time of global climate change, since "lichens are among the most sensitive organisms responding to global warming" (Aptroot and van Herk, 2007).

2. History – Some Debate and Confusion from the Start

Very early on (Wallroth, 1825), there was a clear recognition that lichens contained algae and fungi. Schwendener's dual hypothesis of lichens had been formulated and was meeting resistance early in the second half of the nineteenth century (see Honegger 2000 for a fascinating review of Simon Schwendener's life and contributions). Without going beyond that simple notion at this point, we already have the basis for some debate and confusion about "the nature and the proper treatment of lichens" that extended into the early nineteen century

(Fink, 1913, 1914). At the risk of oversimplifying semantic issues that have kept many people busy for sometime, the fundamental question is, Is a lichen an organism (a species) or is it a composite organism (dual organism) comprised of two or more organisms (two or more species) simply living together? The "vexing problem of the nature of lichens" was by no means settled when Fink wrote his extensive review in 1913 (loc. cit.). Fink's observation that all of the points of evidence "prove conclusively that the lichen is a fungus pure and simple" (Fink, 1914) could perhaps generate a chapter-length discussion even now (regarding the "pure and simple" comments), but as noted by Hill (1994) "lichens are taxonomically fungi, but biologically grouped because they occur in symbiosis with an alga (or cyanobacterium)." On the other hand, the expression of plant form in the lichen thallus can be seen as emerging at the "superorganismal level" (Sanders, 2006) and that observation certainly brings in an interesting, additional perspective on lichens "pure and simple" as their story may be! The basic question of the nature of lichens, becomes important in a practical sense in the taxonomy of lichens. It also relates to the whole topic of the phylogeny of lichens (see e.g., Gargas et al., 1995; Grube and Kroken, 2000; Piercey-Normore and DePriest, 2001; Blanco et al., 2004), but that topic is beyond the purview of this short chapter. Also, the question of whether or not a lichen is an organism begs another question which is, Is the answer to that question the same for every lichen? All of these questions lead to the need for this chapter to review a bit of the basic information about what lichens are, or, more specifically, what is the range of diversity in composition and morphology among all lichens?

3. Lichens – Composition and Morphology

The lichen as a symbiotic relationship between one alga (the photobiont or phycobiont) and one fungus (the mycobiont) is the simplest case and is applicable to ca. 95% of all lichen species (Honegger, 1991). Most commonly, the alga can be a unicellular or filamentous green alga (Chlorophyta, in ca. 85% of lichens) or a cyanobacterium (Cyanophyta or blue-green alga of the phycologists' world, in ca. 10% of lichens). Approximately 3–4% of lichens incorporate both green algae and cyanobacteria simultaneously (Honegger, 1991). Also, there is at least one species of xanthophyte (i.e., a yellow-green alga) and one species of phaeophyte (i.e., a brown alga) that are phycobionts. There also has been at least one report of a lichen-like symbiosis involving *Lemanea*, a rhodophyte (i.e., red alga; Hill, 1992). An aside: the fact that the blue-green algae are prokaryotes has led to the fallacious suggestion that they are not algae and that false notion in turn has led to a debate about the use of "phycobiont" versus "photobiont," as well as consideration of other terms including *inter alia* "cyanolichen," "phycolichen," "chlorolichen," "phaeolichen," and "xantholichen" (Lange and Wagenitz, 2003; Sanders, 2004). Since the term "algae" does in no way denote a natural – or monophyletic – assemblage, the cyanobacteria can certainly continue to be algae and "phycobiont" remains a perfectly useful and appropriate term.

The mycobionts in lichens are mostly (98%) Ascomycetes (forming ascolichens), but some are Basidiomycetes (0.4%, forming basidiolichens) or Deuteromycetes (1.6%) (Honegger, 1991). Together mycobiont fungi comprise ca. 21% of all fungi (Honegger, 1991).

This simple two-partner composition is certainly not the only situation among lichens! Some lichens may have two (or more) phycobionts (Hawksworth, 1988; Hyvarinen et al., 2002). In addition, there are numerous lichenicolous fungi and lichenicolous lichens creating even more complex symbiotic situations (Hawksworth, 1988; de los Rios and Grube, 2000), but we should not let such temporary (sometimes terminal!) associations that are infections complicate the situation. Similarly, the report of diatoms living inside the thallus of *Coenogonium linkii* in Panama and French Guiana (Lakatos et al., 2004) is a fascinating further complication of the symbiotic arrangements that occur in nature, but it doesn't complicate the notion of basic lichen composition. The topic of composition is complicated, though, by the fact that, in rare instances, the actual composition of lichens may change during the life and development of the lichen (Hawksworth, 1988). As noted by Richardson (1999), "The spectrum of interactions is fascinating" and the evolutionary forces at work in the specialization of these relationships are interesting as well (see e.g., Yamamura, 1996; Douglas, 1998).

Regardless of the simple or slightly more complex composition of the lichens, the range of morphologies and physical interactions for lichens is significant. At one end of the spectrum, there are loose associations of fungi and algae such as *Coenogonium* (Meier and Chapman, 1983) that are either considered to be "lichen-like associations" ("mycophycobiosis," Hill, 1992) or not lichens at all (Ahmadjian, 1993) or are considered to be the simplest "borderline," least-developed lichens (Hawksworth, 1988, see also Sanders and Lucking, 2002; Grube and Hawksworth, 2007). These minimalist lichens may represent only a small proportion of all lichen species, but it is interesting to note that perhaps fewer than half of all lichens achieve a complex thallus (Honegger, 1992). Nevertheless, the great majority of lichens have distinctive morphologies and many have highly structured physical relationships with the symbionts. These more structured lichens fall into several categories based on the distinctive morphology.

The four major morphological forms of lichens are crustose, foliose, fruticose, and squamulose. Crustose lichens are generally thin crust-like thalli that closely and tightly adhere to the substrates (example: *Acarospora*). The foliose lichens are somewhat leaf-like with flat sheets of thallus or lobes that are above the substrate to which they are connected by rhizines (example: *Lobaria*), and the fruticose lichens are branched and tubular, somewhat twig-like (example: *Ramalina*). Squamulose lichens are scale-like because the edges of the thalli breakaway from the substrate and rise up (example: *Psora* and the basal portion of *Cladonia*). There are other distinctive morpho-types as well. Filamentose lichens are simply filamentous (example: *Coenogonium*) and leprose lichens are described as powdery (example: *Lecanora*). The "jelly lichens" become gelatin-like when wet (example: *Collema*).

4. The Algal–Fungal Relationships

Having briefly reviewed some of the basics about lichens, we are now ready to broach the topic of relationships, and in so doing, bring in the theme of this volume, *viz.* stress. The classic, simplistic notion about lichens can be paraphrased as follows: *Lichens are produced by a symbiotic association of an alga and a fungus that mutually benefit each other. The alga provides nutrients produced via photosynthesis to the fungus and the fungus offers protection from desiccation and the ability to occupy new habitats to the alga.*

It is the concept of mutual benefits in this partnership that spring to mind whenever lichens are cited as examples of symbiosis. This concept does not conjure up a sense of stressful relationships between phycobiont and mycobiont. This view is not limited to the lay public, but is even evident among many scientists who think that lichens and mutualism are linked (although one would hope that most mycologists, phycologists, and lichenologists all avoid this false generalization!). In a very interesting report on "The Origins of Synergistic Symbiosis," Frank (1995) refers to "mutualisms such as lichens," and this excellent paper is simply one illustration of the fact that even in the scientific literature, the generalized notion that lichens are examples of mutualism *per se* is not uncommon. Yet, interestingly, rather early on, authors had noted that a less than benign relationship could or did exist in lichens (see e.g., Peirce, 1899 and Danilov, 1910). Ultrastructural research on *Strigula elegans* Fée (Müll. Agar.) (Chapman, 1976) made it clear for us that out-right enslavement and "abuse" of the phycobiont was clearly typical of some lichens. Thus, this short chapter is intended to be a brief focus on lichens wherein the symbiosis is indeed a stressful parasitism (from the phycobiont's point of view) and not the textbook story of mutualism in lichens.

Strigula is a folicolous lichen created when an ascomycetous fungus invades or infects the subaerial, folicolous green alga *Cephaleuros* Kuntz (Chapman, 1984). As shown in the ultrastructural study, fungal haustoria penetrated the phycobiont cells and "violated" cells were often shown to have deteriorated. Similar observations on 13 subtropical crustose lichens (Matthews et al., 1989) confirmed penetration of the phycobionts by fungal haustoria in all cases. In all cases, the algal cell wall invaginates ahead of the invading haustorium to some extent, and in some cases, the algal wall appears to thin or disintegrate around the tip of the haustorium. The ultrastructural observations on the condition of the phycobiont cells seems to parallel those made on *Strigula elegans* (Chapman, 1984). That is, haustoria are seen in normal, healthy-appearing algal cells and thus the mycobiont is parasitizing healthy cells and is not simply saprophytic on old dying or dead cells. In *Strigula elegans*, there was a correlation between the amount of haustorial penetration (i.e., size and/or number of haustoria in phycobiont cells) and the amount of algal cell morphological deterioration. Clearly, the alga was being physically harmed by the fungus. In the lichens studied, there is no question that the presence of haustoria indicated a parasitic behavior. Healthy cells are attacked and in terms of stress, this volume's focus, it is quite clear that

the symbiosis is a stressful condition for the algal thallus that is losing viable cells and gaining nothing in return.

One of the "gains" often mentioned for the phycobiont, is access to new habitats occupied by the lichen, but not tolerable to the algal alone. In terms of protection from desiccation and/or high light intensities, there may be many examples where some algae do benefit in terms of expanded habitats *via* lichenization (see e.g., the interesting study of antioxidants and photoprotection in lichens by Kranner et al., 2005), but for foliicolous algae that are lichenized *in situ*, this benefit does not exist. That is, the alga normally grows on the leaf and was growing there before being parasitized, it had not gained any new habitat in the process of being enslaved by the fungus. When fully lichenized, *Cephaleuros* no longer produces specialized zoosporangia that abscise to be distributed by wind, rain, insects, and arachnids to other host plants. Thus, its own very effective asexual reproduction process is closed down. To the authors' knowledge, there is little known about the direct propagation of *Strigula*, but we know of no cases where the lichen occurs in a habitat not already occupied by its phycobiont. It is likely that all lichens in which the phycobiont is a green alga in the order Trentepohliales (including *Trentepophlia* C. P. F. Martius, *Phycopeltis* Millardet, *Cephaleuros*, and the arguable taxon *Physolinum* Printz [Chapman 1984]), constitute examples of fungal parasitism on the phycobionts with no extension of habitat range. So, in all of these cases, basically the alga is harmed and gains no benefits whatsoever from the clearly non-mutualistic symbiosis. The fungus, on the other hand, not only benefits from parasitic consumption of algal cells, but may also benefit from the alga's attack on the host plant. *Cephaleuros* species always grow beneath the cuticle of the host plant and presumably are at least "water parasites;" however, a species like *Cephaleuros parasticus* grows into the host plant tissue and parasitizes the leaf presumably gaining nutrients as well as water. When a parasitic *Cephaleuros* is itself parasitized to form a lichen, one may actually have a case of "hyper-parasitism." There are many of examples of documented clear-cut parasitism of the phycobionts in lichens, and clearly lichens as a group are not perfect examples of mutualism. Nevertheless, the lichen example (i.e., the simplistic and incorrect version) of a fungus and alga living happily together in a mutualistic symbiosis is so useful and so well ingrained, it is unlikely that the "plight" of the enslaved and abused algae in many lichens will ever receive much attention or concern.

5. Concluding Comments

According to Richardson (1999), the first use of the term "symbiosis" in a biological context was in a paper on lichens by Frank (1877) and not, by-the-way, by de Bary (1879 cf. Richardson 1999). Although in these early works, the delineations among mutualism, commensalism, and parasitism were clear, it was apparently the numerous lichen examples of mutualism cited by de Bary (see Goff,

1982; Lewin, 1982) that help lead to the faulty notion that this short chapter tries quixotically to correct. We have a situation in which "lichens are invariably cited as examples of where there is mutual benefit for the partners, although the experimental data to support this are weak" (Richardson, 1999) and ultrastructural studies of many lichens clearly document "phycobiont abuse" (e.g., Chapman, 1976; Matthews et al., 1989; Chapman and Waters, 2002). But like jousting at windmills, changing well-known "facts" is hard to do. As long as the symbiotic nature of lichens continues to generate some measure of interest in, and study of, these fascinating organisms, the over-focus on mutualism in lichens will simply have to be tolerated, like it or not. But we do agree with Ahmadjian (1993) who wrote "The mutualistic myth of lichens hinders our better understanding of these symbioses. To call a lichen association mutualistic is similar to believing that domestic cattle and humans have a comparable relationship because we provide them with food and shelter and increase their populations before we slaughter them." Well put! Now to be fair, it is clear that a mutualistic relationship between algal and fungus can occur (see e.g., Hill, 2001; Kranner et al., 2005), so the Ahmadjian quotation may be a bit extreme; however, the simple and incorrect notion that lichens are all examples of a mutualistic ("stress-free") symbiosis really must be put to rest!

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GREEN BIOFILMS ON TREE BARKS: MORE THAN JUST ALGAE

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1. Introduction

Algae are a very diverse group of organisms ranging in size from unicellular microalgae to giant kelps. They are equally successful in settling aquatic as well as terrestrial and aerial habitats.

Those algae living permanently in terrestrial environments on different substrates exposed to the atmosphere are so called aero-terrestrial algae. Aero-terrestrial algae are divided in a terrestrial and in an aerophytic group (Ettl and Gärtner, 1995). Aerophytic algae cover natural surfaces like tree bark or live epiphytically, e.g., on leaves. They also colonize artificial surfaces like walls, facades, or roofs of houses.

Terrestrial algal species grow in or at the surface of moistly wet soil. Some studies assume an association between algae and higher plants in the rhizosphere (Aleksakhina, 1971; Shtina, 1968; Gollerbach and Shtina, 1969).

Terrestrial algae may be also part of soil crusts, predominantly in semiarid regions such as deserts or steppe (Büdel et al., 2007), but also in temperate climates (Knapen et. al., 2007). Algae living at the soil's surface can be dispersed by air currents and thus can colonize also other habitats. Aerophytic algae are sometimes called airborne algae and spend at least part of their life cycle floating in the air. Aerophytic algae have been detected in heights up to 2000 m and are distributed worldwide by air currents (Reisser, 1999; Sharma et al., 2007) and may also contribute to particulate matter.

Most aero-terrestrial algae have a size of about 5–50 µm and are affiliated to the Chlorophyceae or Cyanobacteria. Some algal species are coccoid, some grow as filaments (Fig. 1b) or as packages (Fig. 2c) in association with other algal cells of that species. In general, aero-terrestrial algae have to be adapted to different and fast-changing environmental conditions as are offered by, e.g., micro-niches in a soil environment. Other survival strategies are the formation of biofilms, soil crusts, or symbiosis formation as photobionts in lichens.

2. Biofilms

Biofilms are associations of different kinds of microorganisms–such as algae, fungi, protozoa, cyanobacteria, and heterotrophic bacteria—that colonize border areas as, e.g., between a solid substrate and water or the atmosphere or between

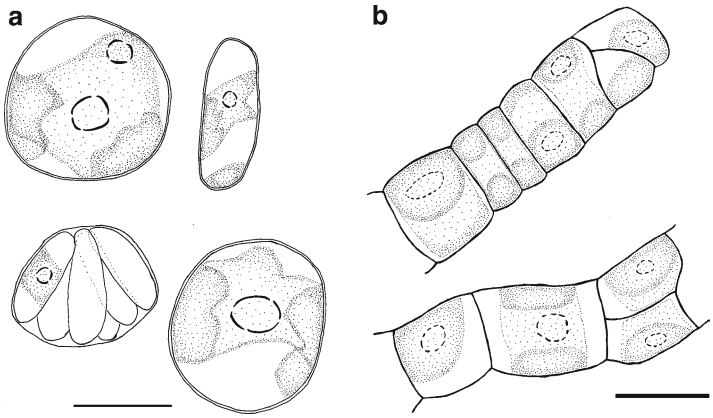

Figure 1. (**a**) Coccoid algal cells of *Elliptochloris sp*. (**b**) Filamentous growing cells of *Klebsormidium montanum* (scale bars: 10 µm).

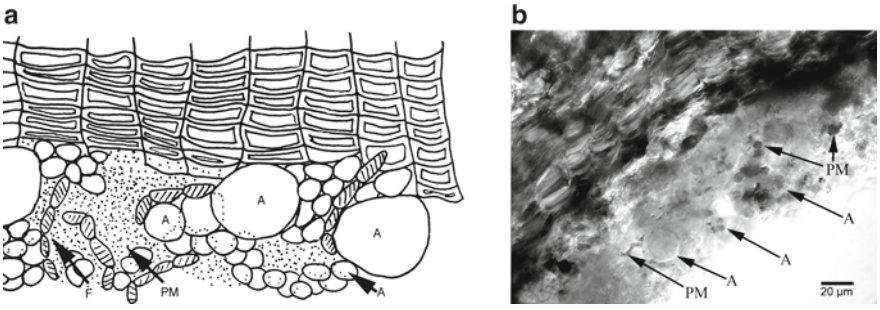

Figure 2. (**a**) Schematic drawing of a green biofilm on tree bark. (**b**) cryo-cut of a green biofilm; A-alga, PM-particulate matter, F-fungi.

the atmosphere and water. In comparison, lichens are always formed by asco- or basidiomycetes and an algal or cyanobacterial photobiont or both. Inside a lichen thallus, algal cells are arranged in dedicated layers to obtain an optimal light regime and gas exchange for photosynthesis (Honegger, 1990, 1991). Algal cells can be carried along by growth processes of the mycobiont (Honegger, 1993).

Assumptions say that most microorganisms are organized in biofilms. Under extreme climatic conditions as in deserts or in the nival and subnival zone in high mountain areas, biofilms on solid substrates represent the main biomass. Biofilms or biofilm-like systems are also important in other habitats and their study will therefore contribute to a better understanding of metabolism in different kinds of ecosystems. Thus, biological soil crusts form a special kind of biofilm. They are mainly formed by cyanobacteria, filamentous or coccoid algal

cells, fungi, and soil particles (Büdel, 2001). The association between those organisms could be of symbiotic or parasitic character.

Crusts formed by algae and fungi are far spread, especially in arid and semi-arid regions and habitats (Büdel, 2001). They can cover extreme habitats like deserts or rock surfaces, can effect water flow and erosion, and enhance the stability of soil aggregates (Eldridge and Greene, 1994). Algae and cyanobacteria can aggregate soil particles to stable macroaggregates (Bailey et al., 1973; Evans and Johanson, 1999). Studies in the Negev Desert in Israel showed a great influence of those crusts on water distribution (Achituv, 2001). They reduce the evaporation rate and slow down the water runoff.

Biofilms best studied until now are part of aquatic habitats. Those associations of different bacteria, protozoa, and algae can colonize water pipes or sewage systems and form medical biofilms that sometimes may cause serious diseases.

In terrestrial habitats that are exposed to the atmosphere, biofilms and biological soil crusts play a key role. As one example, rocks that are freshly exposed to the atmosphere, e.g., after volcanic eruption are colonized by microbial communities first. Those form, for sometime, the main part of the biomass in the newly established ecosystem. As a matter of fact, colonization of freshly established or exposed surfaces is an event more common than usually assumed. Every man-made surface is sooner or later covered by biofilms regardless to the physical or chemical nature of the substrate. The colonization of, e.g., facades depends on mineral grain, cementing material, pores, and fissures. One typical way of colonization starts by fungal hyphae that penetrate the substrate, followed by algae, bacteria, and yeast-like fungi (Gorbushina, 2007). Some biofilms cover the surface, others penetrate the substrate. Especially, the second form may cause serious damages to historical buildings and monuments. Filigree structures may be destroyed by biofilms by overlaying them or peeling them off (Crispim et al., 2003).

Biofilms can be found not only on rocks, monuments, facades, and roof tiles of buildings but settle also on other substrates such as plastics, coated metal, glass, and wood. Görs et al. (2007) could show that terrestrial biofilms on artificial surfaces are composed of a great variety of algal and fungal organisms by measuring the chlorophyll content and doing genetic analyses. Among algae they identified, e.g., *Stichococcus* sp., *Chlorella* sp., and *Lobosphaera incisa*.

3. Algal Biofilms on Tree Bark

In terms of area available for colonization by biofilm-forming organisms, bark of plants presents the most important terrestrial habitat offering a plethora of ecological niches depending on structure and exposition of bark. Accordingly, they are inhabited by a great variety of organisms (Reisser, 2001). Especially, airborne aero-terrestrial algae are able to colonize tree bark. Under due conditions, those micro-algae can form a green biofilm, that is even visible without a microscope. Up to now, the study of those biofilms centered mainly on the taxonomic affiliation of participating algae

whereas data on their ecophysiological features are rather limited. In the following, we want to give an overview on the current knowledge on green biofilms on tree bark supplemented with own studies on interaction between algae and fungi in those biofilms. For this purpose, we define "green biofilms" (Fig. 2) as associations made up primarily by green algae and cyanobacteria. Other organisms such as fungi are always present but do not dominate, neither in term of biomass or structure, so that lichens are excluded by definition.

3.1. TAXONOMY

The taxonomy of green biofilms on tree barks has been studied since the beginning of the twentieth century. For an overview on most common species, see Table 1.

Table 1. Comparison of aero-terrestrial algal species of Europe, North America, and Japan/Korea (cyanobacteria are excluded). The algal species of our own study are presented in correlation to different concentrations and groups of airborne pollutants. Other authors showed no response to air quality.

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Rhodophyta						
Porphyridium purpureum (Bory) Ross						x
Dinophyta						
Rufusiella sp.						x
Chrysophyta						
Bacillariophyceae						
Achnanthes exigua Grunow in Cleve & Grunow				x		
Cocconeis sp.				x		
Hantzschia amphioxys (Ehrenberg) Grunow					x	x
Navicula mutica Kützing				x	x	x
Pinnularia sp.				x		

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Xanthophyceae						
Botrydiopsis intercedens Pascher				x	x	x
Botrydium granulatum Greville.						x
Bumilleriopsis peterseniana Vischer & Pascher						x
Chlorocloster terrestris Pascher						x
Ellipsoidion oocystoides Pascher				x		x
Gloebotrys piriformis Reisigl						x
Vaucheria geminata (Vaucher) De Candolle						x
Vaucheria sessilis (Vaucher) De Candolle						x
Xanthonema debile (Vischer) Silva						x
Xanthonema ulotrichoides (Pascher) Silva				x		
Eustigmatophyta						
Eustigmatos vischeri Hibberd						x
Monodopsis subterranea (J.B. Petersen) Hibberd						x
Chlorophyta						
Chlamydophyceae						
Borodinellopsis sp.					x	
Chlamydocapsa lobata Broady				x	x	

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Chlamydomonas sp.					x	
Chlorococcum hypnosporum Starr				x	x	
Macrochloris multinucleatum (Reisigl) H. Ettl & G. Gärtner						x
Neospongiococcum sp.					x	
Palmellopsis sp.					x	x
Radiosphaera minuta Herndon						x
Tetracystis sp.				x	x	
Chlorophyceae						
Ankistrodesmus sp.					x	
Apatococcus lobatus (Chodat) J. B. Petersen		x		x	x	
Bracteacoccus minor (Chodat) Petrová				x	x	x
Cedercreutziella savoniensis Vischer				x		
Choricystis minor (Skuja) Fott						x
Chlorella ellipsoidea Gerneck	x			x		x
Chlorella fusca Shihira & Krauss						x
Chlorella kessleri Fott & Nováková				x		
Chlorella luteoviridis Chodat						x
Chlorella saccharophila (Krüger) Migula	x			x		x
Chlorella sp.				x	x	

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Chlorella vulgaris Beijerinck			x	x		x
Chlorosarcina sp.				x		
Chlorosarcinopsis pseudominor Groover & Bold				x	x	
Coccomyxa confluens (Kützing) Fott				x	x	
Coccomyxa gloeobotrydiformis Reisigl						x
Coccomyxa subglobosa Pascher		x				x
Coenochloris signiensis (Broady) Hindak		x				
Desmococcus endolithicus Broady & Ingerfeld		x			x	
Desmococcus olivaceus (Pers. ex Ach.) Laundon				x		x
Dictyochloropsis irregularis Nakano & Isagi				x		
Dictyochloropsis reticulata (Tschermak-Woess) Tschermak-Woess						x
Diplosphaera chodatii Bialosuknia em. Vischer		x	x	x		x
Elliptochloris bilobata Tschermak-Woess			x			
Elliptochloris subsphaerica (Reisigl) H. Ettl & G. Gärtner	x	x	x	x		x
Elliptochloris reniformis (S. Watanabe) nov. comb.						x
Ettlia alveolaris (Bold) H. Ettl & G. Gärtner						x

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Fottea stichococcoides Hindák						x
Fritschiella tuberosa Iyengar						x
Geminella sp.					x	
Gloecystis sp.				x		
Haematococcus pluvialis Flotow em. Wille	x					
Interfilum paradoxum Chodat & Topali	x	x	x			
Iwanoffia sp.						x
Jaagiella alpicola Vischer				x		
Leptosira sp.					x	
Lobosphaeropsis lobophora (Andreeva) H. Ettl & G. Gärtner			x			
Lobosphaeropsis pyrenoidosa Reisigl			x			
Muriella sp.					x	
Neochloris pyrenoidosa Arce & Bold			x			
Neochloris sp.					x	
Neochloris texensis Archibald			x			
Oocystis sp.					x	
Phycopeltis arundinacea (Montagne) De Toni						x
Planktosphaerella sp.					x	
Protoderma sp.				x		
Protosiphon botryoides Klebs						x

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Pseudochlorella pyrenoidosa (Zeitler) Lund				x		
Pseudococcomyxa simplex (Mainx) Fott				x		x
Pseudoschizomeris mucosa Broady		x				
Scenedesmus sp.					x	
Scotiellopsis terrestris (Reisigl) Punčochářová & Kalina					x	x
Spongiochloris sp.					x	
Trochiscia aspera (Reinsch) Hansgirg						x
Ulvophyceae						
Microthamnion kuetzingianum Naegeli						x
Pleurastrum sp.					x	
Trebouxia sp.					x	
Trentepholia aurea (Linné) Martius				x	x	x
Trentepholia sp.					x	
Trichosarcina sp.					x	
Ulothrix verrucosa Lokhorst		x		x		x
Charophyceae						
Klebsormidium crenulatum (Kützing) H. Ettl & G. Gärtner		x	x			
Klebsormidium dissectum (Gay) H. Ettl & G. Gärtner	x	x	x	x		x
Klebsormidium flaccidum (Kützing) Silva, Mattox & Blackwell	x	x	x	x	x	x
Klebsormidium mont anum (Skuja) S. Watanabe			x			

(continued)

Table 1. (continued)

	Europe				**North America**	**Japan Korea**
	Own studies			**1) 2) 3) 4) 5)**	**6) 7) 8)**	**9) 10) 11) 12)**
Concentration of particulate matter	**– –**	**0**	**+ +**			
Concentration of ozone	**+ +**	**0**	**– –**			
Collection site	**A)**	**B)**	**C)**			
Species						
Klebsormidium sp.					x	
Klebsormidium sterile (Deason & Bold) Silva, Mattox & Blackwell		x	x			
Klebsormidium subtile (Kützing) Tracanna ex Tell		x				
Stichococcus allas Reisigl		x				
Stichococcus bacillaris Nägeli		x	x	x		x
Stichococcus chorelloides Gritzesco & Péterfi				x		
Stichococcus minutus Grintzesco & Péterfi			x			
Stichococcus sp.					x	
Stichococcus undulatus Vinatzer			x			
Zygnemaphyceae						
Cosmarium sp.						x
Cylindrocystis brebissonii Meneghini						x
Mesotaenium sp.						x
Zygogonium ericetorum Kützing						x

(All taxonomic features are according to Ettl and Gärtner, 1995.)
1) Schlichting, 1975; 2) Vischer, 1960; 3) Steiner and Schulze-Horn, 1955; 4) Reisser and Houben, 2001; 5) Cambra and Hernández-Martiné, 1989; 6) Cox and Hightower, 1972; 7) Wylie and Schlichting, 1973; 8) Graham et al., 1981; 9) Handa et al., 1991; 10) Mrozinska, 1990; 11) Akiyama, 1961; 12) Nakano et al., 1991
+ + High concentration of airborne pollutant; - - Low concentration of airborne pollutant; 0 Moderate concentration of airborne pollutant; (A) rural region; (B) outskirts of a town; (C) city center

Green algal species like *Elliptochloris subsphaerica* Reisigl, *Stichococcus bacillaris* Grintzesco & Péterfi or *Apatococcus lobatus* (Chodat) J.B. Petersen are far spread (Fig. 3).

Brand (1925), Edlich (1936), and Geitler (1942) described one of the common species living on tree bark by light microscopic studies. The current name of that species is *Apatococcus lobatus*. At the beginning of the twentieth century,

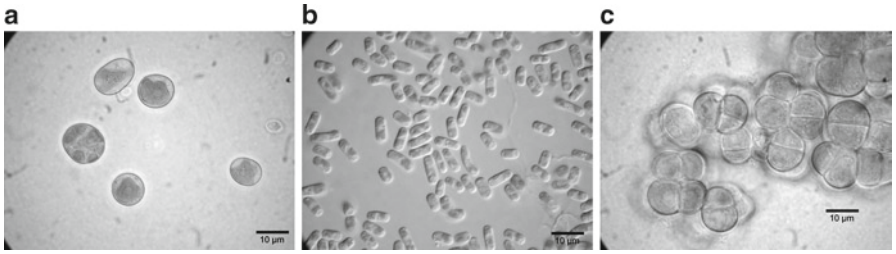

Figure 3. (**a**) *Elliptochloris subsphaerica*, (**b**) *Stichococcus bacillaris*, (**c**) *Apatococcus lobatus*; (scale bars 10 µm).

most algal species on tree barks were known as *Protococcus* or *Pleurococcus*. Brand (1925) started to divide those algal species in those ones living terrestrial, those living aquatic, and in species in between. He started to describe and divide taxonomic groups, like *Pleurococcus*, *Desmococcus*, and *Apatococcus*. Today, *Apatococcus lobatus* is a well-studied aero-terrestrial algal species. It is part of nearly every green biofilm. Cells form typical packages that can be identified by standard microscopic equipment (Fig. 3c).

The chloroplast is parietal and sometimes flabby. By light microscopy, a pyrenoid cannot be identified unequivocally. However, TEM studies show small structures that possibly can be taken for a naked pyrenoid (Gärtner and Ingolič, 1989).

3.2. ECOPHYSIOLOGY

3.2.1. Biomonitoring Features

Only few systematic studies exist on ecophysiological features of green biofilms on tree bark. Schmidt (1927) studied the bark of *Fagus sylvatica*, *Aesculus hippocastanum*, *Robinia pseudacacia*, and *Quercus robur* and noticed that rain water was not taken up by algal biofilms. In laboratory experiments, he realized that algal biofilms were mainly formed by *Apatococcus lobatus* and could only be wetted when rainwater was applied at about 6,000 mm cm^{-2} per hour. His experiments let him conclude that those biofilms could survive only by assimilating vapor of water during seasons of high humidity.

Winter-Günther (1934) noticed that the growth rate of biofilm-forming algae changes according to different light intensities, different humidity, and temperature. She investigated temperatures between 10°C and 30°C and relative humidity between 50% and 100% and assumed that algal cells survive high temperatures (30°C) only in combination with low humidity (50%).

Edlich (1936) was the first to look at the differently structured surface of a tree bark as kind of extreme habitat with various types of microclimates. During the day, there are fast changes between extreme high and low humidity and temperature. He did one of the first studies that examine the physiology of aero-terrestrial algae under natural conditions by lab experiments. Edlich measured temperature and humidity of different structured bark of *Tilia* sp., *Acer pseudoplatanus*, *Quercus* sp.,

and *Ulmus* sp. and monitored temperatures from 15°C to 45°C at the same time around the tree trunk. In his lab experiments, he applied temperatures between −80−20°C and 30–60°C and humidity from 0% to 100% to observe the environmental stress tolerance of biofilm-forming algae.

In our studies (Table 1), we observed that the species composition of green biofilms on trees differed between rural regions (Table 1 – A), moderate polluted collection sites (Table 1 – B), and a higher polluted area (Table 1 – C) (city center of Leipzig, Germany). That study showed that aero-terrestrial algae may be used to detect differences in air pollution (ozone and particulate matter) even on a regional scale (Freystein et al., 2008). The presence or absence of common or exceptional species reflects differences in algal ecology. We assume that some algal species are able to use particulate matter as a kind of nutritional source. Thus, the highest algal diversity was detected at the highest polluted collection site. Similar results were described by Poikolainen et al. (1998) in Finland. They registered that the more nutrient-rich the collection site, the greater the abundance of algae. In conclusion, the establishment of a biomonitoring system based on aero-terrestrial algae should be possible.

3.2.2. Ecophysiological Role of Fungi on Green Biofilms on Bark of Trees

During our studies on green biofilms on tree bark, we soon recognized that in most biofilms, fungal hyphae were present that were extracellularly associated to algal cells (Fig. 4). A survey of the relevant literature showed that the presence of fungal hyphae in green biofilms is mentioned only anecdotally by few authors. An association of algae and fungi was reported by Geitler (1942) who also described intracellular haustoria of an associated fungus in *Apatococcus lobatus*. However, his observations could not be corroborated. Extracellular contact between hyphae and cells of *Apatococcus lobatus* (Fig. 3) was observed by Gärtner (1974) but

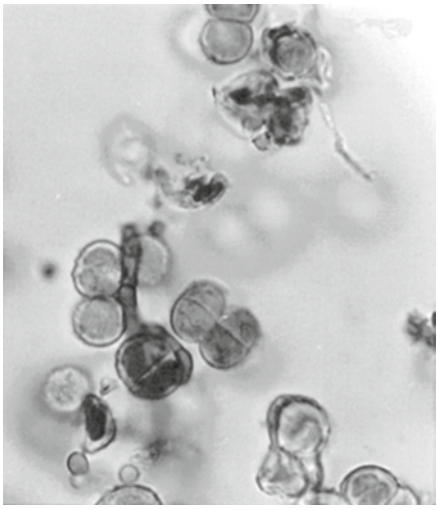

Figure 4. Extracellular contact between hyphae and cells of *Apatococcus lobatus.*

systematic studies are lacking. *Apatococcus lobatus* has never been reported as a photobiont of lichens. In general, those algal species that are common in aero-terrestrial habitats are rarely found as lichen photobionts (Honegger, 1993).

Any information on a physiological interaction between algae and fungi is on tree bark lacking. Our preliminary studies on a potential interaction between algae and fungi suggest indeed some kind of physiological relationship between both organisms (Turian, 1977). We isolated both algae (*Elliptochloris* sp.) and fungi from biofilms and grew them separately as axenic cultures. Experiments showed that algae combined with fungi again grow faster than the isolated algae (Fig 5a). This is corroborated by the fact that axenic algal cultures lost chlorophyll faster than algae in cultures with fungi (Fig 5b).

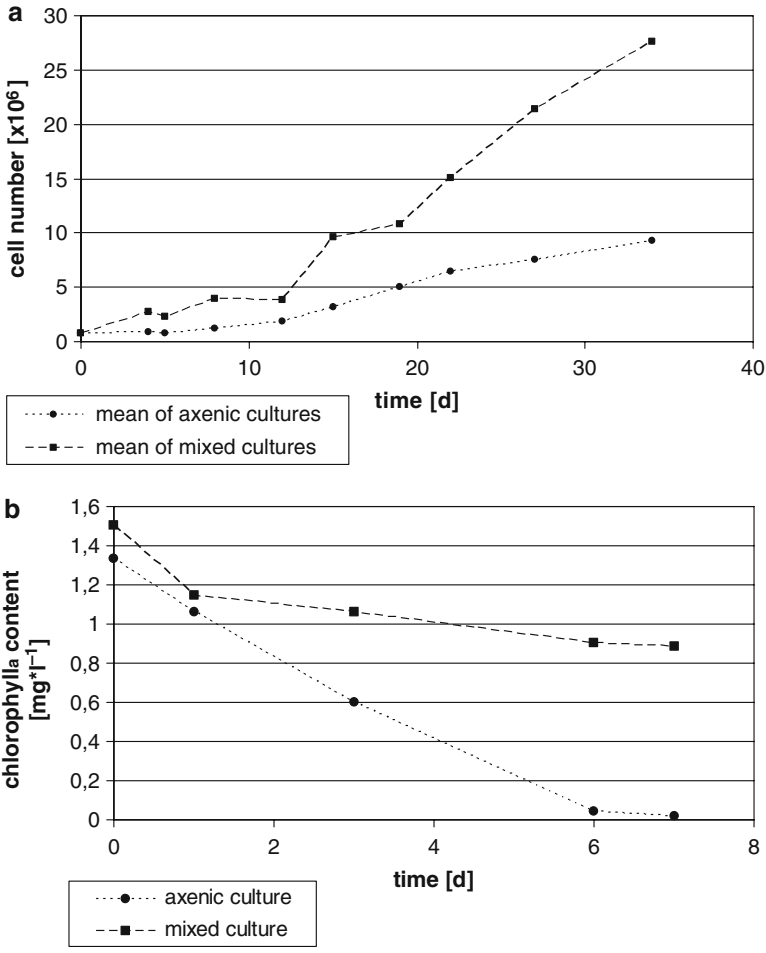

Figure 5. **(a)** Growth of *Elliptochloris* sp. in mixed and axenic culture. **(b)**Chlorophyll a content of axenic and mixed cultures of *Elliptochloris* sp.

Whether observed ecophysiological features justify to speculate about a prelichenization status of observed biofilms remains a matter of discussion and needs further investigation. At any rate, our experiments indicate some kind of cooperation between partners in green biofilms that may enhance their fitness under environmental stress conditions and might be a clue to their evolutionary success and widespread occurrence.

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PART V:
SYMBIOSES AND ASTROBIOLOGY

De Los Ríos
Ascaso
Wierzchos
Sancho
De Vera
Ott

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Asunción de los Rios

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SPACE FLIGHT EFFECTS ON LICHEN ULTRASTRUCTURE AND PHYSIOLOGY

Following the LICHENS 2005 Experiment On-Board the BIOPAN V Space Exposure Facility

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1. Introduction

Growth, survival and metabolic activity of microorganisms are influenced by numerous abiotic environmental factors. Microorganisms, especially when living in association, show an enormous resistance and great capacity for adapting to extreme levels of these abiotic factors. No single terrestrial environment exists that is not inhabited by microorganisms. In contrast, the conditions outside our planet would be very hostile for all life forms known so far, owing to a vacuum, severely desiccating conditions, intense radiation and extreme temperatures (Nicholson et al., 2005). The survival of organisms in the conditions of space has generated much interest for two reasons: first, it is a good way to test the resistance of terrestrial life forms to highly stressful conditions, and second, it also tests the possibility of present, past or future life beyond the confines of the Earth. Two kinds of experiments can be designed to examine the survival of organisms exposed to space conditions: (a) ground experiments simulating the conditions of space or (b) actual space flight experiments. Although costly and much more difficult to design and execute, only space flight experiments can assess the synergetic effects of a lack of gravity and high cosmic radiation doses (Leys et al., 2004).

The earliest experiments on the influence of extreme space conditions were performed on bacteria (Horneck et al., 1994; Mancinelli et al., 1998). This work revealed that some microorganisms are able to survive these conditions when in the dormant state, such as bacterial endospores. In effect, *Bacillus subtilis* can survive years of exposure to the space environment of a low-Earth orbit (Horneck, 1993; Horneck et al., 2001; Rettberg et al., 2002). Some terrestrial organisms have also been able to survive conditions of massive UV and cosmic radiation when protected from direct exposure to solar UV radiation (Horneck et al., 1984; Mancinelli et al., 1998; Mancinelli and Klovstad, 2000). Direct exposure to space conditions has been shown to be rather more lethal, with a survival rate of only 24–40% of halophylic archaea and cyanobacteria observed after 2 weeks in space conditions (Rotchild and Mancinelli, 2001).

Lichens are symbiotic life forms composed of fungi and algae (and/or cyanobacteria) that are tolerant to several stress factors. Thus, extreme desiccation and UV exposure tolerance is greater in the lichen than its isolated partners (Kranner et al., 2005; Vrablikova et al., 2006), indicating the benefits of this type of association. Symbiotic coexistence induces significant metabolic changes resulting from integration of the biochemical pathways of the partners (Provorov and Dolgikh, 2006). In the lichen symbiosis, this could enhance resistance to certain stress conditions. Lichens can withstand absolute dehydration (anhydrobiotic behaviour) and this state increases their resistance to harsh conditions (Cowan et al., 1979). While hydrated thalli are susceptible, for instance, to intense light (Demmig-Adams et al., 1990; Gauslaa and Solhaug 1996, 1999), desiccated lichens seem to be exceptionally resistant to extreme climatic conditions. Lichens could be preadapted to deal with the extreme conditions of space, including vacuum, abrupt temperature changes, and intense UV radiation, especially in a dehydrated state. Hence, lichens could prove to be a good exobiological model for exploring the resistance of symbiotic organisms to extreme conditions including those of outer space. Symbiont cells of three lichen species have in fact been noted to preserve their vitality and germination capacity under extreme space conditions simulated in the laboratory, demonstrating their high tolerance to these conditions (De Vera et al., 2003, 2004). However, until the space flight experiment LICHENS-2005 (Sancho et al., 2007), the survival capacity of lichen symbionts exposed to the real space environment had not been assessed. This experiment addressed the basic question of whether a space vacuum and/or radiation would have any effects on the physiology and ultrastructure of two species of high-mountain lichens.

2. Lichens 2005 Experiment-Biopan V

The LICHENS 2005 experiment was loaded on the BIOPAN exobiology facility of the ESA. BIOPAN is a pan-shaped container for biological experiments in space (Fig. 1a) that is attached to the outer hull of the retrievable Russian FOTON capsule. After a successful flight to low-Earth orbit, the BIOPAN motor-driven lid is opened from the ground control centre, permitting exposure to space conditions of the experiment packages. At the end of the flight, the lid is closed for re-entry. After landing, samples are transported under controlled conditions back to the investigators' laboratories. Over the period 1992–1999, BIOPAN successfully completed four 2-week missions, in which 16 experiments were conducted in the fields of exo/astrobiology, chemical evolution, radiation biology and radiation dosimetry (Demets et al., 2005). A fifth attempt to put BIOPAN (carrying the first LICHENS experiment) in orbit in 2002 failed when the launcher crashed. FOTON M2 and BIOPAN V, carrying the LICHENS 2005 experiment, were launched successfully on May 31, 2005 on board a Soyuz carrier rocket, which remained in orbit for 15.8 days.

The lichens used in this experiment were the bipolar species (occurring in both polar regions) *Rhizocarpon geographicum* and *Xanthoria elegans* collected at

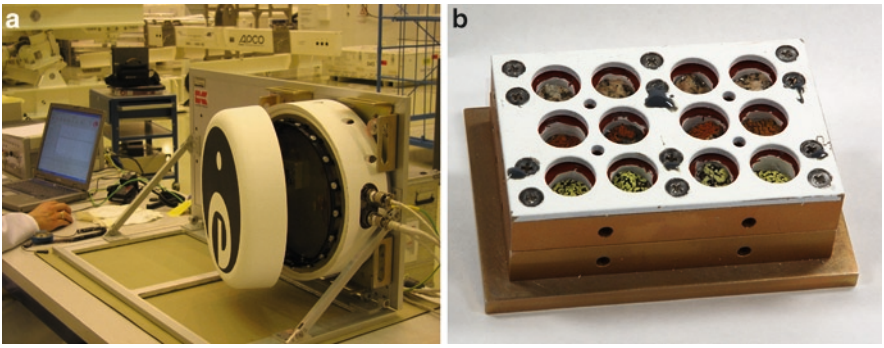

Figure 1. a: Image of BIOPAN-5 opened to load the experiments before the flight. **b**: LICHENS hardware for the space flight in BIOPAN 5.

an altitude above 2000 m in the mountains of central Spain. *R. geographicum* and *X. elegans* were selected on the grounds of their desiccation tolerance and survivability in regions that receive high amounts of UV radiation (Sancho et al., 2007). The experiment LICHENS was accommodated in a rectangular aluminium box consisting of two layers (Fig. 1b), each with 12 sample holes (an upper layer for samples exposed to radiation and a bottom layer for samples kept in the dark). Cut-off filters were placed above the samples in the upper layer to allow the penetration of the following wavelength ranges of light (>170, >280, >320, >400 nm). An identical set of samples was prepared in parallel as a ground control.

During the flight, the lichen samples were exposed to different ranges of the spectrum of extraterrestrial solar electromagnetic radiation, including the highly energetic vacuum-UV (>170 nm) of some 22 MJ m^{-2} and about 3 mGy of cosmic radiation (more details can be found in Sancho et al., 2007). The maximal change in temperature from launching to landing was close to 42°C (−21° to +21°C).

3. Recovery of Photosynthetic Activity After The Space Flight

The LICHENS 2005 experiment allowed us to determine the effects of space radiation and lack of gravity on the photosynthetic capacity of the two high-mountain species. Fluorescence measurements on chlorophyll were used to determine several photosynthesis variables.

Both space-exposed and ground controls of *R. geographicum* and *X. elegans* recovered most of their photosynthetic activity 3 h after being sprayed with water. After 24 h under revitalizing conditions and three sprayings, all samples fully recovered their pre-flight photosynthetic activity (Fig. 2), as revealed by chlorophyll fluorescence (Sancho et al., 2007).

The photosynthetic machinery can be damaged by high UV radiation (Solhaug et al., 2003), although we detected no changes in the photosynthetic

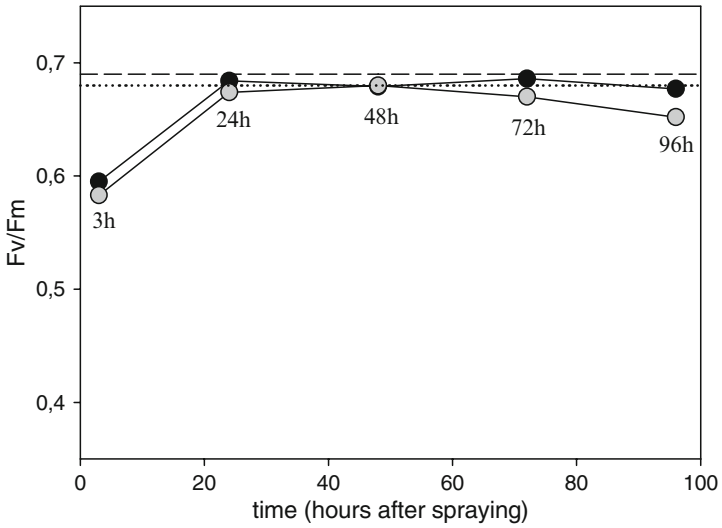

Figure 2. Relative efficiency of photosystem II in *Rhizocarpon geographicum* (black circles) and *Xanthoria elegans* (grey circles) after full exposure to extraterrestrial solar radiation. Maximum values obtained before the flight are indicated by the dashed (*R. geographicum*) and dotted (*X. elegans*) lines.

capacity of the dried lichens of these species, indicating their extreme resistance to 15 days of exposure to space stresses. Moreover, after the intense dehydration induced by the high vacuum, the lichens were able to recover their metabolic activity in a remarkably short time. The photosynthetic machinery of desiccated lichens is protected through the dissociation between their photo system II and light-harvesting complexes (Lange et al., 1989). In addition, it has been also recently discovered that desiccated lichen thalli possess unique and very efficient high-energy thermal dissipation mechanisms (Herber et al., 2006, 2007). Despite these conditions, it cannot be considered that lichens in the desiccated state will be completely resistant to stress conditions (Gauslaa and Solhaug 1996, 1999; Buffoni Hall et al., 2003). Pre-space flight tests performed on *R. geographicum* have also revealed that UV radiation does not impair photosynthetic activity both at atmospheric pressure and in vacuum conditions (De la Torre et al., 2007). Sensitivity to UV radiation varies greatly among organisms. Differences between organisms in their ability to cope with UV radiation are the result of (1) their different capacity to protect themselves against UV light; and/or (2) their different ability to repair any damage incurred (Björn, 2007). The capacity of the present lichens to recover their photosynthetic activity is probably the outcome of the protection mechanisms these species have, consisting of a thick and dense upper cortex and a high concentration of lichen substances, which efficiently screen harmful radiation (Sancho et al., 2007).

4. Vitality and Integrity of Symbiont Cells After the Space Flight

Confocal laser scanning microscopy (CLSM) and live/dead fluorescent assays (Molecular Probes) were used as a fast vitality test, immediately after the samples arrived at the laboratory (Wierzchos et al., 2004). This test revealed that after the space flight, most of the algal cells preserved their high intensity autofluorescence indicating their vitality. Figure 3a shows numerous living cells in the algal layer of the *X. elegans* thallus emitting intense autofluorescence (blue signal) after 2 weeks of exposure to space conditions. However, in the same CLSM image, damaged photobiont cells showing a low level of autofluorescence (pink arrows) can also be

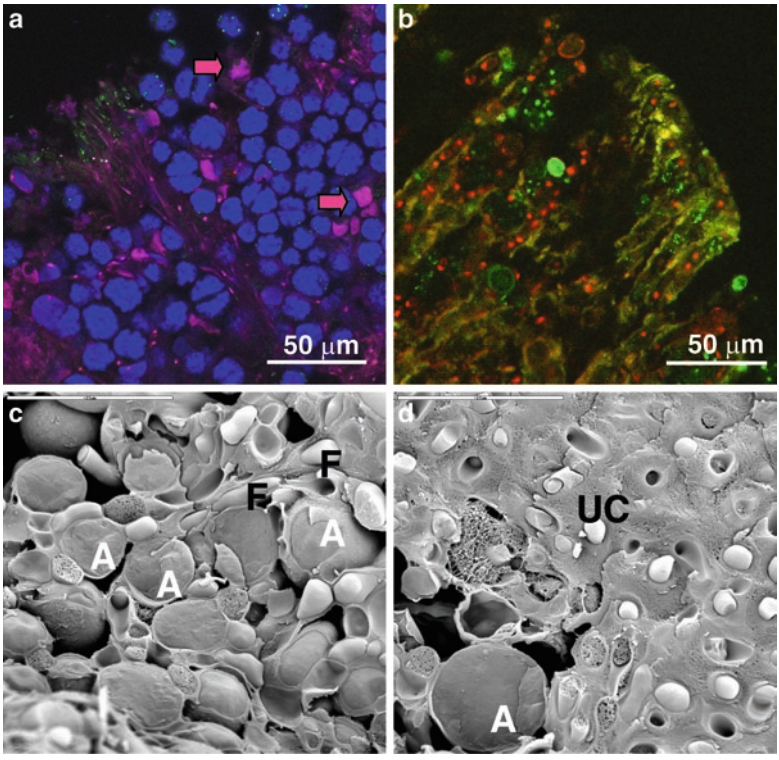

Figure 3. **a–b**: Confocal scanning laser microscopy images of lichen thalli stained using the LIVE/DEAD BacLight kit after the space flight. (**a**) Algal layer of *X. elegans* thallus showing the autofluorescence (in blue) of most of the photobiont cells. Algal cells showing a low level of autofluorescence – probable damaged cells – appear in pink (arrows). (**b**) *R. geographicum* lichen thallus showing an upper cortex comprised of a mixture of dead cells with compromised membranes (nucleus stained red by propidium iodide) and live cells with intact membranes (nucleus stained green by SYTO 9). **c–d**: Low temperature scanning electron microscopy image of the algal layer (**c**) and upper cortex (**d**) of *X. elegans* thalli subjected to space conditions showing preserved cell membrane integrity and no signs of plasmolysis.

observed. The vitality of the fungal partner can be observed in Fig. 3b, in which LIVE/DEAD BacLight viability staining revealed the absence of membrane damage in some of the upper cortex cells of *R. geographicum* (cells with nuclei stained green). Survival rates for the fungal cells in the algal layer of both species were lower than rates for the photobiont cells (Sancho et al., 2007). Low temperature scanning electron microscopy also revealed a lack of signs of loss of cellular integrity and/or severe plasmolysis in the algal and fungal cells forming the algal layer (Fig. 3c). Even the mycobiont cells from the upper cortex, exposed most directly to the space conditions, maintained their cellular integrity (Fig. 3d).

5. Ultrastructural Changes Observed After the Space Flight

Through TEM, we assessed the effects of space conditions on the lichens at the ultrastructural level. Dramatic changes in ultrastructure were not detected, suggesting the survival of most of the lichen symbiotic cells (Sancho et al., 2007), although ultrastructural changes observed in both symbionts, indicate some degree of injury in response to the space flight.

The following ultrastructural changes were noted in the photobiont cells of *R. geographicum*:

- Algal cells exhibited starch granules before the flight (Fig. 4a) that disappeared during the flight or appeared substantially changed (Fig. 4b). Only in lichen samples under the 320 nm filter, were normal starch grains occasionally observed after the flight (Fig. 4c).
- Deformed mitochondria with altered cristae were observed in some of the cells after the flight (Fig. 4d).
- Cytoplasmic lipid bodies were not very abundant before the flight, but some cells showed considerable amounts after the space flight (Fig. 4b). The size of these lipid bodies was variable.
- Electron-dense membranous complexes were frequently observed after the flight, in the cytoplasm (white arrow in Fig. 4c), chloroplast (black arrow in Fig. 4c), mitochondria (white arrow in Fig. 4d) and nucleus. These membranous complexes could be the consequence of lipid loss from cell membranes, indicating some degree of cell senescence.
- After exposure to space conditions, the chloroplasts of some cells exhibited an irregular arrangement of thylakoids (arrowhead in Fig. 4c) and occasionally thylakoidal membranes appeared dilated (arrowhead in Fig. 4b). Cells with disorganised pyrenoids were more frequent after the flight (Fig. 4b).

Figure 4. (continued) grains (S). (**d**): Cell from a thallus exposed to radiation wavelengths above 170 nm exhibiting abnormal mitochondria (M). **e–f**: Transmission electron microscopy image of photobiont cells of *X. elegans* lichen thalli after the space flight. (**e**) Cell from a thallus exposed to radiation wavelengths above 400 nm showing disorganized pyrenoids and dilated thylakoids (arrowhead). (**f**) Detailed image of the irregularly arranged thylakoids in an algal cell from a thallus exposed to radiation wavelengths above 170 nm.

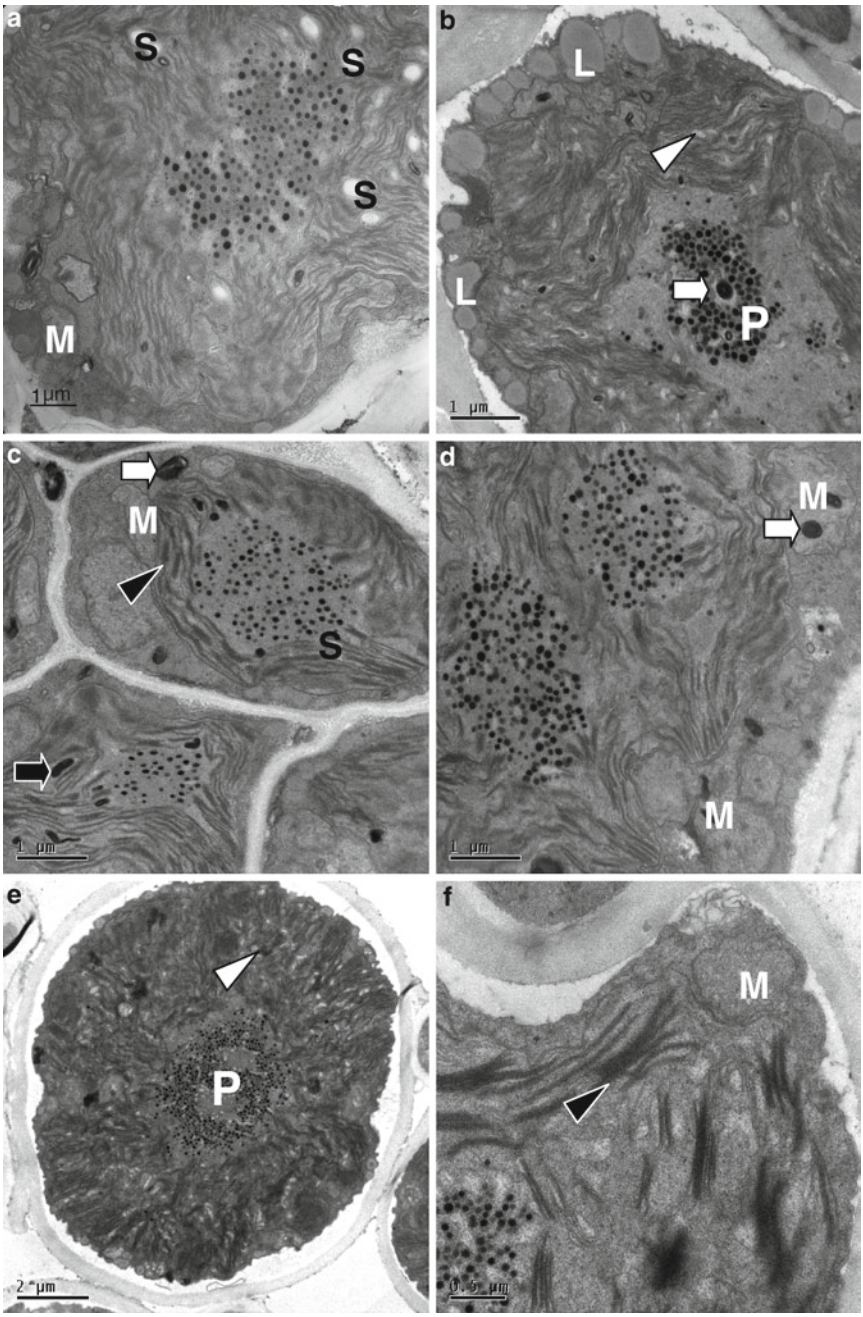

Figure 4. a–d: Transmission electron microscope images of photobiont cells of *R. geographicum* lichen thalli in control specimens and those subjected to different conditions of space. (**a**) Algal cell in a ground control thallus showing the presence of starch granules (S). (**b**) Cell from a thallus in the bottom layer (not exposed) of the box accommodating the experiment showing large amounts of lipid globules, dilated thylakoids (arrowhead) and disorganised pyrenoids (P). (**c**) Cells from a thallus exposed to radiation wavelengths above 320 nm showing many electron-dense membranous inclusions (arrows) and occasional starch

Ultrastructural changes were less evident in the photobiont cells of *X. elegans*. Starch granules were absent and lipid globules were scarce before and after the flight. The electron-dense membranous inclusions observed in *R. geographicum* were not observed in *X. elegans*. However, disorganised and dilated thylakoids (arrowheads in Fig. 4e–f) as well as disorganised pyrenoids (Fig. 4e) were also frequently observed in some photobionts of this species after exposure to space conditions. After the flight, cells with deformed mitochondria were also commonly detected (Fig. 4f).

Electron-dense membranous inclusions in the cytoplasm (arrowheads in Fig. 5a) that were not present before the space flight (Fig. 5b) were often detected in the mycobiont cells of *R. geographicum*, especially those from the upper cortex. These inclusions were similar to those observed in the photobiont cells (Fig. 4c–d). Cells with irregular and abnormally-large mitochondria were also a frequent change noted in this species (Fig. 5c). In some of these mitochondria, the cristae seemed dissolved. The cells of *X. elegans* did not form electron-dense membranous inclusions, but in some of the mycobiont cells, we detected the presence of vacuoles containing electron-dense material (arrows in Fig. 5d–5e), which were not observed in the control samples (Fig. 5f).

The characteristic conditions of space flight are microgravity and radiation. In principle, cosmic and UV radiation and the lack of gravity could interfere with cell processes and the consequent modifications would be reflected by a rearrangement of their ultrastructure. However, the changes observed in response to the time spent in orbit were discrete, and only affected some cells. Chloroplasts in photobiont cells and mitochondria in both symbionts were the organelles in which most ultrastructural changes were detected. In addition, we observed electron-dense membranous inclusions in the *R. geographicum* photobiont and vacuoles with electron-dense material in *X. elegans*, indicating some degree of cell injury. Some or all of these ultrastructural changes observed in the samples could be attributable to the stressful conditions of the space environment, such as massive UV radiation.

Chloroplasts have been described as organelles that are sensitive to UV-B radiation. Alterations such as loss of thylakoid arrangement and/or dilated or wrinkled thylakoids have been reported in plants (Kordyum, 1994) and different groups of algae (Lütz et al., 1997; Poppe et al., 2002; Holzinger et al., 2004; Yu et al., 2005; Holzinger and Lütz, 2006) exposed to UV radiation. Recently, chloroplasts have been reported to be more sensitive to a high dose of gamma rays than other cell organelles (Wi et al., 2007). Similar effects seem to be induced by other environmental stress factors such acid rain (Gabara et al.,

Figure 5. (continued) electron microscopy image of mycobiont cells from *X. elegans* lichen thalli in control specimens and those exposed to different space conditions. (**d**) Cells from the upper cortex of a thallus in the bottom layer of the box accommodating the experiment (not exposed) showing vacuoles filled with electron-dense material (arrow). (**e**) Fungal cell from a thallus exposed to radiation wavelengths above 400 nm displaying vacuoles containing electron-dense material (arrow). (**f**) Cells from a ground control specimen.

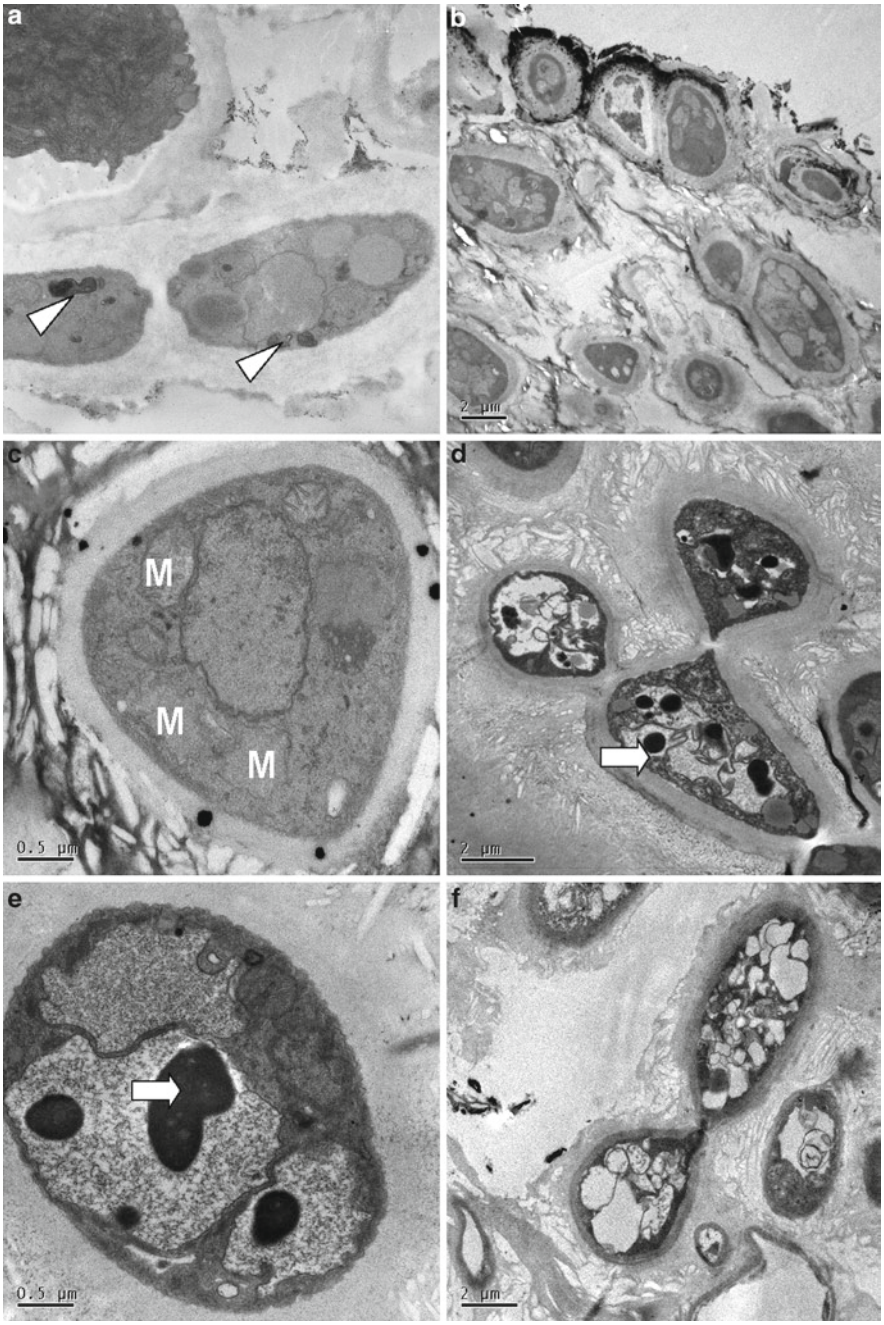

Figure 5. **a–c**: Transmission electron microscopy images of mycobiont cells of *R. geographicum* thalli in control specimens and those subjected to different conditions of space. (**a**) Fungal hyphae from a thallus exposed to radiation wavelengths above 170 nm showing electron-dense membranous inclusions in the cytoplasm. (**b**) Fungal cells from the upper cortex of a ground control thallus. (**c**) Cells from a thallus exposed to radiation wavelengths above 170 nm showing deformed mitochondria. **d–f**: Transmission

2003), indicating that this could be a common response of photosynthetic organisms to different stress situations. The disorganisation of the pyrenoids in lichens has been also reported in laboratory experiments conducted under different stress conditions such as desiccation (Jacobs and Ahmadjiam, 1971; Ascaso, 1978; Brown et al., 1987) or acid rain (Tarhanen, 1998). Densely packed peripheral lipid bodies, such as those observed in some of the photobiont cells of *R. geographicum* after the space flight, have been related in the alpine snow alga *Chlamydomonas nivalis* to shielding of the chloroplast under high irradiation and a potential carbon source during unfavourable climate conditions (Remias et al., 2005). In effect, this mechanism could also help protect photobiont cells in space conditions. Reduced numbers of starch granules is also a common response to exposure to UV radiation (Quaggiotti et al., 2004).

Mitochondrial functions are crucial for maintaining cell homeostasis and are thought to play a role in physiological functions. Ultrastructural changes were observed in the mitochondria of some cells of both species after only 14 days in space. Changes in mitochondrial ultrastructure, such as deformation and dissolution of cristae, have been mainly attributed to UV effects (Poppe et al., 2002; Holzinger et al., 2004; Yu et al., 2005) but have been also reported in response to gravity changes. Thus, larger mitochondria and irregularly arranged cristae, along with condensation of the matrix have been observed in long-term space flight experiments conducted on green algae (Popova, 2003). Increased numbers of mitochondria and changes in crista morphology, indicating altered mitochondrial function, have been reported in other organisms exposed to microgravity (Kordyum, 1994; Schatten et al., 2001). As in the case of the symbiotic cells examined here, other organelles and their components can also be affected by a lack of gravity. Changes in the number, size and morphology of mitochondria in plant cells under conditions of microgravity can be accompanied by progressive cell vacuolisation, increased lipid globule volume, reduced numbers of starch grains, changes in the shape and size of chloroplasts, thylakoid swelling and the appearance of osmiophilic globules in the cytoplasm (Kordyum, 1994).

The presence of electron-dense membranous complexes in different zones of the symbiotic cells of *R. geographicum*, and electron-dense deposits in the vacuoles of fungal cells of *X. elegans*, could also be a consequence of metabolic changes produced as an adaptive strategy against the adverse conditions of space. Membranous electron-dense structures in photobiont cells have been previously observed in lichens subjected to stress conditions (Ascaso and Galván, 1976; Peveling and Galun, 1976). Electron-opaque deposits within vacuoles could indicate vacuolar sequestering of cellular components (Robinson et al., 1998). Deposits similar to those observed in *X. elegans* have been reported in the fungal cells of the lichen thallus after exposure to pollutants (Balaguer et al., 1997; Tarhanen 1998; Tarhanen et al., 2000). These deposits were attributed by the authors to detoxification processes.

6. Conclusions

Two weeks of exposure to real space conditions only partially altered the ultrastructure of symbiotic cells of the lichens *R. geographicum* and *X. elegans*, and these changes did not seem to compromise their physiological functions, or at least their photosynthetic activity. Our findings suggest that lichens may survive the conditions of space even when fully exposed to the massive UV and cosmic radiation previously described as lethal for bacteria and other microorganisms (Mancinelli et al. 1998). The non-dramatic ultrastructural changes revealed by TEM suggest that lichenised fungal and algal cells are not severely damaged and can survive the conditions of the space environment for 2 weeks. Nevertheless, the effects of these ultrastructural changes on the physiology of the lichen thallus and their reversibility need to be further explored. The changes in ultrastructure produced during space flight, besides being determined by the taxonomic position of the microorganisms, could also be influenced by factors such as their previous acclimation to some of these conditions and the time of the exposure to these stress factors. The permanent tolerance of the lichen symbiosis to space conditions will need to be addressed in experiments involving a longer exposure time to space conditions.

Stress factors of outer space such as vacuum, temperature, and radiation are likely to contribute to the loss of viability of the two components of the lichen symbiosis. Both exposure to high-vacuum and solar radiation significantly reduce the possibility of survival of these microorganisms. Solar UV is considered the most immediately lethal component of solar radiation for microorganisms. On the other hand, cosmic radiation is emitted at a lower dose per unit time but has higher penetration power (Nicholson et al., 2005). The temperatures reached during the space flight were moderate relative to the natural Earth-bound conditions experienced by the lichens examined here, and their effects are not unexpected (Sancho et al., 2007). Notwithstanding, the ultrastructural modifications produced in the lichen symbionts are likely to reflect the response to more than a single factor and this response is probably non-specific. Hence, none of the ultrastructural changes detected can be expressly attributed to a certain wavelength of radiation since we noted no clear differences among lichen specimens placed under the different filters (allowing the passage of UV light or not), or between the more exposed samples of the top layer and those of the lower layer (not exposed). Effectively, modifications to the chloroplasts and mitochondria of plant cells are induced by a variety of stress situations (see Tarhanen 1998) indicating a non-specific stress response. In experiments presently underway on samples from a subsequent space flight (Biopan VI), a more detailed analysis of the specific effects of different wavelengths of radiation is being undertaken. Neither can we preclude the possibility of a certain degree of acclimation of the lichen cells during the space flight. For example, the Antarctic red alga *Palmaria descipiens* showed evidence of acclimation to UV radiation,

accompanied by the recovery of previous ultrastructural modifications after 12 hours of exposure to UV light (Poppe et al., 2002).

Lichens are commonly cited as examples of more stress-resistant life forms than other organisms and it seems that they possess several protection strategies to cope with the impacts of harsh environmental conditions. Thus, several reports have described the production of UV-absorbing substances and their deposition in the lichen thallus, preventing or diminishing the penetration of radiation to deeper layers (or entering the cell) (Buffoni Hall et al., 2002; Torres et al., 2004; Vrablikova et al., 2006; Karsten et al., 2007). In the species selected for the present study, it is thought that the build up of rhizocarpic and parietin phenolic acids in the upper cortex could serve to block UV radiation (Solhaug et al., 2003; Gauslaa and Solhaug, 2004). Moreover, all the effects of UV radiation cannot be classified as deleterious in lichens since they have been demonstrated to undergo UV-dependant synthesis of vitamin D, although their requirements for UV light remains unclear (Wang et al., 2001; Björn, 2007). Synthesis of extracellular polysaccharides is another proposed protection mechanisms against factors such as UV radiation, cold temperatures and desiccation (Ehling-Schulz et al., 1997; De los Ríos et al., 2004). Extracellular polymeric substances are important components of the lichen thallus growing under extreme conditions (De los Ríos et al., 2005), such that they could play a role in the response to space conditions and later recovery. In addition, due to their capacity to tolerate absolute dehydration, it could be that a vacuum is not so lethal for lichens. Finally, the survival of these symbiotic cells, lack of dramatic ultrastructural damage and the recovery of their photosynthetic capacity could have been mainly determined by the fact that the lichens were in an anhydrobiotic state before embarking on the space flight beginning. Dry thalli have better photoprotection due to reduced light transmittance through the upper cortex (Büdel and Lange, 1994), greater light reflectance (Gauslaa 1984; Herber et al., 2007) and specific thermal energy dissipation mechanisms (Herber et al., 2006, 2007). Thus, the extreme desiccation induced by a high vacuum could provoke less cellular damage to already dehydrated thalli. Indeed, desiccated lichen thalli have been noted to suffer less ultrastructural damage under desiccation conditions than partially dehydrated thalli (Brown et al., 1987). Owing to their protection strategies, some of the cells under the conditions of this experiment were able to escape damage and maintain their vitality and photosynthetic productivity (Sancho et al., 2007) or perhaps activate the relevant repair mechanisms after wetting, permitting their recovery and survival.

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RESISTANCE OF SYMBIOTIC EUKARYOTES
Survival to Simulated Space Conditions and Asteroid Impact Cataclysms

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1. Symbiosis Among Eukaryotes as Adaptation to Stress During Early Colonization of Earth

Carbon isotope data suggest that microbial life was present on Earth as early as 3.5 Ga ago, and probably even 4 Ga ago, and indicates that biological CO_2 fixation was an early feature (Schidlowski, 2001). The early biosphere was dominated by microbial life forms for a long period, during which they evolved to exploit new niches. For some, this involved interaction between different microbial groups, and now symbiosis represents one of the most successful strategies in evolution (Margulis, 1993). There is now little doubt that eukaryotes arose through uptake of a heterotrophic eubacterial symbiont by an autotrophic archaebacterial host (Martin and Russell, 2003). This milestone in evolution, and the paradigm of the endosymbiont hypothesis, initiated the evolution of the eukaryotic kingdoms of fungi, plants, and animals. Evidence from dating sequence divergence (Wang et al., 1999) suggests that the ancestors of today's plants, animals, and fungi diverged possibly as early as 1.5 Ga ago. Independent of this major evolutionary step, other symbioses arose as exosymbiosis, without the ingestion of one partner. These involve both syntrophic partnerships among prokaryotes, and also associations with or among eukaryotes. Such symbioses are particularly complex in biofilms and biocrusts (Belnap et al., 2001; Flemming and Wingender, 2001), and in associations that are often found in stressful terrestrial habitats that are not amenable to higher plant community development, for instance, due to periodic aridity. In such habitats, lichen symbioses can form the dominant and conspicuous biological elements of the landscape. Lichens can be characterized as a specific exosymbiotic life form that results in an exposed and integrated phenotype of clearly different morphology than that of the constituent organisms alone (Lawrey, 1991; Ahmadjian et al., 1987; Galun, 1988; Grube and Hawksworth 2007). Taylor et al. (1997, 2005) and Yuan et al. (2005) date the first occurrence of the lichen symbiosis from fossil records in the Lower Devonian period (0.6 Ga),

but the evolution of the lichen symbiosis could well pre-date the available fossil records (Lutzoni 2001).

Lichens form a heterogeneous group of organisms represented by a great variety of phenotypes (Henssen and Jahns, 1974; Nash, 1996). Lichens can grow in almost all biomes of the world, and dominate in cold and harsh environments of polar and high mountain regions, often colonizing exposed habitats (Brodo et al., 2001; Kappen, 1973; 1993; Lange et al., 1990, 2000; Poelt, 1969). They also establish successfully in other extreme environments such as deserts and volcanic regions (Büdel and Wessels, 1986; Fahselt, 1995; Lange et al., 1977). The symbiotic state enables the lichen thallus to colonize extreme habitats where the separate bionts would not be able to survive. The dominance of lichens is a result of their poikilohydrous nature and their ability to tolerate a variety of harsh physical environmental conditions including drought, extreme temperatures, and high insolation (including high fluxes of ultraviolet (UV) radiation).

Solar UV radiation (UVR) might have been an important driving force of evolution on Earth, as terrestrial organisms needed to develop protection mechanisms against the deleterious effects of UVR at an early stage (Rettberg and Rothschild, 2002). UV radiation is not only a powerful mutagen, but is potentially destructive for individual cells. In polar and high mountain regions, lichens are challenged by relatively high levels of UVR. The dominance of diverse lichen lineages in such habitats is indicative of the ability of this symbiosis to survive exposure to this high energy radiation (Ott and Jahns, unpublished data). Their high resistance to extreme planetary physical environmental conditions also suggests that lichens could potentially resist even the conditions of outer space or those of cataclysmic asteroid impacts.

To date, the majority of studies of the biological consequences of exposure to the space environment have been performed using viruses, bacterial and fungal spores, and archaea or cyanobacteria (Feofilova, 2003; Horneck, 1993; Horneck et al., 2001a,b,c, Mancinelli et al., 1998; Moeller et al., 2005, 2003; Nicholson et al., 2000; Stetter, 1996). Eukaryotic spores of ferns and mosses and the seeds of higher plants have also been subjected to experiments including exposure to space radiation and vacuum conditions (Kranz et al., 1990; Neuberger et al., 2003; Tepfer et al., 2006). However, there remains very limited knowledge about the potential of the symbiotic association of lichens, or their individual symbionts, to resist simulated and real space conditions (de Vera, 2003, 2004a,b, 2005; de la Torre et al., 2004; Sancho et al., 2007). Sancho et al. (2007) were the first researchers to perform an open space experiment, measuring photosynthetic activity based on the chlorophyll fluorescence of the photobiont cells in a lichen thallus after return from space.

This chapter reviews our results achieved in studies on the resistance and viability of both the entire lichen symbiotic organism and the separated symbionts after exposure to simulated space conditions. The space parameters tested included UV-B and –C radiation, vacuum, low pressure, and combinations of these conditions. Selected lichen species were obtained from different habitats, and included *Buellia frigida*, *Fulgensia bracteata, Peltigera aphthosa, Xanthoria elegans. Buellia frigida*

and *X. elegans* are epilithic lichens that colonize exposed rock faces in the Antarctic. *Fulgensia bracteata* is a typical species of the lichen community colonizing open exposed sites on the calcareous gravel alvar in temperate regions. *Peltigera aphthosa* grows primarily in moss cushions, where it is well protected against high insolation. These lichen species from different habitats were selected in order to compare their degree of adaptation in context of exposure to simulated extreme space conditions. However, the majority of experiments were performed using *X. elegans* as a possible model organism for simulated and real space experiments.

2. Stress Resistance to Simulated Space Conditions and Asteroid Cataclysms

It is crucial to investigate not only the entire lichen thallus but also the isolated symbionts in order to understand the role of symbiotic association in the resistance to space conditions. Most of the research reported so far on the UV effects on lichens focuses on the effects of UV-B radiation on the photosynthesizing symbiont (Bachereau and Asta, 1997; Kappen et al., 1998; Quilhot et al., 1996; Solhaug and Gauslaa, 1996; Swanson and Fahselt, 1997; Lud, 2001; Wynn-Williams et al., 2000), while the influence on the mycobiont has largely been ignored. In our studies, viability tests were completed using the entire lichen thallus and thallus without cortex, on the isolated photobiont and mycobiont, and on apothecia and discharged ascospores, by exposing each of the respective components to simulated space conditions. These studies were performed with *F. bracteata* and *X. elegans*. Independent of the wavelength of UVR and the exposure time, none of the entire lichen thalli investigated showed any significant decrease in viability (Fig. 1).

Exposure of dry lichen thalli of *F. bracteata* and *X. elegans* to UVR (> 160 nm) up to doses of 150 kJ m^{-2} combined with conditions of vacuum, thereby simulating outer space, reduced their viability only slightly. Exposure of separated symbionts of *X. elegans* and the entire thallus to a vacuum pressure of 10^{-5} Pa and to polychromatic UVR (UV A, B, C with 200 nm < λ < 400 nm) or to monochromatic UV-C radiation (λ = 254 nm)—conducted in the space simulation facilities of the German Aerospace Centre (DLR) in Cologne—clearly demonstrated high sensitivity of the isolated photobiont cells, while the mycobiont showed lower sensitivity while being less resistant than the complete lichen thallus (de Vera, 2005; de Vera et al., 2003, 2004a,b, 2008) (Figs. 1, 2).

The isolated algal cells grow in clusters under artificial cultivation conditions. On exposing these clusters to extreme UV-C radiation at a wavelength of λ = 254 nm, most of the nonviable algal cells were found at the surface of the clusters, and not surrounded by a shielding layer. Conversely, cells within the clusters remained viable, appearing to be protected by the surrounding outer cells and their sheath of mucilage, which may strongly inhibit the influence of UVR (de Vera et al., 2003). Similarly, the lower resistance of the mycobiont relative to the entire thallus might be due to the lack of protective layers (mucilage, well-structured cortex, and chemical crystals).

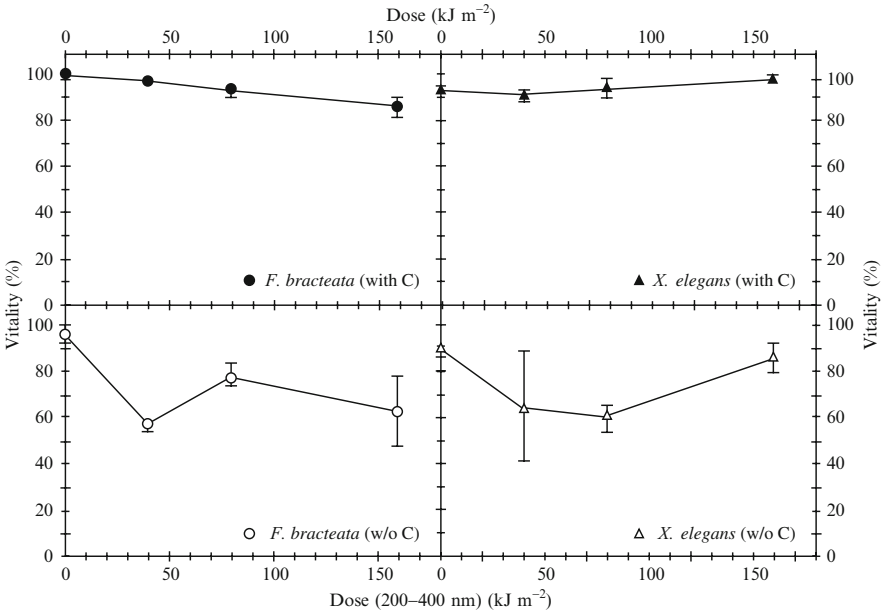

Figure 1. Viability of thalli of *F. bracteata* and *X. elegans* with cortex (top panels) and without cortex (bottom panels) after exposure to UV A, B, C (de Vera et al., 2003).

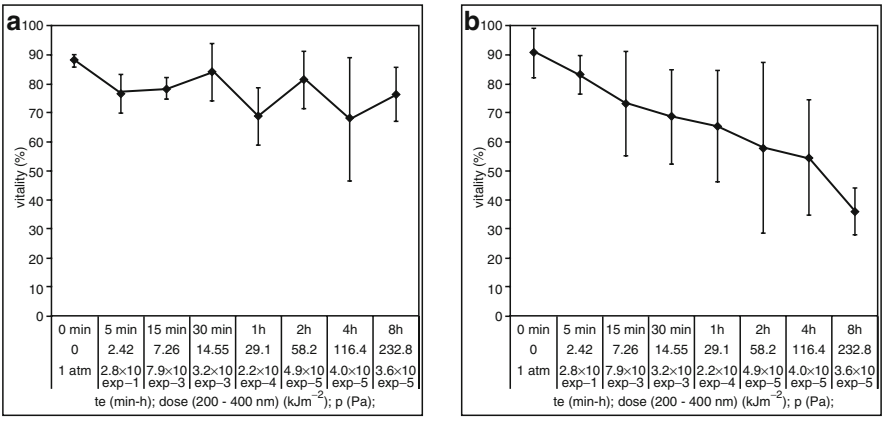

Figure 2a, b. Viability of the mycobiont (**a**) and photobiont (**b**) of *X. elegans* after exposure to space simulation (UV A, B, C and vacuum), time of exposure (t_e), pressure (p) (de Vera et al., 2008).

The intensity of discharge of ascospores following exposure did not appear to be influenced substantially. In each case, the mean total number of ascospores was about 1200 for *F. bracteata* widespread on an area of 2.5 cm on the culture medium and 1031 on an area of 3.2 cm for *X. elegans*. The germination capacity

of the ascospores of either species was not greatly affected by exposure of apothecia to UV-C radiation at λ = 254 nm at the doses tested. Even after a dose of 2.88 kJ m^{-2} of UV-C exposure, the germinative capacity of the ascospores remained greater than 50% (control c. 88%), with the sensitivity of ascospores of *F. bracteata* being slightly greater than those of *X. elegans*.

Further experiments were performed with *X. elegans* to investigate its capacity to survive conditions plausibly similar to those created in asteroid impact events. We suggest that this provides a realistic approach to estimate the survivability that is requisite in the first stage of the hypothesis of lithopanspermia, i.e., the viable transport of eukaryotic life forms between planets (Stöffler et al., 2007; Horneck et al., 2008). For this purpose, the lichens were placed between two 0.5 mm thick gabbro rock material plates in order to simulate an endolithic lichen community. This ensemble was inserted into a container and then exposed to a TNT explosion above a fire plate. The design included a defined distance to the sample device, allowing simulation of well-determined shock pressures (Fig. 3). Testing with Live/Dead staining (Molecular Probes/Invitrogen, Stöffler et al., 2007; Horneck et al., 2008) was used to indicate the presence of physiologically active cells.

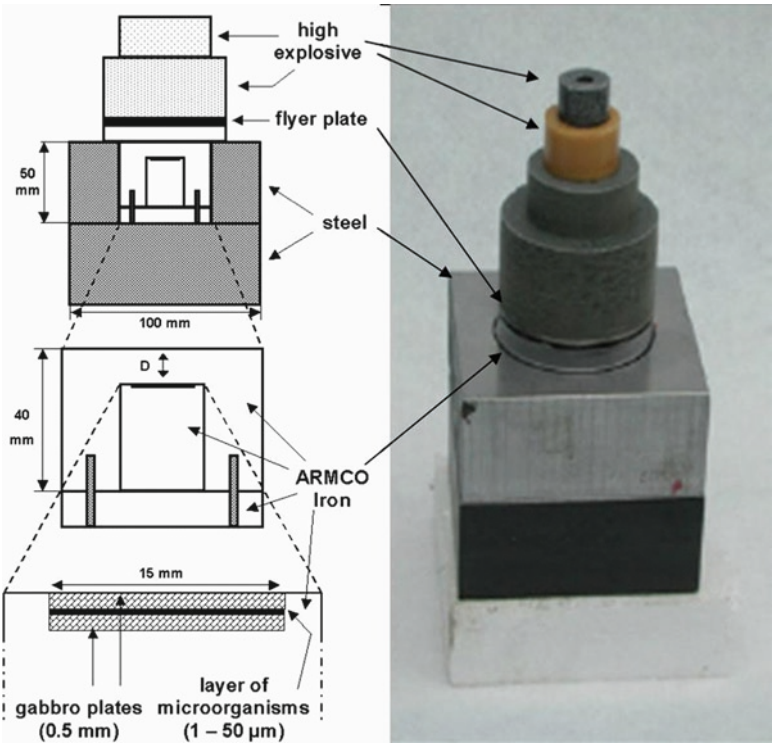

Figure 3. Asteroid impact simulation device (Horneck et al., 2008).

If present, physiological activity is reflected by color changes in the plasma, caused by transport processes from the cell plasma into vacuoles and vesicles where the reaction products with the staining substances (red crystals) are deposited (Horneck et al., 2008). After these shock experiments, the well-ordered structure of the lichen thallus was seen to become less arranged. The highest pressures, of about 50 GPa, were survived by ascospores, which were originally embedded in the fruiting bodies, and some started to germinate after the shock event. It might also be speculated that the water released by damaged cells during the shock event could be an important prerequisite for the subsequent germination of the surviving ascospores. Single photobiont cells embedded in the cellular matrix of thallus, or in the margin of a fruiting body, also demonstrated viability after being exposed to pressure as high as 30 GPa (Stöffler et al., 2007; Horneck et al., 2008). These results are consistent with the possibility of a "direct transfer" by "lithopanspermia," for instance, for the route from Mars to Earth or from any Mars-like planet to other habitable planets in the same stellar system as, although the well-structured thallus is destroyed, single cells of both bionts can survive forces consistent with asteroid impact, including the generative cells of ascospores.

3. Strategies for Resistance to Space Parameters and Asteroid Impacts

Adaptations, which could contribute to endurance of space conditions, can be seen at the anatomical–morphological and physiological–biochemical levels. Removal of the upper cortex (including the gelatinous layer) led to an approximately 40% decrease of the thallus tissue viability, while exposure of the entire thallus to combined conditions of UVR (> 160 nm up to 150 kJ m^{-2}) and vacuum affected viability only to a small extent (c. 15%). However, in young thallus, parts of *X. elegans* where cortex formation was incomplete, the viability of the mycobiont cells was reduced by the full spectrum of UVR. The effect of simulated space conditions on lichens strongly depends on the presence or absence of an epinecral layer, with its component dense mucilage and UV absorbing compounds showing that the anatomical structure primarily serves as a shielding layer. The mucilage layer includes carbohydrates such as the polyol mannitol and the disaccharide trehalose. These are compatible solutes, which protect lichens during desiccation (Jennings and Lysek, 1999), such as will be experienced during exposure to simulated space experiments. The mucilage layer in lichen symbiotic organisms may have analogous characteristics to biological soil crusts (Belnap et al., 2001), where it is hypothesized that a mucilage layer may protect microorganisms from exposure to space-simulating conditions such as UVR (Lütz et al., 1997; de Vera, 2003), and total desiccation caused by vacuum.

Photosynthetically active compounds are shielded by the outer fungal cortex within the structured lichen thallus. This layer concentrates the UV-protective compounds parietin and β-carotene in the epilithic lichen *Xanthoria elegans* (Wynn-Williams et al., 2000), which is therefore characterized by a dark-orange

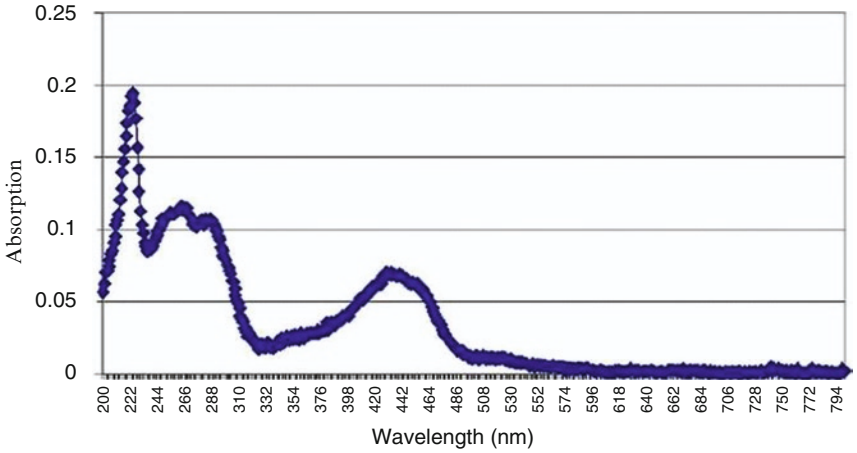

Figure 4. Absorption maxima of the anthraquinone parietin, mainly in the UV spectral range (200–400 nm) (de Vera, 2005).

color (Honegger, 1990; Solhaug, 2003). Parietin absorbs strongly in the UVR spectra (de Vera, 2005) (Fig. 4). Numerous fruiting bodies are formed by this lichen, with abundant production of ascospores under a surface layer of accumulated parietin. The generative part of the lichen symbiotic organism does not appear to be negatively impacted by exposure to simulated space conditions, as the intensity of discharge of ascospores and their germination capacity were not substantially reduced relative to controls. The presence of a shielding layer appears to protect the ascospores against the possible lethal effects of UV-C radiation, even though these wavelengths are not experienced in the lichen's natural (*i.e.*, planetary) environment. Therefore, the production of the UV-screening secondary metabolite parietin by the symbiotic lichen effectively also provides resistance to space parameters (de Vera, 2005).

The secondary metabolites, carotene and emodin, which are formed in the entire vegetative lichen thallus as well as in the apothecia, enhance the UV-shielding effect of parietin (Wynn-Williams et al., 2002; Edwards et al., 2003). This confirms the assumption that an upper cortex with UV-absorbing substances may be of great importance in protecting both symbiont types in the thallus. Chitin, a component in the cell wall of fungi, may also serve as a protectant, with absorbance of UVR at maxima of 200 nm, 210 nm, 300 nm, and 330 nm (van der Drift et al., 1996; Thoss 1999). By analogy, the intense formation of chitin in insects may be responsible for their high resistance to these highly energetic wavelengths of UVR (Berenbaum, 2001; Bletchly and Fisher, 1957; Cork, 1957; Davey, 1919; Lee et al., 1984; Meyer-Rochow et al., 2002; Misra Parvathy Bhatia, 1998; Ross and Cochran, 1963; Upton, 2001; Vernós et al., 1989; Wharton and Wharton, 1957, 1959).

In simulated asteroid impact experiments (Meyer et al., 2005; Stöffler et al., 2007; Horneck et al., 2008), resistance to extremely high temperature and pressure

conditions may be due to the heat and pressure absorption of the protecting rocky substrates, as it is unknown whether any of the protective mechanisms lichens could contribute to resistance to these stresses. The lichen *X. elegans* was highly resistant to exposure to explosive shock waves, both as an entire thallus and as separated photobiont and mycobiont.

4. Differences in Adaptation

Lichen species that are exposed to high UVR in their natural habitat clearly show a higher resistance and only a minor decrease in viability after exposure to simulated space conditions than lichens from more protected habitats (de la Torre et al., 2004, de Vera et al., 2004a,b; Wieners, 2005; Figs. 5a–7c). *Buellia*

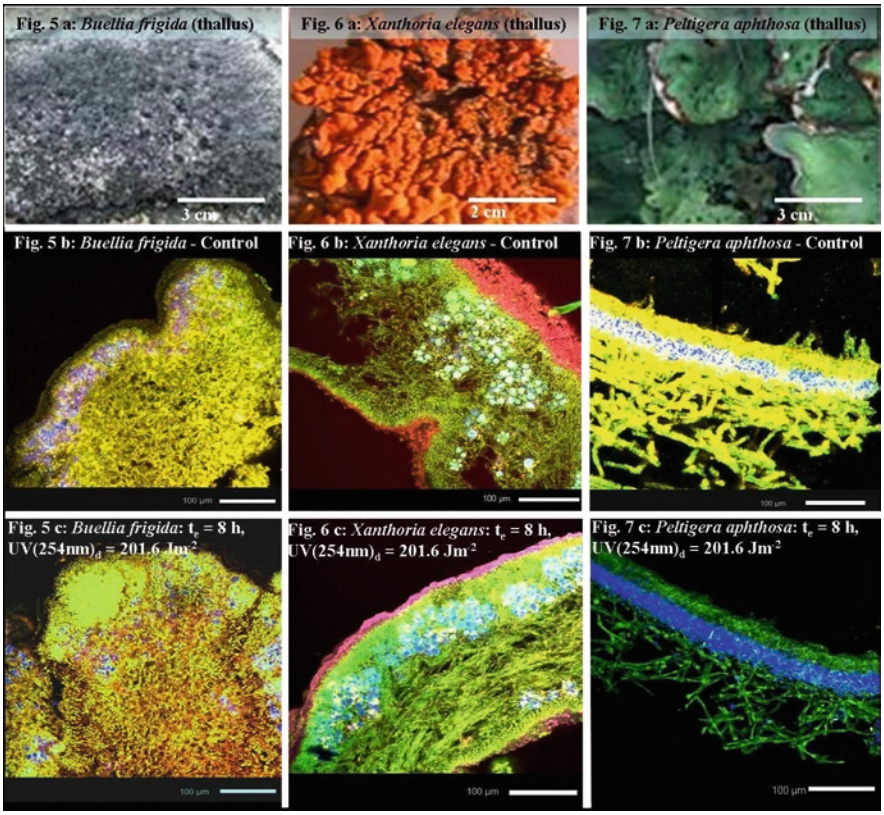

Figure 5a–7a. thallus of (5a) *B. frigida* (6a) *X. elegans* (7a) *P. aphthosa*. **Figure 5b–7c.** lichen stained with LIVE/DEAD-staining kit FUN I. The bright yellow to green colored mycobiont cells and the turquoise to whitish photobiont cells are still viable. (b) Control sample (c) exposed sample to monochromatic UV C (254 nm). t_e = exposure time, d = dose; Fig. 7c: each of the symbionts of *B. frigida* are not stained; the cells are dead.

frigida is an endemic lichen species widespread along the Antarctic Peninsula and across the continental Antarctic, where it is found on some of the most isolated inland rock exposures (Øvstedal and Lewis Smith, 2001). Among the lichen species examined here, *B. frigida* could be expected to be the most resistant to environmental extremes, followed by *Xanthoria elegans.* The latter has a bipolar distribution and reaches altitudes of at least c. 6400 m a.s.l. in the Himalayas. Both species show the highest resistance when exposed to simulated space conditions.

Exposure of *B. frigida* to different doses of UV-C (2.1 J m^{-2}, 5 min to 201.6 J m^{-2}, 8 h) in a desiccated as well as wet state resulted in no significant decrease in viability (relative to controls) in neither the mycobiont nor the photobiont (Fig. 8). A similar result was obtained for *X. elegans*, although there was an obvious, if small, decrease in the viability of the photobiont (to c. 60%, control c. 80%). The response of *P. aphthosa* differed substantially from that of *B. frigida* and *X. elegans.* This foliose lichen species is distributed in boreal to alpine habitats, colonizing primarily humid and shaded habitats, often in association with deep moss cushions. Specific and traceable secondary lichen metabolites are not known (Henssen and Jahns, 1974; Jahns, 1995; Brodo, 2001). Using the same experimental approach, the viability of *P. aphthosa* decreased significantly already after UV treatment at only 2.1 J $m^{-2}$and, after only 1 h exposure, viability was almost 0%. In contrast with *B. frigida* and *X. elegans*, differences in viability between the desiccated and wet state of thalli of *P. aphthosa* were also obvious, with the mycobiont in a desiccated thallus shows the highest, and the photobiont in a wet thallus the lowest, viability (Fig. 9).

5. Conclusions

Our data clearly indicate differences in the studied lichens' responses to simulated space conditions. Depending on the typical environmental conditions of the natural habitat of the lichen, there were different degrees of resistance to different doses of UV-C radiation. These symbiotic organisms have evolved several different and complementary protection mechanisms protecting against the deleterious effects of UVR, including absorption by secondary metabolite crystals and amorphous cell wall components. Both the polar (*B. frigida*) and alpine (*X. elegans*) lichen species were impacted by UV-C radiation only to a minor extent, while the boreal lichen species *P. aphthosa* suffered considerable damage. The lack of UV-absorbing secondary lichen metabolites in the latter species is likely to be one reason underlying the highly lethal effects caused by UVR. The UV-C spectra used in these experiments do not occur under natural conditions on Earth, these wavelengths being strongly absorbed in the upper layers of the atmosphere. We, therefore, suggest that the considerable stress adaptations demonstrated in *B. frigida* and *X. elegans* are of a more general nature, with resistance to simulated space condition being a by-product. It is also likely that the phenotypic structures implicated here as

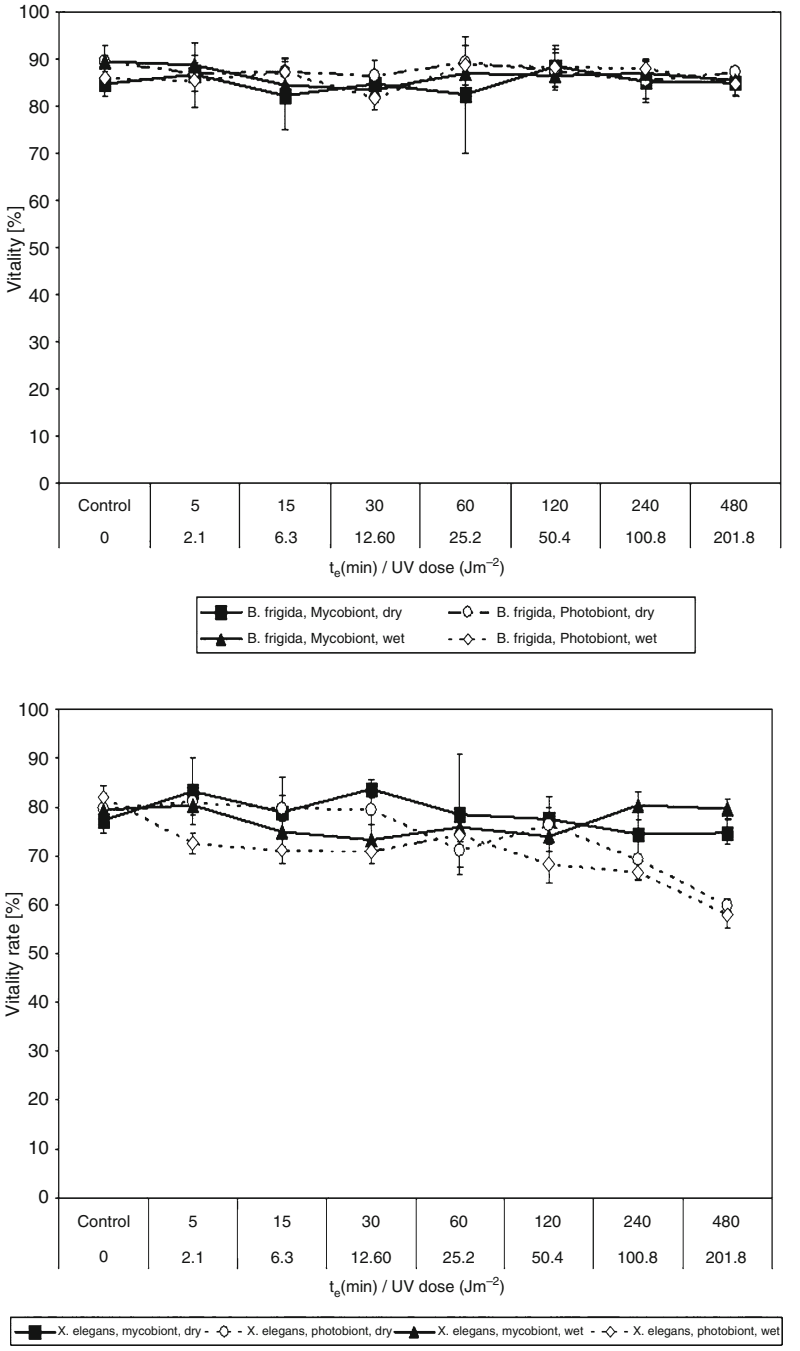

Figure 8. Viability of *Buellia frigida* and *Xanthoria elegans* after exposure to UV-C radiation (λ = 254 nm / each symbiont embedded in the lichen thallus; wet and dry conditions). No significant differences occur.

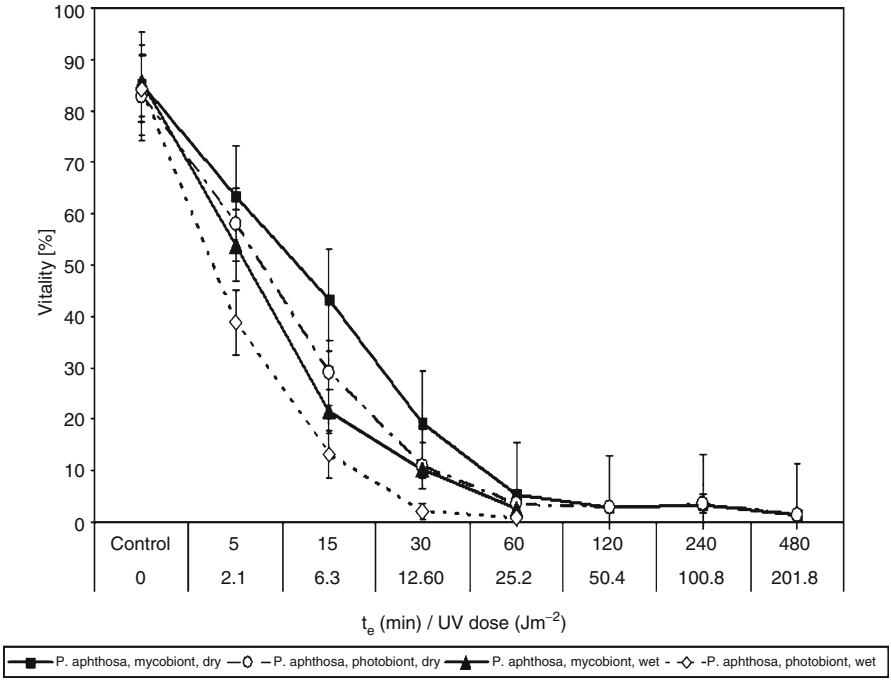

Figure 9. Viability of *Peltigera aphthosa* after exposure to UV-C radiation (λ = 254 nm/each symbiont embedded in the lichen thallus; wet and dry conditions). The viability of the lichen cells is decreasing.

stress adaptations form only a part of a more complex machinery, also involving cytological parameters, which should provide an interesting and fruitful future research topic.

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PART VI:
SUMMARY AND CONCLUSIONS

Grube
Seckbach

SYMBIOSES AND STRESS: FINAL COMMENTS

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In this book we highlight interesting cases of symbioses, in which to certain extent stress effects have been explored. We are aware that the selections do not cover all types of symbioses in which creative investigation has contributed to progress in the recent past. Our main purpose was to promote this exciting area of research with its practical implications and to provoke interest among the next generation of researchers. New questions still await to be addressed about the influence of varying and stressful environmental conditions on the metabolic interplay of symbionts.

We chose to apply the term *symbiosis* in a broad sense, although most chapters in this book focus on what is usually accepted as the mutualistic case, where one species benefits from the other. However, owing to difficulties in the usage of value-laden terminology, we prefer to view symbioses as the long-term intimate associations of organisms that evolve structural or metabolic novelties. Symbiotic association leads to biological complexity and new levels of selection, which act on symbiotic organizations and not on stand-alone organisms. Shifts to symbioses are, in fact, the pacemakers of evolution and responsible for evolutionary radiations: Past shifts to symbiotic life style were at the basis of eukaryote evolution, and switches of partner lineages play important roles in the radiation within kingdoms of life. Facultative symbiotic companions may modulate the fitness of extant species in their specific habitats. Stress plays an important role in initiating the first steps toward symbiotic association and driving adaptations of integrated metabolism in existing symbiotic relationships.

In this volume the editors gathered 33 chapters composed by 60 authors and coauthors from the following countries: Argentina, Austria, Canada, France, Germany, Israel, Italy, Japan, Monaco, Portugal, Slovakia, Spain, and the United States. This book is number 15 in Springer's *Cellular Origin, Life in Extreme Habitats and Astrobiology* series. It complements the first volume, *Symbiosis: Mechanism and Model Systems* (Seckbach 2002). We hope that the readers will garner knowledge and benefit from the new and updated symbiosis information.

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