

# Chapter 1

## Symbiotic Associations: All About Chemistry

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**Abstract** Biology is a challenging discipline because there is so much to know and so much to learn about the details of how living things work. The lessons that symbiosis has to teach are an excellent place to start, because they are some of the most interesting stories that biology has to tell. Symbioses are not just interesting, they are really important in evolution. Eukaryotes owe their existence to a fateful symbiotic encounter between an archaeal host and a bacterial symbiont, the ancestor of mitochondria, more than 1.5 billion years ago. The origin of the plant kingdom roots in a singular symbiotic association between a eukaryotic host and a cyanobacterium that gave rise to the plastid more than 1.2 billion years ago. In order for endosymbioses to become established, the two partners first need to meet and to stay associated over a protracted period of time so that the endosymbiont can evolve into an organelle. Then, in order for two symbiotic partners to remain associated after their first meeting, there has to be some kind of mechanistic benefit or dependence involved that will keep the one cell located next to—or stably maintained within—the other. In microbial symbioses, benefits are typically paid out in the currency of chemical substances. In this chapter, examples are covered that illustrate that principle.

Symbioses are not just interesting, they are really important in evolution. Eukaryotes owe their existence to a fateful symbiotic encounter between an archaeobacterial (archaeal) host and a eubacterial (bacterial) symbiont, the antecedent of mitochondria, more than 1.5 billion years ago (Williams et al. 2013; McInerney et al. 2014). The origin of the plant kingdom roots in a symbiotic association between a eukaryotic host and a cyanobacterium that gave rise to the plastid more than 1.2 billion years ago (Gould et al. 2008; Dagan et al. 2013). That event is called the primary plastid symbiosis, because it led to the establishment of the plastid from a prokaryotic photosynthesizer. Like the origin of mitochondria, the symbiotic origin of plastids occurred just once during all of evolution. Secondary symbiosis, where a eukaryotic alga became engulfed by another eukaryote and became

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reduced to the latter's plastid compartment, is more common in evolution. Many different groups of alga—including the major primary producers in the ocean, the diatoms—are the result of secondary symbiosis (Gould 2012; Stork et al. 2013), as are many important pathogens of humans, including the plastid-bearing malaria parasite *Plasmodium* (McFadden 2014).

The foregoing are examples of endosymbioses, symbioses where one cell came to live within another. In order for endosymbioses to become established, the two partners first need to meet and to stay associated over a protracted period of time so that the endosymbiont can evolve into an organelle. Then, in order for the two partners to remain associated after their first meeting, there has to be some kind of mechanistic benefit or dependence involved that will keep the one cell located next to the other. In microbial symbioses, benefits are paid out in the currency of chemical substances.

What is the nature of the substances that keep symbioses going? That is the topic of this volume: *The Mechanistic Benefits of Microbial Symbionts*. We often think of symbiosis and benefit in terms of mutual benefit, but symbiosis need not benefit both partners. The term symbiosis was coined by Anton de Bary (1878) in the course of his work on lichens and just means “living together,” not “living together for mutual benefit,” which is mutualism, a special case of symbiosis. For this volume, the editor has pulled together experts from many corners of biology to report on progress in their respective fields. Each chapter covers a specific kind of biological interaction and deals directly or indirectly with the nature of the chemical components that mediate that association.

Sometimes living together can be very uncomfortable for one of the partners, particularly in host-pathogen interactions. Such is the case with diphtheria, a severe illness associated with high fever in humans and caused by the facultatively anaerobic Gram-positive bacterium *Corynebacterium diphtheriae*. In Chap. 2, Sheryl Zajdowicz and Randall Holmes report on the interactions of the pathogen *C. diphtheriae* with its own pathogen—a bacteriophage, one that ends up being responsible for pathogenicity of *C. diphtheriae* in humans, because *C. diphtheriae* only produces the diphtheria toxin (a protein) when infected with the phage. The infection of *C. diphtheriae* by bacteriophage causes a genetic modification of the bacterial host, entailing many interactions between gene regulatory proteins that are involved in regulation of the diphtheria toxin expression. Bacteriophages are common “inhabitants” of animals and are usually temperate, meaning that they can be lytic (destroying the bacterial cell) or lysogenic (integrating into the genome and remaining quiescent there). When the phage integrates into the bacterial host genome, they are called prophage. Prophages are very common constituents of genomes, particularly in gammaproteobacteria and Gram-positives. Prophages affect bacterial diversity and can modulate pathogenicity in many bacterial pathogens. In addition to the diphtheria toxin, there are many toxins that are encoded by phage and are responsible for bacterial virulence. Examples of major medical significance include *Vibrio cholerae* or the *Clostridium botulinum* neurotoxin. In *Corynebacterium*, the diphtheria toxin is produced through lysogenization or lytic reproduction of the corynepophage  $\beta$ . Other *Corynebacterium* species can also

synthesize diphtheria toxin. Crystallographic studies of diphtheria toxin reveal different roles played by different parts of the protein during infection. An essential factor for synthesis of the diphtheria toxin is iron, an important bacterial nutrient, and Zajdowicz and Holmes summarize genetic and biochemical work showing how the toxin production is regulated by iron.

In some cases, the nature of the substances that weld organisms into symbiotic associations is not known. In Chap. 3, Kenji Ueda and Teruhiko Beppu examine the interactions of the thermophilic bacterium *Symbiobacterium thermophilum* with *Geobacillus stearothermophilus*, an anaerobic bacterium from the group of the clostridias. *Symbiobacterium thermophilum* cannot be cultured in the absence of *G. stearothermophilus*; the reasons for this dependence are still unknown but progress is being made. Work by the authors has demonstrated the presence of positive factors that promote growth and negative factors that inhibit growth in the coculture. Pure cultures of *S. thermophilum* can be obtained when grown next to *G. stearothermophilus*, separated from it by a dialysis membrane. This technique allowed the identification of diffusible factors that *S. thermophilum* requires from *G. stearothermophilus*. Ultimately they could show that CO<sub>2</sub> was one of the factors supplied and that the CO<sub>2</sub> requirement is not unique to *S. thermophilum*. They describe several other heterotrophic bacteria that require CO<sub>2</sub> concentrations higher than those supplied by air for growth. They also describe growth inhibitory factors that can be isolated from the coculture medium. The use of dialysis membranes provides a means to isolate chemicals required for symbiotic growth.

In other cases, the nature of the substances underlying symbioses is very well known indeed. In Chap. 4, Ivan Oresnik reports on one of the classical examples of symbiosis: the nitrogen-fixing rhizobia that induce root nodules in legumes. Rhizobia are members of the alpha- and betaproteobacteria. Specific bacterial genes are responsible for the intracellular fixation of nitrogen and its mobilization to the host plant via the nodules. Oresnik reviews this well-studied process from the bacterial infection of root-hairs, through differentiation of bacteroids, nodule-induction, and nitrogenase expression. Interactions between other bacteria from the Rhizobiaceae family and legumes are significant for agriculture. *Sinorhizobium meliloti* from the Rhizobiaceae (alphaproteobacteria) colonizes roots of the legume *Medicago sativa*. This complex relationship starts with chemical recognition between the symbiotic partners, which entails binding of a flavonoid that is exuded by *Medicago* roots into the soil. The flavonoid is taken up by *Sinorhizobium* cells, where it binds the transcription factor NodD, which starts a cascade of gene expression that culminates in the formation of nodules and occurs in several steps and diverse morphological differentiation of structural components. The nodule activation is regulated by hormones. A closer view at the particular processes of the symbiosis shows the signal exchange between both symbiotic partners. Rhizobial proteins cause the production of relevant signal factors that mediate the morphological changes in the host plant. The Nod factor, a lipochitooligosaccharide, is the crucial signaling molecule.

Sometimes symbioses involve a cascade of multiple organisms. In Chap. 5, Swati Singh and Steven Forst report on a symbiosis between three organisms,

only two of which reap benefits for which the third ends up paying the price. One partner is *Xenorhabdus nematophilus*, a Gram-negative proteobacterium that lives in a symbiotic association with the nematode *Steinernema carpocapsae*. The nematode in turn infects insects, such as the cutworm—caterpillar larvae of moths that forage on plants and can cause damage to garden and crops. The nematode introduces the bacterium into the insect's body cavity. There, the bacteria undergo a transition to a pathogenic stage, and they multiply. In the insect, *X. nematophilus* induces an elimination of the insect's immune response, and they produce toxic components that kill the insect. The nematode then feeds upon the bacteria in the decaying insect body. These interactions are mediated to a large extent by antimicrobial agents—secondary metabolites—that are synthesized by the bacterium and that act as immunosuppressants in the insect. The regulation of antimicrobial production by the bacteria involves quorum sensing.

Though microbes can spell doom for insects, as in the foregoing cutworm example, most insects strictly require microbial symbionts, in one way or another. Termites are a shining example in that they require anaerobic consortia of microbes in their intestinal tract to digest the cellulose that is their main food source, as David Bignell reports in Chap. 6. Termites were not always cellulose consumers, having evolved from cockroaches in a complex sequence of events that required a number of hefty evolutionary innovations, spanning from physiological transitions to eusociality. Termites have a very important ecological role in tropical and subtropical areas. In Earth history, they have probably played a major role in the terrestrial carbon cycle. They are certainly heavy lifters when it comes to cellulose breakdown, and though they are small, their biomass outweighs that of humans in Africa, South America, and Asia. The degradation of cellulose in the termite gut entails anaerobic consortia that ultimately produce short fatty acids, which the termite can resorb and digest, in addition to methane as a final waste product. Some termites do not perform cellulose digestion in the gut, however, but culture a fungus in their nest instead, and ultimately live from a diet of fungal hyphae. Trophic interactions in termite gut involve a cascade of microbes that live from the waste products of other members, with short fatty acids, CO<sub>2</sub>, H<sub>2</sub> and methane playing major roles. In termites, the substance of symbiosis is food and energy.

Food is also the name of the game in the bacterial symbionts of aphids. Aphids (like us) can only synthesize about half of their amino acids themselves. Yet they feed on plant phloem, which is notoriously poor in amino acids. This is where the bacterial endosymbionts of aphids come in, which live in a specialized organ of the aphid, called the bacteriome, and synthesize the essential amino acids that the aphid cannot extract in sufficient amounts from phloem. The aphid supplies nonessential amino acids in return. If that were not enough, phage plays a role in mediating the bacterial population as well, as Stephanie Weldon and Kerry Oliver address in Chap. 7. They focus on *Hamiltonella defensa*, a known pea aphid symbiont that is a model system for the involvement of phage within a bacteria-insect mutualism. The phage infects the bacteria but can exert a positive influence on these symbioses in terms of insect progeny. Phages are generally seen as pathogens, but here their relationship is mutualistic in many respects, at least with respect to insect fitness.

The role of phage in the *Hamiltonella* system is compared to that in the *Wolbachia* system. We have come to learn that almost all insects have symbionts of some sort, and it well could be that all symbionts will turn out to have phage of some sort, as well.

A fundamentally different kind of symbiosis involving bacteria and insects is described in Chap. 8, though, where Martin Kaltenpoth reviews the biology of the actinobacterium *Streptomyces philanthi* that resides in the glands in the antennae of wasps called “beewolves.” Beewolves belong to the genus *Philanthus* and are distributed more or less worldwide. The name derives from the hunting character of the female adults, which hunt for bees. Inside the beewolf antennae glands, *Streptomyces* help protect wasp offspring by the production of various antimicrobial substances that defend the developing brood against fungal and bacterial pathogens. This particular insect symbiosis has only been known for about 10 years, but the pace of discovery in its investigation has been rapid. Rather than getting a meal in this symbiosis, it is almost as if the wasps obtain medical treatment from their symbiotic streptomycetes. Again chemical substances stand in the foreground of this symbiotic association, but the main function is not nutrition.

In Chap. 9, Antje Lauer reports on the microbial fauna associated with Bryozoa, marine invertebrates that include *Watersipora* species. Bryozoan animals, zooids, grow to less than 1 mm in size but form colonies that can reach many centimeters in diameter. Bacteria form biofilms on *Watersipora*. The nature of the relationship between the bryozoans and their ectosymbionts is still uncertain. A number of bioactive secondary metabolites can be isolated from colonized bryozoans, but it is not clear which organisms within the associations are producing them. Lauer suggests that the epibionts, which can include proteobacteria and cyanobacteria, compete with other microorganisms and stop the fouling of the invertebrate’s surface. The Bryozoa harbor many open questions concerning the function of their microbial symbionts and the variety of bioactive compounds that they produce. It is possible that the epibionts influence the distribution of the Bryozoa and their settlement on different substrates.

Speaking of different substrates, in Chap. 10, Nathan Kirk and Virginia Weis review one of the most gregarious symbionts known: *Symbiodinium*. *Symbiodinium* is a genus of photosynthetic marine dinoflagellates that enters into symbiotic associations with a broad diversity of marine hosts. It can be found as an endosymbiont in ciliates (a group of heterotrophic protists), but it is most commonly found in symbiotic association with animals. These can be reef-building corals, cnidarians (*Hydra*), mollusks, sea anemones, sponges, or members of the Acoelomorpha. They all harbor, and appear to benefit from, *Symbiodinium*. Roughly 20 *Symbiodinium* species are currently known, but only half of them have been described in detail. Most of the known *Symbiodinium* endosymbionts are obligate endosymbionts, though some are facultative, and transmission can be either vertical or horizontal (newly established host-symbiont associations). The diversity of *Symbiodinium* symbiotic associations is only surpassed by the diversity of its unusual genome attributes, which Kirk and Weis also review in their chapter. The symbiotic interactions between *Symbiodinium* and corals are particularly well known and

important for modern ecosystems. For corals, *Symbiodinium* can supply up to 95 % of the coral's carbon (Muscatine and Porter 1977), clearly defining the chemical nature of the association, yet whether or not photosynthesis underpins all of the *Symbiodinium* symbioses is less well understood.

That brings us to the last contribution in this volume, Chap. 11, which sheds more light on symbiosis, biologically generated light, that is. Spencer Nyholm reports on the symbiotic association between the Hawaiian bobtail squid *Euprymna scolopes* and its bioluminescent bacterium *Vibrio fischeri*, which lives in specialized lobes within the animal's body cavity, where it receives all of its nutrients from its host. At night, the squid goes hunting and uses the bacterium's bioluminescence for the purpose of camouflage. The source of *Vibrio's* light is the luciferin-luciferase system, in which ATP is consumed to form luciferyl-AMP, which harbors a mixed anhydride bond between AMP and a carboxyl group of the benzothiazole ring-containing metabolite luciferin; in the presence of O<sub>2</sub>, the complex eliminates AMP and CO<sub>2</sub> to generate oxyluciferin and a photon (Nakatsu et al. 2005). Nyholm points out the many different bioluminescent symbioses involving marine animals and bacteria. Even though the mechanistic benefit of the symbiosis is light, its basis is again chemical.

Looking back into the early phases of eukaryote evolution, what were the chemical benefits that associated the ancestor of mitochondria with its host? One view has it that anaerobic syntrophy was important at mitochondrial origin (Martin and Müller 1998). Anaerobic syntrophy is a well-known principle in microbial ecology: one cell (or species) produces molecular H<sub>2</sub> as a waste product of energy metabolism, and another cell (or species) lives from that H<sub>2</sub> as chemical energy to reduce CO<sub>2</sub> and make ATP (Schink 1997). In that case, the mitochondrial endosymbiont was just a normal facultative anaerobic bacterium, able to respire O<sub>2</sub> or to live anaerobically, and both properties were vertically inherited within the mitochondrial family of organelles. That would explain why anaerobic mitochondria are so widespread in nature, why they share so many overlapping properties with oxygen respiring mitochondria, and why there are still H<sub>2</sub>-producing mitochondria out there among eukaryotic groups (Tielens et al. 2002; Boxma et al. 2005; Müller et al. 2012). Of course, once the mitochondrion became established as an endosymbiont, it became a specialized organelle of eukaryotic energy metabolism. The comparison of mitochondrial respiratory chains with the existing diversity of respiratory chains in proteobacteria is consistent with that view (Degli Esposti et al. 2014).

Many people still think that the main advantage of mitochondria was a roughly sixfold increase in energy yield from glucose: 32 mol ATP per glucose involving O<sub>2</sub>-respiring mitochondria vs. 5 mol ATP per glucose involving anaerobic mitochondria. But if we think about it, that cannot be true: if O<sub>2</sub> respiration was the key to eukaryote complexity, then *E. coli* and all other (facultative) aerobic prokaryotes would have become complex, for the same reason, and cyanobacteria (the first O<sub>2</sub> producers) would have become more complex than eukaryotes. The advantage of mitochondria was the internalization of bioenergetic membranes (as opposed to plasma membrane bioenergetics in prokaryotes) and the roughly 100,000-fold

increased amount of ATP per gene that this afforded to the founding cells of the eukaryotic lineage (Lane and Martin 2010). Thus, while it has now been evident for some time that the common ancestor of eukaryotes possessed a mitochondrion (Embley and Martin 2006), it is now clear why that was so: the lack of true intermediates in the prokaryote-to-eukaryote transition has a bioenergetic cause.

And what about plastids? Traditional reasoning has it that autotrophy—synthesis of carbohydrates from  $\text{CO}_2$  and supply thereof to the host—was the main advantage of plastids. In uniquely original work, that idea was set forth quite explicitly over 100 years ago by Mereschkowsky (1905) in a paper that laid the foundations of endosymbiotic theory. The photosynthate supply idea has been reformulated, rediscovered, warmed over, and reserved many times since. But when we look around at modern symbiotic associations involving cyanobacteria, the main chemical compound delivered from symbiont to host is not carbohydrate; it is nitrogen, nitrogen that is fixed from  $\text{N}_2$  by the cyanobacterial symbionts themselves (Kneip et al. 2007). That prompted the idea that nitrogen might have been the main initial benefit of plastids, and genomic comparisons tend to favor the view that the ancestor of plastids emerged from the nitrogen-fixing forms (Deusch et al. 2008; Dagan et al. 2013).

Symbioses are just plain interesting. They can, however, be so interesting that they become deceptive. A recent example is the sacoglossan sea slugs that capture plastids from their algal prey (Wägele and Martin 2014). The slugs sequester the plastids in the cells of their enlarged digestive gland that resides under the dorsal epithelium, giving the slugs a bright green appearance. Because the plastids can remain in a photosynthetically active state for months within the cytosol of animal cells, and because some sacoglossan species can survive without additional feeding for weeks and months once they have acquired their plastids, it was long assumed, safely, one thought, that the function of the plastids was to provide the slugs with fixed carbon, photosynthate as nourishment (Rumpho et al. 2000). And if photosynthetic feeding is the function of the plastids, then the next logical conclusion is that genes must have been transferred from the algae to the slugs so that the plastids can remain functional in the animal for so long (Rumpho et al. 2008). Great story, but is it really true? When the data was obtained that would actually determine whether any gene transfer had occurred, it was found that no gene transfer had occurred at all (Wägele et al. 2011). Instead, the reason for the ability of the plastids to remain active for long periods in the animal cells appears to reside in the properties of the plastids themselves, possibly a gene in plastid DNA called FtsH (de Vries et al. 2013). Worse yet, the slugs, once they obtain their plastids, live just as long in the dark as they do in the light (Gould et al. 2014), so photosynthesis cannot really have anything whatsoever to do with the mechanistic benefit of the symbiotically sequestered plastids (Christa et al. 2014). That does not detract, however, from the intrinsic fascination of animals that steal plastids from algae and store them for months in the cytosol of their own cells (de Vries et al. 2014) in what appears to be a photosynthetically active state but without mechanistically obtaining benefit from that apparent photosynthetic ability.

In summary, biology is really hard, mainly because there is so much to know and so much to learn about the details of how living things work. Fortunately, the lessons that symbiosis has to teach are an excellent place to start, because they are some of the most interesting stories that biology has to tell.

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