Chapter 4
Evolutionary Success of Prokaryotes

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Abstract How can the evolutionary success of prokaryotes be explained? How did they manage to survive conditions that have fluctuated, with drastic events over 3.5 billion years? Which significant metabolisms and mechanisms have appeared over the course of evolution that have permitted them to survive the most inhospitable conditions from the physicochemical point of view? In a "Red Queen Race," prokaryotes have always run sufficiently fast to adapt to constraints imposed by the environment and the other living species with which they have established interactions. If the criterion retained to define the level of evolution of an organism is its capacity to survive and to yield the largest number of offsprings, prokaryotes must be considered highly evolved organisms.

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4.1 Prokaryotes: An Overview of the Theories of Evolution

In this paragraph, the concept of natural selection as first proposed by Darwin and its role in the evolution of prokaryotes will be first discussed. Studies undertaken after Darwin do not fundamentally question natural selection, but it has been demonstrated to be insufficient to explain the evolution of living beings and, in particular, that of prokaryotes. Afterward, the different theories that have been proposed after Darwin will be briefly described and their application to the prokaryotic world covered.

4.1.1 Darwinian "Natural Selection"

Natural selection, as the driving force of evolution, has been proposed simultaneously and in an independent fashion by Charles Darwin (1859) and by Alfred Russel Wallace (Wallace 1870). Charles Darwin was a naturalist who had built his theory based on the observation of the phenotype of macroorganisms (plants and animals), both living ones and fossils. The mechanisms underlying natural selection were three major ones: variation, heredity, and struggle for existence.

(i) Variation: for Darwin species are not immutable (*I am fully convinced that species are not immutable*). Species evolve "through reproduction with modification," and the species of a given genus are supposed to descend from a common ancestor, even though he considers that the exact character of the common ancestor of a group of organisms is not known (*we never know the exact character of the common ancestor*). This link between all living beings and this concept of a "common ancestor" had not been proposed before Darwin.

(ii) In the framework of natural selection, each variation, which happens in a random fashion, if it is useful for the survival and fitness (maximum number of offsprings) of a living being, is preserved and inherited; conversely, any injurious variation is rejected. Besides, successive modifications that appear are slow and slight and appear in a graduated manner; a great and sudden modification will not occur in the evolution of living beings as conceived by Darwin; for him "Natura non facit saltum" or nature does not make leaps.

(iii) "Struggle for existence": in an ecosystem, the species present (and not individuals) produce more offsprings than can survive given the resources available that are for the most part limited. A struggle will thus occur to obtain these resources. Those that will survive will not necessarily be "the strongest" but those "best adapted" to the conditions of the environment and who will have a success in leaving progeny. The phenotypes emerging at random and yielding the largest number of offsprings will survive. Random variations appear, and among these variations (mutations), those that will spread in the population are those that will result in adaptation to new environmental conditions and will ensure the maximum number of offsprings.

It must be precised that natural selection is not aiming toward "progress" but toward maintenance of species best adapted to a given ecosystem at a given moment; the biotope itself being subject to changes also modifies permanently the conditions to which the species must adapt.

Moreover, species must not only adapt to the changes occurring to their biotope but also to the changes that other species within the ecosystem undergo. Darwin was conscious of the importance of interactions with other species (*if any one species does not become modified and improved in a corresponding degree with its competitors, it will soon be exterminated*).

Is natural selection proposed by Darwin, who was completely ignorant of the microbial world, applicable to prokaryotes?

The concept of natural selection, built on observation of the phenotype of macroorganisms, is applicable to prokaryotes. Indeed, in prokaryotes, as specified by the natural selection concept, each time a "variant," within a population, gains an advantage for survival – be it for the acquisition of resources or adaptation to a natural or anthropogenic modification of the environment – it is maintained and will be propagated in the population. For instance, antibiotic resistance of bacteria and bacterial xenobiotic biodegradation are consequence of evolution via natural selection. However, for Darwin the time scale to grasp evolution was geological; in prokaryotes, the time scale is much faster: the pace of prokaryotes generation (a few hours, days) is completely different from that of macroorganisms which is set in years if not millennia.

Darwin developed the concept of natural selection based on observations, without knowing its underlying mechanisms. The study of prokaryotic evolution has played a capital role in the comprehension of mechanisms involved in the evolution of living beings. What is even more remarkable is that the study of prokaryotes has brought the experimental proof of the action of natural selection and other mechanisms involved in evolution, enabling direct observation and dissection of evolutionary processes (van Ditmarsch and Xavier 2014) (*cf.* "Evolution underway in prokaryotes").

Do discoveries that have followed Darwin's works question natural selection?

Before mentioning the other theories proposed following Darwin, this theory must be first replaced in the context of the knowledge existing at the moment of its elaboration and, second, insist on the carefulness of the author concerning the role of natural selection in the evolution of living beings.

It must be recalled that Darwin was convinced natural selection had been the main, but not the only means causing modification of species (*I am convinced that natural selection has been the main but nonexclusive means of modification*).

He knew the limits to his theory. The most important was his incapacity to find the biological mechanisms at play in descent with modifications. Darwin was convinced that a large field of research would open on knowledge of the causes and laws of variation (*a great and almost untrodden field of inquiry will be opened, on the causes and laws of variation*). It was indeed the case, and the first to propose a theory of heredity compatible with the Darwinian theory was an Augustinian monk Gregor Mendel, 6 years after the publication of *On the Origin of Species* and 17 years before the disappearance of Darwin. After the death of Darwin, a copy of a paper by Mendel was found on his bookshelf, which pages had not been detached, indicating he had not read it. It can thus be said that one answer to the questions he had wrestled with all his life was to be found at home, among his books.

4.1.2 The Synthetic Theory or Neo-Darwinian Theory

The main authors of the synthetic theory, elaborated in the 1930–1948 period, were Fisher (1930), Wright (1931), Haldane (1932), Dobzhansky (1937), Mayr (1942), Simpson (1944), and Huxley (1948). In this theory coupling classical Mendelian genetics and population genetics, biodiversity, and evolution of populations can always be explained by mechanisms of "natural selection," hence the name "neo-Darwinism." However, the scale of observation has changed. Once the laws of heredity had been discovered, the mathematical means were developed to "measure" natural selection.

Genetics was integrated into the Darwinian theory. The concept of "variant" has been replaced with that of "mutant," the result of random mutations that appear in the genetic reservoir. The selective value of a mutant is its adaptation to an environmental change, its propagation in a population, and its capacity to increase its descent (fitness). New alleles, new genotypes, and thus new phenotypes appear at random. If an allele codes for an advantageous "variant," the individuals that carry this allele will reproduce more than those carrying the wild allele that will eventually disappear from the population.

For neo-Darwinians, natural selection remains the most important mechanism to explain evolution of populations. The studies undertaken with prokaryotes have provided convincing arguments in favor of this theory.

1. The first direct experimental demonstration showing any genetic mutation is the fruit of evolutionary chance was done by Luria and Delbrück (1943) who studied resistance of the bacterium *Escherichia coli* B to a bacterial virus α. They asked whether the resistance to bacteriophage in *E. coli* was induced by the presence of phage or if instead it was due to random mutations occurring prior to phage exposure. The virus was plated first on nutrient agar plates and spread over the entire surface of the agar. A few minutes later, a bacterial suspension to be tested was spread over the central part of the plate. Luria and Delbrück noticed that bacteria resistant to the viral infection appeared after a few hours. Two hypotheses concerning the origin of the bacterial resistance could be proposed: (i) the appearance of bacteria resistant to the lytic phages would result from mutations induced in a specific fashion adapted to the presence of viruses. The mutations would thus have appeared not during the growth phase of the populations, but immediately after spreading of the bacteria onto the selectively lethal medium; (ii) the genetic mutations causing the hereditary variation linked to phage resistance would be spontaneous, produced at random during the growth phase of the bacterial population before plating on the selectively lethal containing lytic phages; the viruses would have no role in their occurrence. Luria and Delbrück made fluctuation tests to understand, on the basis of statistics on the distribution of the number of resistant bacteria, if mutations had occurred before (ii) or after (i) exposure of the bacterial population to viruses, in order to determine if the results of these experiences were conform with a Lamarckian explanation or if they were in line with the synthetic theory paradigm. They concluded that "the resistance to virus is due to a heritable change of the bacterial cell which occurs independently of the action of the virus" in accordance with the synthetic theory. This was the first direct demonstration of the random nature of mutation.
2. It is the study of prokaryotes that has permitted to understand the mechanisms underlying mutations.
3. From the studies of prokaryotes, it has been demonstrated that the acquisition of an advantageous allele can also occur through HGT (*cf.* Sect. 4.2.2).

4.1.3 The Neutralist Theory of Molecular Evolution (Genetic Drift)

According to the theory proposed by Motoo Kimura (Kimura and Ohta 1974; Kimura 1986), evolutionary changes, at the molecular level, do not always result in a progressive accumulation of favorable mutations causing, at the phenotypic level, a gradual and continuous evolution of the adaptation to the environment as supposed by Darwinian thought. In a population, not only the appearance but also the diffusion and fixation of mutations – which for this author are mainly but not only strictly neutral or nearly neutral – occur in a purely random fashion, at a regular rhythm over time, whence the notion of "molecular clock." It has nevertheless been

demonstrated, on the one hand, that proteins do not all have the same rate of molecular evolution and, on the other, that the mutation rate is not the same over the genome, thus questioning the concept of molecular clock, as well as that of chance (Chattopadhyay et al. 2009).

Neutral mutations, those that cause neither advantage nor disadvantage in the population, accumulate in the genotype and are not eliminated through natural selection, but are under the control of random "drift," which is a random "drift of genetic frequencies."

The neutralist theory is not opposed to the idea that evolution of forms and functions be governed by Darwinian selection, but it reveals another facet of evolutionary processes. Darwin observed phenotypes, while Kimura studied genes.

Kimura estimated that evolutionary changes, when studied at the molecular level (proteins, DNA), are not the consequence of the action of natural selection, but that of the genetic drift of neutral or nearly mutations; to get established in a population, mutant genes do not inevitably have an advantage.

The neutral theory (or more precisely, the neutral-mutation-random-drift hypothesis) claims that the great majority of evolutionary changes at the molecular level are caused not by Darwinian selection acting on advantageous mutants, but by random fixation of selectively neutral or nearly neutral mutants. The theory does not deny the role of natural selection in determining the course of adaptive evolution, but it assumes that only a minute fraction of DNA changes are adaptive in nature (Kimura 1986).

In the neutralist theory, the size of populations plays an important role (Hallatschek et al. 2007). The smaller the population, the larger the importance of the genetic drift. However this evolution also exists in large populations if the phenomenon is followed over a large number of generations. This situation is that occurring in prokaryotes which generation times are particularly short.

Neutralist Theory and Prokaryotes

(i) Application of the neutralist theory (genetic drift) to small size populations: intracellular pathogenic bacteria? It has been noted that intracellular pathogens or symbionts have a faster mutation rate than their saprotrophic neighbors and that they accumulate deleterious mutations (Moran 1996), effects due to the lack of genetic exchange, and a regular purging mechanism called Muller's ratchet (1964).

(ii) The neutralist theory considers that mutations may affect genes without incidence on the function of the proteins encoded by these genes. Examples of neutral mutations in prokaryotes: ARNr16s, nitrate reductase, etc.

4.1.4 The Theory of Punctuated Equilibria

We will recall that for Darwin, natural selection "can never take a leap, but must advance by the shortest and slowest steps," Natura non facit saltum.

The notion of discontinuity was already present in the theory of mutations proposed by Hugo de Vries, a mutationist evolutionist, who estimated that species

appeared following mutations. The theory of punctuated equilibria (Eldredge and Gould 1973) reinforces the concept of discontinuities in evolution: evolution is not always gradual, moving forward step by step, as proposed by Darwin. It may also occur stepwise, its advance being discontinuous.

Examples Chosen in the Prokaryotic World to Illustrate This Theory

(i) To explain the transition from a cellular organization of the prokaryotic type to a eukaryotic type organization, basically, three "scenarios" have been proposed: *(a)* the "endosymbiotic theory," this theory postulates that mitochondria and chloroplasts were former bacteria that, during evolution, had established a symbiotic relationship with a eukaryotic ancestor, probably heterotrophic and anaerobic (Sagan 1967; Margulis 1970); *(b)* in this scenario eukaryotes result from an association between a bacterium and an archaeon (Martin and Müller 1998; Lopez-Garcia and Moreira 2006); and *(c)* the very controversial hypothesis which suggests that Archaea and Eukarya have evolved from an actinobacterium (Cavalier-Smith 2002). For the three hypotheses proposed, the prokaryotic/eukaryotic transition did not occur progressively on geological scales, but relatively quickly with a massive number of HGTs, for instance, from cyanobacteria to the nucleus of the host (in the endosymbiotic theory) or from deltaproteobacteria to archaea accompanied by the total disappearance of the bacterial genome (hypothesis based on an association between a bacterium and an archaeon).

(ii) Resistance to antibiotics (*cf.*: biosynthesis and resistance to antibiotics) (a bacterium may acquire "at once" the genetic information coding for new properties).

(iii) The association *Wolbachia*/nematodes, arthropodes, and insects (Ioannidis et al. 2013; Moriyama et al. 2015).

(iv) DNA gyrase: the discontinuity may be explained by the acquisition of a mutation that will confer an evolutionary advantage to the mutant population in which it emerged.

4.1.5 The Theory of the Selfish Gene

This theory was proposed by Richard Dawkins (1976). As he proclaims it himself, he is an enthusiastic Darwinian. Even though he considers that much of what Darwin said is, in detail, wrong, he nevertheless estimates that his theory is a different way of seeing, not a different theory.

He considers there are two manners to consider natural selection, from the point of view of the gene and from that of the individual. Well understood, they are equivalent; these are two conceptions of the same truth. One can alternate from one to the other; they are the same neo-Darwinism (*My point was that there are two ways of looking at natural selection, the gene's angle and that of the individual. If properly understood they are equivalent, two views of the same truth. You can flip from one to the other, and it will still be the same neo-Darwinism*).

For Dawkins, the fundamental unit of selection is not the species, nor the individual, but the gene or "replicator" (*the basic unit of natural selection*) which is also the unit of heredity. Natural selection favors genes that control survival machines that develop most harmoniously and yield most offsprings in the environmental conditions present.

Richard Dawkins pushes to the extreme the role of genes in evolution. He sees the evolution of the living world only through the history of genes. For him, "We are survival machines, but 'we' does not mean just people. It embraces all animals, plants, bacteria, and viruses." Genes are selfish, living only for themselves and have an autonomous life. However, if the organism is only a means to carry genes, Dawkins admits their *survival depends on the efficiency of the bodies in which they live and which they helped to build* and that *natural selection favors replicators that are good at building survival machines*.

In prokaryotes, natural selection as defined by R. Dawkins has an important pertinence. One can consider prokaryotes, from the point of view of replication, as particularly efficient machines to transmit their genes to their offsprings.

4.1.6 Natural Selection and HGTs

> ***The tree of life should perhaps be called the coral of life, [its] base of branches dead; so that passages cannot be seen***. (*Life and Letters of Charles Darwin – Volume 1, pg 368*)

The discovery of HGTs, which are particularly intense in prokaryotes, demonstrate that novelty does not emerge only in a vertical fashion "from parents to descent" but also in a horizontal fashion "from neighbor to neighbor." Horizontal transfers of genes (HGTs) are superimposed to vertical transfers (*cf.* 4.3.2 – *Horizontal gene transfers*).

The study of prokaryotes has also revealed a mode of information transfer, unthinkable for Darwin and his successors, until the work undertaken by Griffith on the transmission of hereditary characters in bacteria. Before Griffith, transmission of hereditary characters could only occur from living being to living being. But Griffith showed that information transfer could occur from "dead being to living being" and that the information thus acquired could then be transmitted to offsprings in a stable fashion. These discoveries amplified even more the intensity and complexity of exchanges in the prokaryotes world.

Darwin wanted to propose a scheme that would have highlighted the links that unite all species, hence the notion of the "tree of life." However, the whole of post-Darwinian discoveries demonstrate that the Darwinian tree of life cannot represent completely the evolution of living beings and particularly that of prokaryotes. For that reason, it is necessary to go from the "tree of life" to the "coral of life," a metaphor initially proposed by Darwin himself in his "secret notebooks." The metaphor retained *in fine* by Darwin, that "of a tree which branches spread out through geological times" – this was his time scale – can no longer be retained. If branches spread out, they often also get closer, cross, and even fuse (Fig. 4.1) (Doolittle 1999;

Fig. 4.1 Living beings carrying a very different evolutionary history exchange continually information. If branches spread out, they often also get closer, cross, and even fuse. https://www.pinterest.fr/pin/331436853799089167/

Olendzenski and Gogarten 2009; Koonin and Wolf 2012; Daubin and Szöllősi 2016). Living beings carrying a very different evolutionary history exchange continually information and may even fuse; a strictly genealogical explanation is thus no longer sufficient; it must be coupled to the notion of relatedness between species, which was demonstrated through the study of genes phylogenies, an approach that Darwin could not even imagine.

It should also be noted that the dichotomy between trees and corals with the later fusing while the former would not is false since there are many examples of fused trees in nature.

4.2 The Mode of Reproduction of Prokaryotes: High Rate of Reproduction and Maximal Utilization of Their Genetic Information

4.2.1 Maximal Utilization of Their Genetic Information

The mode of reproduction of prokaryotes through scissiparity is the most efficient to yield, in the shortest time, the highest number of offsprings (fitness). Prokaryotes represent "life at its rawest." They use their genetic information optimally: rate of

replication and translation, this last amplified through coupling of transcription and translation, and their DNA containing up to 90% of coding sequences (strong correlation between genome size and number of ORFs) (Konstantinidis and Tiedje 2004) contrary to DNA of eukaryotes that contain a high percent of noncoding DNA.

Many genomes contain a lot of redundancy, in terms of gene duplications, as well as pseudogenes that seem to have lost any function. Together with repeat sequences and parasitic DNA that seem to bear no function for the organism, the only conclusion can be that bacterial genomes are not always evolving toward optimal efficiency (Land et al. 2015). The presence of such "junk" DNA is one reason for the vast variation in genome size within the bacterial world, although the genome's size is of course also dependent on the number of functional genes and pathways that are present.

4.2.2 Generation Time

Generation times of prokaryotes (the time interval required for the cells or population) to divide are significantly lower than those of eukaryotes. They are highly variable and depend on the physicochemical conditions, available nutrients, and genetic factors.

In laboratory culture, the generation time calculated during exponential growth phase varies according to the species and is evaluated most often under optimal culture conditions. For most known cultured bacteria, generation times range from about 15 to 60 min: approximate generation times were 20, 28, 30, 35, and 50 min for *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Thermus aquaticus*, respectively. However, for some prokaryotes generation times are much higher and vary within a taxon.

The mean generation time of *Rhizobium japonicum* was between 2.8 and 4.1 h for fast-growing isolate and 6.7 and 13.0 h for slow-growing isolates (Keyser et al. 1982). At a pH 5.2 and 88 °C and at optimal factor concentration, the generation time of the archaeal kingdom, *Thermofilum pendens* (order of Thermoproteales), growing in a fermentor, was 10 h (Zillig et al. 1983). *Treponema pallidum*, the syphilis spirochete, doubled every 30–33 h in vivo and 30–50 h in vitro (Lafond and Lukehart 2006). In *Syntrophomonas wolfei*, an anaerobic syntrophic bacterium, the generation times obtained by co-culturing with *Desulfovibrio* and *Methanospirillum hungatei* were 54 and 84 h, respectively (McInerney et al. 1981). It also need to be mentioned that the generation time of an obligately barophilic bacteria (Yayanos et al. 1981), designated MT41 (MT for Mariana Trench), was 25 h at 2 °C and 690 bars; at a pressure of 1035 bars (close to the depth origin), the generation time was about 33 h, and the bacteria were unable to grow at a pressure below 380 bars. All of the examples cited above, which are obtained from growth undertaken in the laboratories (under optimal growth conditions), show a great variability of growth of prokaryotes.

In natural conditions, it is difficult to accurately estimate the generation time of bacteria, because nutrients and other important environmental factors for growth (e.g., temperature, salinity, pH, pressure, etc.) vary widely in time and space. In addition, most frequently (but not always), conditions for optimal growth exist only intermittently. Most often prokaryotes face to "feast-or-famine" conditions. Thus generation times in nature are most of the time well below to those recorded in the laboratory. In addition, growing in pure culture (axenic cultures), which is "absolutely impossible in nature," "protected as it is in its vial" (Winogradsky 1949), the prokaryote is not subjected to the competitive effects of other microorganisms.

In situ, generation time can be extremely high. According to Jannasch (1969) the generation time of four aquatic bacteria calculated from experiments performed in chemostat was slow, between 20 and 200 h. For their part, Wirsen and Molyneaux (1999) studying deep-sea natural microbial populations estimate that the growth rates of natural populations were extremely low (e.g., a doubling time of 629 h). In aquifer, the doubling time of an introduced bacterial strain, *Comamonas* sp. strain S DA001 (isolated from a shallow aquifer), under in situ subsurface conditions, was approximately 15 days; the calculated growth rate can be considered as identical to that of indigenous microbial populations because the strain was injected without nutrients (Mailloux and Fuller 2003). The longer generation times described are those of the endogenous prokaryotic populations living in subsurface depth. The mean generation time of the deep subseafloor microorganisms, calculated from metabolic activity of these populations (e.g., sulfato-reduction), was estimated at ~ 1000 years (Jørgensen and D'Hondt 2006).

4.2.3 Genome Size

The size of bacterial genomes varies from a few hundred nucleotides to >14 000, and it is largely dependent on lifestyle, growth conditions, phylogenetic origins, and nutritional strategies. In Tables 4.1, 4.2, and 4.3 are summarized the main characteristics of the genomes of *Bacteria* and Archaea of the most representative species of free-living organisms and organisms which are enable to have a free-living state. Unlike eukaryotes, the genome size variation in bacteria translates almost directly into biochemical, physiological, and organismal complexity because the majority of sequences are functional protein-coding regions.

Note that some prokaryotes contain more DNA than some eukaryotes. For instance, if the average genome size of fungi is 43.30 Mbp, some strains have a genome size below this average such as *Candida caseinolytica* (9.18 Mbp), *Hansenula polymorpha* (8.97 Mbp), and *Wallemia sebi* (9.82) (Mohanta and Bae 2015). In the same way, the genome size of *Ostreococcus tauri*, the smallest free-living eukaryote, marine alga, is 12.56 Mb (Derelle et al. 2006).

Finally, let's note that genome size of several prokaryotes is smaller than the larger known viral genomes at 2.5 Mbp (Philippe et al. 2013).

Table 4.1 Comparison of genomes of *Bacteria*

Organisms	Genome size (bp)	GC content (%)	Number of coding sequences	References
Mycobacterium genitalium	580,076	31.7	475	McCutcheon and Moran (2011)
Rickettsia prowazekii	1,111,523	29.0	835	McCutcheon and Moran (2011)
Pelagibacter ubique Strain HTCC1062	1,309,000	29.7	1354	Grote et al. (2012)
Methylophilaceae Strain HIMB624	1,333,209	35.37	1381	Hugget et al. (2012)
Prochlorococcus Strain MED4	1,657,990	30.8	1,716	Rocap et al. (2003)
Prochlorococcus Strain MIT9313	2,410,873	50,7	2,275	Rocap et al. (2003)
Synechococcus Strain WH8102	2,430,000	59.4	2,525	Dufresne et al. (2005)
Prochlorococcus Strain MIT9303	2,680,000	50	3,022	Scanlan et al. (2009)
Escherichia coli	4,639,675	50.8	4,145	McCutcheon and Moran (2011)
Anabaena variabilis ATCC29413	7,105,752	41.4	5,710	Thiel et al. (2014)
Rhodococcus sp. Strain RHA1	9,702,737	67	9,145	McLeod et al. (2006)
Sorangium cellulosum Strain S_0ce56	13,033,779	71.38	9,367	Schneiker et al. (2007)

Table 4.2 Comparison of genomes of Archaea

Organisms	Genome size (bp)	GC content (%)	Number of coding sequences	References
ARMAN-4	800,887	34.8	916	Baker et al. (2010)
ARMAN-2	999,043	47.2	1,033	Baker et al. (2010)
Methanothermus fervidus strain V24S^T	1,243,342	31.64	1,311	Anderson et al. (2010)
Staphylothermus marinus	1,570,485	35.7	1,610	Anderson et al. (2009)
Methanococcus Jannaschii	1,664,976	31.4	1,682	Bult et al. (1996)
Thermofilum pendens	1,781,889	57.6	1,883	Anderson et al. (2008)
Methanosarcina acetivorans	5,751,492	42.7	4,524	Galagan et al. (2002)

Table 4.3 Comparison of genomes of host-dependent prokaryotes

Organisms	Genome size (bp)	GC content (%)	Number of coding sequences	References
Candidatus Nasuia deltocephalinicola	112,031	16.6	137	Bennett et al. (2016)
Candidatus Tremblaya princeps	138,927	58.8	121	McCutcheon and Moran (2011); McCutcheon and von Dohlen (2011)
Candidatus Hodgkinia cicadicola	143,795	58.4	169	McCutcheon and Moran (2011)
Candidatus Carsonella ruddii	159,662	16.6	182	McCutcheon and Moran (2011)
Candidatus Zinderia insecticola	208,564	13.5	202	McCutcheon and Moran (2010)
Candidatus Sulcia muelleri strain CWSS	245,530	22.4	227	McCutcheon and Moran (2011)
Nanoarchaeum equitans	490,885	31.6	552	Waters et al. (2003)
Candidatus Moranella endobia PCVAL	538,000	43.5	411	Martínez-Cano et al. (2015)
Candidatus Blochmannia floridanus	705,557	27.38	583	Gil et al. (2003)

4.2.3.1 Free-Living Prokaryotes

According to Land et al. (2015), a "typical" bacterial genome is around 5 million bp and encodes about 5000 proteins; however, different lineages of free-living bacteria – most of them in marine environments – evolved reduced genomes (Table 4.1).

Pelagibacter ubique (strain HTCC1062) is a bacteria whose genome has long been considered as the smallest genome of an autonomously replicating free-living in nature (1,308,759 base pairs, 1354 protein code genes, GC% 29,7) (Giovannoni et al. 2005; Grote et al. 2012) (*cf.* Table 4.1). This strain is an ultramicrobacteria with an average cell volume (enclosed by outer membrane) of 0.037 ± 0.011 μm^3 (Zhao et al. 2017a). This strain cultivated in the laboratory (Rappe et al. 2002) thrives at low nutrient concentrations (oligotrophic conditions) characteristic of open oceans. *P. ubique* is the most studied members of the clade SAR11. All SAR11 bacteria (α-proteobacterium) are obligate aerobic and chemoheterotrophic, with full respiratory electron transport systems and the light-dependent proton pump proteorhodopsin. They have an ancient evolutionary origin, likely a Precambrian origin (Giovannoni 2017). It is the most abundant group of heterotrophic bacteria in the

oceans: in some regions SAR11 clade represents 50% of the total surface microbial community and 25% of the subeuphotic microbial community, and this global abundance in the oceans worldwide would be 2.4 × 10^{28} cells (Morris et al. 2002). However, presently, there is no consensus about biomass and activity of the clade in nature, largely due to variable results obtain as function of the method used to evaluate these two parameters. Today, SAR11 is one of the most successful competitors for dissolved organic compounds in euphotic zone and dark ocean and plays a major role in ocean carbon cycle. SAR11 members are have likely undergone genome streamlining, an evolution that leads to minimization of cell structure and complexity (small cell size, reduced genome) in large populations of free-living microbes in nutrient-poor environment (Giovannoni et al. 2005; Grote et al. 2012). In SAR11 large populations, genome streamlining is thought to benefit cells by lowering the cost of replication and optimization of transport systems that are essential to competition in the open ocean; in some cases surface-volume ratios might also drive streamlining. According to the streamlining hypothesis, the following characteristics are consistent with natural selection acting to economize cellular metabolism. For detailed information concerning evolution, abundance, diversity, biogeography, activity, biochemistry, and metabolism of SAR11, see Grote et al. (2012) and Giovannoni's review (Giovannoni 2017).

More recently, several species with lower genome size than *P. ubique* have been described. For instance, a novel clade of an uncultured marine *Actinobacteria* has been described by Ghai et al. (2013) using a combination of metagenomic approaches, flow cytometry, and FISH. The term of subclass "*Candidatus* Actinomarinidae" was proposed to denominate this group of prokaryotes. A member of these *Actinobacteria* named "*Candidatus* Actinomarina minuta" has an average cell volume of ~ 0.013 μm^3 and a genome size estimated in the range of 823–1029 kb.

The clade OM43, related to Type 1 methylotrophs, is another group of marine and freshwater bacteria with a small genome. The most investigated strains were HTCC2181 et HIMB624 which formed a monophylic lineage within the family *Methylophilaceae*, with a genome size of 1,304,428 bp (Giovannoni et al. 2008) and 1,333,209 bp (Huggett et al. 2012), respectively. These heterotrophic strains cannot oxidize methane but oxidize C1 compounds methanol and formaldehyde as sources of carbon and energy.

Bacteria endowed with small genome have been detected in groundwater such as the candidate phyla SR1, WWE3, TM7, and OD1 with a genome size equal to 1,777,760 bp, 878,109 bp, 845,464 bp, and 693,528 bp, respectively (Kantor et al. 2013; Luef et al. 2015).

Marine picocyanobacteria, the most abundant photosynthetic free-living organism on Earth, with two genera, *Prochlorococcus* and *Synechococcus*, have larger genomes. The genome size of *Prochlorococcus* ranges from 1.66 to 2.68 Mbp (Dufresne et al. 2003; Rocap et al. 2003; Scanlan et al. 2009). Rocap et al. (2003) describe two ecotypes within the genus: a high-light-adapted ecotype (1,657,990

bp, one of the smallest known genomes of any oxygenic phototroph), which is the most abundant in surface waters, and a low-light-adapted ecotype with a larger genome (2,410,873 bp), dominating in deeper waters. *Synechococcus* genome size ranges from 1.64 to 2.68Mb (Scanlan et al. 2009). According to Scanlan et al., the genome size of *Prochlorococcus* and *Synechococcus* is still small compared to the average genome size of other sequenced cyanobacteria (5,33 ± 3,69 Mb). For instance, the genome sizes of *Anabaena variabilis* (Thiel et al. 2014) and *Acaryochloris marina* (Swingley et al. 2008) were 7.1 and 8.3, respectively. Finally, let's note that some free-living bacteria are endowed with a largest genome such as myxobacterium *Sorangium cellulosum* strain S_0ce56 (Schneiker et al. 2007) and strain So0157-2 (Han et al. 2013) with a genome reaching 13,033,779 pb and 14,782,125 pb, respectively.

Concerning the genome size of free-living Archaea (Table 4.2), some are endowed with a small genome such as uncultivated ARMAN lineages (archaeal Richmond Mine acidophilic nanoorganisms) with a genome size ranging from 800,887 to 999,043 bp; ARMAN cells have volumes of 0.009–0.04 μm^3, as determined by cryoelectron microscopy (Comolli et al. 2009). It was suggested that these organisms were at least partially dependent on (an)other community members for basic metabolic building blocks. Other archaea have larger genome size. This is the case of hyperthermophilic strain *Thermofilum pendens* with a genome size of 1,781,889 bp (Anderson et al. 2008) or *Methanosarcina acetivorans* with a genome containing 5,751,492 bp which is by far the largest known archaeal genome and unique among the Archaea in forming distinct multicellular structures (Galagan et al. 2002).

4.2.3.2 Host-Dependent

Endosymbiotic and intracellular parasites living in a protected and nutrient-rich environment of their host have many features in common, including massive gene losses according to the Darwinian principle (many molecules can be obtained from the host) (Table 4.3). Bacteria with the smallest genomes are *Candidatus* Tremblaya princeps, *Candidatus* Hodgkinia cicadicola, and *Candidatus* Carsonella ruddii with genome sizes of 138,927, 143,795, and 159,662 bp, respectively (McCutcheon and Moran 2011). Many highly reduced genomes have been described in bacteria symbionts of several insect lineages (Martínez-Cano et al. 2015). This is the case of two bacteria that are hosted by the insect *Planococcus citri*. These two named bacteria *Candidatus* Moranella endobia (*β-Proteobacteria*) and *Candidatus* Tremblaya princeps (*γ-Proteobacteria*) live symbiotically and are associated by forming an unprecedented organization: *Ca.* M. endobia lives inside *Ca.* T. princeps (Thao et al. 2002). Another intimate and specific association deserves to be reported, the one between two Archaea, *Nanoarchaeum equitans* and *Ignicoccus hospitalis*, where *N. equitans* grows only in co-culture with *I. hospitalis* (Waters et al. 2003; Podar et al. 2008).

4.2.3.3 How Minimal Bacterial Gene Set Is Necessary to Exist?

The analysis of smallest prokaryotic genomes leads to a question of fundamental biology: what is the minimum gene number necessary for an independent life, required for reproduction and self-maintenance under given environmental conditions, for a free cell exist? This question can be approached by comparative genomics.

From the comparison of genome sequencing of *Haemophilus influenzae* and *M. genitalium*, Mushegian and Koonin (1996), the pioneers in identification of minimal gene set, estimated that 256 gene were needed to sustain the existence of a modern-type cell. Gil et al. (2004) proposed gene set composed of 206 protein-coding genes necessary, but probably not enough, to maintain a cell alive in a realistic environment. So, Gil and Peretó (2015) revisited the minimal gene set by adding gene to improve cell viability and new genes for RNA processing and metabolism. In 2016, Ye et al. (2016) proposed a simplified bacterial gene set by comparative genomics and supplemented it by neo-construction of a bacterial approximately minimal metabolic network. From this procedure, they proposed a simplified bacterial gene that preserves both self-replication and self-maintenance systems including 327 genes and requiring 431 reactions.

4.2.3.4 Artificial Minimal Cell

The simplified molecular machinery of organisms with small genomes may be used to aid in the design of live artificial minimal cells in the laboratory. An artificial cell could be considered "alive" if three criteria are met: *self-maintenance* (*metabolism*), *self-reproduction*, and *capable of Darwinian evolution*. Two approaches, conceptually different, can be implemented to synthetize artificial cell (Gil et al. 2004; Juhas 2016; Martínez-García and de Lorenzo 2016; Ye et al. 2016; Glass et al. 2017) (see also the site www.ees.lanl.gov/protocells): (i) the top-down approach (genome downsizing, reprogramming existing cell with simple genome) starts from existing organism (viable "natural" cell) and sequentially removes genes, until no more genes can be removed without severe growth impairment or loss of viability; beyond this genome simplification, growth is no longer possible; (ii) the bottom-up approach (de novo synthesis) consists of to synthesize artificial cell from scratch using non-living organic and inorganic materials.

Major results in the construction of a minimal synthetic cell, using a top-down approach, were obtained by the J. Craig Institute using *Mycoplasma*, a group of bacteria characterized by lack of cell wall, obligate parasitic lifestyle, metabolic simplicity, and small genome. Indeed, C. Venter's team designed, synthetized, and assembled the genome of *Mycoplasma mycoides* (1,078 kbp) and then transplanted it into a recipient cell, the cell of another bacteria, *Mycobacterium capricolum*. The new cell obtained, called JCVI-syn1.0, was controlled only by the synthetic

chromosome (901 genes), was capable of self-replication, but was not a truly "synthetic" cell because its synthetic genome was put into an existing cell (Gibson et al. 2010). In 2016, Hutchison et al. of the C. Venter Institute designed and synthetized a genome that contains 531,560 bp and 473 genes, appointed JVCI-syn3.0 (428 genes were deleted compared to the genome of JVCI-syn1.0); this genome is smaller than that of any free-living replicating cell. For instance, *M. genitalium*, one of the smallest known natural free-living bacteria, has a genome size 580,070 bp and a total 470 predicted coding regions (Fraser et al. 1995). JVCI-syn1.0 and JVCI-syn3.0 showed that they have similar colony morphologies, with smaller colony size for JCV-3.0; moreover, their generation time was different: JCVI-syn 3.0 has a doubling time of 173 min, while it is 63 min for JVCI-syn1.0. An unexpected result emerged from the analysis of JVCI-3.0 genes: if most were assigned into different functional group (cell membrane structure and function of genome information, expression and preservation, cytosolic metabolism), 149 genes (31.5% of the genome) were of unknown biological functions, suggesting the presence of undiscovered functions that are essential for life. If the work to produce a minimal set of gene necessary for the cell to survive has been focused on mycoplasma, "the quest for the minimal bacterial genome" was also undertaken at other prokaryotes (e.g., *E. coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Pseudomonas putida*, *Streptomyces avermitilis*, *M. pulmonis*, *M. pneumoniae*) (Martínez-García and de Lorenzo 2016; Glass et al. 2017).

Concerning the studies devoted to construction of artificial cells using a bottom-up approach, many have been focused on self-replicating lipid vesicles (Hanczyc et al. 2003; Rasmussen et al. 2004; Chiarabelli et al. 2009). Rasmussen et al. used liposome with a lipophilic peptide nucleic acid (PNA) anchored on his surface. Rasmussen's artificial cell was capable of self-replication using light as a source of energy.

Many difficulties must be overcome in the bottom-up approach due, largely, to the complex interactions between genes such as the lack of detailed information on gene networks: gene can become essential only when isolated from other genes, not integrated into the gene network. Another problem is the high number of possible combinations of genes from a gene pool. In addition, if the artificial cell cannot support life, it is difficult to know what (s) gene (s) is (are) absent(s) in order to obtain a self-replicating system.

In conclusion, the exact composition of the universal genome remains unresolved, and we are still unable to create life in the laboratory. Furthermore, the construction of minimal cell has a potential for medical application and provide large and valuable informations in the understanding of life. Furthermore, if gene set will be able to sustain the main vital functions of a hypothetical simplest bacterial cell, according to Gil et al. (2004), "one must keep in mind that this kind of research has little relevance for the study of the origin of life." The contribution of these studies on the knowledge of the origin of life remains open.

4.3 Rate of Evolution of Prokaryotes: Intensity and Propagation of Mutations Coupled to an Extraordinary Capacity to Exchange Genetic Information

4.3.1 Mutations

Darwin saw evolution as a two-step process. The first being a random process producing "variations," favorable or unfavorable for an organism, the second maintaining the favorable variations by the process of "natural selection." The notion of "variation" was reformulated in terms of "mutations" within the framework of modern synthetic theory (synthesis of Mendelian genetics and Darwinian evolutionism). Indeed, although DNA replication is a very reliable and very effective mechanism, the prokaryotic genome still undergoes permanent and hereditary modifications: mutations. A mutation corresponds to an alteration of the genetic material, the consequence of a lesion in the DNA, which occurs randomly from an evolutionary point of view; moreover, any mutation is caused by a malfunction of one of the DNA polymerases.

The first evidence that genetic mutations occur randomly was provided by Luria and Delbrück (1943), using *E. coli* as a biological model. These authors demonstrated that *E. coli* cells in the presence of phages resisted viral infection but that the presence of phages had no role in the emergence of resistant bacteria; the genetic mutations appeared spontaneously during the exponential phase of *E. coli* before contact with the virus.

The major consequence of the rapidity of reproduction of prokaryotes is their ability to produce a large number of mutants which will be subjected to the action of "natural selection." These mutations may be favorable, unfavorable (even lethal), or neutral in terms of survival and reproduction of an organism in a given biotic and abiotic environment. Most of the errors that occur in a genome are neutral or deleterious: neutral mutations will have no effect on the reproduction of organisms; deleterious mutations will be eliminated by "natural selection." But if a mutant benefits from a favorable mutation that allows him to live in a new environment, the advantage gained is such that the mutant will dominate the rest of the population. This is why the fight against pathogenic prokaryotes is an endless struggle; every time a new antibiotic is discovered and used, a mutant will appear that will resist this new product and will be able to grow in its presence. More generally, at each environmental change, a mutant appears which will adapt, and this capacity of adaptation explains the durability of the prokaryotes during the 3.5 billion years of evolution with a conquest of all biotopes, even the most extreme ones.

There are two types of mutations: *spontaneous mutations* and *induced mutations*. These two major classes of mutations have a completely different origin

4.3.1.1 Spontaneous Mutations

Spontaneous mutations result from a natural process. Indeed, during DNA replication, DNA polymerases PolI, II, and III occasionally make incorporation errors (replication is semiconservative) that they spontaneously correct. If this is not enough, a mismatch repair system (MMR) is used. In the latter case, the spontaneous frequency of mutations is of the order of 10^{-9} to 10^{-11}. These values attest to the very high stability of the DNA molecule which is essential for the organism to be genetically stable. But mutations, which are part of the natural evolution and occur at random on the nucleotide sequence, are also essential because they allow the organism to evolve "if diversity is essential to survival, and if mutagenesis is required to generate such diversity, perhaps mutagenesis has been positively selected for throughout evolution" (Radman 1999).

4.3.1.2 Induced Mutations

The second type of mutations, induced mutations, involves the action of an exogenous agent (mutagen). In principle, whether it is of physical, chemical, or biological origin, this mutagen acts, like the spontaneous mutations, at random on the genome. The SOS system (in reference to the naval Save Our Souls distress signal) is the typical example of an inducible mutator system. It is a coordinated response to DNA damage; it ensures the repair of DNA and the survival of the bacterium in response to strong environmental stresses that alter the genome (genotoxic attacks) (d'Ari 1985; Cox 2003; Fuchs et al. 2004; Nohmi 2006; Erill et al. 2007; Żgur-Bertok 2013; Baharoglu and Mazel 2014; Madigan et al. 2015; Gillings 2017). The SOS system is triggered by UV irradiation, chemicals or oxidative compounds, acids, organic mutagens, some antibiotics, high pressure, reactive oxygen species, etc. The SOS system, which is induced when normal repair systems cannot repair DNA damage, was first described in *Escherichia coli* (Radman 1975).

How does this system work? Two key proteins govern the SOS response: RecA (an inducer) and LexA (a repressor). As soon as the DNA is damaged (Fig. 4.2), the DNA polymerase stops recopying the genome. The triggering of the SOS system is recognition by the RecA protein (Fig. 4.2) of portions of the genome in the form of single-stranded DNA, the appearance of which is caused by aggression. RecA binds to this DNA (nucleofilament) and changes its conformation. Activated RecA has new properties, notably the ability to hydrolyze LexA, which is the brake of the SOS system. The cleavage of LexA leads to the depression of the SOS genes (LexA regulon). The key element is the presence of single-stranded DNA: as long as it is present, RecA retains its LexA-degrading properties, and the intensity of the SOS response depends on the number of single-stranded DNA portions. The SOS system encodes several proteins involved in various DNA repair mechanisms, in particular polymerase V (an enzyme encoded by the *umu*C, *umu*D genes) and polymerase IV

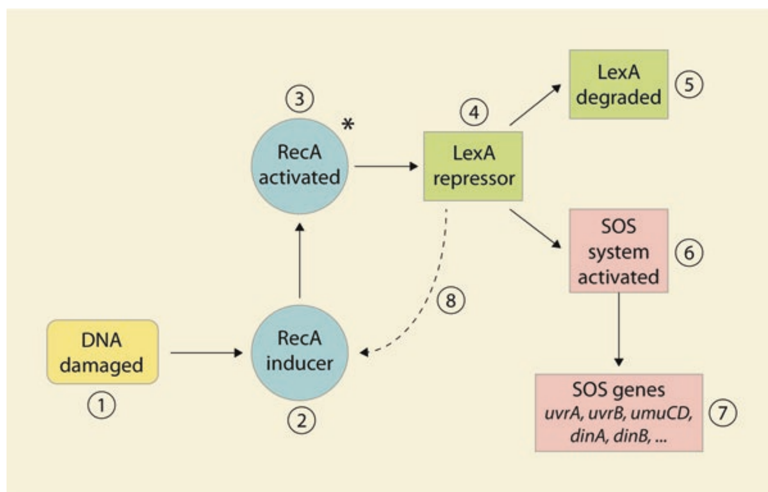


Fig. 4.2 The SOS system. DNA damage (1) activates RecA (2,3)
RecA★ activated hydrolyzes LexA (4,5). Proteolysis of LexA leads to coordinate derepression of LexA regulated genes (LexA regulon) (6,7). Rec A is expressed at a low level even in the presence of the LexA repressor (8) (Modified and redrawn from Nohmi, 2006; Żgur-Bertok (2013); Baharoglu and Mazel 2014; Madigan et al. 2015)

(an enzyme encoded by *din*B). Rec A is expressed at a low level even in the presence of the LexA repressor. Polymerases V and IV have the property to bypass the lesions at the level of the DNA sequence (translesion synthesis). They continue DNA replication despite lesions and thus allow the survival of the bacterium, unlike a "normal" polymerase that no longer works when it encounters DNA damage. However, these polymerases which are not characterized by high copy fidelity make copying errors and thus produce an increase in the rate of mutations in the damaged genomic regions. These polymerases are, on the one hand, able to cope with DNA lesions and, on the other hand, to increase the frequency of mutations in the event of stress. This increase in mutations rate generates genetic diversity and adaptation (e.g., antibiotic resistance) and potentiate bacterial survival and adaptation to changing environments. The disappearance of stress causes the SOS system to stop, and the rate of mutation returns to a normal mean value.

SOS response is a widespread and ancient trait of bacteria. Indeed, almost all bacterial phyla harbor a *lexA* gene with characteristic SOS boxes (Erill et al. 2006; Žgur-Bertok 2013; Baharoglu and Mazel 2014).

In addition, potentially carcinogenic substances generally induce an SOS response in bacteria. This correlation is the basis of several genotoxicity and carcinogenicity tests, the most widely used of which is the test developed and improved by Bruce Ames and his team (Maron and Ames 1983).

4.3.1.3 Hypermutators

Another property of prokaryotes, which explains their evolutionary success, is the presence in nature of bacteria that have a rate of spontaneous mutations 100–1000 higher than a normal or wild-type cell. These bacteria are referred to as hypermutators (Taddei et al. 1997; Tenaillon et al. 1999; Oliver 2005; Denamur and Matic 2006; Woodford and Ellinton 2006; Jayarama 2009; Oliver and Mena 2010; Hammerstrom et al. 2015; Lindgren et al. 2016). This hypermutability can be perceived in real time (see Chap. 6). The appearance of mutators is rare in a stable environment but increases in stressful situations where only bacteria that rapidly acquire one or more mutations to cope with the effect of stress will be able to survive. Mutators have been found in different populations – *E. coli*, *Salmonella enterica*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, etc. – with a frequency ranging from 0.1% to 60%. The ability of hypermutability is linked to damage affecting DNA repair and replication fidelity. The hypermutability that will create biodiversity is a considerable selective advantage for the mutator when a prokaryotic population faces a change in the environment: the possibility of possessing a mutant that will resist change – for example, the arrival of an antibiotic – will be considerably higher than in a wild-type strain. Indeed, it has been demonstrated, in vivo and in vitro, that treatment with an antibiotic leads to the selection of mutators whose resistance to the antibiotic is higher than that observed in non-mutators. A large number of mutators are found in hospitals. The mutator will grow faster than wild strain that has a low rate of mutations. But the increase in mutation capacity also involves genes essential for the survival of the bacterium, i.e., the number of lethal mutations will also be higher in the mutator than in the wild-type strain. In other words, the mutator adapts faster, but it is under threat of the appearance of a lethal mutation and is condemned in the long term. What is its future? Gene transfer between the mutator and the wild-type strain (reacquisition of a wild-type allele of an anti-mutator gene from a mutator bacteria via a horizontal gene exchange) or a reversion of the mutator may occur. Another possibility exists: the mutator can transmit the favorable mutation to the wild-type strain. In conclusion, despite the innumerable deleterious mutations that occur in hypermutability, the tiny proportion of favorable mutations confers such an advantage for the mutator that it overcomes the rest of the population.

4.3.2 Horizontal Gene Transfers

Prokaryotes live and evolve in a large variety of biotopes where physical, chemical, and biological conditions change rapidly in time and space. Beyond the modulation of enzyme production and activity, mutations that are frequent in prokaryotes will permit them to cope with these permanent fluctuations. However, adaptation capabilities of prokaryotes cannot be apprehended solely as ability to mutate. Indeed, on top of mutations – which are a source of diversity at the genetic level – the

evolutionary success of prokaryotes can be explained also by their extraordinary capacity to exchange genetic information through horizontal transmission (also named lateral transfer). This mode of transmission, with no relation to cell division, is a particularly efficient source of dissemination of genes or alleles carrying an evolutionary advantage. Contrary to vertical transmission of genes (from parent to offspring; from mother to daughter), there is gene transfer from a “donor cell” to a “recipient cell,” cells that can have no close parenthood in horizontal transfers (HGTs). Under certain conditions, the genetic material acquired through HGTs may integrate into the genome of the “receptor” cell and become an element of its genetic heritage; it will thereafter be transmitted vertically. Such horizontal exchanges, which were certainly very common when life appeared on Earth, are still today a major mode of rapid adaptation of prokaryotes to environmental changes they face constantly in contemporary ecosystems that are very frequently perturbed by human activities (Gillings 2017). HGTs have played and will continue to play an essential role in the evolution of prokaryotes (Ochman et al. 2000; Gogarten et al. 2002; Pál et al. 2005; Boto 2010; Brochier-Armanet and Moreira 2015; Koonin 2016).

4.3.2.1 Horizontal Transfer Mechanisms

Acquisition of DNA from another organism by horizontal transfer can occur through several mechanisms. The three main ones are transformation, conjugation, and transduction (Lorenz and Wackernagel 1994; Koonin et al. 2001; Redfield 2001) (Frost et al. 2005; Daubin and Szöllősi 2016). Two other modes of transfer must also be mentioned: outer membrane vesicles (Berleman and Auer 2013; Biller et al. 2014) and nanotubes (Dubey and Ben-Yehuda 2011; Pande et al. 2015).

During *transduction* (Fig. 4.3), a bacteriophage (virus capable of infecting bacteria) causes a unidirectional transfer of DNA from one cell to another. During a normal infection cycle, the bacteriophage injects its genetic material into the bacteria and takes over the host cell replicative machinery to make new viral particles, which are liberated into the environment as a consequence of lysis of infected cells (lytic cycle). In the case of transduction, the bacteriophage will transfer DNA from one parasitized bacterium to another one. Two types of transduction exist: generalized transduction and specialized transduction.

In the first case, the phage injects its genomic DNA into the host cell where it is replicated by the parasitized cell replicative machinery, which DNA is degraded and fragmented. Such fragments of the host DNA are occasionally packaged into “phage particles,” and, together with normal phages, they are liberated in the medium following lysis of the parasitized cell. Phage particles containing host DNA can then infect new bacterial cells and inject them with host DNA.

In the case of specialized transduction, “temperate” phages can integrate into the bacterial chromosome. Once integrated, the phage DNA will be replicated together with the host chromosome and be transmitted to the two daughter cells.

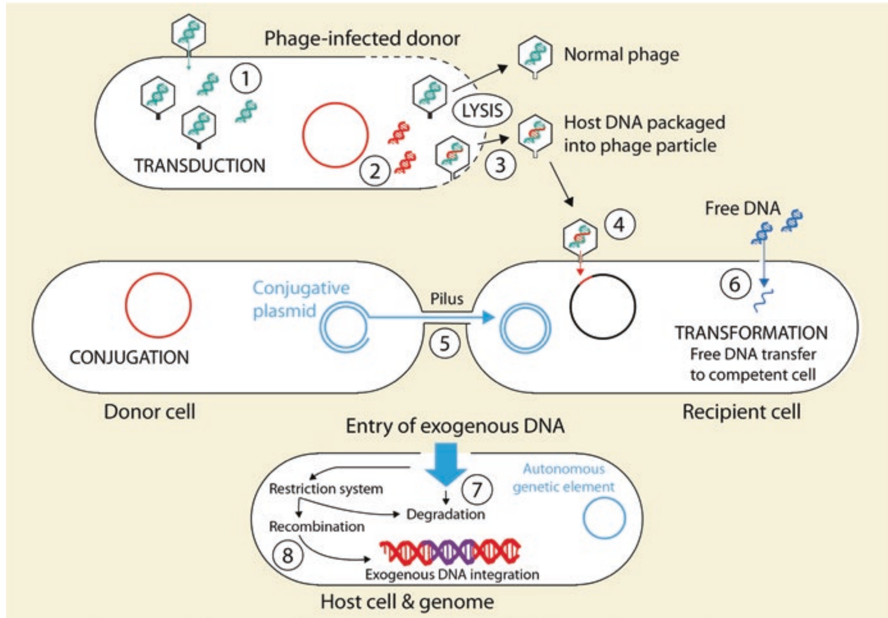


Fig. 4.3 Horizontal transfer mechanisms and the fate of transferred genetic material
DNA transfer by transduction: In bacterial cells infected by the phage, DNA fragments from the host are occasionally packaged into "particles" and following cellular lysis are transferred to new bacterial cells (1, 2, 3, 4)
DNA transfer by conjugation: DNA transfer imposes a direct cell-to-cell contact via a pilus. DNA penetrates into "recipient cells" via the pilus as a single strand (5)
DNA transfer by transformation: Competent cells can take up free DNA present in the environment (6)
Fate of the transferred DNA: The DNA that has been transferred to the host cell is either degraded or fragmented by restriction systems (7). It can integrate into the host genome or be maintained stably in the "recipient cell" as an "autonomous genetic element" (8)

In the case of transformation and conjugation, HGTs can only occur between species sharing the same biotope; on the contrary, transduction may occur between species living in spatially separated biotopes.

Restriction systems have emerged over the course of evolution because of the high risk represented by phages that can cause lysis of the bacterial host. These systems recognize a given sequence of four or more nucleotides and also whether that sequence is methylated or not. In a constant race between phages and their bacterial hosts, phages will mutate to avoid the recognized sequence, while the bacterial host will also mutate to increase the level of restriction enzymes or to change the recognized sequence. Other known defense systems are adsorption inhibition, CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins) systems, and abortive infection (Samson et al. 2013).

Phages are usually highly specific; they will recognize one strain but not another closely related one within a given species. A phage will recognize and bind to a receptor on the cell surface, such as a protein (OmpA), or a lipid (LPS), a pilus, or a flagellum (Rakhuba et al. 2010). The well-known phage λ uses, for instance, a transporter, the bacterial maltose LamB for docking onto the bacterial cell surface, and injects its DNA inside the cytoplasm (Chatterjee and Rothenberg 2012). Given this high specificity, the genetic distance between donor and receptor is generally quite small. Several mutations in the *E. coli lamB* gene, for instance, will result in modifications of the range of phage mutants that can infect the host (Werts et al. 1994).

During *conjugation* (Fig. 4.3), two cells get in contact, and their DNA is transferred from one cell to the other via a conjugative plasmid (capable of transferring a copy toward another bacteria); DNA goes from one cell to the other through a hollow tube called pilus, which ensures a temporary bridge between two cells; this exchange has been visualized by Babic et al. (2008). The plasmid DNA forms a double strand (though some are linear), the two strands are separated, and one of the two strands penetrates within the receptor cell, and the complementary strand is then synthetized. In parallel, the DNA complementary strand left in the donor cell is also complemented. At the end of the conjugation process, there is a copy of the plasmid in each of the donor and receptor cells. Some plasmids, called episomes, after their integration to the chromosome, can cause the transfer of fragments of the chromosomal DNA. The cells containing a plasmid integrated to the chromosome are called HFR (high frequency of recombination). Some conjugative plasmids can transfer informations between phylogenetically distant organisms (e.g., from Gram-negative bacteria to Gram-positive bacteria). The size of plasmids is very variable (1 kilobase to more than 1 megabase). The main plasmids are those that confer resistance to antibiotics, virulence plasmids, and metabolic plasmids that carry genes coding for enzymes that degrade some aromatic compounds (toluene), pesticides (atrazine), etc. The conjugation process has been described by Lederberg and Tatum (1946) who wrote that "these experiments imply the occurrence of a sexual process in the bacterium *Escherichia coli*." Indeed, conjugation that implies a physical contact during the exchange of DNA has sometimes been considered as a form of sexuality; however, it is not a true one: there are no cell division and no fusion of two gametes.

Free-naked DNA is abundant in the environment, originating essentially from lysed cells from DNA excreted by living cells. This DNA can survive naked in substrates such as soil for several years, being protected by clay micelles (Paget and Simonet 1994). During natural *transformation* (Fig. 4.3), free DNA penetrates within a cell, as a single strand, inasmuch as the receptor cell is competent (presence of specialized proteins on the surface of the cell that make it competent) (Lorenz and Wackernagel 1994; Chen and Dubnau 2004; Thomas and Nielsen 2005; Johnsborg et al. 2007).

The first to demonstrate the process of bacterial transformation was Fred Griffith (Griffith 1928) who was studying virulence in "pneumococcal types." He studied

two strains with different phenotypes. One strain was called "smooth" which cells were surrounded with a capsule made up of sugars and which, injected to mice, caused the death of these animals. Another strain called "rough" was not virulent and was devoid of capsule, the determinant of virulence. Griffith injected into these mice a mixture of "smooth" cells killed by a heat treatment of living "rough" cells. Following this injection, he noticed the death of his mice, and the only bacteria recovered in the mice were living "smooth" bacteria. What had occurred? The dead "smooth" bacteria had transmitted to "rough" bacteria a "transforming principle" that had resisted the heat treatment that was necessary to synthesize the capsule. The acquired "smooth" character was transmitted from generation to generation by bacteria that had inherited the "transforming principle."

From Griffith's experiences, two concepts can be drawn that were two important steps in the history of genetics. The first one is that a hereditary character can be exchanged between bacteria (e.g., the capacity to synthetize a capsule) through a transmission route different from the one known at that time (from mother cell to daughter cells): vertical transmission. He had discovered a new mode of transmission of hereditary characters: horizontal transmission. The second concept, even more surprising, was that the ability to synthesize a capsule could be transmitted from dead bacteria to living ones, a transmission from the dead! The discovery of the chemical nature of this "transforming principle" would have to wait for 16 years until Avery, MacLeod, and McCarty (1944) would demonstrate that the "transforming principle" was DNA. Dead bacteria had transmitted DNA – in other words the gene responsible for the synthesis of the capsule – to living bacteria.

Afterward, transformation was demonstrated in cells belonging to the same species but also in prokaryotes belonging to very different taxonomic (*Bacteria* and Archaea) and trophic (photolithotrophs, chemolithotrophs, heterotrophs, methylotrophs) groups. Generally the state of competence is only reached for a transitory period, and the percent of bacterial cells capable of transformation is low: *Vibrio parahaemolyticus*, 1.9×10^{-9}; *Bacillus subtilis*, 3.5×10^{-2}) (Lorenz and Wackernagel 1994). It must be underlined that it is possible to transform noncompetent cells into competent cells through artificial methods (e.g., electroporation).

HGTs can also occur through *outer membrane vesicles* (OMVs), structures that are between ~50 and 250 nm in diameter. OMVs intervene in different processes: virulence, quorum sensing, biofilm formation, redox reaction, cellular defense, and HGTs. This type of exchange, produced by a wide range of taxa, is particularly important in aquatic ecosystems. For example, a culture of *Prochlorococcus*, which is a numerically dominant marine cyanobacterium (population of ~1027 cells), releases continuously lipid vesicles that contain proteins, RNA, and DNA. Such vesicles carry DNA, from diverse bacteria, which have been identified in coastal and open-ocean samples. They could be the main vectors for horizontal gene transfer in marine systems (Biller et al. 2014).

DNA transfer can also occur between neighboring cells via *nanotubes* that permit direct cell-to-cell contact and exchange of cytoplasmic elements such as protein, metabolites, and DNA (*cf*. Chap. 1)

4.3.2.2 The Fate of Transferred Genetic Material

The DNA transferred may not be integrated into the "recipient genome," thus not resulting in a new genetic combination. It can be degraded or fragmented by endonucleases present in the cytoplasm of the receptor cell (restriction system of the host) (Fig. 4.3(7)). If the DNA escapes the host-defense systems, the exogenous DNA integrates into the host genome through recombination (homologous, illegitimate, homology-facilitated illegitimate combination) (Fig. 4.3(8)). There is then formation of a new heritable genome within the "recipient cell." It must be underlined that only a low proportion of HTG-transferred DNA is fixed in a durable manner in populations (Thomas and Nielsen 2005). The transferred genetic material will be maintained in its host only if it represents a benefit for the host.

4.3.2.3 The Prokaryotes Concerned by These Exchanges

Different techniques are implemented to detect and quantify HGTs. Such quantification is hard because the estimates obtained can be very variable from one technique to another; in particular, the different approaches do not identify the same types of HGTs (Brochier-Armanet and Moreira 2015; Daubin and Szöllősi 2016; Chan et al. 2017). Keeping in mind this problem, it is nevertheless possible to conclude that HGTs percentages can be very variable as a function of the species studied and of their function (Nakamura et al. 2004). HGTs are few in some species (0.5% in *Buchnera* sp. APS; 1.9% in *Mycoplasma genitalium* G-37); on the contrary, they are numerous in other species (10.5 in *Halobacterium* sp. NRC-1, 13% in *Synechocystis* sp. PCC6803, 24.1% in *Chlorobium tepidum* TLS, 25.2% in *Methanosarcina acetivorans* C2A) (Nakamura et al. 2004).

Besides, if horizontal transfers occur most frequently between closely related species, they are also possible between evolutionary distant organisms (Gram-negative bacteria/Gram-positive bacteria), comprising organisms belonging to different domains. In this manner, Archaea genomes code for proteins characteristic of *Bacteria* and vice versa. For example, the genome of the hyperthermophilic bacterium *Thermotoga maritima* contains around 8–11% of archaeal hyperthermophilic genes (Zhaxybayeva et al. 2009). The presence of Archaea genes in *T. maritima* would be the result of HTGs between organisms that share a given biotope, hot thermal springs. Thus, through HGTs, in a single step, barriers corresponding to million years of evolution are crossed.

4.3.2.4 Consequences on the Definition of Prokaryotes Genome: Core Genome and Pan Genome

Studies of comparative genomics have shown that the number of genes present in bacterial strains belonging to the same species may be very variable. This situation has led to distinguish in the genome of bacteria, a pan genome that comprises the

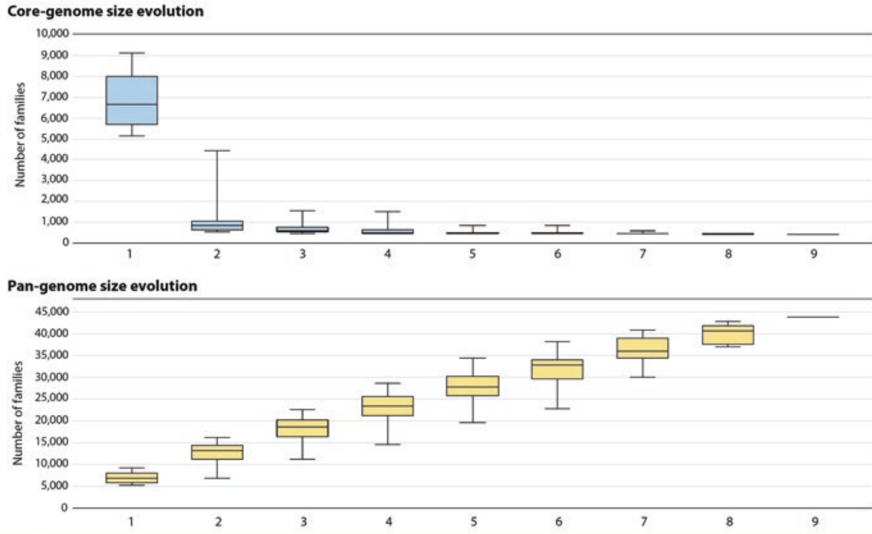


Fig. 4.4 *Frankia* core genome and pan-genome evolution according to the number of sequenced genomes
On the X-axis is the number of *Frankia* genomes analyzed in the data set. In the top "core genome," the first box represents the average number of genes (and the standard error) in the group of nine genomes studied. The second box represents the average number of genes shared (80% AA threshold) between pairs of the nine genomes studied. The third box represents the average number of genes shared (80% AA threshold) between triplets of the nine *Frankia* genomes studied, etc.
In the bottom "pan-genome" graph, the first box represents the average number of genes (and the standard error) present in the group of nine *Frankia* genomes studied. The second box represents the average number of genes (and the standard error) shared between pairs of genomes in the group of nine genomes studied plus those not shared by the pairs. (Normand P., personal communication)

whole of genes present in all individuals of a species as well as a core genome that comprises only those genes present in all strains identified in a given species.

For example, studies realized by Rasko et al. (2008), who have analyzed the genome of 17 strains of *E. coli*, have revealed a core genome of ~2200 genes and a pan genome containing more than 13,000 genes. Such elasticity can correspond to thousands of genes as in *Frankia* where genomes with less than 2% distance in the 16S rRNA genes were shown to have genomes ranging from 5.4 to 9Mb (Normand et al. 2007).

It is generally admitted that genes that constitute the core genome, which have been conserved over the course of evolution, are essential. Those genes outside the core genome should be considered dispensable. They would be necessary only to survive under certain environmental conditions particular to a group of strains.

The number of sequenced genomes is "dramatically" increasing in the last 10 years. In the case where multiple genomes of the same species are available, it is possible to calculate the pan and core genomes; in addition, if the size of the core genome remains constant, the pan genome continues to rise with the addition of more genomes (Fig. 4.4).

4.3.2.5 HTGs and Adaptive Evolution of Prokaryotes

Genes that are transferred horizontally generally code for metabolic functions other than the fundamental molecular processes such as DNA replication, transcription, and translation. There are nevertheless numerous exceptions to that rule, and, precisely, certain ribosomal genes show evident traces of horizontal transfer (Brochier et al. 2000).

The dissemination of functions acquired through HGTs has permitted prokaryotes to adapt rapidly to changes of their environment and represents an important selective advantage. Among the main functions are antibiotic resistance, virulence transmission, conquest of new biotopes (or ecological niches), and capacity to catabolize synthetic compounds.

The best known function is resistance to antibiotics (Dobrindt et al. 2004; Juhas et al. 2009). The mode of transfer varies as a function of the species (conjugation, natural transformation, transduction), but its main characteristic is the speed of dissemination of resistant mutants (*cf.* Chap. 6). Efficiency of dissemination can be explained in part only by the clustering of resistance genes. As a matter of fact, those genes acquired through HGTs are not distributed homogeneously over the length of the genome, but are clustered on plasmids and particular chromosomal regions called genomic islands (GIs) (Langille et al. 2010). Thus, through GIs, acquisition of all resistance genes can occur as a single step, and the "receiving" strain will become multiresistant. Besides, as they move from one host to another, GIs can acquire new resistance genes, and thus, bacteria that are resistant to antibiotics will become more and more efficient. Emergence of resistance to antibiotics by pathogenic bacteria has become a major public health challenge. A real "arms race" has begun between scientists attempting to elaborate new antibiotics and bacteria that develop means of resistance to these new molecules.

Another genetic transfer concerns the transfer of virulence functions by pathogenic bacteria (adhesins, toxins, invasins, protein secretion systems, iron uptake systems, modulins, etc.). The genes transferred that are responsible for virulence are localized on large regions of chromosomal and plasmidic DNA (pathogenicity islands) (Hacker and Kaper 2000). For example, the uropathogenic *E. coli* (UPEC) that is the main cause of "urinary tract infection" (UTI) represents around 40% of nosocomial UTI; UPEC virulence factors are frequently encoded on pathogenicity islands (Brzuszkiewicz et al. 2006). Usually, in closely related nonpathogenic neighbors belonging to the same genus or the same species, these genes are absent. In *Agrobacterium*, the agent of crown gall in a number of plants, the virulence genes are also plasmid-borne, but the plasmid appears to be lost frequently, in particular as a result of heat as occurs in tropical soils (Krimi et al. 2002); this plasmid that modifies the fitness of the bacterial host can later be reacquired through conjugation.

HGTs also play a role in the conquest of new biotopes through the acquisition of new metabolic capacities. For example, it has been demonstrated that a strain belonging to genus *Thermus* that grew only under strict aerobiosis had acquired the capacity to grow under anaerobiosis using nitrate as electron acceptor (acquisition of a respiratory nitrate reductase gene cluster). The capacity to respire nitrate can be

transmitted through conjugation, thus perming aerobic strains to colonize anoxic biotopes containing nitrate (Ramírez-Arcos et al. 1998).

Some prokaryotes have acquired the capacity to resist, or even to use as nutrient source, man-made toxic compounds. Many examples can be cited. Bacteria have acquired gene cassettes capable to confer precipitation of heavy metals and of radionuclides that through HGTs can be disseminated within contaminated biotopes (Moënne-Loccoz et al. 2015). HGTs are also involved in degradation of lindane (Berger et al. 2015), atrazine (Soulas and Martin-Laurent 2015), etc. that represent a significant source of carbon in soil.

4.3.2.6 HGTs and the "Universal Tree of Life"

After millions of year of evolution, the genomes of prokaryotes have become true "mosaics." The extent of HGTs and their importance in the evolution of prokaryotes are still a hotly debated question: their precise identification and quantification remain always difficult, since besides HGTs, other modifications of genomes through vertical evolution occur; some authors thus tend to minimize the role of HGTs in genome phylogeny in modern cells versus primitive genomes (Kurland et al. 2003).

However, important in certain species and negligible in others – HGTs complicate the definition of "species" in prokaryotes (*cf.* Chap. 2) and also the representation of relations between species in phylogenetic trees: in such trees, if branches split, others through HGTs fuse and sometimes form new branches. Darwin himself, before adopting the vision of a "universal tree of life," had proposed a representation of evolution of species in the form of a "coral of life": "The tree of life should perhaps be called the coral of life, [its] base of branches dead; so that passages cannot be seen" *(Life and Letters of Charles Darwin – Volume 1, pg 368)*. Presently, the concept of a "universal tree of life" is yielding to that of a reticulated tree, a network forming a web- or net-like pattern (Doolittle 1999; Kurland et al. 2003; Olendzenski and Gogarten 2009; Koonin and Wolf 2012). The intensification of the genomes sequencing effort should permit to build a reliable and detailed history of genomes. The concept of phylogenetic trees should not be abandoned, but one must remain aware that the evolutive history of genes can be very different from the lineal descent of the cells that carry them, "prokaryotic evolution and the tree of life are two different things" (Bapteste et al. 2009).

4.4 Advantages to Have a Small Size

4.4.1 Dissemination

Due to their small size, the spread of prokaryotes is planetary, in the four interconnected spheres of the Earth (atmosphere, hydrosphere, lithosphere, (deep)biosphere). Indeed, prokaryotes are continuously transported over long distances by winds,

currents (due to the constant circulation and mixing of the oceans), the living beings (due to the migrations over thousands of kilometers for some species, including man), and the clouds. Due to this dispersion, prokaryotes are present on the whole planet Earth, in particular in some biotopes inaccessible to plants and animals: deep biosphere and clouds.

(i) *The deep biosphere* includes a variety of subsurface habitants in continental realm (mines, deep aquifer systems) and marine realm (sediments and igneous rocks) (Schrenk et al. 2010; Biddle et al. 2012; Colwell and D'Hondt 2013); deep biosphere extends down to around 2.5 km below the ocean floor (Inagaki et al. 2015). Migration of prokaryotes in deep biosphere can take place in different ways: capture in sediments during their formation, migration from the surface by natural geological process or drilling operations, and percolation from the surface through fissures in the rock. It should be noted that a transfer of microbial communities from deep subseafloor to overlying water should be considered (Hoshino et al. 2017). The biomass of deep biosphere biomass is estimated according to the authors from 3.55×10^{30} cells (Whitman et al. 1998) to 2.9×10^{29} cells (Kallmeyer et al. 2012); whatever the estimate proposed, the deep biosphere biomass is one of the largest biospheres on Earth.

(ii) *The atmosphere*. The prokaryotes (fungi and yeasts too) play an important role in the formation of clouds. They are present in the atmospheric water phase (fog and clouds), where they are capable to survive and develop in spite of harsh conditions encountered in the atmosphere (desiccation, low temperature, solar radiations, presence of oxidizing agents, oligotrophic conditions, human pathogens, etc.) (Morris et al. 2014; Pöschl and Shiraiwa 2015; Fröhlich-Nowoisky et al. 2016).

Aerosolized from virtually all surfaces (soil and water surface, vegetation), prokaryotes (and others microorganisms) are transported in altitude, where residence time in atmosphere can range from several days to weeks, long enough for cells to travel between continents (Burrows et al. 2009). The prokaryotes will then fall back to the surface of the Earth, via the rain or the snow, where they will be able to regrow. The total bacterial concentration in atmospheric waters varies from 10^3 to 10^5 cell ml^{-1} (Delort et al. 2010). The diversity of bacterial population found in clouds is great (Amato et al. 2007b; Bottos et al. 2014). For instance, from samples collected at the Puy de Dôme summit, 28 genera have been described and were found to belong to *Actinobacteria*, *Firmicutes*, *Proteobacteria* (*Alpha*, *Beta*, and *Gamma* subclasses), and *Bacteroidetes* phyla and mainly to the genera *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, and *Arthrobacter* (Amato et al. 2007b); total bacterial count were about 1×10^5 cells ml^{-1} of cloud water, and less than 1% were cultivable (Amato et al. 2005). However, the measures of the ATP concentration in cloud samples support the conclusion that a large majority of cells are likely viable but noncultivable (VBNC) and remain alive in clouds (Amato et al. 2007c). Isolated bacteria were able to degrade various organic substrates such as formate, acetate, lactate, methanol, formaldehyde, as well as H_2O_2, a precursor to oxidant species in clouds (Amato et al. 2005, 2007a; Vaïtilingom et al. 2013).

4.4.2 Surface-Volume Ratio

A small size has another advantage. The exchanges with the outside environment – the rate at which nutrient passes into and out of the cell by passive diffusion, facilitated diffusion, or active transport – are easier in a small cell than in a large cell. So, for a given amount of resource to support growth, smaller size increases the cellular metabolic rate and growth rate. Consequently the population of small cells is higher than of large cells, which in turn can affect the evolution. Indeed, a large population will have a high rate of mutations ("the raw material of evolution") which will allow a more rapid adaptation to changes in environmental conditions and better exploitation of resources. How to explain the advantage of cells with a small size? This advantage is mainly due to the fact that these cells have a surface area greater than that of large cells; they have a higher surface-to-volume ratio (Young 2006; Moya et al. 2009). For example, a coccus with a radius equal to 1 μm, its S/V ratio will be 3 (surface area ($4\pi r^2$) of 12.6 m^2/volume ($4/3\pi r^3$) of 4.2 μm^3). For a coccus with a radius equal to 2 μm, S/V ratio will be equal to 1.5 (surface area = 50.3μm^2/volume = 33.5 μm^3) (Madigan et al. 2015). For some cells with different sizes such as *Pelagibacter ubique* (S/V: 0.31/0.014), *Escherichia coli* (S/V: 6.28/1.3), and *Epulopiscium fishelsoni* (S/V: 151.000/3.10^6), S/V ratio ($\mu m^2/\mu m^3$) is equal to 22, 4.8, and 0.05, respectively (Young 2006). *P. ubique*, one of the most successful and numerous life forms on the planet, which has a very high S/V ratio (S/V: 22), fits the model in which natural selection optimizes the surface-to-volume ratio to provide appropriate transport rates in oligotrophic environment (low-nutrient conditions) (Grote et al. 2012). At the opposite end of the spectrum is the giant bacteria, *Epulopiscium fishelsoni* (S/V: 0.05), a symbiont that spends the entire of its life in the intestinal tract of a tropical marine fish, *Acanthurus nigrofuscus* (Angert et al. 1993). *E. fishelsoni* overcomes the disadvantage of a very low S/V ratio in three ways: *(i)* life in nutrient-rich environment; *(ii)* the inner membrane contains many invaginations which increases transport across the membrane; and *(iii)* the bacteria use extreme polyploidy and contain multiple copies of its genome; each cell has between 50,000 and 120,000 copies of the chromosome (Mendell et al. 2008; El-Hajj and Newman 2015).

In environment (seawater, freshwater), the consumption of prokaryotes by bacterivores varies according to the predator and the prey; however, longer and shorter cells escape more frequently when grazed by protists. So, Pernthaler et al. (1996) proposed a model that divides freshwater bacterioplankton into four size ranges of different vulnerability to size-selective protistan grazing: small cells (<0.4 μm) weakly affected by protist grazing, "grazing-vulnerable" bacteria (0.4 and 1.6 μm), "grazing suppressed" (1.6 and 2.4 μm), and "grazing-resistant" (>2.4 μm) fractions of the bacterioplankton (Young 2006; Pernthaler et al. 1996).

4.5 Through Evolution, Prokaryotes Have Developed an Array of Mechanisms to Ensure Their Survival Under Adverse Conditions

4.5.1 Life of Prokaryotes Under Dormancy and Starvation Conditions

Sergei Winogradsky (1856–1953) drew attention to the fact that in soils *la grande majorité des germes, à un moment donné, est à l'état de vie latente, une minorité seulement à l'état actif* (Winogradsky 1949). Numerous works have confirmed these observations. Indeed, when environmental conditions become unfavorable for growth, that are in *état de vie latente* are unable to grow, but are not dead can regrow with the appearance of favorable conditions. This process is known as "dormancy," a strategy to cope with environmental stress, which is defined operationally as "reversible state of metabolic shutdown" (Stevenson 1978; Roszak and Colwell 1987; Kaprelyants et al. 1993; Jones and Lennon 2010; Lennon and Jones 2011; Wang et al. 2014a; Aanderud et al. 2015). Dormancy, "a reversible state at low metabolic activity, in which cells can persist for extended periods without division", refers to two kinds of cells: cells forming specialized structures (spores, cysts) that formed the most durable dormancy present in a limited number of bacterial species (*cf*. part 4.5.4) and non-spore-forming cells which are vegetative cells which enter in a dormant state (Kaprelyants et al. 1996). Dormancy includes three steps: *(i)* initiation, in response to environmental unfavorable changes, *(ii)* microorganism at rest where dormant cell exhibits a wide range of phenotypes (spore-forming and non-spore-forming bacteria), and *(iii)* "resuscitation" from dormancy (revival of dormant cells and spores) (Lennon and Jones 2011). A portion of prokaryotic population in nature is not active, and in some soils, the active biomass may be less than 10%. For example, in soil from Argentinean Pampa, Alvarez et al. (1998) compared the active biomass (kg ha^{-1}) versus the total biomass (kg ha^{-1}) as a function of soil management: plow tillage (total biomass, 295; active biomass, 28.4), no-tillage (total biomass, 414; active biomass, 35.3), and pasture (total biomass, 1114; active biomass, 4.19). In the same way, in soils collected near Uppsala (Sweden), growing microorganisms fraction is generally small (5–20%) in soils with no recent addition of substrates (Stenström et al. 2001). In deep biosphere – proposed as the largest reservoir of biomass on planet Earth (Whitman et al. 1998) – the hypothesis proposed by Jørgensen (2011) postulates that only 1% of the cells are active and that the other cells can persist for a long time without transforming into vegetative cells.

The main reason that leads the cells to dormancy is the energy deficiency where cells enter in a physiological state known as starvation-survival as defined by Morita (1990) "physiological state resulting from the amount of nutrients available for growth and reproduction being insufficient." Microbiologists, the most of time, investigate bacterial growth under optimal conditions (carbon and energy sources, optimal pH, temperature, Eh, salinity, aeration conditions, etc.), using axenic culture that are absolutely impossible in nature (Winogradsky 1949). The cells, thus sub-

tract from their natural biotic and abiotic environment, are placed under artificial conditions, often radically different from the natural habitat of the organisms. But, in natural environments, prokaryotes are frequently subject to alternation of feast (in rare instance) and famine (starvation stage) like in open oceans that are continuously oligotrophs with only brief periods of nutrient inputs. Indeed, the amount of dissolved organic matter in the sea is usually less than 1 mg of C/liter for surface water and 0.5 mg of C/liter for deep water (Novitsky and Morita 1977); on the other hand, standard laboratory media contain nutrients at concentrations that are very high, e.g., > 2000 mg of C/liter (Roszak and Colwell 1987). A feast and famine diet was also proposed by Jørgensen and Boetius (2007) in deep-sea bed, where bacteria live under nutrient-limiting conditions and extreme energy limitation (Hoelher and Jørgensen 2013). The period of starvation can last for long period of time. For example, Novitsky and Morita (1977) studied the survival of a psychrophilic marine *Vibrio* (Ant-300) under long-term nutrient scarce and observed that during starvation, 50% of the starved cells remained viable for 5–7 weeks and a portion of the population (10^3 cells/ml) remained viable for more than 1 year (viability was determined by the spread-plate technique). Another example of long-term starvation is this of marine bacterium *Alteromonas denitrificans* which has survived for up to 7 years in unsupplemented synthetic seawater (Nissen 1987). The main starvation responses of bacteria include formation of periplasmic spaces, decrease of size, change in shape (from a rod to a coccus), and a sharp decrease of the endogenous respiration; the endogenous respiration of the starving cells Ant-300, a psychrophilic marine vibrio, during the first days of starvation, decreased over 80%, and, after 7 days, respiration has been reduced to 0.0071% (Novitsky and Morita 1977). During starvation a part of the population survives, and the other part dies. The recycling of dead cells might play a key role in the maintenance of long-term survival (Takano et al. 2017). The survival under starvation conditions has been well documented in many different bacteria: Ant 300 *Alteromonas denitrificans*, *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Erwinia amylovora*, and *Azospirillum brasilense*. For more informations concerning the cellular (morphology, size, motility), physiological, biochemical, and genetic changes accompanying starvation, refer to previous works (Koch 1971; Kjelleberg et al. 1993; Mukamolova et al. 2003; Peterson et al. 2005; Gonzàlez-Escalona et al. 2006; Lerner et al. 2010; Santander et al. 2014).

4.5.2 Ultramicrobacteria: Prokaryotes Adapted to the Oligotrophic Way of Life

At (very) low substrate concentrations, many prokaryotes are, as described above, able to survive in such conditions by becoming inactive, and during this “rest period,” the cells are nonculturable. In contrast, other prokaryotes have a lifestyle adapted to nutrient-depleted environments and are capable to grow at (very)low

substrate concentrations. This is the case of different marine bacteria, called "ultramicrobacteria," which are able to grow in natural seawater which is characterized by extremely low nutrient concentrations (oligotrophic conditions), compared with media commonly used for studies of bacterial growth (Jannasch 1967). For these oligotrophic bacteria, starvation is the normal state unlike copiotroph bacteria which grow only at high concentration of nutriments and are inactive in natural seawater where they are able to survive. In 1981, Torella and Morita described two types of free-living heterotrophic bacteria in freshly collected marine water samples, which can passed through a 0.3 µm-pore-size filter used for the entrapment of bacteria. The first one, of small size and in a state of low metabolic activity, displays, upon inoculation onto a nutrient-rich agar, an increase in their size and in their growth rate. The second type of cells, designed by Torella and Morita (1981) as "ultramicrobacteria" (less than 0.1 μm^3 in volume), formed very small microcolonies and displays a slow rate; these cells do not increase in size even in the presence of high concentration of organic matter (nutrient medium). Ultramicrobacteria maintained similar size and volume regardless of their growth conditions; these bacteria were adapted to the low-nutrient conditions of the seawater. So there is a fundamental difference between ultramicrobacteria (UMB) and starvation forms: UMB retain their small size even when exposed to media with high nutrient concentration contrary to starvation forms that regain their full vegetative size and metabolic activity when flooded with nutrients. Ultramicrobacteria that can pass through a 0.2 µm filter (referred to as 0.2 µm filterable bacteria) (Hood and Macdonell 1987) belong to *femto*bacterioplankton (size: 0.02–0.2) according to the size classification of planktonic microorganisms proposed by Sieburth et al. (1978). Even if it is difficult to isolate ultramicrobacteria, different strains have been isolated and cultured: *Sphingomonas* sp. Strain RB2256 (Fegatella and Cavicchioli 2000), *Candidatus* Pelagibacter ubique (in SAR11 clade) (Rappe et al. 2002), *Polynucleobacter necessarius* (Hahn 2003), *Herminiimonas glaciei* sp. nov. (Loveland-Curtze et al. 2009), etc. Possessing a small size provides many benefits: protection against predation by bacterivorous nanoflagellates and a surface/volume reduction that optimizes exchanges with nutrient-poor environment.

4.5.3 Viable But Nonculturable State

4.5.3.1 Definition and Characterization

To withstand to adverse environmental conditions, in addition to spore formation, many bacteria possess the ability to enter in a state qualified of viable but noncultivable (VBNC) (Roszak et al. 1984; Roszak and Colwell 1987; Colwell 2000; Oliver 2010; Oliver and Mena 2010; Fernández-Delgado et al. 2015; Pinto et al. 2015; Ding et al. 2017; Zhao et al. 2017b). VBNC state has been described for the first time by Xu et al. (1982), from Rita Colwell's laboratory, in *E. coli* and *Vibrio cholerae*; these two bacteria incubated in sterile water at low temperature without

nutriment supplements were able to persist for several days in such conditions but were unable to form colonies on agar plate, thus demonstrating that "a significant proportion of the nonculturable cells were, indeed, viable." The term VBNC was coined by Roszak and Colwell (1987) "for those bacterial cells with detectable metabolic function, but not culturable by available methods." Indeed, VBNC bacteria escape detection by standard methods for testing samples for viable bacteria (viability is equated with cultivability a single cell yielding a visible colony on the surface of nutrient agar plate). However, nonculturable on routine culture media on which they would normally grow does not mean dead, and VBNC cells demonstrate many general characteristics as a kind of viable cells. VBNC cells sustain certain functions like uptake and incorporation of amino acids into proteins, active metabolism and respiration, ATP levels and membrane potential remain high (membrane integrity), gene transcription with specific mRNA production, antibiotic resistance, plasmids are retained, etc. (Lleò et al. 2000; Yamamoto 2000; Oliver 2010; Li et al. 2014; Pinto et al. 2015).

Some authors proposed an alternative to VBNC terminology: "active but nonculturable (ABNC) state", e.g., active cells but nonculturable (ABNC) cells "exhibit measurable activity but fail to grow to a detectable level" (Kell et al. 1998), and non-growing but metabolically active (NGMA) state (Manina and McKinney 2013).

VBNC cells present some changes from culturable cells besides the inability to growth: change in shape, cell wall, and membrane composition, including proteins, fatty acids and peptidoglycan, gene expression, etc. (Pinto et al. 2013). For example, a change in the fatty acid composition of the membrane is observed in *Vibrio vulnificus* into VBNC state, suggesting a change in membrane fluidity (Day and Oliver 2004) and presence of a specific protein profile of VBNC in *Enterococcus faecalis* (Heim et al. 2002). With respect to morphology, most of the time, VBNC cells are reduced in size and changed their morphology. *Vibrio cholerae* under VBNC state change from rod to coccoid cells (Chaiyanan et al. 2007). VBNC cells can stay alive over long periods of time, even under continued stress, and many species have the ability to "resuscitate" (Cf. Sect. 4.5.3.5).

Finally, note that since the definition of the VBNC state is proposed by Xu, the interpretation of VBNC state has been controversial within the scientific community. For example, Bogosian et al. (1998) think that the return to the cultivable state is due to the presence of the few culturable cells that remain in sample and "that the nonculturable cells were dead and that the apparent resuscitation was merely due to the growth of the remaining culturable cells." Other authors propose that in adverse conditions, some cells excrete organic molecules into the surrounding medium which are used by the other members of the populations, thus ensuring cell survival until better time comes (Arana et al. 2004) (Cuny et al. 2005). Subsequently, Manina and McKinney (2013) proposed that the "death of a fraction of cells releases molecules that can be scavenged by the survivors" and the death of "altruistic" members of the population release molecules that can be used by "survivors."

Little is known about understanding the molecular control of VBNC state, and the mechanism by which bacteria adjust in the VBNC state remains unclear. Nevertheless, many proteins seem implicated into the VBNC state induction.

In *E. coli*, EnvZ/OmpR system regulates the synthesis of the major outer membrane proteins OmpF and OmpC (Li et al. 2014). EnvZ has no effect on survival (determinated by plate count), but is involved in the entry into VBNC state: indeed, *envZ* mutants were found not to enter in VBNC state (Darcan et al. 2009). Glutathione S-transferase (GST) (Abe et al. 2007) and catalase KatG (Oliver 2010) are involved in the VBNC state in *Vibrio vunificus*.

Regulators factors RpoS (Boaretti et al. 2003; Liu et al. 2010a; Kusumoto et al. 2012) and OxyR (Cuny et al. 2005; Li et al. 2014) seem to be important for the induction of VBNC state.

4.5.3.2 Detection

Various methods to bypass agar culture have been developed (Kell et al. 1998; Khan et al. 2010; Oliver 2010; Manina and McKinney 2013; Davis 2014; Ramamurthy et al. 2014; Fernández-Delgado et al. 2015; Ayrapetyan and Oliver 2016; Léonard et al. 2016; Ding et al. 2017; Zhao et al. 2017b). The most commonly used methods are as follows:

– Direct viable count (DVC) was first described by Kogure et al. (1979). The Kogure procedure is based on acridine orange staining after elongation of cells in the presence of DNA-gyrase inhibitors such as nalidixic acid. Many bacterial pathogens became resistant to nalidixic acid necessitating modification of Kogure's method. For example, Joux and Lebaron (1997) used an antibiotic cocktail instead of nalidixic acid alone, including 4 quinolones (nalidixic, piromidic, and pipemidic acids and ciprofloxacin) and one ß-lactam (cephalexin); 100 marine strains isolated from 2 coastal areas and natural marine communities were screened for their sensitivities to these antibiotic cocktail. The combination of antibiotics resulted in higher viable counts for all water samples revealing the existence of a large and unexpected number of viable cells in coastal marine areas.
– Double staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4′,6-diamino-2-phenylindole (DAPI), this method makes it possible to count and differentiate between dead cells and living cells. The DAPI allows the detection of all the cells, the CTC, and the enumeration only of the living cells.
– LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) is based on double two nucleic acid stains: membrane-permeable green-fluorescent SYTO® 9 (stains both live and dead bacteria) and membrane-impermeable red-fluorescent propidium iodide that penetrates only the bacteria with damaged membranes and reduces the SYTO® 9 fluorescence when both the stains are used. Other kits are available (e.g., RedoxSensor™, Green Vitality Kit).
– BacLight Kit (molecular probes), which combines two nucleic acid stains, membrane-permeable SYTO9 (green) and membrane-impermeable propidium iodide (red), to identify "live" cells (which stain green) versus "dead" cells (which stain red).

– Multiparameter (or polychromatic) flow cytometry (MP-FCM) is a powerful tool for rapidly analyzing cell populations on a cell-by-cell basis and provides the opportunity to obtain information in real time. Light scatter and fluorescence properties are recorded simultaneously as cells cross one by one one or several laser beams. Indeed, a very large number of cells can be measured rapidly, typically up to several thousands of cells per second. Analysis of cells by MP-FCM after staining with artificial fluorescent dyes (such as DiOC6(3) as illustrated in Fig. 4.5) provides information on the cell characteristics (size, shape, granularity) and damage addressed at the single-cell level, especially for quantitative analysis of metabolism and physiological state of VBNC cells by measuring multiple cellular parameters on each cell simultaneously: membrane integrity, membrane potential, metabolic activity (respiratory activity and intracellular enzymatic activity such as dehydrogenase, esterase), and detection of damaged DNA. It assumed that cells having intact membrane are alive and those with damaged membrane potential are dead or theoretically dead. Figure 4.5 shows an example of the investigation of the membrane potential of a culture of bacteria, thanks to the carbocyanine DiOC6(3).
– Molecular Detection-Based Methods

Molecular biology techniques can also be used to determine VBN bacteria, and molecular methods for more effective detection and quantification of these cells have increased. The most used methods are the following:

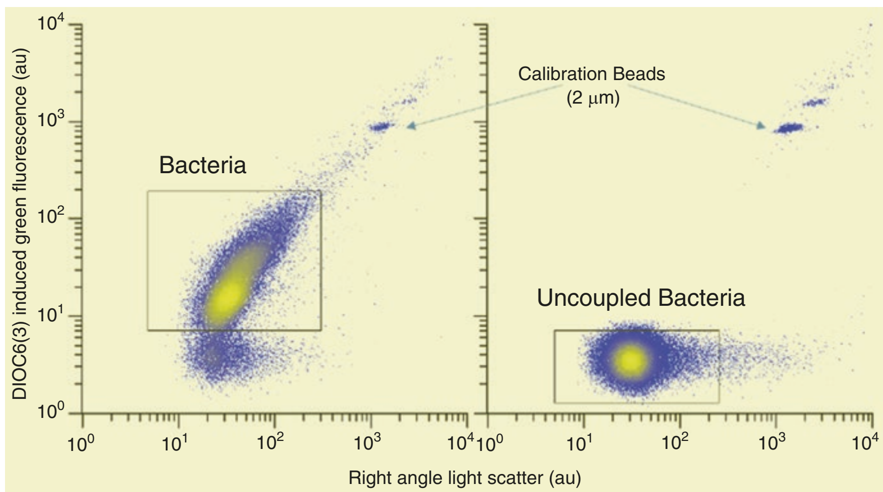


Fig. 4.5 Detection by flow cytometry
On the left, a culture of *Pseudomonas nautica* stained with carbocyanine DiOC6(3). On the right, the same culture with the membrane potential of the bacteria abolished by the uncoupler CCCP and stained with DiOC6(3). (Gregori G., personal communication)

Real-time polymerase reaction (RT-PCR) and quantitative PCR (RT-qPCR) based on detection of mRNA which have been proposed as markers for cell viability because they are very unstable molecules with very short half time inside the cells, there are only present in viable cells. Yaron and Matthews (2002) carry out gene amplification and have demonstrated that RT-PCR can serve as a detection method for *E. coli* O157:H7 cells. Using PCR, *rfbE*, *fliC*, *stx1*, *stx2*, *mobA*, *eaeA*, *hlyC*, and *A* 16S rRNA were amplified, whereas RT-PCR amplified only the 16S rRNA, *rfbE*, *stx1*, and *mobA*. Their result suggests that 16S rRNA, *rfbE*, *stx1*, and *mobA* are good targets for the detection of the presence of viable *E. coli* O157:H7 in sample containing nonculturable cells, and *rfbE* gene is the most appropriate target for detection of *E. coli* O157:H7. RT-PCR method is very specific: the targeted genes can only be used for the detection of *E. coli* O157:H7. Furthermore, 10^6 cfu were required for the detection of *rfbE*, and 10^7 cfu were needed for the detection of *fliC* and *stx1* transcripts. Using the RT-PCR microelectronic array technique, Liu et al. (2008) increased the sensitivity of the method: *rfbE* and *fliC* genes were detected with 50 VBNC cells in liter of river.

The detection of live cells can also be entreprised using viability dyes in combination with DNA amplification; the technology is based on sample treatment with the photoactivatable, and cell membrane impairment, nucleic acid-intercalating dyes ethidium monoazide (EMA) or propidium monoazide (PMA) followed by light exposure prior to extraction of DNA and amplification (Fittipaldi et al. 2012). For instance, Nogva et al. (2003) and Rudi et al. (2005) have developed a concept for quantification of viable and dead cells. The viable/dead stain ethidium monoazide (EMA) is used in combination with real-time PCR to inhibit amplification of DNA from dead cells that have taken up EMA; EMA is a DNA-intercalating dye that enters bacteria with damaged membranes; EMA penetrates dead cells and binds to the DNA, permitting differentiation between viable and dead bacteria. Despite the success of the method, some practical limitations have been identified, especially when applied to environmental samples.

4.5.3.3 Species Enter the VBNC State

Since the works of Xu, the number of pathogen and nonpathogen species known to enter the VBNC state constantly increases; 85 species of bacteria in VBNC state were reported (Kell et al. 1998; Oliver 2010; Li et al. 2014; Pinto et al. 2015; Zhao et al. 2017b), including pathogenic bacteria for humans: *Salmonella enterica*, *Vibrio cholerae*, *E. coli* (e.g., O157:H7 EHEC), *Campylobacter jejuni*, *Vibrio vulnificus*, *Shigella dysenteriae*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, etc.

4.5.3.4 The Stressful Conditions Triggering the VBNC State

Many stressful conditions, prevalent in natural environment constantly fluctuating, can trigger transition to VBNC state (Li et al. 2014; Fernández-Delgado et al. 2015; Wu et al. 2016; Ding et al. 2017; Zhao et al. 2017b). The most stressful conditions are as follows: low nutriment concentrations, temperatures outside those that are permissive to cell growth, elevated or lowered osmotic concentrations, elevated salinity, extreme pH, oxygen stress, solar radiation, sulfur dioxide, low redox potential, heavy metal, presence of food preservatives, etc.

4.5.3.5 Return to Cultivable State: "Resuscitation"

Cells in VBNC state can recover their "culturability," in a process termed "resuscitation," by a restoration of more favorable conditions for bacterial growth (e.g., temperature upshift, rich medium). The term was first presented by Roszak et al. (1984). The definition proposed by Kell et al. (1998) will be retained, a term used "to denote transition of cells from 'nonculturable' to culturable states with respect to a given medium." The ability of a bacterium to "resuscitate" can be considered as a truly survival strategy. For instance, *Vibrio vulnificus* has an optimal growth temperature at 37 °C. In an artificial seawater microcosm incubated at 5 °C, *V. vulnificus* responds to this temperature downshift by entering to a viable but nonculturable state (Fig 4.6). The bacteria recovered culturability after a temperature increase (22 °C);

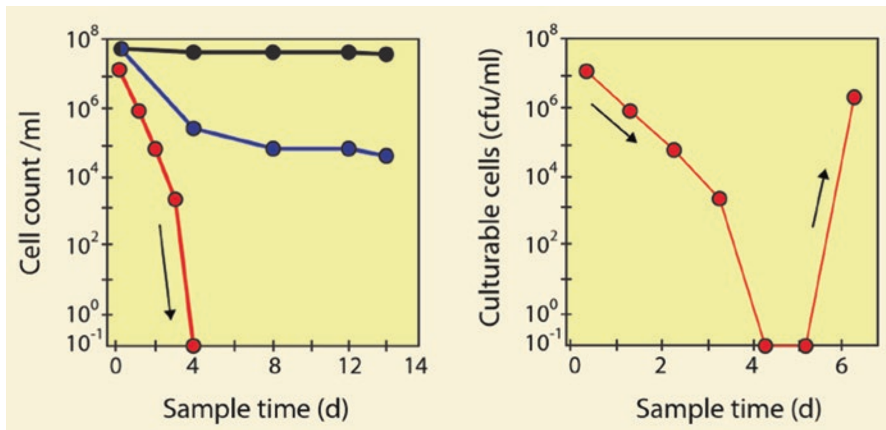


Fig. 4.6 Loss and recovery of culturability of *Vibrio vunificus*
The bacteria entered into the viable but nonculturable state in an artificial seawater microcosm incubated at 5 °C (downward arrow) and recovered culturability after a temperature increase (upward arrow). Total cell counts (acridine orange staining method), direct viable counts (method of Kogure et al. 1979), and plate counts on HI (heart infusion broth) agar in CFU per millimeter are represented by black, blue, and red circles, respectively. (Modified and redrawn from Whitesides and Oliver 1997)

the temperature upshift would release the bacteria from low-temperature stress, promoting "resuscitation" to the original cell state (Nilsson et al. 1991; Whitesides and Oliver 1997). Amel et al. (2008) claimed that VBNC cells of *Vibrio fluvialis* were able to "resuscitate" to the culturable state up to 6 years of incubation in marine sediment. Many bacteria require specific conditions to transit from a VBNC state to cultivable state (Zhao et al. 2017b). *Micrococcus luteus* cells are able to "resuscitate" from a state of nonculturability dormancy in the presence of supernatants taken from the stationary phase of batch cultures of the organism (Kaprelyants et al. 1993). *Vibrio cholerae* O1 cells in the VBNC state can be resuscitated by introduction in the intestines of human volunteers; the demonstration of a "resuscitation" in the gut supports the proposition that viable but nonculturable bacterial enteropathogens may pose a potential threat to health (Colwell et al. 1996). *Legionella pneumophila* Philadelphia JR32 in VBNC state "resuscitated" and regained pathogenic potential during intracellular residence within *Acanthamoeba castellanii* (Steinert et al. 1997), and the data of García et al. (2007) showed that *Legionella pneumophila* that became nonculturable after chlorine treatment "resuscitated" in co-culture with *Acanthamoeba polyphaga*. All the same, conversion of VBNC *V. cholerae* O139 and *V. cholerae* O1 cells to the culturable state by co-culture with eukaryotic cells (CHO, Caco-2, T84, HeLa, and intestine 407 cell lines) was reported by Senoh et al. (2010). The work undertaken on the *Micrococcus luteus* supernatant made it possible to isolate and characterize a protein (*a muralytic enzyme*), named Rpf (resuscitation-promoting factor) which is capable of very low concentration (activity at picomolar concentrations), to "resuscitate" nonculturable forms cells of *M. luteus* (Mukamolova et al. 2003; Li et al. 2014). If *M. luteus* seem to contain only one *rpf* gene, other species contain several *rpf*-like genes: *Corynebacterium glutamicum* (*2 rpf*), *Mycobacterium leprae* (3 *rpf*), *Mycobacterium marinum* (4 rpf), *Mycobacterium tuberculosis* H37Rv (5 *rpf*), and *Streptomyces avermitilis* (6rpf) (Ravagnani et al. 2005). Other proteins, called Sps (Stationary phase survival), like Rpf proteins, control bacterial culturability (Ravagnani et al. 2005).

4.5.3.6 VBNC State a Potential Threat to Health

The majority of bacteria studied in a VBNC state are pathogenic bacteria present in the environment in food and drinking water. So, VBNC pathogens can pose a serious risk to food safety and public health (Li et al. 2014; Ramamurthy et al. 2014; Zhao et al. 2017b). Indeed, VBNC cells which are characterized by a loss of culturability on routine agar escape detection by conventional plate count techniques leading to an underestimation of total viable cells in environmental, food, water, or clinical samples. VBNC cells are potentially a threat to human health because some strains remain virulent in VBNC state.

Vibrio vulnificus is an estuarine bacterium responsible for 95% of all seafood-related deaths in the United States; can enter in VBNC state and remain virulent, at least for some time; and is capable of causing fatal infections following in vivo

resuscitation; however, it must be specified that virulence decreases significantly as cells enter the VBNC state mice. As *V. vulnificus* became nonculturable, virulence was determined by employing CD-1 5 to 9 weeks old (Oliver and Bockian 1995). VBNC cells of *E. coli* 0157:H7 (enterotoxigenic *Escherichia coli*), one of the most common food-borne and waterborne pathogens, retain the ability to express both stx1 (Shiga-like toxin gene1) and stx2 (Shiga-like toxin gene2) genes; Shiga-like toxins (Stx) are responsible for the major symptoms of hemorrhagic colitis and hemolytic uremic syndrome (Liu et al. 2010b). *Vibrio cholerae* can be resuscitated by introduction in the intestine of human volunteers (Colwell et al. 1996). From the analysis of all these works, it seems that in the VBNC state, the virulence is maintained in numerous pathogenic bacteria and infection can be initiated in certain strains and under certain conditions. So, VBNC state constitutes an important reservoir of pathogens. Indeed, pathogens in VBNC state have been found to be responsible for many latent infections, which may show up after months or even years.

4.5.4 Spores

Several prokaryotes belonging to the genera *Bacillus*, *Clostridium*, and *Sporosarcina* produce during a process called sporulation a highly differentiated structure named endospore, the purest form of prokaryote dormancy. In response to unfavorable conditions (starvation, desiccation, etc.), vegetative cells of spore-forming bacteria undergo an asymmetric cell division, resulting in two genetically identical daughter but morphologically distinct compartments, a larger "mother cell" and a smaller "forespore," that undergo different cell fates; the smaller of the compartment develops into a mature spore, capable of protecting the genome of the mother cell. The spore is released in the environment, when where it can persist for very long periods of time. Once favorable conditions return, the spore germinates and initiates a rapid growth, and the bacteria return to the vegetative state within minute to > 24 h (Setlow 2013, 2014a, b); spore germination required a number of spore-specific proteins (Gould 2006; Setlow 2013, 2014b). Spores, which are a long-term survival strategy, are very resistant to environmental stresses as any cell found on Earth: extreme heat, UV and γ, chemicals (acids, bases, oxidizing agents, alkylating agents, aldehydes, and organic solvents), drying, and nutrient depletion (Gould 2006; Setlow 2006, 2014a, b). Due to the ability of spores to withstand to harsh environments, the capacity of spores to survive to extraterrestrial environments has been considered, and experiments have been undertaken to test spore resistance under simulated Mars surface environment (Hagen et al. 1964; Nicholson et al. 2009; Moeller et al. 2012). Morphologically the Sps constituted by a central core containing DNA, ribosomes, and most spore enzymes (Fig. 4.7). The core is partially dehydrated (water content represents 25–50% of wet weight) and contains huge amount (~10% of the total spore dry weight) of a spore-specific molecule, the pyridine-2,6-dicarboxilic acid (dipicolinic acid, DPA) (Setlow 2014a). The core is protected by several concentrically arranged protective layers. To know the

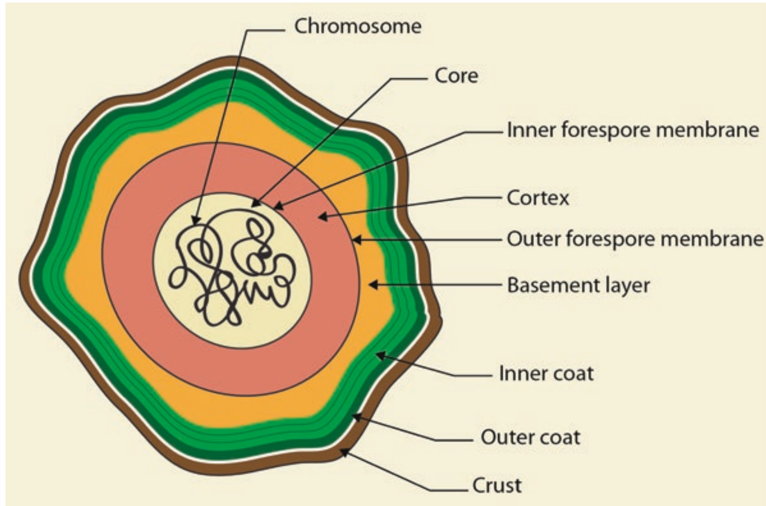


Fig. 4.7 Schematic spore structure
The multiple layers of the spore serve to protect the genome housed in the partially dehydrated central core which contains the DNA, RNA, and most enzymes. The core is surrounded by the inner forespore membrane, (ii) the protective cortex, (iii) the outer forespore membrane, and (iv) the spore coat. The spore coat consists of four layers: basement layer, inner coat, outer coat, and crust. (Modified and redrawn from McKenney et al. 2013).

physiological, physiological, and genetic mechanisms involved in the sporulation, refer to previous works (Driks 2002; Gould 2006; Galperin et al. 2012; McKenney et al. 2013; Tan and Ramamurthi 2013; Al-Hinai et al. 2015). Studies on endospores have a theoretical and applied interest. Many sporulating bacteria are pathogenic. Thus, they are of great importance in food, industrial, and medical microbiology. In addition, spore formation is an excellent model for understanding the molecular basis of developmental biology, including transcription regulation, intercellular signalling, membrane remodelling, intercellular communication, subcellular protein localization, and morphogenesis.

Other resistance structures exist in prokaryotes:

(i) Akinete, a survival structure of cyanobacteria.
(ii) *Azotobacter* and *Myxobacteria* cysts. For instance, when exposed to unfavorable conditions (e.g., starvation), cells of *Myxococcus xanthus* aggregate to form structure called fruiting bodies (multicellular structures) within which some cells differentiate into spherical, heat-resistant spores (Licking et al. 2000).
(iii) *Actinobacteria* exospores (exospore-forming filamentous *Streptomyces coelicolor*).

Spores, which are not a reproductive state but a survival state, can remain in their dormant state for years and yet return to active growth. Examples of spores able to survive for extended periods are common (Sneath 1962; Gest and Mandelstam

1987; Kennedy et al. 1994). For instance, Jacotot and Virat (1954)reported that spores of *Bacillus anthracis* from flask sealed in Louis Pasteur laboratory in 1888 were still alive 68 years later. *Clostridium aceticum* is an obligatory anaerobic spore bacteria isolated by Wieringa in 1939. The spores of this organism, which remained dormant in dry soils for more than 30 years (from 1947 to 1981), suspended in a complex medium containing fructose as energy and carbon source, were revived with growth occurring within 12 h (Braun et al. 1981). Viable *Thermoactinomyces* endospores were collected from Vindolanda site (England), where they had been deposited between 1850 and 1890 years previously (Unsworth et al. 1977).

Furthermore, increasing studies have reported the existence of prokaryotes of different origins (rocks, sediments, salt deposits, extinct animals), which could possibly have been preserved and have been survived for geological period of time. Examples are described in the following paragraph.

4.5.5 Prokaryotes Can Survive for Very Long Period Geological Time Scale (?)

The possible presence of viable prokaryotes in geological formations has been specifically studied in salt deposits (Dombrowski 1963; Kennedy et al. 1994; Grant et al. 1998; McGenity et al. 2000; Winters et al., 2015). Dombrowski (1963) was the first to announce isolation and culture from rock salt, and he describes the morphological and physiological characters of these Paleozoic bacteria. In order to avoid introduction of contaminants from surface site, he develops a rigorous method for isolation and cultivation of bacteria from Permian deposit. These results lead him to the assumption that the discovered bacteria are living representative of this geological period. Since Dombrowski's works, many studies have brought a large body of evidence that would prove the presence of long-term survival of halobacteria inside fluid inclusions of halides, and viable halophilic archaea would have been isolated from ancient salt deposit of the Permian and Triassic ages (McGenity et al. 2000; Vreeland et al. 2000; Stan-Lotter et al. 2002). For instance, Vreeland et al. (2000) announced the isolation and growth of a halotolerant spore-forming *Bacillus* species from a brine inclusion within a salt crystal of Permian Salado Formation in New Mexico. The geological age of the Salado is at least 250 Myr old. Crystal which measured 3.5 × 3.5 × 2.5 cm and contained living bacteria was collected 569 m below the surface. All precautions were taken to ensure that the samples were extracted under aseptic conditions and avoid contamination. The fluid recovered from the crystal was inoculated into casein-derived amino acid medium supplemented with 20% (W/V) NaCl. The strain isolated was designed 2-9-3; the complete 16SrRNA was sequenced and deposited in GeneBank; the sequence showed that the organism was most similar to *Bacillus marismortui*

(99% similarity) and *Virgibacillus pantothenticus* (97.5%); this ancient prokaryote is not significantly different from modern isolated prokaryote (Vreeland et al. 2000). For Vreeland et al. (2000), the strain "was present in a hypersaline during the late Permian, trapped inside a crystal at that time, and survived within the crystal until the present."

Some works are in favor of a microbial life present in the intestinal tract or in the abdomen of extent animal species. A nearly complete and remarkably well-preserved skeleton of a Pleistocene mastodon (*Mammut americanum*) was discovered in Licking County (Ohio). From a mass of plant found in the animal and interpreted as intestinal contents of the mastodon, Lepper et al. (1991) announced the isolation of enteric bacteria (*Enterobacter cloacae*). For the authors, these facultative anaerobic bacteria would be the survivors or descendants of the intestinal microflora of the mastodon.

In 1995, Cano and Borucki (Cano and Borucki 1995) using stringent, aseptic, and controlled conditions claimed that a bacterial spore isolated from abdominal content of extinct bees (*Proplebeia dominicana*) was preserved for 25–40 million years in buried Dominican amber. Spore was revived, cultured, and identified. So, a putatively ancient *Bacillus* sp. was obtained, coded BCA16. The characteristics (enzymatic, biochemical, DNA) are most closely related to extant *Bacillus sphaericus*.

Despite all the precautions taken in isolating these bacteria, all these results were viewed with scepticism, and serious arguments were made in favor of the possibility of contamination by modern bacteria during sampling and/or subsequent handling (Kennedy et al. 1994; Gutiérrez and Martin 1998; Graur and Pupko 2001). Even for samples retrieved from 2800 m depth, a very inaccessible place, the possibility that water may seep into the porous rocks during long time period, contaminating the drill sample, cannot be ruled out.

These results on the longevity of prokaryotes in geological samples – rocks, sediments, salt deposits, soils, and extinct animals – are obtained from a substantial number of samples by independent research teams; it emerges that further works are needed to validate definitively the presence of bacteria in old geological samples through the techniques described above (Parkes 2000).

The presence of microbial cells in a geological sample can also be demonstrated by the presence of a metabolic activity. Thus, Morono et al. (2011) demonstrated that carbon and nitrogen assimilation activities are maintained into microbial cells from 219-m-deep lower Pleistocene (460,000 years old); microorganisms in these old deep subseafloor sediments were alive and maintained potentials for metabolic activities and growth. For their part, Røy et al. (2012) measured an aerobic microbial respiration in 86-million-year-old deep-sea red clay, 0.001 micromole of O^2 liter^{-1}year^{-1}at 30 m depth, proving that microbial community can subsist in such sediment without fresh supply of organic matter for million years. Using a molecular technique targeting specifically rRNA, Schippers et al. (2005) demonstrated that a large fraction of the subseafloor prokaryotes are alive in very old (16 million years) and deep (> 400 m) sediments.

4.5.6 *Storage of Organic and Inorganic Compounds*

When resources are abundant, above what cells need to grow ("feast" periods), many prokaryotes accumulate intracellular storage polymers that will be used as endogenous energy reserves and/or sources of carbon to be used during starvation ("famine" periods) (Guerrero and Berlanga 2007; Madigan et al. 2015). Such a strategy constitutes a prokaryotic way to increase survival in the ever-changing environment and represents a Darwinian selective advantage. Endogenous organic and inorganic compounds include (i) glycogen a polymer of glucose; (ii) elementary sulfur (S_0) stored from oxidation of H_2S by sulfur chemolithotrophs to form microscopically visible granules (Fig. 4.8a) (when reduced sulfur source becomes limiting, S_0 is oxidized to sulfate (SO_4^{2-}), and the granules disappear); (iii) polyphosphate, used as sources of phosphate for nucleic acid and phospholipid biosynthesis or used directly to make ATP; (iv) polyhydroxyalkanoates (PHAs) (Fig. 4.8b–d) (poly-ß- hydroxybutyric acid (PHB), formed from ß-hydroxybutyric acid units, is probably the most common type of PHA and the best studied); (v) cyanophysin which is synthesized by cyanobacteria and some heterotrophic bacteria; and (vi) bacterioferritin, a protein that stores Fe^{2+} and thus makes it available when the supply is low and prevents the Fenton effects that causes an oxic shock.

Many prokaryotes, *Bacteria* and Archaea, produce PHAs. The occurrence of 91 different PHA constituents reflects the low substrate specificity of polyhydroxy alkanoic acid synthetases which are the key enzymes of polyhydroxyalkanoic acid biosynthesis (Steinbüchel and Valentin 1995). As a function of the number of carbon atoms of the monomer, PHAs are classified as short- (3-5 C-atoms), medium- (6 or more C-atoms), and chain-length PHAs (Mezzina and Pettinari 2016).

PHAs accumulate as intracellular granules in the form of inclusion bodies in amorphous state, as water-insoluble inclusions. Prokaryotes are able to accumulate as much as 80% of their dry weight in PHA. The size of PHA granules mostly comprises between 100 and 500 nm in diameter, and their number which varies as a function of organisms is between 5 and 10 granules per cell. Inclusion bodies contain approximately 97.5% PHA, 2% proteins, and 0.5% lipids, although some estimates of the lipid contents are considerably higher (Pötter and Steinbüchel 2005). Inclusion bodies are coated with a monolayer of phospholipids and granule-associated proteins that play a major role in biogenesis and intracellular degradation of PHAs.

Four types of granule-associated proteins are found (Steinbüchel et al. 1995; Pötter and Steinbüchel 2005; Grage et al. 2009).

(i) Polyester or PHA synthases (PhaC) which are the key enzymes of PHA biosynthesis (Rehm 2003; Peters and Rehm 2005).
(ii) PHA depolymerases (PhaZ) consist of two groups: the intracellular depolymerases (PhaZ) which degrade the amorphous PHA within granules and the extracellular depolymerases, which are secreted by most bacteria to utilize denatured PHA present in the environment from, for example, other nonliving cells.

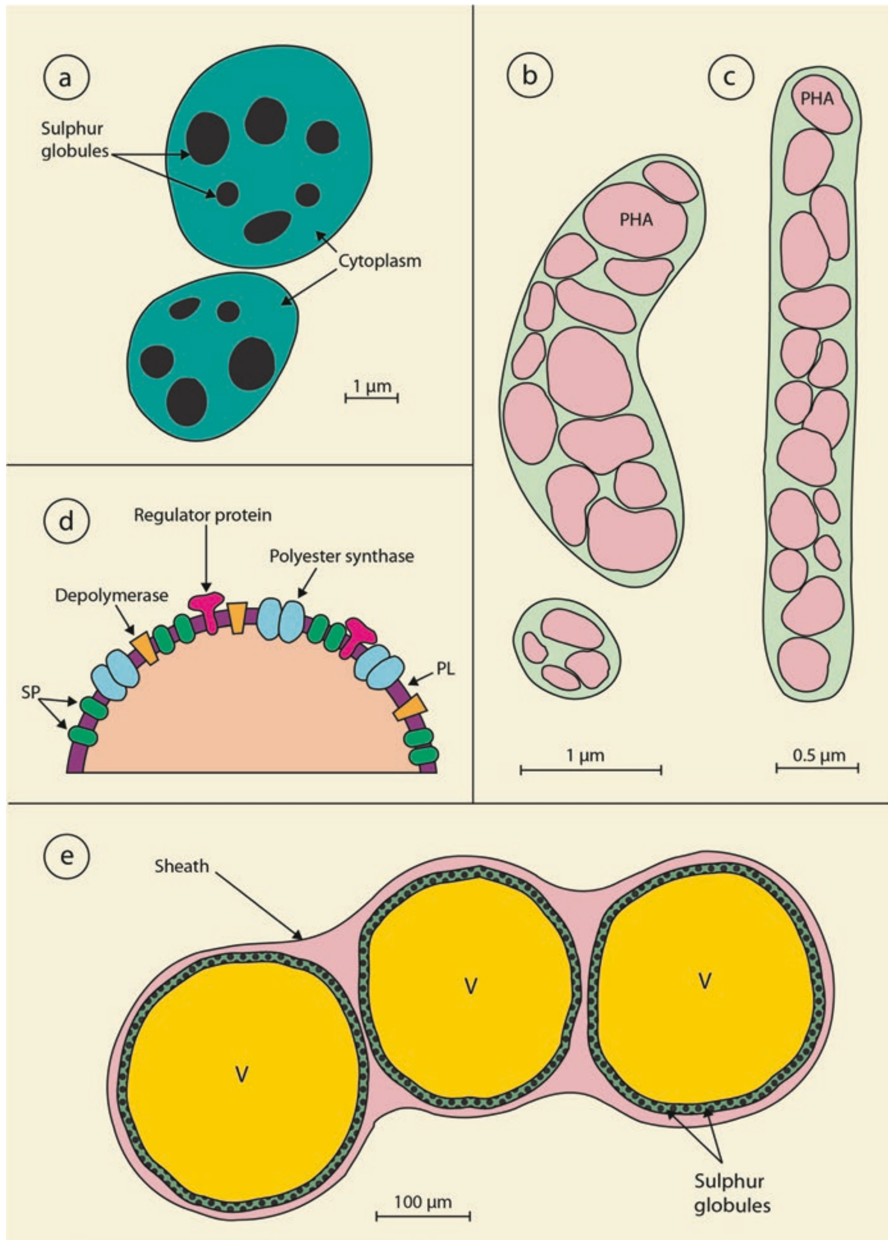


Fig. 4.8 Schematic representation of intracellular storage of organic and inorganic compounds (**a**) Bacterial intracellular sulfur globules in *Chromatium buderi* (Maki 2013); (**b**) PHA granules of the strain MAT-28 from Ebro Delta microbial mats (Guerrero and Berlanga 2007); (**c**) PHA granules of the strain *Wautersia eutropha* H16 (Tian et al. 2005): (**d**) organization of PHA granule. PHA granules are surrounded by a phospholipid membrane (PL), with embedded or attached proteins: PHA synthase, PHA depolymerase, PHA-specific regulator proteins, structural proteins (SP), additional proteins with as yet unknown functions (Rehm 2003); (**e**) *Thiomargarita namibiensis*. Most of the biovolume is occupied by a large, central vacuole (V) surrounded by internal deposit of sulfur globules (SG). Cytoplasm is restricted to a thin outer layer (green). Cells are organized in chains held together by a mucus sheath (Schulz et al. 1999). (Modified and redrawn from Schulz et al. 1999; Rehm, 2003; Tian et al. 2005; Guerrero and Berlanga 2007; Maki 2013)

(iii) Phasins (PhaPs) are noncatalytic proteins which are thought to be the major structural proteins of the membrane surrounding the inclusion, forming an interphase between the content of PHA granules (hydrophobic) and the cytoplasm content (hydrophilic) (Mezzina and Pettinari 2016).
(iv) The regulator proteins (PhaR) involved in PHA granule synthesis and phasin production (Peters and Rehm 2005; Pötter and Steinbüchel 2005).

PHAs are interest for industrial and biomedical applications (Pötter and Steinbüchel 2005; Chen 2009; Grage et al. 2009; Kourmentza et al. 2017).

A remarkable example of prokaryote able to withstand drastic changes in its environment, thanks to the accumulation of reserve substances, is that of *Thiomargarita namibiensis* (Fig. 4.8e). This giant bacterium, the largest bacterium ever discovered, visible to the naked eye, has a spherical shape and formed typical chain which contained on average 12 cells. Most cells have a diameter of between 100 and 300 µm, but some cells may have a diameter of up to 750 µm. This bacterium was discovered 1997, in Namibian shelf sediments, during a cruise aboard the R/V Petr Kottsov (Schulz et al. 1999). Each cell contains a large central vacuole, which represents about 98% of the cell volume, that it fills with high concentrations of nitrate (0.1–0.8M). The rest of the cell contains sulfide granules localized in the thin layer of cytoplasm. These sulfur globules, capable of reflecting the light, explain the name given to this bacterium "sulfur pearl of Namibia." *Thiomargarita namibiensis* that couples nitrogen cycle and sulfur cycle is able to live under oxic (O_2-rich waters) and anoxic conditions (sulfide-rich marine sediments). Its strategy is as follows: when buried in sediments, cells oxidize H_2S to S^0 anaerobically by reducing NO_3^- stored in the vacuole to ammonium (NH_4^+). They then store the S^0 as intracellular granules. When turbulent waters mix cells into the overlying oxic water column, where H_2S is lacking, they switch to the aerobic oxidation of stored S_0. The energy they gain from S^0 oxidation is used to refill their vacuole with NO_3^- from the water column so they will be able to survive under nitrate starvation for long periods (40–50 days).

4.6 The Conquest of All Biotopes by Prokaryotes and Their Role in Their Functioning

Biotopes have varied characteristics, among which varied sources of energy, various electron acceptors, and prokaryotes have evolved to colonize an amazing range of such biotopes on Earth. These biotopes undergo fluctuations, the amplitudes and periods of which vary. The microbes present have thus had to evolve to withstand the *short-term modifications* of these physicochemical parameters in these biotopes. Microbes thus have been selected to have adaptive mechanisms that allow them to face *immediate changing conditions of their habitat* (nutrient levels, thermal or osmotic shock, pH and oxygen levels, prolonged dehydration, UV irradiation, etc.).

4.6.1 Fluctuating Parameters in the Physical World and the Need for Homeostasis

Besides having contrasted physicochemical parameters, biotopes vary a lot in the amplitudes and rate of fluctuations for these parameters. Between a mammalian colon where temperature, acidity, salinity, and concentration of organic compounds fluctuate only marginally and a desert topsoil that sees temperature ranging between +40° and −20°, there is thus a whole array of biotopes offering contrasted conditions.

Homeostasis is the tendency of biological entities to maintain intracellular conditions as stable as possible in order to permit the hundreds of enzymes and metabolites present to interact in a sustainable fashion. All enzyme and cell constituents have optima for physicochemical parameters; these optima may not be similar for all enzymes and other determinants; therefore the cells strive to maintain these parameters close to the optima in order to permit most enzymes to function most of the time at an efficiency as high as possible.

Parameters fluctuate in general following a driver such as the sun with its diurnal and annual cycles. Other drivers are geysers and volcanoes, tides, and of course more recently anthropic inputs. Such cycles generate in general coupled fluctuations such as temperature, aridity, and UV flux, complicating the necessary biochemical adaptations. Yet bacteria have evolved to colonize and thrive in all such biotopes, even if at different concentrations.

4.6.1.1 Temperature

Temperature is the most pervasive parameter, one that microbial cells cannot escape contrary to a few large eukaryotes that can control it. Due to their small size, microbial cells will rapidly see their intracellular temperature follow that outside, and cells can only adapt the cell functioning to it. Some biotopes such as the mammal gut or the deep oceans have very stable temperature, while others such as topsoils, especially desert ones, fluctuate a lot, and those cells selected in these biotopes have evolved mechanisms to cope with it. One type of adaptation is selection of lipids, proteins, enzymes, and RNA that function better at a given temperature (Barabote et al. 2009); this process occurs over millions of years and can be accelerated by lateral gene transfer.

Temperature adaptation is also a short-term event implying HSPs (heat-shock proteins) that can renature proteins which structure has been modified by heat (Arsene et al. 2000). Cold-shock proteins are RNA chaperones that upon a cold shock will release mRNAs that will in turn modify the protein pattern of the cell (Phadtare and Inouye 2004).

Prokaryotes produce complex structures, spores, able to withstand temperatures (cf. 4.4.4). For instance, endospores present in *Firmicutes* constitute a complex structure, based on about 500 genes, many of which are conserved, that permit a

bacterial culture to survive exposure to 100 °C (Galperin et al. 2012). *Actinobacteria* also produce spores which resistance to heat is modest, with mesophilic actinobacterial spores being inactivated typically by exposure to 50 °C (Fergus 1967); however, these actinobacterial spores can withstand long episodes of desiccation typical of soils.

4.6.1.2 Light

Light is a source of energy that has been harnessed by many lineages, collectively called photosynthesis. Photosynthetic lineages appeared presumably among the first microbes on Earth, and the known photosynthetic machineries are based on a conserved set of components comprising a light-harvesting pigment antenna, a reaction center, and an electron transport system (Stanier et al. 1986). Bacterial photosynthesis can be oxygenic or not, using varied chlorophyll structures, resulting in different chemistry.

The wavelength used by photosynthetic organisms is centered around 550–650 nm, but solar radiation comprises a much wider spectrum of wavelengths, many of which are hard to cope with, especially as their wavelength is close to the UV (400 nm to 100 nm, shorter than that of visible light but longer than X-rays). Such light in general accompanies the harvestable light, and so photosynthetic microbes have developed ways to protect themselves against it. UV light is absorbed by double bonds such as those present in DNA, and this causes damages like cyclobutane-pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4PPs) that are cytotoxic and mutagenic (Sinha and Hader 2002). Adaptation to these challenges comprises DNA repair mechanisms that are not specific to light adaptation but are especially necessary for microbes thriving in lighted areas.

Other adaptations to light are the synthesis of pigments, such as melanins or carotenoids (Figs. 4.9 and 4.10), that absorb short wavelength light and prevent fragile cell constituents from being exposed. Melanin-pigmented microbes are often found on stone surfaces exposed to light where they form dark stains (Sghaier et al. 2015).

Light is a powerful signal that indicates the coming switch from night cold and wet conditions to hot and dry ones. Many bacteria have evolved bacteriophytochrome triggers that detect this signal and use direct protein-protein interaction to synthesize light-harvesting antennae (Giraud et al. 2002).

4.6.1.3 Nutrients (Electron Donors)

Electron donors vary a lot in the various biotopes microbes have colonized. Microbes are divided into heterotrophs that catabolize organic compounds synthesized typically by autonomous organisms and autotrophs that can synthesize compounds using energy sources such as light or chemical compounds. Those microbes able to transform light into chemical energy are called photosynthetic (above). Those able

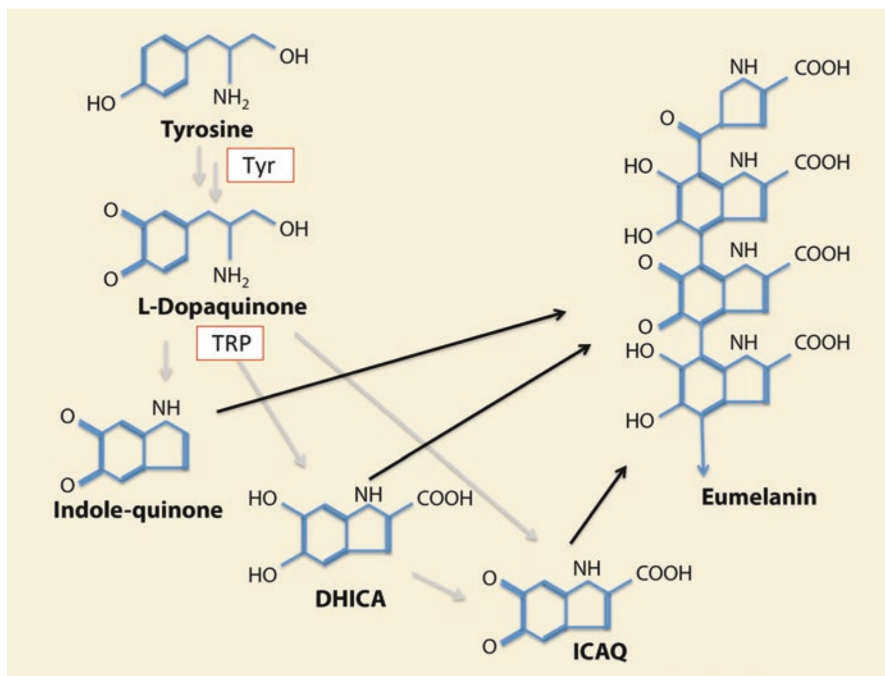


Fig. 4.9 Biosynthetic pathway for synthesis of melanin pigments
The amino acid tyrosine is transformed by tyrosinase into L-dopaquinone and DOPA, then into indole-5,6-quinone (IQ) and indole-2-carboxylic acid-5,6-quinone (ICAQ) by TRPs (tyrosine-related proteins), and finally into eumelanin by poorly characterized enzymes. Melanin is a broad term to describe brown to black pigments found in a large array of bacteria and fungi

to use energy-rich chemical compounds are autotrophs, and among the compounds used contain nitrogen (nitrate, nitrite), sulfur (sulfide), iron, hydrogen, etc. These microbes must also have ways to fix carbon to synthesize cell constituents; they do so using rbc or use organic compounds as building blocks and are then called facultative autotrophs.

The oxidation of reduced compounds has the potential to yield energy, the amount of which depends on the redox difference as indicated in Fig. 4.11.

4.6.1.4 Nutrients (Electron Acceptors)

The most prevalent electron acceptor is oxygen; it has the most positive redox and thus produces the most energy and is the preferred acceptor when present. Initially the term "oxidation" was coined by Lavoisier who recognized that compounds were chemically modified by contact with this gas. Aerobic bacteria couple redox reaction and transfer electrons toward oxygen; they thus need oxygen. Aerobic bacteria have thus developed dedicated electron transfer systems to harness the chemical

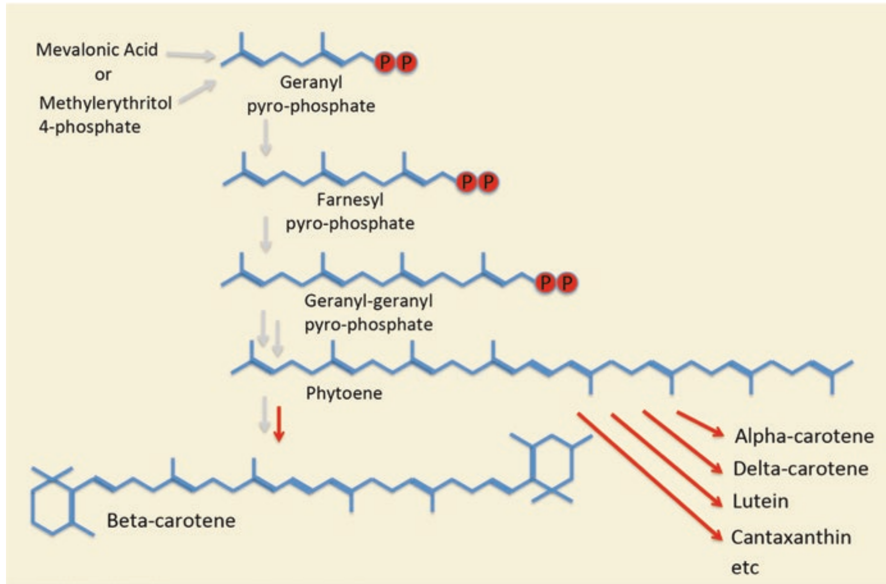


Fig. 4.10 Biosynthetic pathway for synthesis of carotenoid pigments
Two characterized pathways, the mevalonate and the methyl erythritol phosphate ones, produce dimethylallyl diphosphate, which is then transformed into geranyl-pyrophosphate, then farnesyl-pyrophosphate, then geranyl-pyrophosphate, and the 40C phytoene and beta-carotene and a range of modified derivatives, accounting for the various yellow to red hues seen in nature

energy necessary for cell functioning. Typically, cells will use organic acids or hexoses to feed the TCA cycle, which comprise dehydrogenases that oxidize organic compounds and funnel the resulting electrons to membrane-bound quinones and cytochromes. These will then bind oxygen and transform it into water.

However oxygen is a highly reactive compound, with a tendency to form daughter molecules such as peroxides, superoxide, hydroxyl radical, and singlet oxygen which together are called reactive oxygen species (ROS). These ROS interact with different cell constituents; peroxide, for instance, is a well-used antibacterial compound that modifies the membrane lipids and causes cell death. Cells exposed to peroxide, for instance, pathogens of eukaryotes, typically have developed peroxidases and catalases that transform peroxide into water. Superoxide ions also present in eukaryotic tissues can be metabolized by superoxide dismutases that yield hydrogen peroxide, which must then be dealt with by peroxidases.

The Fenton reaction involves free iron that reacts with hydrogen peroxide forming a hydroxyl radical and a hydroxide ion, which is why bacteria exposed to hydrogen peroxide have developed mechanisms to minimize the level of free iron such as bacterioferritins to store iron in an inactive form and to replace iron by other metals in proteins.

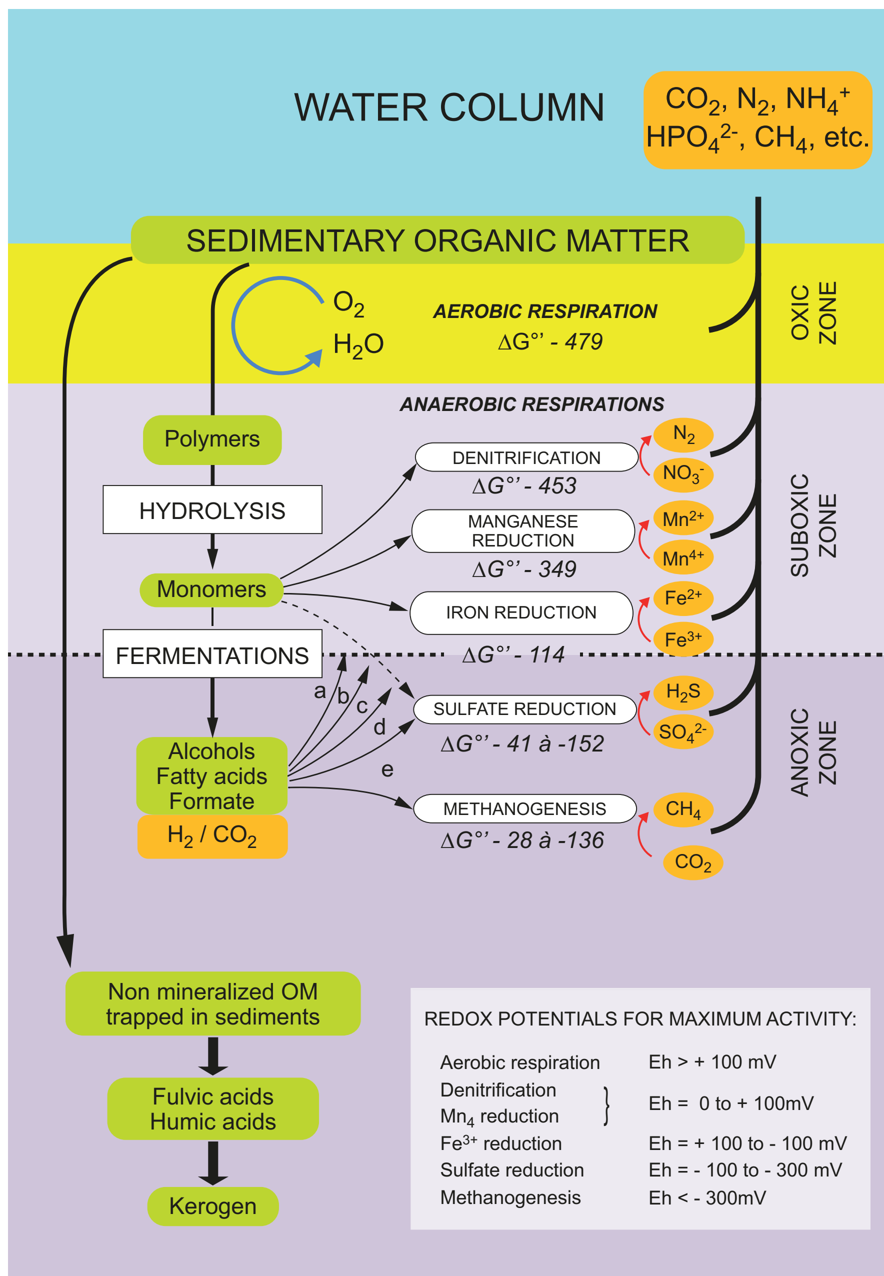


Fig. 4.11 Organic matter transformation in aquatic sediments
$\Delta G^{0'}$ values are expressed in KJ.mole^{-1} of oxidized acetate (Thauer et al. 1977). For fermentations, $\Delta G^{0'}$ values range from +70 to −260 KJ.mole^{-1} according to the fermented substrate. Interspecies hydrogen transfers are included. Alcohols and fatty acids are also utilized by denitrifiers (**a**), Mn (**b**), iron (**c**), and sulfate reducers (**d**) as indicated by cartouches from top to bottom. Sulfate reducers (**d**) and methanogens (**e**) use also hydrogen and formate

Anaerobic bacteria and facultative aerobic bacteria use other electron acceptors such as nitrate, nitrite, sulfate, manganese, iron oxides, carbon monoxide, or fermentation. Some of these compounds are toxic to cell constituents such as nitrite or manganese. Nitrite, which can be recovered from saltpeter on stone surfaces in caves lying next to soil or urinals, for instance, has been used for centuries to cure meats because it is a powerful antibacterial compound. The reaction starts with nitric oxide (NO) that interacts with superoxide (O2·−) to form peroxynitrite (ONOO−). The chemical basis for its toxicity is poorly known, but it is considered to stem from the formation of reactive nitrogen species (RNS) that interferes with biological processes by interacting with protein cofactors such as Fe-S clusters, heme, and lipoamides (Zhang et al. 2013).

4.6.1.5 Acidity

Several definitions of acidity exist, and most are based on the proportion of protons in a solution, a parameter that influences the proton gradient between the outside and the inside of cells. Microbial cells respire essentially by coupling oxidation of an electron donor with an electron transport chain which comprises expulsing protons outside the cells. This proton gradient is then tapped by a chemiosmotic process that allows protons back inside cells coupled with ATP synthesis. This process is in general adapted to neutral conditions, but bacteria are known to have colonized acidic and basic biotopes such as deep-sea hydrothermal vents (Bernhardt and Tate 2012).

Adaptation to an acid biotope entails either modification of the hydrogenase or local alkalinization. Local alkalinization has been described in *Helicobacter pylori* that causes stomach ulcers. This bacterium can resist highly acidic stomach content using a urease enzyme that cleaves urea, releasing ammonium that will locally neutralize hydrochloric acid (Marshall et al. 1990).

Since high pH (commonly understood as above pH 8.5) may denature DNA, destabilize membrane lipids, and denature enzymes, adaptation to an alkaline biotope is essential and has been shown to occur in two manners. Bacteria need to establish a proton gradient; therefore they may cause local acidification of the medium or bring about modification of the wall. Na+/H+ antiporters are integral membrane proteins that catalyze Na^+ uptake and proton efflux, which have been described as permitting cells to cope with high pH biotopes (Wang et al. 2014b). Use of acidic wall polymers (galacturonic, glutamic, phosphoric acids, and proteins) is another strategy that results in an acidic matrix that creates a buffer and protects the plasma membrane (Janto et al. 2011).

4.6.1.6 Water

Water is considered the best indicator for the possibility of life, and deserts are basically places where water is rare. Water is the solvent in which chemical reactions take place and osmolytes outside cells constitute a powerful force that makes water

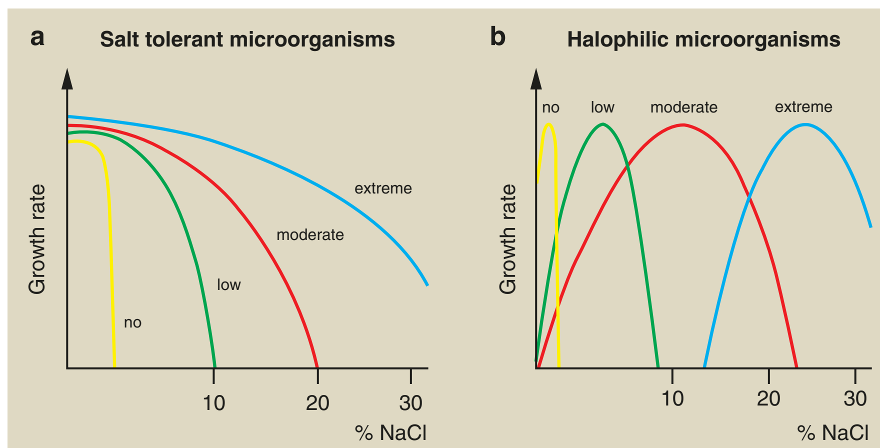


Fig. 4.12 Bacterial growth as a function of the NaCl concentration
(**a**) Salt tolerance in bacteria and archaea depends on the concentration of NaCl they can withstand: low salt tolerance, 0–8%NaCl; moderate salt tolerance, 0–20% NaCl; and extreme salt tolerance, 0–30% NaCl. (**b**) Halophilic bacteria and archaea for which are presented the lowest, highest, and optimum concentrations of NaCl in which they can develop: lowly halophilic, 1–3 – 8% NaCl; moderately halophilic, 2–3 – 10–12 – 20–25% NaCl; and extremely halophilic, 12–15 – 25–30 – 30% NaCl

difficult to get. Water abundance fluctuates typically over the day/night cycle or over the yearly cycle in terrestrial biotopes. Osmolytes that will play a major role will vary depending on the biotope, with NaCl salt being the most prevalent in seawater, but it is always accompanied by other ions, and typical seawater contains Cl^- 0.566 ($mol.kg^{-1}$), Na^+ 0.486, Mg^{2+} 0.055, SO_4^{2-} 0.029, Ca^{2+} 0.011, K^+ 0.011, etc. Besides seawater, there are biotopes that contain significantly higher concentration of salts such as the Dead Sea. Halophiles (salt lovers) are microbes that thrive in salted water, and they are characterized as slight, moderate, or extreme (Fig. 4.12).

4.6.1.7 Nitrogen

Nitrogen is a major constituent of cells, being part of DNA, RNA, walls, and proteins. There are animated discussions on the evolution of nitrogen in the primitive Earth atmosphere with some hypothesizing ammonia and others proposing high concentrations of cyanide (Silver and Postgate 1973; Miller and Schlesinger 1983). It is now abundant in the biosphere but in a form that most cells cannot metabolize, dinitrogen. Bacteria and archaea have developed systems for the fixation (reduction) of dinitrogen into ammonia with a very conserved protein, nitrogenase. This enzyme can in a reducing environment devoid of oxygen transform dinitrogen into ammonium ions. In order to provide this reducing environment, cells need to exclude oxygen which can be done with thick-walled cells such as heterocysts in

cyanobacteria (Wolk 2000) or with multi-lamellate hopanoid walls in *Frankia* (Berry et al. 1993). Other bacteria develop clumps where cells on the outside deplete oxygen by respiring it allowing others at the center of clumps to fix nitrogen.

4.6.1.8 Other

Microbial cells need minimal amounts of oxygen, nitrogen, carbon, hydrogen, manganese, magnesium, calcium, iron, nickel, and in some cases, need maximal amounts of these. Oxygen, carbon, and nitrogen have been described above, and we will briefly describe the others below.

Iron is a constituent of some enzymes in the form of iron-sulfur clusters that are situated at the active core of nitrogenase and hydrogenase.

4.6.2 Types of Molecular Regulation and the Search for Efficiency

4.6.2.1 Transcriptional

The best understood mode of regulation is that at the transcriptional level where a transcriptional regulator, a small molecule that has affinity to a DNA sequence, has evolved to be modified by an environmental parameter, in such a way as to modify its affinity to DNA. There are negative regulators, such as Fur (ferric uptake regulator), that utilizes Fe^{2+} as a corepressor and represses siderophore (a compound to chelate iron) synthesis in pathogens as long as iron is present by binding to DNA promoters upstream of the siderophore synthesis genes and preventing their synthesis (Venturi et al. 1995). Conversely, OxyR is a positive regulator in *Escherichia coli*, negatively autoregulated, that in the presence of hydrogen peroxide will see its affinity for DNA increased and will induce resistance to subsequent lethal doses of H_2O_2 by increasing transcription of 30 proteins among which catalase, glutathione reductase, and an alkyl hydroperoxide reductase (Christman et al. 1989).

There are families of transcriptional regulators, such as the Crp, LuxR, MerR, and TetR, that vary in their structure and mode of interaction with the transcriptional apparatus. Typically bacterial genomes have hundreds of transcriptional regulators belonging to various families, the proportion of which varies as a function of the biotope (Santos et al. 2009).

Transcriptional regulation is widely represented in bacterial cells because it is an energetically cheap way to modulate cells response. However, it is a slow process, whereby the cells will see the level of a compound be modified, the transcriptional regulator will start transcription of the genes of the regulon into mRNAs, and these mRNAs will then be translated into proteins that will sometimes need to be processed to be functional and will then modify the cells physiology.

4.6.2.2 Translational

Translational regulation is the control exerted by a cell on the level of proteins from a given level of mRNA. The mechanisms involved comprise the recruitment of ribosome on initiation codons of messengers, sequestration of messengers by proteins, or modulation of elongation or termination of protein synthesis (Kozak 1999). Instances of regulation at this level are few.

4.6.2.3 Posttranslational

Posttranslational regulation refers to the control exerted on synthesized proteins by reversible or irreversible modifications. Proteins are made up of the 20 standard amino acids that are linked into chains on ribosomes using mRNA as matrix. On such raw proteins can be attached various functional groups such as phosphate, acetate, amide, methyl, glycosyl, uridyl, or lipid groups. Nonstandard amino acids can also be added such as selenocysteine, pyrrolysine, and N-formylmethionine. Maturation of enzymes, such as proteolytic cleavage, and formation of disulfide bridges will also modify the protein functions.

Nonenzymatic protein modifications can be made on proteins such as glycation (addition of a sugar molecule) and denaturation; these will modify their ability to function.

Signal transduction systems constitute the best studied part of the posttranslational control systems. These are based on proteins that are modified by environmental parameters and that can in turn modify other proteins in order to modify their function. Histidine protein kinase systems are the system most common in the bacterial world that consists of two proteins, a membrane-embedded histidine kinase protein (HK) and an intracellular response regulator (RR) that interact through the transfer of a phosphoryl group from the HK protein onto the RR protein. The phosphorylated RR protein will in turn become active until it is dephosphorylated either by the HK or by a dedicated phosphatase. Among the environment stimuli detected by HK proteins are the concentration of nutrients, the redox and the osmotic potentials, temperature, or pH. A good example is the chemotactic behavior of motile cells that can feel the concentration of nutrients. *E. coli*, for instance, has rotating flagella that switch the sense of rotation from the clockwise to the counterclockwise following a signal. Chemical compounds are sensed by transmembrane CheA proteins that autophosphorylate and then transfer the PO^{4-} to CheB and CheY that in turn interfere with the flagella Fli protein and trigger a switch in the sense of rotation which in turn modifies the direction of swimming.

4.6.3 Biodiversity and Adaptation of Extremophilic Microorganisms Inhabiting Extreme Environments

Our anthropocentric vision of life leads us to consider that extremophilic microorganisms occupy niches that are inhospitable to humans due to their harsh physicochemical conditions. These conditions are considered inhospitable nowadays, yet they may have been of primary importance for the emergence of life more than 4 billion years ago. This is true for microbes growing optimally at high (thermophiles/hyperthermophiles) or low (psychrophiles) temperatures, high (alkaliphiles) or low (acidophiles) pH, high saline conditions (hyperhalophiles), as well as high hydrostatic or lithostatic pressures (piezophiles), high levels of ionizing radiation, etc. (Cayol et al. 2015). In addition, numerous habitats may exhibit various combinations of extreme conditions, where growth of only poly-extremophiles is possible (e.g., thermohalophiles, thermoalkaliphiles, thermoacidophiles). It is quite fascinating to imagine that such microorganisms may have prevailed in the anoxic primitive atmosphere under extreme conditions with many-facetted coping strategies at the onset of life. While there are several proposed *scenarii* involving extremophiles at the origin of life, much attention has been paid to thermophilic/hyperthermophilic anaerobes as being the first microorganisms possibly occurring on Earth (Cayol et al. 2015; Westall et al. 2018). In this chapter, we will focus on microbial diversity of extremophiles and how they have adapted their physiology to their respective habitats with a peculiar emphasis on halophiles/hyperhalophiles, psychrophiles, alkaliphiles, acidophiles, piezophiles, and thermophiles/hyperthermophiles.

4.6.3.1 Halophiles/Hyperhalophiles

The ionic composition, pH, and total salt concentration reaching saturation level (34% v/v) are variable from one hypersaline ecosystem to another, whatever its origin (terrestrial, subterranean, or marine), thus allowing the establishment of a large microbial biodiversity including aerobes and anaerobes (Ollivier et al. 1994; Cayol et al. 1995; Oren 2008; Oren 2013) (Fig. 4.13). While algae (e.g., *Dunaliella* spp.), bacteria (e.g., *Halomonas* spp.), and archaea (members of the family *Halobacteriaceae*) are significant representatives of aerobes in these environments, anaerobes, which are also of importance for mineralizing organic matter in hypersaline sediments, comprise fermentative (e.g., members of the family *Haloanaerobiaceae*), homoacetogenic (e.g., *Acetohalobium arabaticum*), sulfate-reducing (e.g., *Desulfohalobium* spp.), and phototrophic bacteria (e.g., *Ectothiorhodospira* spp.), together with methanogenic archaea (e.g., *Methanohalophilus* spp.) (Ollivier et al. 1994). Since life at high salt concentrations requires a high-energy investment to counteract the effect of osmotic pressure, the free-energy changes associated with some reactions performed by anaerobes (oxidation of hydrogen or fermentation of acetate by methanogens, oxidation of acetate by sulfate-reducing bacteria) are not sufficient to allow growth, and, consequently,

Fig. 4.13 Retba Lake (Lac rose, Sénégal)
The salinity of the water is 340 g/L; it is thus a hypersaline lake in Sénégal inhabited by a wide range of aerobic and anaerobic prokaryotes

such metabolic activities do not occur *in situ* (Oren 1999). In contrast, methylotrophic methanogenesis is performed in anaerobic sediments, thus making hypersaline habitats one of the very rare environments where competition between sulfate-reducing bacteria (SRB) and methanogens does not exist since SRB are known not to use methylated compounds as electron donors (Ollivier et al. 1994). More opportunities are offered to aerobes for living in high saline conditions because of the energy provided by oxygen respiration. However, it will be seen below that energetics are not the only constraints for using one strategy instead of another in order to grow in the presence of high salt concentration.

Salts outside cells exert an osmotic pressure that attracts intracellular water from the outside to the inside and, thus, makes transport of constituents inside cells more difficult. In this respect, whatever the type of metabolism, all these microbes have to maintain cell turgor despite the high osmotic pressure exerted by the high salt concentration in the medium. This is achieved by two major strategies. One is the salting-in strategy where the uptake of potassium and chloride from the environment is followed by their accumulation inside cells, which is correlated to extracellular salt concentrations (Oren 1999; Oren 2008; Ma et al. 2010). This osmoadaptive strategy has been demonstrated many times in aerobic members of the order *Halobacteriales* (e.g., *Halobacterium*, *Haloarcula*, *Haloferax*, and *Halorubrum* spp.), as well as in anaerobic members of the order *Halanaerobiales* (e.g., *Halobacteroides* and *Halanaerobium* spp.) and in one member of the *Bacteroidetes* (e.g., *Salinibacter ruber*). The other one, called the "compatible solute strategy," consists in the accumulation of low-molecular-weight highly soluble organic solutes through uptake

and/or biosynthesis in the cell cytoplasm (Oren 1999; Empadinhas and DaCosta 2008; Oren 2008; Ma et al. 2010; Shivanand and Mugeraya 2011). These organic osmolytes comprise amino acids (e.g., proline), amino acid derivatives (e.g., glycine betaine), and also sugars (e.g., trehalose) or sugar alcohols (glycerol). Glycine betaine is produced by a large range of bacteria comprising the phototrophic anaerobic *Ectothiorhodospira* spp. and cyanobacteria, domain *Bacteria*, as well as by the methylotrophic methanogenic *Methanohalophilus* spp., domain *Archaea*. Apart from glycine betaine, these methanogens may accumulate beta-glutamine, beta-glutamate, and *N*-epsilon-acetyl-beta-lysine. Other osmolytes such as sucrose and trehalose have also been shown to accumulate in osmotically stressed cyanobacteria. It has been recently demonstrated that trehalose/2-sulfotrehalose biosynthesis and glycine-betaine uptake are widespread mechanisms for osmoadaptation in the *Halobacteriales*, thus demonstrating that a given microorganism may use the two strategies mentioned above to face osmotic stress (Youssef et al. 2014). Glycerol was shown to be the major compound produced by *Dunaliella* spp. to grow under hypersaline conditions, and ectoin, a cyclic amino acid, was first identified within the cytoplasm of *Ectothiorhodospira* spp. Other organic osmolytes such as L-alpha-glutamate, L-proline, hydroxyectoine, and alpha-glucosylglycerol have been also identified in hyperhalophilic microorganisms. It is clear from these results that despite its energy cost, most hyperhalophiles use the compatible solute strategy to keep their cytoplasm isosmotic with the external medium. This may be related to the fact that cells using the salting-in strategy require extensive adaptation of intracellular enzymatic machinery and, notably, to maintain protein conformation and activity in the presence of high salt concentration (Lanyi 1974; Oren 2008), while the compatible solute strategy does not interfere with intracellular enzymatic activities and cellular processes (Grant 2004) but is most energetically costly. The resistance to or requirement for salt of *Halobacteriaceae* proteins result in an excess ratio of acidic to basic amino acids (DasSarma and Arora 2001; Ma et al. 2010) including aspartic acid and glutamic acid (Hoff 2009) that may interfere with the maintenance of physiological intracellular pH. In addition, unsaturated ether lipids have been often observed in members of this family (Ma et al. 2010), which may help in facing osmotic stress. In addition to *de novo* synthesis of compatible solutes, microorganisms have also the ability to take them up when they are present in the culture medium which is less energy costly and thus more favorable for cell growth (Oren 1999). Such solutes have been synthesized by other microbes, and their uptake by others thus constitutes a case of scavenging.

4.6.3.2 Psychrophiles

Most terrestrial and marine environments on Earth are cold, and, thus, microorganisms inhabiting them and developing under these extreme conditions of temperature, called psychrophiles, are of primary importance in governing all the existing biogeochemical cycles in nature (D'Amico et al. 2006; Cayol et al. 2015; Cavicchioli

Fig. 4.14 An example of a typical cold ecosystem in Antarctic (Photography: courtesy from Jean-Claude Marx)

2016). Psychrophiles have been recovered from various ecosystems including sea ice, snow packs and glaciers, Arctic and Antarctic Oceans, Antarctic subglacial lakes, deep-sea waters, atmosphere, clouds, and permafrost (Margesin and Miteva 2011; Cayol et al. 2015) (Fig. 4.14). More attention has been paid to them in recent decades since other planets (e.g., Mars) or satellites in our solar system (Europa), which might have been conducive to the emergence of life on them (Price 2007; Cayol et al. 2015), exhibit low-temperature conditions similar to those encountered on our planet. It is noteworthy that polyextremophily may apply to psychrophiles living at high hydrostatic pressure (piezopsychrophiles) or high salinity (halopsychrophiles). Despite being less plausible, because most probably hot conditions have prevailed in the early Earth (Westall et al. 2018), it has even been suggested that a psychrophile was the first living microbe some 3.5 billion years ago (Price 2007).

All these microorganisms show adaptations which may be different from one another for growing at temperatures close to 0 °C with some of them having the ability to maintain activities at subzero temperatures down to – 20 °C and even less, in sea ice and possibly in permafrost (Margesin and Miteva 2011; Cayol et al. 2015; Cavicchioli 2016). The limiting factor for enzymatic activity is the availability of free water, which may occur below 0 °C depending on the presence of various salts and pressure conditions. The challenge for psychrophiles is to maintain cell integrity and functioning homeostasis particularly while water is freezing. This is possible in unfrozen water inside the permafrost soil; brine pockets resulting, for example, from marine salt increases; and channels within the ice where dissolved organic

matter is available. In addition to appropriate physicochemical conditions favoring lowering of the water freezing temperature, microorganisms have also developed remarkable strategies that will be discussed below to face low temperatures when thriving in such extreme environments (for reviews, see D'Amico et al. 2006; Orfaniotou et al. 2009; De Maayer et al. 2014; Cayol et al. 2015; Cavicchioli 2016). Microbial ecology studies based on cultural and molecular approaches have reported that bacteria, archaea, yeasts, filamentous fungi, and also algae colonize these habitats. Most cold habitats are considered as highly oxygenated because of the high solubility of gases at low temperatures. In this respect, many aerobes performing either photosynthesis or oxidation of various organic electron donors are present in ice, snow, and deep-sea environments. However, anaerobic populations are also well represented, notably in permafrost which is known to transfer huge quantities of methane to the atmosphere during summer thaw, thus impacting significantly, together with anthropogenic activities, global warming. Among aerobes, the most commonly reported microorganisms include members of the *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Actinobacteria* and the *Cytophaga-Flavobacterium-Bacteroides*, and photosynthetic cyanobacteria within the domain *Bacteria*, Crenarchaeota involved in ammonium oxidation (nitrification), and at least one euryarchaeotal representative, *Halorubrum lacusprofondi* (D'Amico et al. 2006; Margesin and Miteva 2011; Cayol et al. 2015). Anaerobes comprise members of the *Deltaproteobacteria*, the *Firmicutes*, and the *Euryarchaeota*. They are found mainly in Arctic and Antarctic lakes, in cold soils, and in permafrost. As examples of oxygenic bacteria, *Alteromonas*, *Glaciecola*, *Colwellia*, and *Polaribacter* spp. were found in Arctic and Antarctic seawaters, while *Gammaproteobacteria* (e.g., *Photobacterium*, *Colwellia*, *Psychromonas*, *Moritella* spp.) occupy deep-sea waters. *Pseudomonas*, *Polaromonas*, and *Arthrobacter* spp. have been recovered from permafrost samples. Methanotrophs such as *Methylobacter*, *Methylosinus*, and *Methylomicrobium* are active in Arctic soils and permafrost where they participate in the regulation of methane emissions into the atmosphere (D'Amico et al. 2006; Margesin and Miteva 2011; Cayol et al. 2015). Surprisingly, the thermophilic aerobic hydrogen-oxidizing proteobacterium *Hydrogenophilus thermoluteolus* was detected in accretion ice in the subglacial Lake Vostok, Antarctica, where only its survival but not growth seems possible. Its presence was explained (Lavire et al. 2006) by possible seismotectonic activity episodes releasing debris along with microbial cells present within deep faults in the bedrock below the lake, where deep thermolysis of water could yield hydrogen, a source of electrons for *H. thermoluteolus*, as well as oxygen. Anaerobic activities in cold environments can be attributed to methanogens (Euryarchaeota), sulfate-reducing bacteria (*Deltaproteobacteria*), fermentative bacteria (*Firmicutes*), and possibly others which have yet to be discovered as most attention has been paid by scientists to aerobic microorganisms so far. We may expect ongoing metagenomic studies to deliver more significant information on the different anaerobic trophic groups that may participate in biogeochemical cycles in cold environments. It is known that sulfate reduction occurs in deep-sea sediments and should be mainly the result of *Desulfovibrio* spp. activity (Kaneko et al. 2007; Margesin and Miteva 2011). *Desulfotalea psychrophila*, which is able to

grow by reducing sulfate to sulfide at *in situ* temperatures below 0 °C, was isolated from permanently cold Arctic sediments. In spite of the fact that *Clostridium* strains do not appear to be acclimatized to deep-sea sediments, with only spores occurring *in situ*, several of them have been isolated from Antarctic microbial mats where they may be active. *Clostridium psychrophilum* has a growth temperature optimum of 4 °C, while *C. frigoris*, *C. lacusfryxellense*, and *C. bowmani* have higher optimal growth temperatures ranging from 4 to 16 °C (Spring et al. 2003). Methanogens have been isolated from different locations in Antarctica (Ace Lake), in Alaska (Skan Bay), and in Europe (Soppen Lake, Switzerland). Their optimum growth temperature ranges from 18 to 35 °C with *Methanococcoides burtonii* being the only methanogen known so far to develop under 0 °C. They comprise hydrogenotrophic (*Methanogenium frigidum*, *M. marinum*), methylotrophic (*Methanococcoides burtonii*, *M. alaskense*), and acetoclastic archaea (*Methanosarcina baltica*, *M. lacustris*) (Margesin and Miteva 2011; Cavicchioli 2016). The hydrogen-oxidizing and acetate-fermenting capacities of cold-adapted methanogens suggest that complete mineralization of organic matter under anaerobic conditions producing methane may occur down to temperatures around 0 °C. The existence of such oxidative anaerobic processes at high negative temperatures needs further investigation.

All proteins have optimal temperatures at which attachment to substrate, to cofactors, and conformational changes permit them to fulfill their function. Other cell constituents, such as lipid membranes, are also capable of exchanging with proteins at temperatures that control rigidity and fluidity. Moreover the freezing of water around 0 °C makes movement of solutes impossible and generates reactive oxygen species. To grow at cold temperatures, psychrophiles have to adapt physiologically (for reviews, see D'Amico et al. 2006; De Maayer et al. 2014; Cayol et al. 2015; Cavicchioli 2016). This is achieved for most of them by an increased synthesis of membrane unsaturated fatty acids, polyunsaturated fatty acids, and branched fatty acids, as well as also sometimes by a decrease in the length of hydrocarbon chains which increase membrane fluidity. In addition, to avoid the deleterious effects of ice formation within cells, psychrophiles withstand freezing by synthesizing several types of proteins involved in various cellular processes including transcription, translation, or protein folding. Synthesis of antifreeze proteins allows lowering the freezing temperature of water. Response to cold temperature in psychrophiles may also result in the production of "cold-shock proteins" together with "cold-acclimatization proteins." Accumulation of osmolytes (sugars, polyols, amino acids, polyamines) may help microorganisms to face osmotic stress when high concentrations of salt are present in cold environments. Cell cryoprotection may be improved by the production of exopolysaccharides which have been found in Arctic and Antarctic marine bacteria and which are used for attachment of microorganisms to supports, thus facilitating in particular biofilm formation, concentration of nutrients, protection against unfavorable physicochemical conditions, and water retention. Due to the high solubility of oxygen at low temperatures, psychrophiles have also to protect themselves against reactive oxygen species that they may detoxify by a high production of antioxidant enzymes (e.g., catalase, superoxide dismutase). Finally, studies on a number of psychrophilic protein structures revealed a reduction

in arginine and proline, while proteins from cold-adapted archaea have a higher content of glutamine and threonine. As demonstrated above, the strategies used by psychrophiles to thrive in a cold environment are varied and may therefore be different from one microorganism to another. Proteins play an important role within these strategies and may be the result of constitutive expression, upregulation, or downregulation of genes in response to cold exposure (De Maayer et al. 2014).

4.6.3.3 Alkaliphiles

Alkaliphilic microorganisms are divided into two groups. While obligate alkaliphiles are defined as those growing only at pH values of nine and above, facultative alkaliphiles may grow under alkaline conditions as well as at neutral pH (Yumoto et al. 2011; Cayol et al. 2015). Some of them may develop under other extreme physicochemical constraints (e.g., high temperature and/or high salinity) making them polyextremophiles (e.g., halophilic alkalithermophiles like *Natranaerobius thermophilus*) (Mesbah and Wiegel 2008; Wiegel 2011). They inhabit a wide range of environments including soda lakes (Fig. 4.15), underground alkaline waters, soil

Fig. 4.15 Alkaline crater lake (pH around 10) of Rincon de Parangueo in the state of Guanajuato, Mexico (Photography: courtesy of Manon Bartoli)

samples where ammonification is carried out, the guts of insects, alkaline industrial effluents, and the terrestrial or marine alkaline ecosystems with alkalinity resulting from serpentinization reactions from mantle rocks with water (Grant 1992; Jones et al. 1998; Grant and Sorokin 2011; Yumoto et al. 2011). Most attention has been paid by microbiologists to soda lakes, where the microbial diversity has been widely described (Grant and Sorokin 2011). However there is an increasing interest in serpentinized ecosystems (e.g., Lost City) that exhibit physicochemical conditions which might have been of primary importance for the emergence of life more than 4 billion years ago (Kelley et al. 2005; Russell et al. 2014; Westall et al. 2018). Primary production in soda lakes consists in aerobic (e.g., cyanobacteria) and anaerobic photosynthetic bacteria (e.g., *Ectothiorhodospira* spp.) which may predominate in highly saline alkaline lakes where availability of oxygen is limited due its low solubility in saturated saline waters. These primary producers deliver organic matter to the environment, which may be used by a wide range of aerobic and anaerobic microorganisms within the domains *Bacteria* and *Archaea* (Grant and Sorokin 2011; Yumoto et al. 2011; Cayol et al. 2015). Among aerobic bacteria, numerous representatives of the *Gammaproteobacteria* have been isolated from these extreme environments and include proteolytic (e.g., *Halomonas* spp.), sulfur-oxidizing (e.g., *Thioalkalivibrio* spp.), methanotrophic (e.g., *Methylomicrobium* spp.), and nitrate-reducing microorganisms (e.g., *Alkalispirillum-Alkalilimnicola* group). The *Alphaproteobacteria Nitrobacter alkalicus* was demonstrated to be involved in the nitrogen cycle by oxidizing nitrite to nitrate. A great number of aerobic archaea belonging to the order *Halobacteriales* (e.g., *Natronobacterium*, *Natronococcus*) have been identified in soda lakes where their high content in carotenoids is responsible for the red color of these lakes. Anaerobic microorganisms comprise fermentative bacteria belonging to the phylum *Firmicutes* (e.g., *Natroincola* and *Tindallia* spp.), sulfate-reducing members of the orders *Desulfovibrionales* and *Desulfobacterales*, and methylotrophic (e.g., *Methanosalsus* and *Methanohalophilus* spp.) or hydrogenotrophic methane-producing archaea (*Methanobacterium* sp.), with hydrogen being poorly or not at all used by the latter at high pH values. An anaerobic poly-extremophile, the halophilic, alkalithermophilic bacterium *Natranaerobius thermophilus*, was isolated from soda lakes of the Wadi El Natrun, Egypt. It is noteworthy that, to date, the anaerobic oxidation of acetate has never been demonstrated by any microorganism indigenous to alkaline environments, thus suggesting that complete mineralization of organic matter in the absence of oxygen still remains an enigma in soda lakes. Microbial studies that have been performed in soil samples and also in the gut of insects revealed the presence of numerous alkaliphilic *Bacillales*, while an industrial process conducted under alkaline conditions (e.g., production of the indigo fermentation liquor) identified *Alkalibacterium* spp. as the main microbial components of the liquor. Molecular analyses of alkaline groundwaters indicate also the presence of *Bacillus* spp. together with *Alkaliphilus*, *Natronoincola*, and *Anaerobranca* spp. Molecular surveys were performed in submarine alkaline hydrothermal ecosystems driven by serpentinization processes (Brazelton et al. 2010; Suzuki et al. 2013; Tiago and Verissimo 2013; Quéméneur et al. 2014; Postec et al. 2015). Such processes result

in the production of hydrogen and methane by alkaline fluids originating from carbonate chimneys. These studies highlighted the presence of anaerobic members of the order *Methanosarcinales* as the main archaeal representatives possibly involved in methane production or oxidation together with sulfate-reducing bacteria. Within the *Bacteria*, evidence for the presence of anaerobic microorganisms of the *Firmicutes*, as well as aerobic *Gammaproteobacteria*-oxidizing sulfide and methane, has been provided. Few culture-dependent studies have yet been performed from serpentinite-containing environments. They have led to the isolation of heterotrophic aerobes comprising *Actinobacteria*, *Bacillus*, and *Staphylococcus* spp. (Tiago et al. 2004) and one hydrogenotroph isolated from the continental serpentinizing springs at the Cedars (CA, USA) which has been proposed as a novel genus "*Serpentinomonas*" within the *Betaproteobacteria* (Suzuki et al. 2014). It is only recently that the first anaerobic bacteria belonging to the *Firmicutes* have been isolated from such alkaline ecosystems. They include the hydrogen-producing bacteria *Alkaliphilus hydrothermalis*, *Acetoanaerobium pronyense*, and one *Clostridium* sp., all originating from the Prony hydrothermal field in New Caledonia (Mei et al. 2014; Ben Aissa et al. 2015; Bes et al. 2015).

A high external pH is destructive for cell machinery and integrity and especially for the ATP-generating proton-motive force, which is why alkaliphiles have to maintain their cytoplasmic pH at values much lower than that of the exterior environment. The mechanisms that allow microorganisms to maintain pH close to neutrality despite pH variations (acidic or alkaline pH) possibly occurring in their culture medium are known under the term "pH homeostasis" (Krulwich et al. 2011a). Such mechanisms have been discovered in aerobic *Bacillus* cells, in particular (Preiss et al. 2015). One of the major challenges for the growth of alkaliphiles is to sustain a proton-motive force in low proton concentration environments as is also the case in alkaline ecosystems (for reviews, see Krulwich et al. 2011a, b). To achieve this goal, these microorganisms increase ATP synthase activity with proton entrance in the cytoplasm being coupled to ATP generation. The energy released during the respiratory stage benefits the Na^+/H^+ or the $Na^+(K^+)/H^+$ antiporters which help also in internalizing H^+ into the cells, thus leading to acidification inside the cells. Such strategy involves cation/proton antiporters which have to allow more protons entering the cells than sodium/potassium ions, in particular, in order to maintain suitable internal pH for alkaliphiles to grow. Alkaliphiles therefore play a subtle game in the transport of cations and protons which should be favorable for proton import into the cells. Nevertheless, to ensure this bioenergetic process, the availability of cations (Na^+ or K^+) should not be limited. In this respect, the entry of Na^+, if necessary, may be activated by Na^+/soluble symporters or through the MotPS sodium ion channel that power motility. In addition, studies on aerobic alkaliphilic *Bacillus* spp. demonstrated that they possess a specific secondary cell wall polymer associated with the peptidoglycan and specific components in the cell membrane (e.g., cardiolipin, squalene) which may be helpful to trap protons at the membrane surface, thus favoring the functioning of a proton-motive force in low H^+ -containing environments, such as alkaline ones (Padan et al. 2005; Mesbah and Wiegel 2008). Experiments conducted with the anaerobic *Clostridium paradoxum* demon-

strated that, in contrast to aerobic *Bacillus*, it used a Na^+-coupled ATP synthase rather than a H^+-coupled ATP synthase, thus reducing sodium toxicity and cytoplasmic proton loss that would occur in aerobic alkaliphiles using a H^+-coupled ATPase (see above).

4.6.3.4 Acidophiles

Acidophiles thrive in acidic environments with pH values ranging from 1 to 5. While extreme acidophiles grow optimally at pH below 3.0, the optimum pH for growth of moderate acidophiles is considered to be between pH 3.0 and 5.0. Acidic ecosystems occur in both subaerial and subsurface environments with low pH resulting most often from anthropogenic activities (e.g., acid mine drainage) (Johnson 1998, 2012; Cayol et al. 2015). Acidophilic microorganisms also inhabit natural ecosystems such as sulfuric springs (Fig. 4.16), volcanic ecosystems, and rivers with the Rio Tinto being one of the most studied sites from a microbiological point of view (Gonzalez-Toril et al. 2003; Sanchez-Andrea et al. 2012; Aguilera 2013; Cayol et al. 2015). In all these habitats, the acidification process mainly results from the oxidation of reduced sulfur compounds (e.g., elemental sulfur, sulfide) into sulfuric acid by a variety of aerobic or facultative anaerobic microorganisms belonging to the domains *Bacteria* and *Archaea* (Cayol et al. 2015). Interestingly, due to low pH, metal solubility is improved, and, consequently, acidophiles have been reported to resist high metal concentrations making them good candidates to be used in biotechnology (e.g., biomining, recovery of metals, bioremediation of metal-rich solid and liquid wastes) (Dopson and Holmes 2014). Among these metals, iron is recognized as the most abundant one in acidic environments, and numerous ferrous-oxidizing and ferric-reducing prokaryotes have been detected by molecular or cultural approaches in these extreme environments (Cayol

Fig. 4.16 "Los Azufres," an acidic lake (pH <3) in the state of Michoacan, Mexico (Photography: courtesy of Agnès Hirschler)

et al. 2015). It is now clearly accepted by the scientific community that, in the absence of light, reduced sulfur compounds together with ferrous iron are the primary sources of energy for providing organic matter in acidic environments. Hydrogen is also available often in these environments as an electron donor and may contribute to the overall energetic processes within such extreme habitats. Within the domain *Bacteria*, members of the genus *Acidithiobacillus* are known to oxidize both Fe(II) and sulfur compounds (e.g., *A. ferrooxidans*, *Sulfobacillus* spp.) or only sulfur compounds (e.g., *A. caldus*, *A. thiooxidans*). *Leptospirillum* spp. can grow only by oxidizing reduced iron and are considered together with *Acidithiobacillus* spp. of ecological significance in these habitats. The ability to use ferric iron as an electron acceptor has been reported for the oxidation of inorganic (e.g., elemental sulfur) or organic material (reduced carbon compounds) by *A. ferrooxidans* and *Acidiphilium* spp., respectively (Johnson 1998, 2012; Cayol et al. 2015). A moderately thermophilic weakly acidophilic anaerobic bacterium, using organic compounds as substrates and belonging to the genus *Athalassotoga,* was recently demonstrated to also reduce ferric iron (Itoh et al. 2016). Thermophilic members of the order *Sulfolobales*, domain *Archaea*, obtain energy by oxidizing elemental sulfur or sulfidic minerals (e.g., *Sulfolobus* spp.) under aerobic conditions, while others make it by reducing ferric iron (e.g., *Acidianus* spp.). Within the same domain, *Ferroplasma* spp., order *Thermoplasmatales* (e.g., *F. acidiphilum* and *F. acidarmanus*), have the ability to oxidize iron. Finally the anaerobic *Stygiolobus azoricus* and the facultative anaerobic *Acidianus* spp. were the only extreme thermoacidophiles reported to oxidize anaerobically hydrogen by reducing elemental sulfur (Johnson 1998; Cayol et al. 2015). Although these thermophilic sulfur-reducing archaea have been shown to grow under extreme acidic conditions below 3.0, cultural-dependent studies have led only to the isolation of moderate acidophilic sulfate-reducing bacteria so far. They all belong to the phylum *Firmicutes* with the exception of *Thermodesulfobium narugense* growing at pH ranging from 4.0 to 6.5 with an optimum at 5.5–6.0 (Mori et al. 2003). They include *Desulfosporosinus* spp. which incompletely oxidize their substrates. This is the case of *Desulfosporosinus acidiphilus* growing optimally at pH 5.2 using hydrogen, lactate, pyruvate, glycerol, glucose, and fructose as electron donors (Alazard et al. 2010). It is noteworthy that neither hydrogenotrophic nor acetoclastic acidophilic methanogens have been isolated to date. However recent experiments that have been performed in peatlands (pH 4.5) have demonstrated that moderate acidophilic or acidotolerant methanogens acting as acetate or hydrogen scavengers during syntrophic oxidation of butyrate, ethanol, or propionate do exist (Schmidt et al. 2016). Eukaryotes are also present in acidic environments, notably in the presence of light. However they are generally considered as acidotolerant since they do not exhibit optimum growth at low pH. They include algae, yeasts, filamentous fungi, and amoebae (Aguilera 2013; Cayol et al. 2015).

Altogether, these results demonstrate that the oxidoreductive processes making use of iron are very effective under extremely acidic conditions, while that of sulfur compounds is still a matter of debate as reduction of the latter has been mainly established only at pH values > 3.0. They also indicate that most probably complete

anaerobic mineralization of organic matter in very low pH environments (< 3.0) does not occur through sulfate reduction or methanogenesis but rather through reduction of ferrous iron. However further experiments are needed to clarify the functions of the sulfur cycle in particular in acidic environments.

To face acidic conditions, acidophiles have developed different strategies with the aim of maintaining their cytoplasmic pH at near-neutral values (pH homeostasis) to avoid cell destruction (for reviews, see Baker-Austin and Dopson 2007; Krulwich et al. 2011a, b). These strategies may or may not require energy to be operational (active regulation or passive regulation, respectively). The most important mechanisms of pH homeostasis in acidophiles are to restrict the entrance of protons which could intensify cellular protonation known to be deleterious for cells. Moreover, this restriction results in a pH difference (delta) across the cytoplasmic membrane, which is essential for the functioning of the proton-motive force to produce ATP through the F_0F_1 ATPase. To sustain an appropriate proton-motive force at low pH, microorganisms possess a cell membrane highly impermeable to protons. This is the case for acidophilic archaea (e.g., *Thermoplasma*, *Ferroplasma*, *Sulfolobus* spp., etc.) which have a cell membrane composed of tetra-ether lipids. In contrast, despite the fact that tetra-ether lipids have been already detected in bacteria (e.g., diabolic acid within the membrane of *Thermotogales* (Damsté et al. 2007)), the bacterial cell membrane has been recognized to contain essentially ester lipids. Consequently, the cell membrane structure is of primary importance in allowing acidophiles to develop in their extreme habitats. Accordingly, some members of the genus *Leptospirillum* were shown to have numerous genes for cell membrane biosynthesis in their genomes. Another way to reduce the proton influx into the cells is to create a reversed membrane potential, which may be activated by the entrance of potassium ions (Baker-Austin and Dopson 2007). In this respect, many acidophilic prokaryotes were shown to have a high number of putative cation transporters that may counteract the proton influx. The excess of protons can also be removed by active pumping, as well as by using proton-driven secondary transporters. Other possibilities include the production of cytoplasmic buffer molecules to maintain the intracellular pH around 7.0. Such buffer molecules include organic compounds containing basic amino acids, and also mineral ones, such as phosphoric acid whose pH is not much affected by the addition or removal of protons. Despite organic acids are known as uncouplers to be toxic as substrates to be used under acidic conditions, heterotrophic acidophiles do exist, thus demonstrating that the latter have implemented unique strategie(s) with respect to pH homeostasis. Analysis of the genomes of some extreme acidophiles revealed the presence of several genes involved in DNA and protein repair. In addition, chaperones characterized by protein refolding were found to be highly expressed when acidophiles were exposed to a drop in pH. Finally, the pH stability of proteins at low pH may result from their content in iron. In this respect, we may expect acidophiles to use one or a combination of the strategies mentioned above to grow optimally under acidic to extreme acidic conditions, but future research in this field will provide more information on additional mechanisms that may be used by prokaryotes to circumvent the toxic effect on growth due to external low pH values.

4.6.3.5 Piezophiles

The deep biosphere includes the lithosphere, the deep oceans, and the ocean crust (Whitman et al. 1998; Cayol et al. 2015). These environments share one common feature, namely, high hydrostatic pressures (up to 1 100 atm or 110 Megapascal (MPa)). The organisms living in them and growing preferentially under high hydrostatic pressure are called "piezophiles" (defined as exhibiting optimal growth at pressures > 0.1 MPa). Hyperpiezophiles have been defined as those displaying optimal growth at pressures > 60 MPa (Allen and Bartlett 2004). The great majority of microorganisms isolated and characterized as piezophiles are psychrophilic facultative anaerobic bacteria mainly affiliated to *Gammaproteobacteria* and belonging to five genera: *Photobacterium*, *Shewanella*, *Colwellia*, *Psychromonas*, and *Moritella* (Simonato et al. 2006; Campanaro et al. 2008; Aono et al. 2010; Wang et al. 2015). These bacteria have been all isolated from deep marine sediments with the exception of the obligate piezophile *Colwellia* sp. MT-41, isolated from a shellfish collected in the Mariana Trench at 10 476 m depth, which is capable of developing only at pressures between 38 and 103 MPa (Yayanos et al. 1981). Regarding the mesophilic bacteria, they are represented by three species of sulfate-reducing bacteria belonging to the genus *Desulfovibrio* (*D. profundus*, *D. piezophilus*, and *D. hydrothermalis*) and two *Pseudomonas* strains (BT1 and MS300) (Bale et al. 1997; Kobayashi et al. 1998; Kaneko et al. 2000; Pradel et al. 2013; Amrani et al. 2014). Piezophilic thermophiles or hyperthermophiles belonging to the *Archaea* have been mainly isolated from deep-sea hydrothermal vents. They belong mainly to the *Thermococcales* order (e.g., *Thermococcus*, *Pyrococcus*, and *Palaeococcus*). A single obligate piezophile is known so far: the hyperthermophilic *Pyrococcus yayanosii* CH1, isolated from the site "Ashadze" on the Mid-Atlantic ridge at 4 100 m depth (Michoud and Jebbar 2016). Others are affiliated to the *Methanococcales* (Huber et al. 1982; Jones et al. 1983). High pressure impacts the activity of cells and cellular components and reduces the activity of numerous key processes, eventually leading to cell death of piezosensitive organisms (Simonato et al. 2006). Biochemical and genomic studies yield a fragmented view on the adaptive mechanisms in piezophiles. According to the genera investigated, piezophilic adaptation requires either the modification of a few genes, a more profound reorganization of the genome, the fine tuning of gene expression, or a stress-like physiological cell response. Global analyses, performed on few species (e.g., *Desulfovibrio hydrothermalis*, *D. piezophilus*, *Photobacterium profundum*, *Pyrococcus yayanosii*) at the genomic, transcriptomic, or proteomic levels, suggest that adaptation to high pressure is diffused in the genome and may concern only a small fraction of the genes (Le Bihan et al. 2013; Amrani et al. 2014, 2016; Michoud and Jebbar 2016). The most significant finding in studying the biochemistry of piezophilic bacteria is the discovery of *de novo* synthesis of polyunsaturated fatty acids (PFA), their proportions reaching as much as 70% of the total fatty acids in the membranes of these bacteria (Allen and Bartlett 2004; Abe 2013). The high proportion of PFA may increase the fluidity of the membrane at high hydrostatic pressures. A second major finding is the accumulation of solutes in the bacteria, which may play the expected role of "piezolyte." Similar to

the mechanisms reported for microorganisms in response to osmotic or heat stresses, the piezolytes may play the role of protein-stabilizing solutes. For example, glutamate has been shown to accumulate in the piezophilic bacteria of the *Desulfovibrio* genus when pressure increases (Amrani et al. 2014, 2016). In *P. profundum*, accumulation of β-hydroxybutyrate has been reported (Martin et al. 2002).

At the genomic level, Campanaro et al. (2005) have highlighted 27 genomic regions specific to the genome of the piezophilic *P. profundum* SS9 strain, by comparison with the genome of the piezosensitive *Photobacterium* 3TCK et DSJ4 strains. These regions contain, for the greater part, genes encoding phage proteins and integrases, as well as genes for (*i*) the energy metabolism (e.g., gene encoding the NAD(P)H oxidoreductase), (*ii*) the metabolism and the transport of the tryptophan, and (*iii*) the synthesis of flagella and the assembly of pili. These specific regions may have been acquired by horizontal transfer. Comparison between 141 orthologous proteins of the piezophilic archaea *Pyrococcus abyssi* and the piezosensitive *P. furiosus* revealed a significant substitution of amino acids tyrosine and glutamine by the amino acids arginine, glycine, serine, valine, and aspartate in *P. abyssi* (Di Giulio 2005). The rate of polar amino acids was positively correlated to the pressure increase, whereas a negative relation has been established between molecular weight of amino acids and resistance to high hydrostatic pressure. Similar substitutions have been evidenced by comparing the piezophilic *D. piezophilus* to the piezosensitive *D. salexigens* (Pradel et al. 2013).

At the transcriptomic and proteomic levels, the energy metabolism has been evidenced as one of the most important cellular processes involved in high-pressure adaptation (Le Bihan et al. 2013; Pradel et al. 2013; Amrani et al. 2014, 2016). Enzymes produced by high-pressure-adapted bacteria have been shown to be more functional under high-pressure conditions than at atmospheric pressure. In this context, the expression or the structure (e.g., due to amino acids replacements) of enzymes of the metabolism may be modified in piezophilic bacteria, favoring their activity and their stability under conditions of high hydrostatic pressure. For example, a pressure-resistant terminal oxidase has been revealed in *Shewanella violacea* (Tamegai et al. 2011). In the same bacterium, pressure-regulated promoters and differentially expressed cytochromes were identified. In *P. profundum*, a TMAO reductase is more abundant in the cells at highest pressures (28 MPa) (Le Bihan et al. 2013). Finally, in the anaerobic sulfate-reducing bacterium *D. piezophilus*, transcriptomic and biochemical analyses have shown that the metabolite cycling (H_2/Formate) is an important mechanism required for energy conservation at high hydrostatic pressure (26 MPa) (Amrani et al. 2016).

4.6.3.6 Thermophiles/Hyperthermophiles

The discovery in 1965 of the first thermophilic bacterium *Thermus aquaticus* in the geothermal springs of Yellowstone marked the beginning of a new era dedicated to the study of extremophilic microorganisms, among which thermophiles and hyperthermophiles are probably the most documented ones. By definition, thermophiles

grow at high temperature, with temperature optima higher than 45 °C and often above 55 °C, and hyperthermophiles thrive fastest at temperatures higher than 80 °C (Gottschal and Prins 1991; Stetter 1999). Thermophiles are found in both *Bacteria* and *Archaea*, while, to date, hyperthermophiles are mostly affiliated to the archaeal domain. The highest temperature limits for life ever recorded are held by two archaea: *Pyrolobus fumarii* exhibiting a maximal growth temperature of 113 °C (Blöchl et al. 1997) and *Methanopyrus kandleri* strain 116 proliferating at 122 °C under high pressure (20–40 MPa) where water remains liquid (Takai et al. 2008). Among *Bacteria*, the maximal growth temperatures ever documented are 95 °C and 100 °C, for *Aquifex pyrophilus* and *Geothermobacterium ferrireducens*, respectively (Huber et al. 1992; Kashefi et al. 2002). The upper temperature limit for life of prokaryotes has not yet been defined but is likely to lie in the region of 132 °C, since peptide and hydrogen bonds, two major chemical linkages governing macromolecules structures and interactions, are destabilized at this temperature. Even if life is not constrained only by temperature but rather by a range of physical and chemical parameters that act both individually and together, it is important to determine what the upper temperature for life is in order to delimit life's boundaries in hot environments and in Earth's subsurface. Such information would help in understanding in particular when and where life might have evolved on the Hadean Earth.

Thermophiles inhabit natural hot environments and may adapt to various physicochemical conditions (high hydrostatic pressure, high salinity, high pH, etc.). So far they have been recovered from water-containing natural marine and terrestrial biotopes and from artificial anthropogenic-driven systems. Hyper-/thermophiles have been recovered from marine biotopes such as shallow and deep-sea hydrothermal vents (located along the mid-ocean ridges and in back-arc basins), coastal shallow volcanic systems, marine volcanoes, and deep subseafloor sediments (the average geothermal gradient in the lithosphere is ca. 25 °C km^{-1}) (Fig. 4.17). Numerous hyper-/thermophilic taxa have also been isolated from high-temperature biotopes on land, such as hot springs, mud volcanoes, solfataric fields, petroleum

Fig. 4.17 A small hydrothermal chimney at Rainbow Hydrothermal Field (Mid-Atlantic Ridge) constituting a privileged habitat for aerobic and anaerobic hyperthermophiles (Credit photo: IFREMER – Victor 6000. EXOMAR cruise 2005)

reservoirs, and deep subterranean habitats (Cayol et al. 2015). Artificial high-temperature biotopes from which thermophiles have been isolated are various and include systems associated with human activities of extraction, transformation, and treatment of materials or wastes at elevated temperatures, such as wastewater treatment plants, smoldering coal refuse piles, sugar refineries, paper mills, hot water pipes, or domestic or industrial boilers. Surprisingly, very few thermophilic *Archaea* and *Bacteria* have been isolated from hot and arid deserts, these ecosystems being rather inhabited by mesophilic species exhibiting tolerance mechanisms to desiccation.

Thermophilic and hyperthermophilic prokaryotes available in clonal cultures are diversed in terms of phylogenetic diversity and biochemical and physiological features. However, the microorganisms cultivated so far represent only a fraction of the whole taxonomic, genomic, and phenotypic diversity of hyper-/thermophiles (Hedlund et al. 2015). Indeed, it is currently difficult to estimate the taxonomic richness and the physiological diversity of hyper-/thermophiles as most of the diversity of thermophiles is yet-uncultivated and remain to be explored. Taxa comprising hyper-/thermophilic representatives that have been detected only by molecular approaches include, for example, taxonomic groups such as “Aigarchaeota” (HWCG I, *Hot Water Crenarchaeotal Group I*), “Korarchaeota,” “Calescamantes” (EM19), and “Acetothermia” (OP1) (Hedlund et al. 2015). In the past decade, previously uncultivated taxa were successfully isolated as a result of information gathered from molecular- and/or genome-based methods and/or to geochemical modelling of environmental conditions. This is notably the case for *Aciduliprofundum boonei*, an obligate thermoacidophilic sulfur- or iron-reducing heterotroph, which is the first cultured representative of the DHVE2 (*Deep-sea Hydrothermal Vent Euryarchaeotic 2*) lineage, a widespread euryarchaeotal lineage at deep-sea hydrothermal vents (Reysenbach et al. 2006). To date, less than 1000 thermophilic or hyperthermophilic species, available in axenic cultures, have been formally described (source: *List of Prokaryotic names with Standing in Nomenclature* (Parte 2014)). Efforts continue to isolate novel thermophilic taxa, even if cultivation is time-consuming. Apart from cultivation, genome sequencing efforts and progress in obtaining high-quality draft genomes from metagenomics data have impacted the taxonomic classification of these microorganisms. Some thermophile-containing lineages were reclassified or have been proposed for reclassification. This is the case for the mesophilic to moderately thermophilic *Epsilonproteobacteria* known as primary producers in deep-sea hydrothermal vent systems, where they represent most often the dominant bacterial lineage in the warm area of the deposits (Campbell et al. 2006). On the basis of a comparative genomic analysis performed on more than 600 genomes of *Bacteria*, it was recently proposed to reclassify the class *Epsilonproteobacteria* to a novel phylum named “Epsilonbacteraeota” (Waite et al. 2017). Based on current evidence with regard to archaeal and bacterial thermophiles, there are phyla containing merely thermophiles, while there are phyla containing only thermophiles and mesophiles. A great number of phylum-level lineages containing many thermophiles branch off deeply in phylogenetic trees (Stetter 2006), thus indicating that thermophilic life probably appeared early on Earth. In terms of metabolism, known hyper-/

thermophiles cover a wide range of energy-yielding reactions and can enter the food web at various levels, from the primary production to the terminal degradation of organic matter. Cultivated thermophiles involved in primary production are chemolithoautotrophs using a variety of inorganic electron donors (H_2, Fe^{2+}, S°, $S_2 0_3^{2-}$, H_2S) and respiring various electron acceptors, ranging from oxygen to sulfate, and including nitrate, nitrite, ferric iron, other sulfur species, and carbon dioxide. Six different carbon fixation pathways are present among thermophiles: the reductive pentose phosphate cycle (Benson-Calvin cycle), the reductive tricarboxylic acid cycle (rTCA), the reductive acetyl-CoA pathway (Wood-Ljungdahl), the 3-hydroxypropionate bicycle (3-HP), the 3-hydroxypropionate/4-hydroxybutyrate cycle (3-HP/4-HB), and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle (Hügler and Sievert 2011). Several mixotrophs were also described among known thermophiles. With regard to heterotrophic hyper-/thermophiles, there are different various pathways of fermentation and types of respiration that have been reported in yet-cultivated taxa. In recent years, numerous progresses were notably done regarding the description of thermophiles with specialized metabolisms such as carboxydotrophy (Slepova et al. 2006) or sulfur compound disproportionation (Slobodkin et al. 2013), for example. The metabolic potential of yet-uncultivated thermophiles is obviously diverse as well, as revealed by metagenomics and single-genomic data analyses of hot environments, ranging from autotrophy ("Acetothermum," from a subterranean gold mine in Japan) (Takami et al. 2012) to obligate fermentation ("Korarchaeum," from Obsidian Pool, Yellowstone National Park, USA) (Elkins et al. 2008) or respiration ("Calescibacterium," from Great Boiling Spring, Nevada, USA) (Rinke et al. 2013). The discovery of an unexpected diversity of novel methyl-reducing methanogenic archaeal lineages, including those which do not belong to the Euryarchaeota (Vanwonterghem et al. 2016) and inhabit hot habitats (Sorokin et al. 2017), suggests that there are still novel lineages of thermophilic methanogens to discover.

Thermophilic and hyperthermophilic prokaryotes thrive at temperatures much higher than those tolerated by most other prokaryotes and have implemented strategies to maintain the integrity of their enzymes and genomes. A recent review on protein stability pointed out that despite much research in the field, adaptive mechanisms still remain only partially decrypted (Pucci et al. 2017). It appears that a combination of different factors that vary from one protein to another one are involved in thermostability. Nevertheless some general trends emerge clearly; they concern amino acid interactions (i.e., hydrophobic effect, salt bridges, disulfide bridges, etc.) and 3D protein structure (i.e., rigidity, loop shortening, oligomerization, etc.) (Pucci et al. 2017). These organisms can also accumulate small molecules called compatible solutes (Pais et al. 2009) that interact with the protein surface and so enhance their rigidity. Nucleic acid DNA and mostly RNA are highly sensitive to temperature. Several mechanisms that contribute to DNA stability have been identified in hyperthermophilic prokaryotes. The presence of a reverse DNA gyrase that promotes positive supercoiling of DNA (while DNA gyrase from mesophiles produce negative supercoiling) (Forterre et al. 1996; Lulchev and Klostermeier 2014; Lipscomb et al. 2017) is known to ensure a better stability of DNA to temperature.

Single-stranded DNA-binding proteins (Giaquinto et al. 2007) may contribute to the stabilization of nucleic acids and DNA-binding proteins in a similar way to that already reported for histones within the eukaryotes (histone-like proteins), thus contributing to the compaction of the DNA molecule and to its stability (Orfaniotou et al. 2009). Higher G+C% contents in tRNAs and rRNAs of many thermophiles were also reported and are likely to contribute to the thermostability of the genomes (Wang et al. 2015). Maintaining the functionality of the membranes at high temperature is also crucial. A recent review (Siliakus et al. 2017) pointed out that the presence of tetra-ether monolayers in the archaeal membrane is a highly efficient mechanism and that this feature is shared by some hyperthermophilic bacteria. Hopanoids are also found in high concentrations in membranes of thermophilic bacteria and may be involved in maintaining the integrity and fluidity of membranes at high temperatures (Caron et al. 2014).

In recent years, genetic tools have been developed for various thermophilic archaeal and bacterial models (Yu et al. 2001; Thiel et al. 2014; Lipscomb et al. 2017). In the coming years, genetic approaches (gene deletions, mutagenesis, etc.) will obviously contribute to a better understanding of life at high temperature.

Finally, mechanisms of adaptation of microorganisms to harsh and changing physicochemical conditions have in all likelihood developed early in the evolution of life as the primitive atmosphere has been recognized as being anaerobic, slightly reduced to neutral, and hot with marine ecosystems being slightly acidic (see Chap. 3). This is without considering alkaline conditions that resulted from prevailing serpentinization reactions following the contact of water with crustal rock some 3–4 billion years ago (see Chap. 3). In this respect, the first existing microorganisms already had to face extreme physicochemical conditions. We may expect that some of these mechanisms were essential to prokaryotes during the course of evolution whatever their type of metabolism whether aerobic or anaerobic. Extremophiles do not generally use a single strategy; instead they use several strategies for growth in various conditions. These strategies are different from one microorganism to another to maintain the functional integrity of their enzymes and genome. This has been made possible, in particular, by the accumulation of small molecules (organic solutes such as amino acids, cations, etc.) in the cytoplasm, quite often by modification to the lipid composition of membranes (presence of larger amounts of unsaturated fatty acids), and by the emergence of membrane carriers in order to regulate the exchange with the external environment. In some cases, there is evidence that the 3D protein structure and the amino acid content in proteins have evolved to permit cells to thrive in extreme environments. One noticeable mechanism called "pH homeostasis" is a remarkable adaptation of microorganisms to restricting the entrance of protons (acidophiles) or internalizing them in cells (alkaliphiles), thus helping in keeping the proton-motive force for ATP generation operational, despite low or high external pH conditions, respectively. Extremophilic prokaryotes may also use a set of up- and downregulation of genes to adapt to inhospitable conditions. It is noteworthy that the strategies that extremophilic microorganisms use do not allow some metabolisms due to energetic constraints (e.g., methanogenesis from $H_2 + CO_2$ or from acetate in hypersaline ecosystems, etc.). Due to the specific

adaptive mechanisms that microbes have acquired, extremophiles and/or microbes that are highly resistant to unfavorable conditions will have facilities to sustain life for the next few billion years despite the important climatic changes expected to occur on our planet on geological time scales (increasing luminosity of the sun resulting in water loss and aridification of the planet).

It thus appears that microbes have colonized all regions of our planet where liquid water was present, whatever the temperature, the pH, the pressure, the presence of ionizing radiation or metal ions, or the salt concentration. It now remains to be seen whether other celestial bodies, some of which have or had liquid water, do harbor microbial life. This will likely be one of the challenges of microbial ecology for the coming century.

4.7 The Functional Redundancy in Favor of the Evolution Success of Prokaryotes

Some properties seem universally required for life. One of the essential points is the absolute necessity for all living things to transduce energy from their environment. The availability of chemical energy is controlled by the environment of an organism and by the set of potential chemical reactions to support life. On a global scale, life is sustained by a flux of energy – either visible light or from chemical sources – throughout the metabolic networks. Due to their high metabolic versatility, only the microorganisms possess such a wide range of possibilities and continues to expand as we explore more of the microbial world.

From a much-cited study from 1998 (Whitman et al. 1998), the number of prokaryotes and the total amount of their cellular carbon on Earth are estimated to be 4–63 10^{30}cells and 350–550 Pg of C, equal to between 60% and 100% of the estimated total carbon in plants, and inclusion of prokaryotic carbon in global models will almost double estimates of the amount of carbon stored in living organisms. In addition, Earth's prokaryotes contain 85–130 Pg of N and 9–14 Pg of P or about tenfold more of these nutrients than do plants and represent the largest pool of these nutrients in living organisms. There are typically 50 million bacterial cells in a gram of soil and 1 million bacterial cells in a milliliter of sea water (Whitman et al. 1998). In the global subseafloor, sedimentary microbial abundance could reach $2.9 \cdot 10^{29}$cells [corresponding to 4.1 Pg of C and~0.6% of Earth' s total living biomass]. This estimate of subseafloor sedimentary microbial abundance seems much lower than previous estimates of subsea (Kallmeyer et al. 2012). Thus, prokaryotic carbon would equal to 60–100% of the total plant carbon and potentially could double the total amount of carbon stored in living organisms.

Microorganisms represent the predominant mode of life on Earth; they obtained their energy and nutrients required for growth and maintenance from enzyme-mediated redox reactions, i.e., successive transfers of electrons and protons from a relatively limited set of chemical elements. Metabolic reactions can be classified as either assimilatory, in which chemicals from the environment are absorbed to build

cellular components, or dissimilatory, in which chemicals from the environment are reacted to gain energy and the products are released back into the environment. All organisms require sources of carbon (and other structural elements) as well as sources of external energy. That is, the environment must supply a reduced compound (an electron donor) and an appropriate oxidized compound (an electron acceptor) to complete an energy-yielding reaction. Consequently, the vast majority of the fluxes between the different oxidation states of the major elements (H, C, N, O, S) are the result of microbial catalyzed redox reactions. A wide variety of redox couples can be used by various microorganisms to obtain energy, and new ones continue to be discovered periodically. The outcomes of the reactions of the five major elements connected by electron transfers are driven by thermodynamics, but selected by biologically mediated metabolisms. The biogeochemical cycles (H, C, N, O, S) are coupled via microbiologically catalyzed electron transfer reactions. Thus, biogeochemical cycles based on abiotically and biologically driven redox reactions and the feedbacks between microbial metabolisms and geochemical processes create the average redox condition of the oceans and atmosphere.

The evolution of the network of non-equilibrium redox reactions that are the sources of energy of the life on Earth remains largely unknown. However, microorganisms have probably determined the basic composition of Earth's atmosphere since the origin of life and created the breathable, O_2-rich air (Lyons et al. 2014). Indeed, 4.5 billion years ago, free oxygen was at level mostly less than 0.001% of those present in the atmosphere today. During the first half of its evolutionary history, a set of metabolic processes involving exclusively microbes has dramatically modified the surface chemistry of Earth. A most conspicuous expression of this is the accumulation of oxygen. Still, considerable controversy and debate surround when atmospheric oxygen first began to accumulate. The primitive atmosphere of Earth was mainly composed of CO_2 and N_2 with some H_2O and CH_4. Besides the oxygen production, microbes control also the atmospheric concentrations of a number of important greenhouse gases (Conrad 1996). For example, prokaryotes use CO_2 as source of carbon for their biomass synthetize or release CO_2 as a product of decomposition but are also the primary producers of CH_4 and N_2O that are strong greenhouse gases (Shoun et al. 1992). It is usually conceded that the atmospheric greenhouse effect must have been higher in the past to offset reduced solar luminosity, but the levels of atmospheric carbon dioxide and other greenhouse gases required remain speculative. A 1993 model by Kasting (1993) estimates that carbon dioxide (CO_2) levels in Earth's early atmosphere must have been 10 times to as much as 10,000 times today's level. For Kasting volcanic gases released at depth from mid-ocean ridge, hydrothermal vents could have contained appreciable concentrations of CH_4. Indeed, if the oxygen fugacity of the upper mantle was low, most of the carbon released from the mid-ocean ridge vents should have been in the form of CH_4 instead of CO_2, as it is today. The presence of this much CH_4 in the prebiotic atmosphere could have had important implications for the origin of life because it would have also permitted formation of HCN by the mechanism suggested by Zahnle (1986). This abiotic production of "organic material" fuels a long debate. Some authors argue that primeval life was based on anaerobic microorganisms able to use a wide

inventory of abiotic organic materials (i.e., a heterotrophic origin), whereas others invoke an organization thrived on simple inorganic molecules such as CO_2 (i.e., an autotrophic origin).

Today, nitrogen and oxygen are by far the most common atmospheric gases; dry air is composed of about 78% nitrogen (N_2) and about 21% oxygen (O_2). Argon, carbon dioxide (CO_2, 400ppm), and many other gases are also present in much lower amounts; each makes up less than 1% of the atmosphere's mixture of gases. In the twentieth century, humans began to have an enormous impact on the global biogeochemical cycles by developing industrial processes, by implementing new agricultural practices, and by burning fossil fuels. The primary greenhouse gases in Earth's atmosphere are water vapor, carbon dioxide, methane, nitrous oxide, and ozone. The atmospheric concentration of carbon dioxide increased from 280 ppm in 1750 to 406 ppm in early 2017, and the concentration of methane has increased by about 150% during the same period. Methane is a strong greenhouse gas with a global warming potential 84 times greater than CO_2 in a 20-year time frame. Nitrous oxide has also a significant global warming potential, considered over a 100-year period; nitrous oxide has 298 times the atmospheric heat-trapping ability of carbon dioxide (CO_2).

Thus, the planet has been subjected to extraordinary environmental changes, from bolide impacts and global glaciations to massive volcanic outgassing, and despite their antiquity, microorganisms maintain their fundamental role in biogeochemical cycle functioning and in maintaining the biosphere. The origin and evolution of metabolic pathways allowed primitive cells to become more chemically independent from the prebiotic sources of essential molecules. It is reasonable to assume that during the early stages of cell evolution, the primitive metabolism was based on a limited number of rudimentary (i.e., unspecific) enzymes. Several mechanisms such as the divergence and duplication or horizontal gene transfers may account for a rapid expansion of metabolic abilities. Thus, success in the evolution of prokaryotes could be explained by a great metabolic diversity and versatility which is expressed through the biogeochemical cycles that they were the only ones initially to ensure the functioning.

4.7.1 The Carbon Cycle

4.7.1.1 Different Autotrophic CO_2 Fixation Pathways

Autotrophic CO_2 fixation represents the most important biosynthetic process in biology permitting to build all cell material solely from inorganic carbon and thus providing the organic carbon for heterotrophic organisms. Moreover, the latter oxidize organic carbon back to inorganic carbon, completing the carbon cycle.

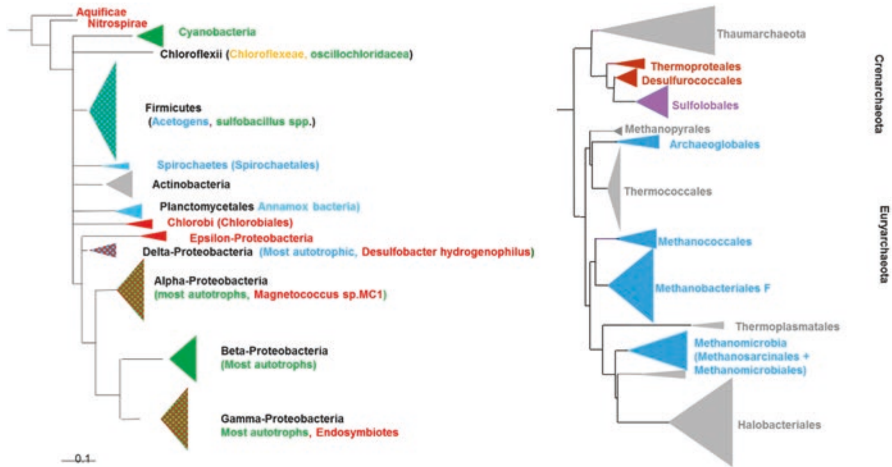
The O_2-resistant Calvin-Benson-Bassham (*CBB*) cycle has long been considered as the only CO_2 fixation pathway, occurring primarily in the first tens of meters of the water column, with oxygenic photosynthesis as the main energy input (Bassham et al. 1950). The characteristic enzyme involved in the *CBB* cycle (or reductive

pentose phosphate cycle) is ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Quayle et al. 1957), which catalyzes the primary carboxylation of ribulose 1,5-bisphosphate, yielding two molecules of 3-phosphoglycerate. The *CBB* cycle probably evolved in cyanobacteria, and it is the only carbon fixation pathway operating in eukaryotes (algae and plants) as a result of the endosymbiotic acquisition of a cyanobacterium that evolved into the chloroplasts. Overall, the phylogenetic diversity of bacterial groups using the CBB cycle is rather limited (Fig. 4.18a). Besides cyanobacteria, the CBB cycle occurs in photo- and (aerobic) chemoautotrophic *Alpha*-, *Beta*-, and *Gammaproteobacteria*. The key *CB* cycle enzyme, RubisCO, is the most abundant protein in the world (Ellis 1979), as it can comprise up to 50% of the total soluble protein in chloroplasts of a C3 plant or in bacteria using this cycle (Wildman 2002).

Five alternatives to the CBB pathway have been discovered in the last 60 years for CO2 fixation (Fig. 4.18a): the reductive tricarboxylic acid (*rTCA*) cycle, the reductive acetyl-CoA (or Wood-Ljungdahl, *WL*) pathway, the 3-hydroxypropionate (*3-HP*) bicycle, and the recently described 3-hydroxypropionate/4-hydroxybutyrate (*3-HP/4-HB*) and dicarboxylate/4-hydroxybutyrate (*DC/4-HB*) cycles (Ljungdahl 1986; Buchanan and Arnon 1990; Berg et al. 2007; Huber et al. 2008; Zarzycki et al. 2009). The importance of these CO_2 fixation pathways has only started being recognized during the last decade (Raven 2009; Berg 2011; Hügler and Sievert 2011), and our knowledge of dark CO_2 fixation outside hydrothermal vents is still in its infancy. Only an in-depth study to track these alternative pathways in the dark pelagic ocean will unequivocally change our understanding of the carbon cycle and energy flow in the ocean.

Different pathways are expected to be found in the O_2-rich seawater or in particle O_2 gradients. The *rTCA* cycle is essentially a reversal of the oxidative TCA cycle or Krebs cycle (Fig. 4.18b). The *rTCA* cycle is present in quite diverse groups of bacteria; however, due to the oxygen sensitivity of the enzymes 2-oxoglutarate and pyruvate synthase, the cycle appears to be restricted to anaerobic or microaerophilic bacteria (Fig. 4.18). Some pathways indeed harbor O_2-sensitive enzymes (*WL*, *rTCA*, *DC/4-HB*), while the others also function under fully aerobic conditions (*CBB*, *3-HP*, *3-HP/4-HB*) (Hügler and Sievert 2011). The anaerobic WL, rTCA, and DC/4-HB require 1-5 ATPs for the synthesis of pyruvate, against 7–9 ATPs with the *CBB*, *3-HP*, and *3-HP/4-HB* pathways. It is likely that dark ocean organisms adapt to use the most energetically efficient pathway, depending on their immediate physical-chemical environmental conditions. A partition of CO_2 fixation pathways is indeed observed at hydrothermal vent sites according to temperature, which is inversely proportional to the O_2 concentration (Hügler and Sievert 2011).

Even the assimilation of the simplest carbon molecule such as CO_2 leads to the introduction of a large metabolic redundancy. The further distribution of life drove the first organisms into diverse ecological niches, confronting them with different problems; this led to the appearance of new metabolic strategies. Not only were the conditions different, the organisms diversified as well, thus creating additional factors that influenced their metabolism. Life at the thermodynamical limit does not favor the usage of metabolic schemes with high-energy demands. The reversibility



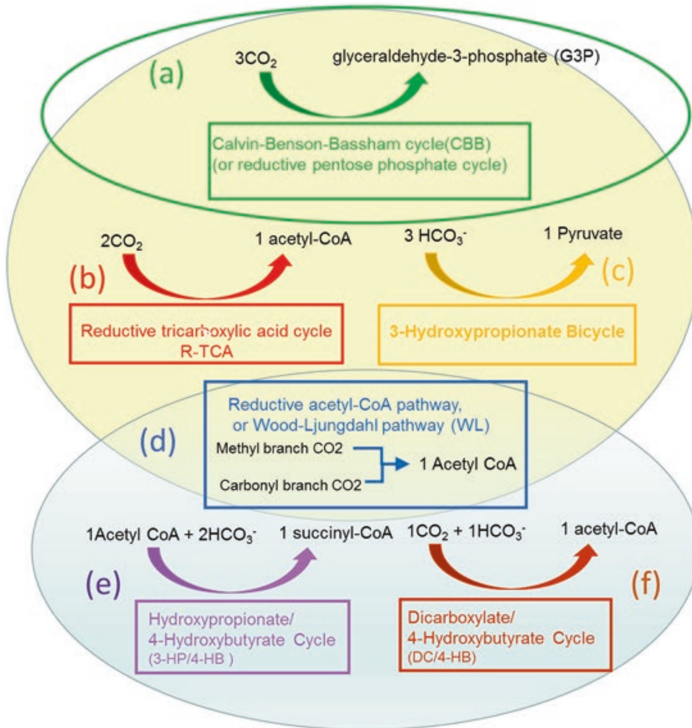


Fig. 4.18 The six pathways of autotrophic CO_2 fixation
(**a**) CBB, (**b**) rTCA, (**c**) 3-HP, (**d**) WL, (**e**) 3-HP/4-HB, and (**f**) DC/4-HB. Carbon balances are reported for each metabolic pathway. The same color is used to write the phylum and its associated metabolic patway, (Adapted from Hugler and Sievert (2011) and Berg (2011))

of the reductive acetyl-CoA pathway and the *rTCA* cycle may give additional metabolic plasticity to the organisms possessing these pathways. However, the usage of a more energetically efficient pathway is not necessarily advantageous, since it ensures the higher growth yields but may result in slower growth rates.

4.7.1.2 Methanogenesis and Methane Oxidation

Methane and its photochemical products deserve also our special attention because their roles as greenhouse gases may very well have helped to keep the early Earth habitable. Once life had arisen on Earth, the atmospheric CH_4 abundance should have risen because biological sources of CH_4 would have been available. In the absence or near absence of oxygen and sulfate, a greater amount of labile organic matter is available for microbial methane production (methanogenesis).

Methanogenic archaea are strict anaerobes that produce methane (CH_4) as the major product of their energy-conserving metabolism. All methanogenic archaea characterized so far belong to the Euryarchaeota and are distributed among five taxonomic classes, i.e., Methanopyri, Methanococci, Methanobacteria, Methanomicrobia, and Thermoplasmata. Currently, no methanogenesis has been found in bacteria or eukaryotes.

Several methanogenic pathways that rely on various substrates have been described (Fig. 4.19):

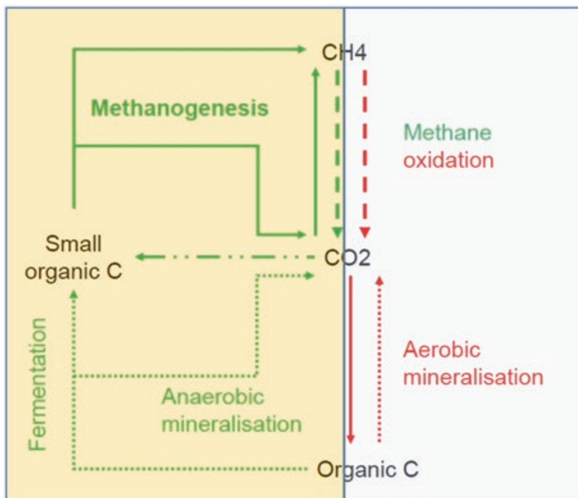


Fig. 4.19 Schematic representation of the prokaryotic carbon cycle
Red and green arrows indicate aerobic and anaerobic pathways, respectively; (1) hydrogenotrophic methanogenesis is a lithotrophic process resulting from the reduction of CO_2 with H_2 as electron donor; (2) formatotrophic methanogenesis, an organotrophic process supported by degradation of formate, acetate, and methylated compounds; (3) methane oxidation can occur via the aerobic or anaerobic pathway

(a) CO_2 reduction with hydrogen (hydrogenotrophic methanogens) or formate (formatotrophic methanogens) as electron donors or methanol reduction with hydrogen. Thermoplasmata reduce methanol with hydrogen (Paul et al. 2012).
(b) Fermentation of acetate (acetotrophic methanogens).
(c) Dismutation of methylated compounds (methylotrophic methanogens) such as methanol, methylamines, dimethyl sulfide (DMS), or methanethiol (Liu and Whitman 2008; Ferry 2010).

Whereas most cultivated methanogens reduce CO_2 with hydrogen, only members of Methanosarcinales have the ability to produce methane from the fermentation of acetate and the dismutation of methylated compounds and might also use methylamines as methanogenic substrate (Poulsen et al. 2013). Although acetate fermentation is performed by only a few cultivated methanogens, this process could account for up to two-thirds of the methane released to the atmosphere by archaeal methanogenesis; the reduction of CO_2 accounts for the rest of the archaeal contribution to atmospheric methane, with minor amounts of methane produced by the dismutation of methyl compounds (Ferry 2010).

Methanogens have been isolated from various anoxic environments (e.g., rice paddies and peat bogs; freshwater, marine, and hypersaline sediments; hydrothermal vents; deep subsurface habitats; the gastrointestinal tract of various animals) and are usually abundant where electron acceptors such as NO_3^-, Fe_3^+, and SO_4^{2-} are in short supply. Hydrogenotrophic methanogens and acetogenic bacteria have similar requirements, including anoxic conditions, a source of H2 as electron donor, and a source of CO_2 as electron acceptor. But methanogenesis occurs preferentially at low H_2 concentrations and at a pH lower than 7; it can also be performed at high temperatures (Thauer et al. 2008).

Syntrophic interactions enable methanogenesis when methanogenic substrates are limiting and methanogenic archaea established in various syntrophic partnerships. This process involves the transfer of electrons from a fermentative organism to the methanogen via a carrier molecule, such as H_2 or acetate. This transfers between two organisms enabling growth on otherwise thermodynamically unfavorable reactions. The methanogens use the carrier molecule as electron donor for energy conservation, and the fermentative organism gains energy from the redox reaction that produces the electron carrier only if the methanogens oxidize the carrier molecule, keeping the carrier at a low concentration.

Methanogenic archaea are a major source of CH_4 emissions, but some of their closest relatives in turn play a critical role in controlling these emissions by oxidizing CH_4 back to CO_2. Methane-oxidizing archaea (AMNE) are strict anaerobes, all belonging to single taxonomic class, the Methanomicrobia of the Euryarchaeota. Twenty years later, AOM was suggested to be a cooperative metabolic process mediated in marine environments by associations between anaerobic methanotrophic archaea (ANMEs) and sulfate-reducing bacteria (Knittel and Boetius 2009).

Today, at least four new ways in which microorganisms achieve anaerobic oxidation of methane (AOM) have been described (Fig. 4.20). Three of these implying two or more syntrophic partners (a, b); in the other two cases, a single microorganism performs both reactions (c,d).

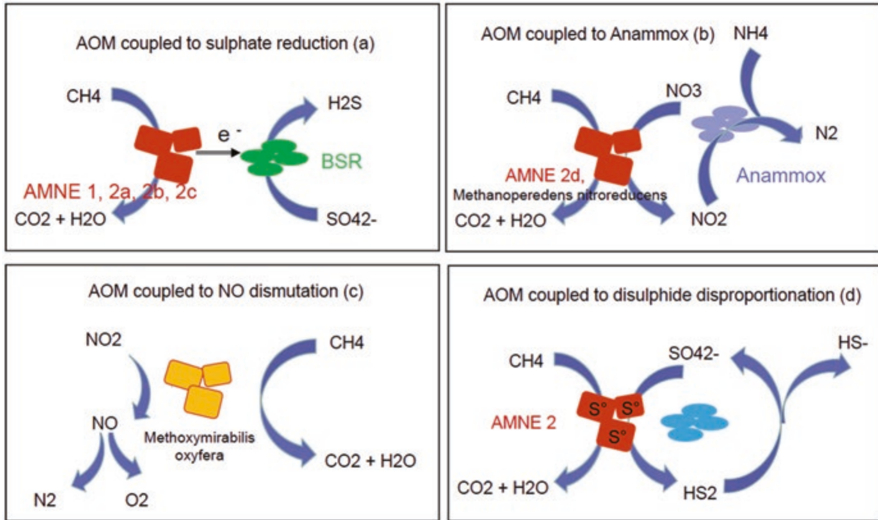


Fig. 4.20 The four known pathways of microbial anaerobic methane oxidation
(**a**) Obligate associations between two or more microbial partners, one performing oxidation of methane into carbon dioxide and water (ANME) and the other performing the reduction (here, a sulfate-reducing bacteria which convert sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S))
(**b**) *Candidatus* "Methanoperedens nitroreducens" can oxidize methane anaerobically through reverse methanogenesis by using nitrate as terminal electron acceptor. Nitrite produced by ANME is reduced to dinitrogen gas through a syntrophic relationship with an anaerobic ammonium-oxidizing bacterium (anammox), with both reactions performed by a single microorganism
(**c**) The bacterium *Methylomirabilis oxyfera* converts nitrite (NO2) to nitric oxide (NO) and then dismutates NO into nitrogen and oxygen as diatomic gases. The bacterium then uses the resulting O_2 to support methane oxidation
(**d**) ANMEs oxidizes methane (as in a) but also reduces sulfate to disulfide which can be used by *Deltaproteobacteria* to yield sulfide (HS^-) and sulfate. (Modified and redrawn from Joye 2012 and Haroon et al. 2013)

(a) The anaerobic oxidation methane (CH_4) into CO2 by methanotrophic archaea (ANMEs) occurs in cooperation with sulfate-reducing bacteria, which convert sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S). The mechanism of energy exchange between the ANMEs and the sulfate-reducing bacteria is unknown (Knittel and Boetius 2009).
(b) The independent AOM by *Candidatus* "Methanoperedens nitroreducens" through reverse methanogenesis by using nitrate as the terminal electron acceptor. Nitrite produced by ANME-2d is reduced to dinitrogen gas through a syntrophic relationship with an anaerobic ammonium-oxidizing bacterium (Haroon et al. 2013).
(c) The O2 produced by the dismutation of NO into diatomic gases by the bacterium *Methylomirabilis* is used by the same microorganism to support methane oxidation. Indeed, "*M. oxyfera*" bypassed the denitrification intermediate

nitrous oxide by the conversion of two nitric oxide molecules to dinitrogen and oxygen which was used to oxidize methane (Ettwig et al. 2010).

(d) Some ANMEs oxidize methane but also reduce sulfate to disulfide (HS_2^-). Zerovalent sulfur compounds (S^0) are formed during AOM through a new pathway for dissimilatory sulfate reduction performed by the methanotrophic archaea. Hence, AOM might not be an obligate syntrophic process but may be carried out by the ANME alone. Furthermore, we show that the produced S^0 – in the form of disulfide – is disproportionated by the *Deltaproteobacteria* associated with the ANME (Milucka et al. 2012).

Anaerobic methane oxidizers exert a strong control over ocean CH_4 emissions, in the oceans more than 50% of the gross annual production of CH_4 could be consumed by anaerobic methanotrophs before CH_4 is even released to ocean waters (Reeburgh 2007).

4.7.2 *The Nitrogen Cycle*

The form(s) of nitrogen, the rate of accretion, and the secondary atmosphere arising from volcanism controlled the prebiotic nitrogen cycle. Planetary accretion models generally assume that nitrogen was delivered to the protoplanet as solid (ice) NH_3, amino acids, and other simple organics. Because ultraviolet oxidation of atmospheric NH_3 (in equilibrium with NH_4^+ in the oceans) would have formed N_2 (Kasting 1982), N_2 gas remained the dominant form of nitrogen in the atmosphere (Canfield et al. 2010). Oxygen rose to its modern levels over the last 550 million years (Berner 2006). With the oxygenation of the ocean, NO_3 became the dominant nitrogen species, with minor concentrations of NH_4^+ and NO_2^- in the water column.

The nitrogen cycle is comprised of a series of redox reactions for transforming nitrogen compounds. Dinitrogen is relatively inert and may be directly used only by some microorganisms in a process called nitrogen fixation that converts inorganic nitrogen into biologically available substrates: ammonium (NH_4^+) and its conjugate acid, ammonia (NH_3). This is the main mechanism for the introduction of nitrogen into the biosphere. Ammonium and ammonia can also be converted to nitrate (NO_3^-) and nitrite (NO_2^-) during the nitrification, an aerobic process which is performed by specialized microorganisms. During denitrification, the nitrate is transformed into gaseous compounds: nitric oxide (NO), nitrous oxide (N_2O), and finally dinitrogen (N_2) which is quickly released back to the atmosphere. It is important to note that different processes can lead to the same product (Herbert 1999). Although many of the pathways in the microbial nitrogen cycle were described more than one century ago, additional fundamental pathways have been only recently discovered due to the rapid advances in molecular ecology and isotopic approaches (Fig. 4.21).

A curious feature of the modern terrestrial nitrogen cycle is that nitrogen fixation and nitrogen loss are largely balanced. This issue deals only with the nitrogen fixation and processes by which dinitrogen fixation is lost from the ecosystems.

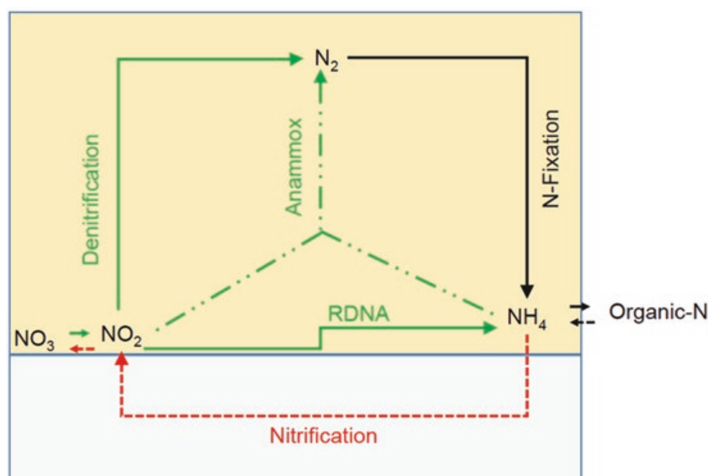
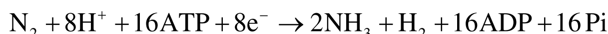


Fig. 4.21 Schematic representation of the nitrogen cycle

4.7.2.1 Dinitrogen Fixation

N_2 fixation is the process by which N_2 gas is reduced into two molecules of ammonia. The reaction consumes cellular energy, and the overall reaction is:



$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16\,Pi$$

This reaction is catalyzed by the nitrogenase enzyme complex, which is extremely oxygen sensitive.

Nitrogen limitation would have provided strong selection pressure for the evolution of biological N_2 fixation to allow anoxygenic phototrophs to fully make use of the reducing substrates available to them in the environment. Indeed, the ability to fixN_2 evolved early in biological evolution, possibly in anaerobic photoautotrophs, all of which are in the domain *Bacteria* (Canfield et al. 2010)

The capacity for N_2 fixation occurs in bacteria and archaea of diverse physiologies (including anaerobes, facultative aerobes, aerobes, and phototrophs), and although nitrogenase is thought to be an ancient enzyme, it is not uniformly distributed and is present in perhaps a few hundred cultivated species. Cyanobacteria (mainly *Trichodesmium* species) have long been considered the main N_2 fixers in the open ocean and in many marine ecosystems. However, the importance of other diazotrophic groups such as gamma- and deltaproteobacteria has been now demonstrated. These microorganisms are diversed in terms of phylogeny but also of metabolism. Indeed, multiple sources of carbon and energy can be used by diazotrophs, which can be autotrophic or heterotrophic, phototrophic (anoxygenic photosynthetic cyanobacteria or bacteria), or chemotrophic.

Many diazotrophs have had to develop various strategies to protect the dinitrogenase complex of the oxygen. Photosynthesis and N_2 fixation can be uncoupled during the daily cycle, with fixation occurring at night. In this case, ATP required for fixation is provided by respiration and not through photosynthesis. This process has been demonstrated in *Gloeothece* sp., *Synechococcus*, *Microcoleus chthonoplastes*, and *Oscillatoria* under natural conditions; another mechanism exists in some filamentous cyanobacteria that have specialized cell structures for N_2 fixation, the heterocysts, which promote a spatial uncoupling of the two types of metabolism. The formation of heterocysts requires a significant change in vegetative cells. Indeed, the cell wall is modified, and narrow junctions appear between heterocysts and vegetative cells.

To prevent the irreversible inactivation of enzymes, other strategies consist to increase the respiratory activity, to inactivate of the enzyme when the partial pressure of oxygen becomes too high. This changed the conformation of the FeS-protein which binds to dinitrogenase in the presence of oxygen (known as conformation protection or "switch-off-switch-on" phenomenon).

The majority of marine N_2 fixation has historically been attributed to the filamentous, non-heterocystous cyanobacteria *Trichodesmium* spp. resident in the warm, stratified, and nutrient-depleted regions of the surface ocean (Carpenter 1983; Capone et al. 1997). Additionally, indirect evidence such as teledetection (Westberry and Siegel 2006) and geochemical modelling (Deutsch et al. 2007) describes geographic distributions of N_2 fixers, including *Trichodesmium* spp., that differ from our expectation of oligotrophic dominance. Finally, a number of both in situ and culture-based studies raise the possibility that sensitivities of marine diazotrophs may be different than previously thought. So, the possibility that N_2 fixation occurs in environments beyond the surface waters of the oligotrophic gyres cannot be excluded.

4.7.2.2 Dinitrogen Production

Low-oxygen environments are of particular interest for nitrogen transformations because they are the sites of fixed N loss via denitrification and anammox that are important ecological processes to maintain nitrogen equilibrium.

4.7.2.2.1 Denitrification

Denitrification is an anaerobic respiration process where nitrate is used as the terminal acceptor of electrons. The energy efficiency of denitrification is lower compared to aerobic respiration. Denitrification yields 24 molecules of ATP for one molecule of reduced. During denitrification, the transformation of nitrate to dinitrogen is carried out by four steps during which the initial nitrate (NO3) is successively reduced to nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), up to the production of molecular nitrogen (N_2). The process involves four enzymes: dissimilatory nitrate

reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductases. For each step, there are several possibilities.

The first step of denitrification, dissimilatory reduction of nitrate to nitrite, can be performed by two types of dissimilatory nitrate reductases which have a different location in the cell: one is periplasmic (Nap) and the other is membranous (Nar). One or both enzymes are present without phylogenetic determination. The role of the periplasmic nitrate reductase (Nap) is poorly understood, and different physiological functions have been proposed. Periplasmic nitrate reductase may help to maintain the redox balance in cells when adapting to some environmental changes, including the transition between aerobic and anaerobic conditions. Because Nap is not sensitive to oxygen, some denitrifiers can realize aerobic denitrification by coupling this enzyme to nitrite reductase or to nitric oxide reductase. This is particularly favorable for microorganisms that thrive in natural environments subject to changing conditions in oxygen availability.

The nitrite transferred into the periplasmic space can get in contact with nitrite reductase, a periplasmic enzyme which catalyzes the second step of denitrification that is the reduction of nitrite to nitric oxide. There are also two types of nitrite reductases which are very different in their structure, but have a similar function: one is made of cytochrome cd1 (Nir-cd1) and the other of copper (Nir-Cu). Until now, these two types of nitrite reductases were never found simultaneously in the same organism.

Nitric oxide reductase, membranous cytochrome bc complex, and nitrous oxide reductase were responsible of the two final steps of denitrification allowing the reduction of nitric oxide (NO) to nitrous oxide (N_2O) and then to dinitrogen (N_2).

In addition to the unconventional activities by classical wellknown nitrifiers and denitrifiers and the discovery of novel N metabolic pathways in new organisms, a short circuit of the nitrification/denitrification has also been proposed recently. In marine sediments, which typically contain relatively high manganese levels, N_2 gas can be produced by the oxidation of ammonia by manganese oxide (Fernandes et al. 2015; Aigle et al. 2017).

4.7.2.2.2 Anammox

A completely novel process, in which nitrite is used as the electron acceptor for the anaerobic ammonium oxidation in nitrogen gas as the final product in the presence of ammonia, has been more recently described. Next to ammonium, organic and inorganic compounds can be used as alternative electron donors, e.g., propionate, acetate, and formate. At least for "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Scalindua" spp., it has been shown that, besides nitrite, iron and manganese oxides can also be use as electron acceptor.

Anammox was recently recognized as one of the major sinks for fixed inorganic nitrogen in coastal sediments and the anoxic waters of basins isolated from oxygenated deep circulation. Globally, 30–50% of the total nitrogen loss occurs in oxygen-minimum zones (OMZs) and is commonly attributed to heterotrophic denitrification.

There has been, until now, no published direct evidence for anammox in OMZs. However, the extremely low concentration of ammonium could indicate that anammox bacteria also play an important role in the nitrogen removal from OMZ waters (Kuypers et al. 2005).

All organisms responsible for this novel metabolism have been identified as relatives of *Planctomyces*. So far, the capability of anammox is limited to a very specific order of the Brocadiales, while denitrification occurs in bacteria, archaea, and even eukaryotes (van Niftrik and Jetten 2012). Anammoxifiers are anaerobic chemolithoautotrophic microorganism with an unusual morphology.

The cytoplasm in anammoxifiers was thus proposed to be divided into three cytoplasmic compartments separated by single bilayer membranes. The outermost compartment, the paryphoplasm, occurs as an outer rim, defined on its outer side by the cytoplasmic membrane and cell wall and on the inner side by the intracytoplasmic membrane. The middle compartment, the riboplasm, contains ribosomes and the nucleoid. Finally, the innermost ribosome-free compartment, the anammoxosome, occupies most of the cell volume and is bound by the anammoxosome membrane. In addition to the cell plan, anammox bacteria contain also atypical membrane lipids named ladderanes.

Anammox bacteria do not conform to the typical characteristics of bacteria but instead share features with all three domains of life, *Bacteria*, Archaea, and Eukarya, making them extremely interesting from an evolutionary perspective.

Anoxic ammonia oxidation, whether it results directly in N2 formation (as in anammox) or in nitrate production (when linked to manganese reduction), would introduce new links into the aquatic and sediment N cycle.

4.8 Conclusion

The study of the evolution of prokaryotes confirms that all discoveries done after Darwin validate his natural selection theory. The post-Darwinian theories – neo-Darwinism, neutralist evolution, punctuated equilibria, and selfish gene – are not fundamentally opposed to Darwin's proposals; they enrich it and lead to incremental revisions. For instance, the discovery of mutations and horizontal gene transfers (HGTs), and in a more general fashion of mobile elements, contradict both a progressive vision of evolution and the notion of heredity as conceived by Darwin: a purely vertical heredity (our classifications will come to be, as far as they can be so made, genealogies). As Albert Jan Kluyver famously wrote, "From elephant to butyric acid bacterium – it is all the same" to underline the profound unity of life's biochemical mechanisms, and since it is simpler to work with bacteria than mammals, evolution will continue to be most profitably studied in prokaryotes.

In this chapter were presented all the characteristics that explain the evolutionary success of prokaryotes, their role in the evolution of the geosphere, the biosphere and its functioning, and their ability to colonize all biotopes, including the most extreme ones:

- A mode of reproduction with a very high efficiency: a high rate of reproduction and an optimal utilization of their genetic information.
- A short generation time within large populations results in a high mutation rate, and haploidy makes each mutation the origin of a new lineage, thus permitting a rapid expression of mutations. The importance of mutations in prokaryotes is amplified by the presence in populations of constitutive and inducible mutators.
- An extraordinary capacity to exchange genetic information and integrate in their genome genetic information originating from other organisms through horizontal transmission. These horizontal gene transfers (HGTs) – through conjugation, transduction, and transformation – often very important may occur between evolutionarily very distant species, even belonging to different domains of life (*Bacteria*, Archaea, Eukarya). HGTs will result in the acquisition of new adaptive capacities: resistance to antibiotics, capacity to deal with artificial substances such as xenobiotics that never existed in nature before the advent of man, etc.
- The small size of their cells and their large populations have allowed prokaryotes to conquer more biotopes than other domains. Presently, they represent the most important biomass in the living world.
- Through evolution, prokaryotes have developed an array of mechanisms to ensure their survival. The survival capacity under unfavorable conditions is not limited to the emergence of mutators (constitutive or inducible). Prokaryotes possess adaptive mechanisms that allow them to face the constantly and rapidly changing conditions of their habitat (nutrients levels, thermal or osmotic shock, increase or drop of pH and oxygen concentration, prolonged dehydration, UV irradiation, etc.).
- Certain prokaryotes live under oligotrophic conditions which is their normal lifestyle (ultramicrobacteria), especially in the open ocean.
- Moreover, under adverse conditions, some prokaryotes are able to survive, sometimes for very long periods of time: (I) by utilizing precious intracellular reserves, (II) by the emergence of forms of resistance (spores, cysts, fruiting bodies), or (III) by undergoing conversion to a dormancy or viable but noncultivable (VBNC) state. If dormancy is a reversible state ("resuscitation"), the notion of viable but noncultivable cellular forms is still a matter of debate; only some strains of VBNC are known to recover a normal physiology when environmental conditions become favorable again.
- Prokaryotes have also evolved to be able to live continually under the most extreme life conditions using various strategies to adapt to in situ harsh physicochemical conditions with the aim to maintain cell integrity and functioning. They include (i) the accumulation of small molecules (organic solutes such as amino acids, cations, etc.) in the cytoplasm, (ii) a higher content of unsaturated fatty acids within the membrane lipid composition, (iii) the use of membrane carriers for regulating exchanges with the outside environment, (iv) the modification of the 3D protein structure and the protein amino acid content as well, (v) the setting up of up- and downregulation of genes to face such drastic conditions, and possibly (vi) other unknown mechanisms to be discovered. Such strategies might have been of first importance to facilitate the emergence of life on the planet where extreme physicochemical conditions prevailed some 3.7–3.8 billion years ago.

Over geological times, prokaryotes have acquired all the functions that are necessary to biogeochemical cycles functioning, enabling them to ensure all transformations of chemical elements, and, in the past and present, almost all reactions on Earth's surface are catalyzed by prokaryotes. Furthermore, their biogeochemical functions have created environmental conditions in terrestrial habitats in which eukaryotic life forms could appear, grow, and diversify, thus showing the essential and indispensable role of prokaryotes in the evolution of the living world. If all living eukaryotes should disappear following a natural or man-made catastrophe, from protists to *Homo sapiens*, biogeochemical cycles should continue to function with prokaryotes. Life would continue, and a new evolutionary cycle involving microbes would then start. On the other hand, in a bacteria- and archaea-free world, most biogeochemical cycling would cease, and "Life would not longer remain possible in the absence of microbes" (Louis Pasteur).

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