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The Discovery of Archaea

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1.1. Introduction

The discovery of archaea by Carl Woese and his collaborators was one of the greatest breakthroughs in 20th-century biology. This finding went largely unnoticed by many biologists, particularly those focused on eukaryotes, and remains unknown to nearly the entire general public. By unveiling a previously unknown segment of the living world, the discovery of archaea has, for the first time, allowed us to consider the history of life on Earth in its entirety. This discovery has spectacularly revived debates on the nature of the last common ancestor of current living beings and the origin of eukaryotes (our origin). Challenging the prokaryote–eukaryote dichotomy was one of Carl Woese's major endeavors. This struggle appears far from over, especially as the emphasis is once again on phenotypic similarities between archaea and bacteria at the genomic level. The scenario favored by most biologists continues to reflect the classical view that eukaryotes emerged from the association of different prokaryotes.

These reflections recently led me to write a review titled "Carl Woese: Still ahead of our time" on the 10th anniversary of his death (Forterre 2022a). In this context, it seems particularly important to revisit the history of the discovery of

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Biology of Archaea 1,
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archaea to understand the intricacies of the major debates which continue to captivate 21st-century evolutionists.

1.2. Prokaryotic–eukaryotic dichotomy

In the late 1960s, molecular biologists imposed the division of the living world into two major groups: eukaryotes and prokaryotes (Sapp 2005). This classification was long considered a reflection of the history of life. The simpler prokaryotes were thought to have appeared first, followed much later by eukaryotes. The term "prokaryote" ("before the nucleus") reflects this idea. Quickly, the terms "prokaryotes" and "bacteria" became synonymous for most biologists. The term "prokaryote", which originally referred to a type of cellular structure (absence of a true nucleus), eventually took on an evolutionary connotation: all bacteria were believed to be close relatives, descending from the same ancestral "prokaryotic" bacterium. Prokaryotes were considered "primitive" compared to eukaryotes (Stanier and Van Niel 1962; Whittaker 1969). Most biologists accepted that eukaryotes descended from prokaryotes similar to current bacteria, possibly through a symbiotic merger of several different bacteria (Margulis 1975) (Figure 1.1(a)).

For many biologists, the molecular mechanisms of prokaryotes were assumed to be similar across the group, and studying one bacterium at the molecular level was thought sufficient to understand them all! Consequently, discoveries made on the model bacterium *Escherichia coli* were believed to be applicable to all bacteria. This view, still tacitly accepted by many biologists and taught in many universities, proved to be false. The work initiated in the late 1970s by the American molecular biologist Carl Woese revealed this (for reviews and books summarizing the history of this work, see Woese (1981), Forterre (2007, 2016, 2022b), Sapp and Fox (2013) and Quammen (2018)).

In the early 1970s, Carl Woese set himself the goal of constructing an evolutionary tree of life that would include microorganisms, bacteria and protists. Until then, microorganisms had never been integrated into a comprehensive universal phylogeny, as these were primarily based on the fossil record and the comparison of phenotypic characteristics. However, this approach was only feasible for "macro-organisms", mainly animals and plants. Consequently, the evolutionary models of microorganisms could only be based on theoretical considerations, primarily focused on the presumed evolution of metabolic types (from anaerobiosis to aerobiosis) and certain morphological characteristics considered more or less "primitive". For instance, mycoplasmas were sometimes regarded as the most primitive living beings because they lacked a cell wall (Figure 1.1(a)).

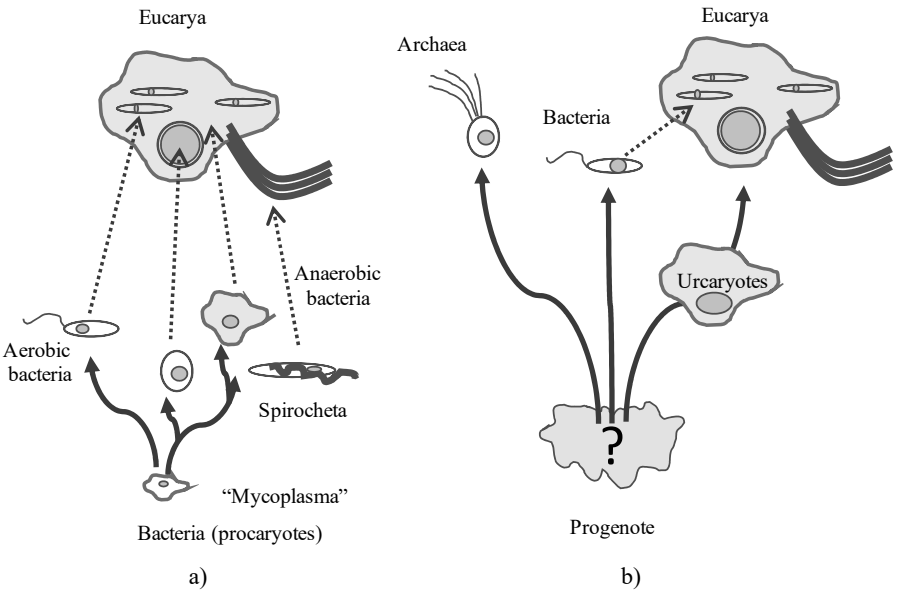


Figure 1.1. *Two visions of evolution in the late 1970s*

COMMENT ON FIGURE 1.1.– *(a) According to Lynn Margulis, eukaryotes result from the fusion of various types of bacteria, assimilated to prokaryotes (Margulis 1975). (b) According to Carl Woese, three kingdoms of prokaryotes emerge from an ancestor more "primitive" than current prokaryotes, the progenote. Eukaryotes originate from an extinct prokaryote lineage, the urcaryotes (Woese and Fox 1977).*

1.3. Two domains for prokaryotes: archaebacteria and eubacteria

1.3.1. *Ribosomal RNA as a molecular marker: a historical choice*

To establish a classification of microorganisms on an objective basis that reflects their actual, rather than presumed, kinship relationships, Carl Woese and his collaborators at the University of Urbana in Illinois began in the early 1970s to determine the kinship relationships between different bacteria by comparing the RNAs present in the small subunit of their ribosomes, specifically the 16S ribosomal RNA (16S rRNA) (Figure 1.2(a)). Woese and his team chose ribosomal RNAs because these informational macromolecules are present in all organisms (with the exception of viruses, which will be discussed in Chapter 2). Additionally, the various functions they perform have been conserved throughout evolution,

suggesting a “neutral” mode of evolution unaffected by changes in function. In other words, these macromolecules would have consistently evolved at the same rate across different cell lineages. This concept allows for the use of a molecular clock, meaning that the differences observed at the sequence level would directly reflect the evolutionary distances between organisms.

Why 16S rRNA rather than 5S or 23S rRNA? When Carl Woese began his research program in the early 1970s, studying all three ribosomal RNA molecules simultaneously was not feasible. He chose 16S rRNA because it contains more sequence information than the smaller 5S rRNA, while being easier to analyze than the (larger) 23S rRNA. Since the development of PCR and massive genome sequencing, the advantage of 16S rRNA over 23S rRNA has disappeared. Today, it is possible to quickly obtain the sequences of both molecules. However, this was not the case in the early 1970s. At that time, DNA sequencing was not yet developed, and RNA sequencing was a very lengthy and complex technique. Carl Woese and his collaborators overcame this difficulty by using a technique developed by the English biochemist Frederick Sanger (Sanger et al. 1965). The idea was to determine the evolutionary distance between two organisms by comparing the catalogs of oligonucleotides derived from the digestion of their RNA by a specific ribonuclease, ribonuclease T1. The genetic fingerprint of an organism corresponded to all the oligonucleotides identified in it. These oligonucleotides, previously made radioactive, were separated from each other by chromatography according to their size and composition, and then visualized by autoradiography (Figure 1.3(a)). It was then necessary to compare the catalog of the studied species with those of all the other species already analyzed.

This procedure required extensive development work, which explains Carl Woese’s choice of an RNA molecule of intermediate size, neither too large nor too small (Figure 1.3(c)). First, the organisms had to be grown in large quantities to isolate their 16S (or 18S in eukaryotes) rRNAs. These molecules were then digested with ribonuclease T1, which systematically cuts the RNA sequence to the right of a G. For example, the portion of an RNA molecule corresponding to the sequence AUUUCGUUGAAU would have yielded the three oligonucleotides: AUUUCG, UUG and AAU. The different oligonucleotides present in the mixture obtained (a few dozen) were then separated by two-dimensional chromatography on very large sheets of paper. The cells from which the 16S RNA molecules were derived had been cultured in a medium containing radioactive phosphorus (P-32). The radioactive oligonucleotides then appeared as black spots on the photographic films placed for some time above the sheet of paper (Figure 1.3(a)). The arrangement of these spots was characteristic of the species studied. The oligonucleotides corresponding to each spot could then be eluted and their sequence identified by a

combination of chemical and enzymatic methods. Fortunately, this laborious last step quickly became unnecessary, as the positions of the spots were reproducible from one analysis to the next, allowing for the visual identification of the corresponding oligonucleotide.

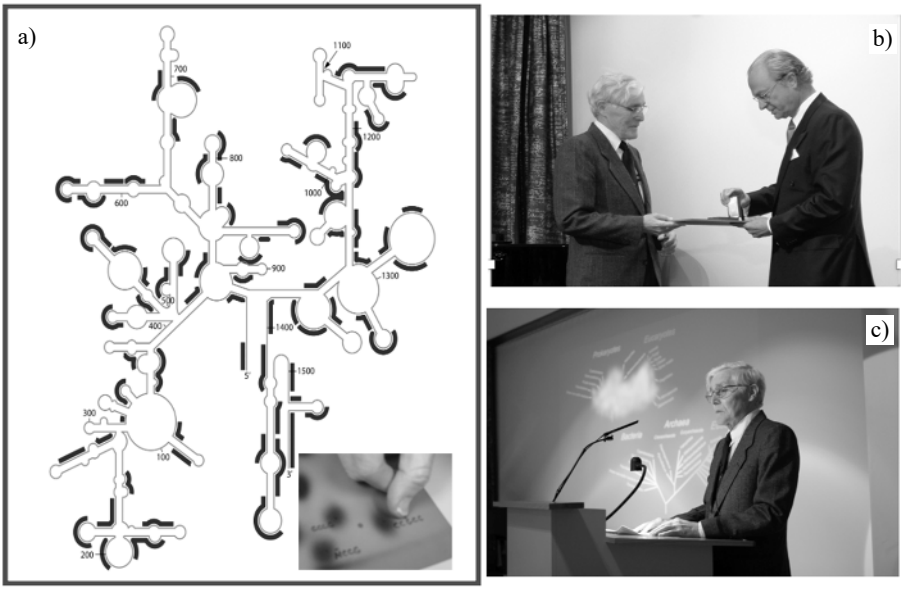


Figure 1.2. *Carl Woese and the Triumph of Reductionism*

COMMENT ON FIGURE 1.2.– *(a) The secondary structure of 16S rRNA, highlighting variable regions useful as an evolutionary marker in blue. The inset depicts Carl Woese's finger pointing to a specific spot of the archaea, corresponding to an oligonucleotide containing a modified base. (b) Carl Woese receives the Crafoord Prize in 2003 from the hands of the King of Sweden, Gustav XVI. (c) Lecture by Carl Woese at the symposium organized for the 30th anniversary of the 1977 publication; the two-domain, prokaryotic/eukaryotic tree was replaced by the three-domain tree, with the archaea sister group of eukaryotes.*

Many small oligonucleotides (AG, CCG, UAAG, etc.) were found by chance in the catalog of all species and were therefore not useful for analysis. To mitigate their influence, Carl Woese chose to consider only oligonucleotides that were at least six letters long (AUUUCG in the previous example). Under these conditions, if the same oligonucleotide appeared in two different species, it was likely inherited from

a common ancestor, rather than occurring by chance. Consequently, the closer two species were, the greater the number of large oligonucleotides they shared.

To quantify the similarity between two organisms A and B, Carl Woese and his colleagues devised a similarity coefficient, S_{AB}, calculated as the number of large oligonucleotides common to both species (N_{AB}) multiplied by two and divided by the total sum of the large oligonucleotides present in the catalog of each species, N_A and N_B, that is, $S_{AB} = 2N_{AB}/(N_A + N_B)$ (Figure 1.3(c)). When two species were identical, $N_A = N_B = N_{AB}$, S_{AB} equaled 1, while the coefficient approached 0 as the species grew more distant. Below 0.1, the value of S_{AB} was no longer significant, as it was statistically expected to always obtain, even by chance, a few common oligonucleotides of at least six nucleotides.

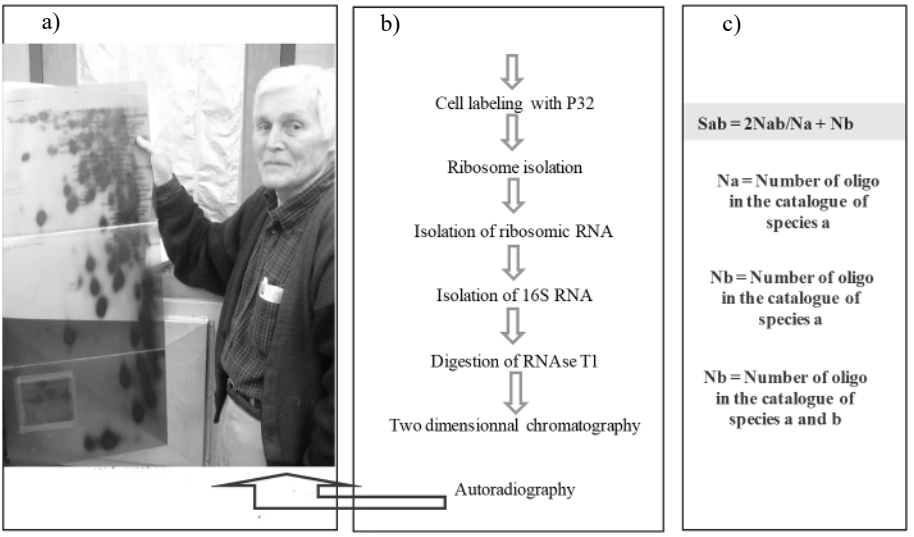


Figure 1.3. *Analysis of 16S rRNA by the Oligonucleotide Method. (a) Carl Woese showing the film of a two-dimensional chromatogram and diagram. The black spots correspond to the positions that the radioactive oligonucleotides occupied on the chromatography sheet. (b) Protocol of the experiment. (c) Method of calculating the SAB between two species, a and b*

Initially, by comparing the catalogs obtained with bacterial 16S rRNAs and those obtained with 18S rRNAs from three eukaryotes, this new approach allowed Carl Woese and his team to rediscover the separation between “prokaryotes” and eukaryotes. Pairs of bacteria or eukaryotes always had S_{AB} scores above 0.1 (e.g. 0.25 between *Escherichia coli*, a Gram-negative bacterium, and *Bacillus firmus*, a

Gram-positive bacterium, or 0.33 between humans and the yeast *Saccharomyces cerevisiae*). However, when comparing a bacterium and a eukaryote, S_{AB} scores were equal to or less than 0.1, which was below the threshold where the values obtained could be considered significant (Table 1.1).

The work of Carl Woese's team challenged several misconceptions. For instance, microbiologists had become accustomed to classifying bacteria according to Gram staining. This classification proved to be irrelevant from an evolutionary standpoint; indeed, the Gram-negative cyanobacterium *Aphanocaspa* was as distant from *Escherichia coli* as it was from the Gram-positive *Bacillus firmus*, with an S_{AB} of 0.26 in both cases. Gram-negative bacteria were even grouped with Gram-positive bacteria. This was the case with mycoplasmas, the smallest known bacteria at the time, characterized by the absence of a cell wall. Some considered mycoplasmas to be possible descendants of the first bacteria that appeared on the planet (Figure 1.1(a)). The analysis of their 16S rRNAs showed that mycoplasmas actually originated from Gram-positive bacteria by losing their cell wall (Woese et al. 1980). This evolution by simplification was not surprising, as mycoplasmas are intracellular parasites living inside eukaryotic cells.

Furthermore, using 16S rRNA, Carl Woese and his team were able to demonstrate that chloroplasts in green plants originated from ancient bacteria, as predicted by Lynn Margulis a few years earlier (Zablen et al. 1975). The comparison between the 16S rRNA of chloroplasts and that of cyanobacteria, their ancestors, yielded an S_{AB} value of 0.31, while the comparison between the 16S rRNA of chloroplasts and that of plants resulted in values below 0.1. The same result was independently obtained by Ford Doolittle's team in Canada (Bonen and Doolittle 1975), indicating that the approach initiated by Carl Woese had already gained widespread adoption.

1.3.2. *Atypical ribosomal RNA of methanogenic "bacteria"*

The big surprise came when Carl Woese became interested in methanogenic "bacteria", that is, methane producers. Methanogens are strict anaerobic organisms that were very difficult to culture. In 1976, for the first time, four strains of methanogens had just been successfully cultured in the laboratory of Ralph Wolf, who worked at the same institute as Carl Woese. When George Fox, a postdoctoral researcher in Carl Woese's team, analyzed the 16S rRNA of the first Gram-positive methanogenic "bacterium", *Methanobacterium thermoautotrophicum* ΔH, in May 1976, he noticed that the distribution of spots corresponding to radioactive oligonucleotides on the photographic film was very different from those of all the bacteria they had studied so far, including Gram-positive bacteria. Many spots typically observed in bacteria were absent, while new, previously unseen spots were

present (inset in Figure 1.2(a)). For a time, George Fox even wondered if he had sequenced the correct rRNA or if he had properly separated the 16S and 23S rRNAs before digesting the 16S rRNA with ribonuclease T1. However, when he repeated the experiment on the other three species of methanogens (one of which was Gram-negative) during 1976, he obtained the same result. The spots absent in the first methanogen studied were also absent in the other three; the new spots that appeared were common to all four methanogens. Today, we know that some of the specific spots observed by George Fox corresponded to oligonucleotides with modified bases specific to archaea (inset in Figure 1.2(a)).

This initial visual impression revealing the peculiarities of methanogenic "bacteria" was confirmed by the calculation of S_{AB} values. In pairs formed by one of the four methanogenic "bacteria" and any other bacteria or eukaryotes, the S_{AB} values ranged from 0.05 to 0.13, which was not significant (Table 1.1). According to these analyses, methanogens, presumed to be bacteria, did not show a more detectable kinship with other bacteria than with eukaryotes. In contrast, the S_{AB} values for methanogen pairs were significant, ranging from 0.24 to 0.51 (Table 1.1). Therefore, the four methanogens formed a group of prokaryotes as evolutionarily distinct as bacteria, on the one hand, and eukaryotes, on the other hand.

1.3.3. *The concept of archaeobacteria*

To explain this unexpected observation, Carl Woese proposed abandoning the old binary division of the living world between eukaryotes and prokaryotes, replacing it with a partition into three major "primordial kingdoms" (*urkingdoms*): eukaryotes, classical bacteria and a third group corresponding to "methanogenic bacteria" (Woese and Fox 1977) (Figure 1.1(b)). A name had to be found for this third group. Carl Woese chose "archaeobacteria[1]" – literally, "ancient bacteria" – based on the anaerobic nature of methanogenic bacteria and their requirement for an atmosphere rich in carbon dioxide and hydrogen. It is known that the atmosphere of early Earth, about 4 billion years ago, did not contain oxygen. Most geologists believe that this primitive atmosphere was rich in carbon dioxide. It was therefore tempting to imagine that methanogens could have already populated such an environment. Additionally, the low S_{AB} value observed between certain pairs of methanogens (0.24) was of the same order as those observed between the most distantly related bacteria (Table 1.1). According to this result, methanogens had already diversified very early in the history of life on Earth, indicating that their appearance dates back to the very early stages of evolution. While proposing the

1 This name was suggested to Carl Woese and George Fox by their collaborators Linda Magrum and David Nanney (Woese and Fox 1977).

new name "archaeobacteria" for the primordial kingdom represented by methanogens, Carl Woese also suggested the name "eubacteria" for "classical" bacteria and "urcaryote" for the lineage of prokaryotes that led to eukaryotes with the emergence of the nucleus (Figure 1.1(b)). The discovery by Carl Woese and George Fox thus challenged the prokaryote–eukaryote dichotomy and proposed a new vision of the universal tree of life (Figure 1.2(c)).

1.3.4. *The grouping of halophilic and thermo-acidophilic "bacteria" with methanogens within archaeobacteria*

Were all archaeobacteria methanogens? This seemed unlikely if archaeobacteria truly represented a primordial kingdom on par with bacteria and eukaryotes. Carl Woese quickly devised a strategy to identify other types of archaeobacteria based on the available knowledge about methanogenic "bacteria". One notable characteristic of methanogens caught his attention: their natural resistance to most antibiotics known to inhibit bacterial growth. Carl Woese identified a small number of other "bacteria" in the literature, which were also known to be insensitive to most antibiotics and prioritized analyzing their 16S rRNAs. Initially, in 1978, Woese and his collaborators demonstrated that the 16S rRNA of an aerobic "bacterium" called *Halobacterium halobium* was very similar to that of methanogens (Magrum et al. 1978). This halophilic (salt-loving) microorganism had long been known for its ability to grow in environments with very high salt concentrations, up to 4 M NaCl, a value close to saturation (Figure 1.4(c)). To compensate for the osmotic pressure of such an environment, *H. halobium* accumulates equal concentrations of KCl in its cytoplasm. All its proteins are thus adapted to function in these extreme halophilic conditions, which had greatly intrigued biochemists (Lanyi 1974).

Furthermore, *H. halobium* could perform a unique type of non-chlorophyll photosynthesis based on a single protein called "bacteriorhodopsin". This protein formed a purple-colored membrane on the cell surface (see Figure 1.4(b)).

Other "halobacteria" without bacteriorhodopsin exhibited red pigmentation due to bacterioruberins, proteins that protect them from light exposure at the surface of salt lakes. At first glance, *H. halobium* did not appear to share any similarity with the four methanogenic archaeobacteria that led Carl Woese to propose the concept of archaeobacteria.

However, the analysis of their 16S rRNAs revealed that *H. halobium* was particularly close to one of these four methanogens, *Methanosarcina barkeri*, with an S_{AB} of 0.34. We now know that halophilic archaea have indeed evolved from a subgroup of methanogenic archaea, which includes *M. barkeri* (see Chapter 5).

In 1979, still searching the literature for microorganisms known to be naturally resistant to antibiotics, Carl Woese's team identified a third type of archaeobacteria: thermo-acidophiles. These were two atypical microorganisms discovered a few years earlier by the American microbiologist Thomas Brock, namely, *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*. *T. acidophilum* had been isolated from coal mine heaps where heat-generating combustion reactions occurred (Darland et al. 1970). It thrived in the laboratory in a very acidic medium (pH 1) and at 60°C. On the other hand, *S. acidocaldarius* was found in Yellowstone Park in the United States (Brock et al. 1972). It was slightly less acidophilic than *T. acidophilum* (with an optimal pH around 3), but it set records for thermophily at the time, with a maximum growth temperature of 85°C! *T. acidophilum* and *S. acidocaldarius* were aerobic, like *H. halobium*, and acidified their environment by producing sulfuric acid through the oxidation of elemental sulfur. The S_{AB} values between *T. acidophilum* and the four methanogens ranged from 0.19 to 0.23, and those between *S. acidocaldarius* and the four methanogens ranged from 0.13 to 0.19 (Table 1.1). These sometimes very low values, especially in the case of *S. acidocaldarius*, reinforced the idea that archaeobacteria were indeed very ancient organisms.

Compared species	Range of S_{AB} values
Eukaryotes among themselves (3)	0.29–0.36
Eukaryotes (3) and bacteria (6)	0.05–0.11
Bacteria among themselves (6)	0.20–0.34
Methanogens (4) and bacteria (6)	0.05–0.13
Methanogens (4) and eukaryotes (3)	0.06–0.11
Methanogens among themselves (4)	0.24–0.51
Methanogens (4) *and Halobacterium*	0.28–0.34
Halobacterium and *Thermoplasma*	0.23
Methanogens (4) and *Thermoplasma*	0.19–0.23
Methanogens (4) and *Sulfolobus*	0.13–0.19
Sulfolobus (S), *Thermoplasm*a (T) and *Halobacterium* (H)	0.13–0.19
STH and eukaryotes (3)	0.05–0.13
Methanogens (4) or STH and eukaryotes (3)	0.05–0.13

Table 1.1. *Ranges of S_{AB} values obtained by comparing different species with each other. The numbers in brackets indicate the number of species tested for a given group. These values were taken from the review published by Woese in 1981*

The grouping of methanogens with *H. halobium*, *S. acidocaldarius*, and *T. acidophilum* in the new kingdom of archaeobacteria validated the prediction of Carl Woese and George Fox that the third group of living beings on Earth were also characterized by a great variety of organisms and lifestyles (for the first review summarizing all this work, see Fox et al. (1980)).

1.4. The living trilogy

1.4.1. *The concept of archaeobacteria confirmed by the uniqueness of their membrane phospholipids*

If archaeobacteria truly represented a primordial kingdom, equivalent to bacteria and eukaryotes, their biochemistry and/or molecular biology should exhibit unique characteristics distinguishing them from the other two "primordial kingdoms". This prediction was soon confirmed. In the 1960s, Canadian researchers discovered a new type of membrane phospholipid in *H. salinarium* (Kates et al. 1965). Unlike those in bacteria and eukaryotes, which are composed of a glycerol molecule linked to two long linear fatty acid chains by ester bonds, *H. salinarium* phospholipids consist of a glycerol molecule linked by ether bonds to two complex long fatty alcohol chains called isoprenols. These chains are characterized by the presence of a methyl group (CH_3) replacing a hydrogen atom every five carbon atoms in the chain (see Chapter 2). Additionally, and most surprisingly, the lipids of *H. salinarium* exhibited a reverse stereochemistry compared to those known in lipids in bacteria and eukaryotes[2].

How might these atypical lipids benefit halophilic microbes? Initially, we might think they provide an advantage to halobacteria for living in environments with very high salt concentrations. However, in 1972, American biochemist Thomas Langworthy discovered the same type of lipid in *T. acidophilum* and, two years later, in *S. acidocaldarius*. Consequently, it became evident that these lipids were characteristic of organisms being identified as "extremophiles". The concept of archaeobacteria proposed by Carl Woese suggested another hypothesis: could these distinctive lipids be a signature of archaeobacteria? If so, methanogens, though not extremophiles, should also possess this type of lipid. No one had previously considered studying the lipids of methanogens, assuming they were similar to those of classical bacteria. Alerted by Carl Woese to the significance of this question, Thomas Langworthy discovered these atypical lipids in the four methanogen strains studied at the time, even though three of them were neither halophilic nor thermophilic (Tornabene and Langworthy 1979). Thus, the lipids discovered by

2 The central carbon of glycerol, which forms the backbone of phospholipids, is indeed asymmetric.

Kates and his collaborators in the 1960s in *H. halobium* were indeed present in all archaeobacteria and could be considered a marker of this "primary kingdom".

1.4.2. *German biochemists: champions of the archaeobacteria concept*

It took some time before Carl Woese's discoveries were recognized and began to significantly influence the scientific community. A German microbiologist specializing in bacterial cell wall chemistry, Otto Kandler, played a major role in drawing the attention of some of his compatriots to the importance of archaea. In the mid-1970s, Otto Kandler made a surprising observation: unlike all the bacteria he had studied until then, the cell wall of methanogens did not contain peptidoglycan (Kandler and Hippe 1977). One of the four species of methanogens studied by Carl Woese, *Methanobacterium thermoautotrophicum*, did have a rigid wall composed of a peptidoglycan-type polymer, but it was chemically different from those previously studied in bacteria. When Otto Kandler, while visiting the United States, learned that Carl Woese had shown that methanogenic "bacteria" were not bacteria, he immediately recognized the significance of this discovery and became one of Carl Woese's first disciples. Back in Germany, Otto Kandler encouraged the community of microbiologists in his country to take an interest in archaeobacteria, which explains why, even today, German researchers are at the forefront of research on archaea.

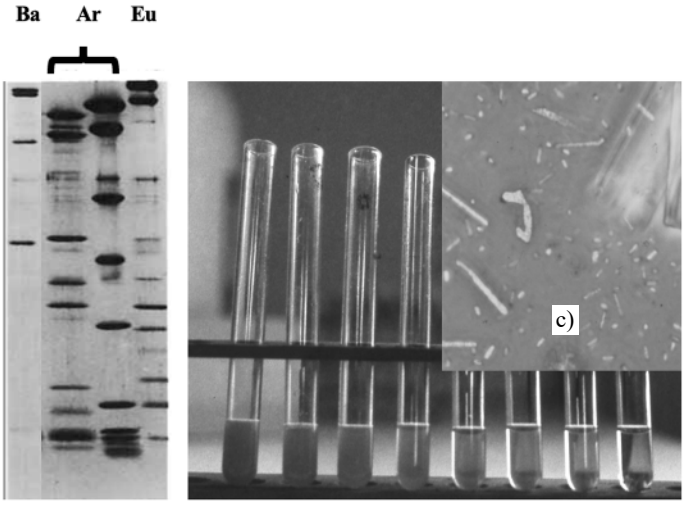


Figure 1.4. *Revealing the evolutionary link between "archaeobacteria" and eukaryotes*

COMMENT ON FIGURE 1.4 – *(a) Electrophoresis gel (SDS-PAGE) of RNA polymerases from a bacterium (*Escherichia coli*),* two archaea (Ar) *Sulfolobus acidocaldarius and Thermoplasma acidophilum, and a eukaryote (Eu) Saccharomyces cerevisiae. Each band corresponds to a subunit (extracted from Figure 5 of Prangishvili et al. 1982). (b) Inhibition of Halobacterium halobium growth by increasing doses of aphidicolin (from left to right) (Forterre et al. 1984). The typical red color of these archaea disappears. (c) Pleomorphic cells of H. halobium (phase-contrast microscopy). Salt crystals can be seen in the upper left, indicating the high concentration of NaCl (4 M) in the culture medium.*

Otto Kandler succeeded in convincing Wolfram Zillig, a biochemist already internationally renowned for his work on RNA polymerases, to abandon bacteria and eukaryotes and devote himself exclusively to "archaeobacteria" (Albers et al. 2013). He also encouraged Karl Stetter, a young microbiologist who had completed his thesis under Kandler's supervision, to join Wolfram Zillig for his postdoctoral fellowship. In the late 1970s, these two researchers isolated and characterized the RNA polymerases of the two aerobic archaeobacteria available at the time, *H. halobium* and *S. acidocaldarius*. The results were spectacular; not only were these RNA polymerases very different from those of bacteria (e.g. they were resistant to rifampicin, an antibiotic used in the treatment of tuberculosis), but they were also much more complex (Zillig et al. 1979). In fact, the RNA polymerases of archaeobacteria resembled those of eukaryotes much more than those of bacteria. Bacterial RNA polymerases are formed by the association of four subunits, whereas those of archaea and eukaryotes are composed of about a dozen subunits (Figure 1.4(a)). Until then, it was thought that our RNA polymerases were more complex than those of bacteria because we (eukaryotes) were considered "higher" organisms compared to prokaryotes. However, here were archaea – prokaryotes – with RNA polymerases as complex as ours. This discovery heralded a paradigm shift.

1.4.3. *The evolutionary link between archaeobacteria and eukaryotes and the introduction of the term archaea*

Not only did the RNA polymerases of eukaryotes and archaeobacteria resemble each other in the number of subunits, but more importantly, they were evolutionarily closer. Indeed, by collaborating with André Sentenac's laboratory in Saclay near Paris, Wolfram Zillig demonstrated in 1983 that antibodies directed against the RNA polymerase of the yeast *S. cerevisiae* recognized the RNA polymerases of archaea but not those of bacteria (Huet et al. 1983). This was the first of a series of discoveries highlighting a close evolutionary relationship between the molecular mechanisms of archaea and those of eukaryotes. It was quickly realized that while

archaeobacteria were resistant to most antibiotics effective against bacteria, they were sensitive to certain inhibitors previously considered specific to eukaryotes, such as diphtheria toxin, which inhibits translation, aphidicolin, an inhibitor of DNA polymerases (Figure 1.4(b)) and antitumor drugs acting on DNA topoisomerases (Kessel and Klink 1980; Forterre et al. 1984; Sioud et al. 1987).

The evolutionary proximity between "archaeobacteria" and eukaryotes led Carl Woese to propose in 1990 to replace the term "archaeobacteria" with "archaea" (see Chapter 2)[3]. With this name change, Carl Woese hoped that "archaeobacteria" would no longer be considered by most biologists as merely old bacteria or somewhat bizarre bacteria (extremophiles), but rather as representatives of a distinct "domain", with the term domain replacing that of "primordial kingdom".

By the late 1990s, the evolutionary link between archaea and eukaryotes was confirmed through the sequencing of the first archaeal genomes, specifically those of *Methanocaldococcus jannashii* and *Archaeoglobus fulgidus*, both isolated in the 1980s by Karl Stetter's team (see section 1.5.1). It became clear that for all major "informational" mechanisms (translation, transcription and DNA replication), archaea were much closer to eukaryotes than to bacteria (Forterre 1997; Woese and Olsen 1997). This link, which will be illustrated by numerous examples throughout this book, remains at the center of a scientific controversy regarding the topology of the universal tree of life: are archaea our sisters or our ancestors (see Chapter 3)?

This evolutionary connection between archaea and eukaryotes led to unexpected discoveries in laboratories studying archaea. For instance, the discovery and characterization of type II DNA topoisomerase in archaea led to the identification of the protein SPO11, which triggers genetic recombination during meiosis in eukaryotes by cutting parental chromosomes (Bergerat et al. 1997). Additionally, it led to the discovery of a topoisomerase responsible for the size of plant cells, and thereby the size of the plants themselves, by enabling the formation of polyploid giant cells (Sugimoto-Shirasu et al. 2002).

1.5. Archaea and high-temperature living

1.5.1. *The discovery of anaerobic hyperthermophilic archaea*

In the 1980s, while continuing their work on RNA polymerases and transcription in archaea, Wolfram Zillig and Karl Stetter embarked on a search for new families

3 In the remainder of this chapter and in the other two chapters authored by me, I will henceforth capitalize the names of the three domains.

of archaea, focusing on thermophiles. The first thermophilic archaea identified, *Thermoplasma* and *Sulfolobus*, were aerobic. Wolfram Zillig and Karl Stetter decided to search for thermophilic archaea that were anaerobic. Their reasoning was straightforward: if archaea were truly ancient, thermophilic archaea must have existed early on primitive Earth, which they envisioned as hot and anaerobic, since the atmosphere of primitive Earth lacked oxygen. They hoped that the descendants of these hypothetical archaea still inhabited the hot springs scattered across our planet. Wolfram Zillig and Karl Stetter chose to start their search in the numerous hot springs of Iceland (Figures 1.5(a) and (b)). From their first expedition in 1981, cultures conducted in the absence of oxygen allowed them to isolate four new anaerobic thermophilic microbes, which, unlike the previously known thermophilic archaea, grew at neutral pH (for reviews, see Stetter (2006, 2013) and Forterre (2007, 2016)). 16S rRNA analysis and RNA polymerase characterization of these anaerobic, neutrophilic thermophiles confirmed that they were indeed archaea.

The first isolated archaeon was a new methanogen, *Methanothermus fervidus*, which had an optimum growth temperature of 83°C and could grow up to 97°C, close to the boiling point of water (hence its Latin name, *fervidus*, meaning boiling) (Figure 1.5(c)). Next, they isolated two new archaea that were not methanogenic but were anaerobic. The first, rod-shaped and capable of forming branched structures, was named *Thermoproteus* in reference to the Greek god Proteus, who could take multiple forms (Figure 1.5(c)). Its optimum growth temperature was 88°C, higher than that of *Methanothermus*, and it could also grow up to 97°C. The second archaeon had spherical cells that could have initially been mistaken for *Sulfolobus*. However, instead of converting sulfur into sulfuric acid like *Sulfolobus*, it transformed it into hydrogen sulfide (H_2S). This archaeon was named *Desulfurococcus*, meaning “the coccus that reduces sulfur”.

Finally, they isolated anaerobic thermophilic archaea that did not resemble either spheres or rods but had rather long and very thin filaments, which they named *Thermophilum*.

In the 1990s, due to the optimal growth temperatures of over 80°C exhibited by these new organisms, Karl Stetter proposed the cell and named them “hyperthermophile”. Analysis of their 16S rRNAs and RNA polymerases revealed that *Thermoproteus*, *Desulfurococcus* and *Thermophilum* were phylogenetically closer to *Sulfolobus* than to other archaea known at the time. This marked the first indication of a fundamental division within archaea, with one lineage comprising *Sulfolobus*, *Thermoproteus* and *Desulfurococcus*, and another grouping *Thermoplasma* with methanogenic and halophilic archaea (Prangishvili et al. 1982).

One year later, during an expedition to the volcanic island of Vulcano in Italy, Karl Stetter and Wolfram Zillig discovered anaerobic hyperthermophilic archaea that were evolutionarily closer to certain methanogens (*Methanococcales* and *Methanobacteriales*) than to *Sulfolobus* and other anaerobic thermophiles (Zilig et al. 1983). These organisms included *Thermococcus celer* (literally, the swiftly moving thermal shell, from *celer*, meaning "speed" in Greek) and *Pyrococcus furiosus* (the furious burning shell). These two closely related organisms were grouped within the order Thermococcales. Thermococcales are currently associated with methanogenic archaea, *Thermoplasma*, and halophilic archaea within the phylum *Euryarchaeota* (from the Greek *Euryos*, meaning diversity), while *Sulfolobus* and its related hyperthermophiles are grouped within the phylum *Crenarchaeota* (from the Greek *Crenos*, meaning source/origin) (Woese et al. 1990) (see Chapter 2).

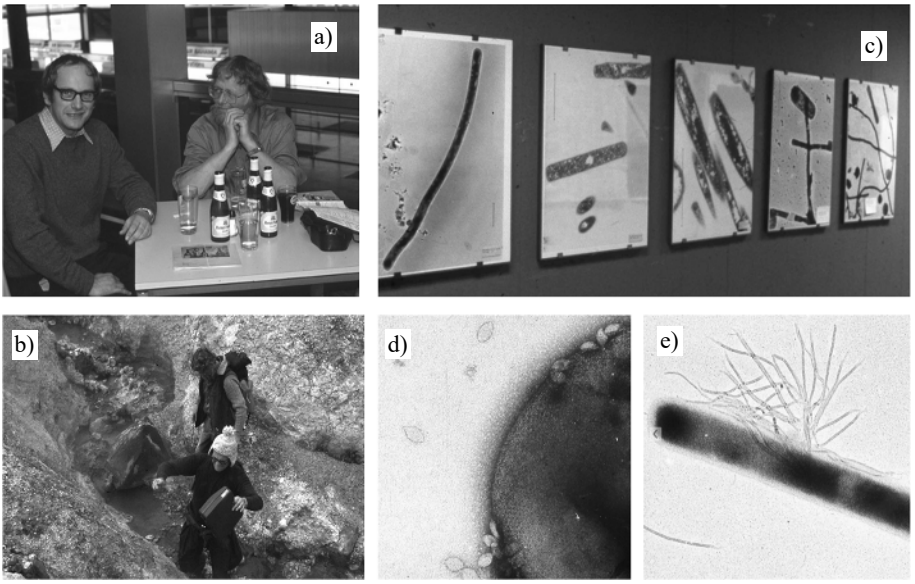


Figure 1.5. *The discovery of hyperthermophilic archaea and their viruses*

COMMENT ON FIGURE 1.5.– *(a) Karl Stetter (left) and Wolfram Zillig (right) at Reykjavik airport during their first expedition to Iceland in 1981. (b) The same individuals sampling a hot spring during their Icelandic expedition. (c) Photos displayed in the hallway of Wolfram Zillig's laboratory at the Max Planck Institute of Martinsried depicting hyperthermophilic archaea discovered in Iceland in 1981, with two photos of Methanothermus fervidus (left) and Thermoproteus* tenax *(right). (d) Sulfolobus shibatae cell surrounded by particles of the SSV1 virus.*

(e) Thermoproteus tenax cell surrounded by particles of the lipothrixvirus TTV1 (transmission electron microscopy).

From 1982 until the early 2000s, Karl Stetter, who became a professor at the University of Regensburg, continued his field research independently, exploring various volcanic areas around the globe with his students and postdoctoral researchers, and describing an extraordinary variety of hyperthermophilic archaea. Most of these new archaea belonged to the phylum *Crenarchaeota* (*Staphylothermus*, *Pyrobaculum*, *Pyrodictium*, *Pyrolobus* and *Ignicoccus*), but there were also some *Euryarchaeota* (*Archaeoglobus and Methanopyrus*). All these archaea were anaerobic, with a few exceptions, such as *Pyrobaculum aerophylum*, which was microaerobic. Karl Stetter and his team even discovered two hyperthermophilic bacteria, *Thermotoga maritima* and *Aquifex pyrophylus*, capable of dividing between 80 and 90°C (Stetter 2006, 2013).

One of the last fascinating discoveries of Karl Stetter's team was *Nanoarchaeum equitans*, a miniature archaeon living in symbiosis on the surface of a *Crenarchaeota* named *Ignicoccus hospitalis* (Huber et al. 2002). To Karl Stetter, *Nanoarchaeum equitans* seemed to be "riding" its "hospitable" host. This discovery foreshadowed the revelation of a whole group of miniature and parasitic archaea, the DPANN (see Chapter 2), which would be uncovered through metagenomic approaches 10 years later.

In the late 1990s, Karl Stetter was joined by other teams in the hunt for hyperthermophilic archaea. A Japanese team succeeded in isolating a strictly aerobic hyperthermophilic archaeon, *Aeropyrum pernix* (Sako et al. 1996). In France, Daniel Prieur's team in Brest specialized in isolating hyperthermophilic archaea from the walls of hydrothermal vents. These vents, or chimneys, appear black or white depending on the minerals in the plume and are located at faults formed by plate tectonics on the ocean floor. Sample collection was facilitated by the submarine Nautile owned by Ifremer (French Research Institute for Exploitation of the Sea). In 1993, Gael Erauso, a student of Daniel Prieur, isolated *Pyrococcus abyssi* from a sample taken from a smoker in the Pacific Ocean north of the Fiji Islands (Erauso et al. 1992). *P. abyssi* would later become a preferred model for the French community studying archaea (Forterre 2022a).

The discovery of hyperthermophilic archaea and bacteria has spurred biochemists to investigate the traits that enable these organisms to thrive at temperatures lethal to other life forms. This research led to the discovery of reverse gyrase in *Sulfolobus* in 1984, a type 1 DNA topoisomerase capable of introducing

positive supercoils into DNA in vitro (Kikuchi and Asai 1984; Forterre et al. 1985), and to the identification of positively supercoiled DNA in a virus of this archaeon, the SSV1 virus (see Figure 1.5) (Nadal et al. 1986). Reverse gyrase has been found in all hyperthermophiles without exception, whether archaea or bacteria. Its exact role remains unclear, as some hyperthermophiles also possess gyrase, which negatively supercoils their DNA (Guipaud et al. 1997). Nevertheless, reverse gyrase appears to play a crucial role at high temperatures, as it is the only protein specific to hyperthermophiles (Forterre 2002; Catchpole and Forterre 2019). The discovery of archaea has particularly benefited research on DNA topoisomerases, especially in France, with the identification of new families and activities of these enzymes which manipulate DNA topology. Archaea have indeed become a veritable goldmine for researchers in this field (Garnier et al. 2021; Forterre 2022b).

1.5.2. *The race for the thermophilia record*

All the hyperthermophilic archaea previously mentioned had maximum growth temperatures between 80 and 100°C, except for *Pyrodictium* and *Pyrolobus*, which broke all records by growing at temperatures above the boiling point of water. How was this possible? In 1982, Karl Stetter isolated from underwater hot springs off the island of Vulcano, a Crenarchaeon that would hold the temperature record for nearly 10 years, *Pyrodictium occultum*, meaning "burning fingers" (Figure 1.6). The optimal growth temperature of this archaeon, whose cells resembled a hand with fingers, was 105°C, and it continued to divide at 110°C under pressure (Stetter 1982). It originated from hot springs located 10 m deep, where the water remains liquid above 100°C.

In 1989, Karl Stetter's team discovered a hyperthermophilic methanogenic archaeon (a Euryarchaeon) capable of dividing up to 110°C, which he named *Methanopyrus kandleri* in honor of his former thesis supervisor, Otto Kandler (Huber et al. 1989). In 1997, the same team isolated a Crenarchaeon from a sample of underwater smokers off the coast of Iceland, whose optimal growth temperature was 106°C and which continued to divide at 113°C, *Pyrolobus fumarii*, meaning "the burning lobe of smokers" (Blöchl et al. 1997). According to Karl Stetter, this archaeon still holds the temperature record.

Since then, however, two hyperthermophilic archaea with even higher maximum growth temperatures have been described. The first, in 2003, by an American laboratory, could divide up to 121°C (Kashefi and Lovley 2003). The second, described in 2008 by a Japanese team, was a new strain of *M. kandleri* that could divide up to 122°C, but only under pressure (Takai et al. 2008). However, no other laboratory has reproduced these results, which remain controversial.

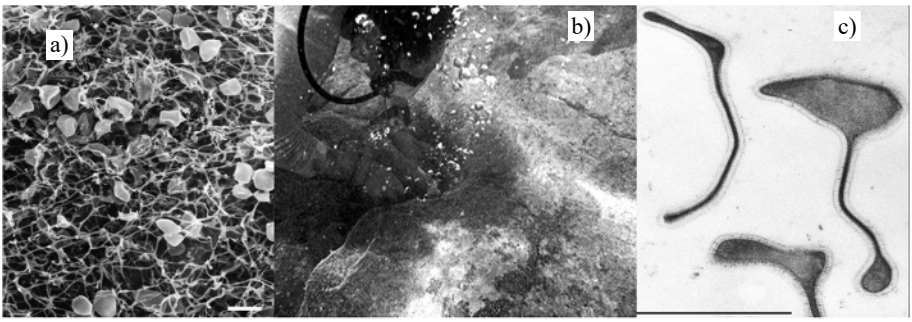


Figure 1.6. *Discovery of the first archaea with an optimal growth temperature above 100°C, Pyrodictium abyssi*

COMMENT ON FIGURE 1.6.– *Pyrodictium abyssi cells observed by scanning (a) and transmission (c) electron microscopy. (b) Karl Stetter sampling underwater hot springs off Vulcano beach in Italy. It was from these samples that he isolated this archaeon in 1982.*

It is true that some proteins are still active after being exposed for several tens of minutes at 120°C or even 140°C, but this is not the case for biological membranes. Vesicles formed with lipids of hyperthermophilic archaea quickly lose their impermeability to ions if the temperature exceeds 105°C, which corresponds to the optimal growth temperatures of *P. occultum* and *P. fumarii*, but also of the strain "121" and *M. kandleri* (Van de Vossenberg et al. 1995). It therefore seems difficult to imagine organisms capable of growing at temperatures well above 110°C. The maximum temperature at which an organism can divide on our planet, therefore, remains, in my opinion, an open question that has yet to be rigorously resolved.

The pursuit of the most thermophilic organism has sometimes led to errors. For instance, in 1982, the journal *Nature* published an article by two American researchers who claimed the discovery of bacteria capable of dividing at 250°C under 350 atmospheres in black underwater smokers (Baross and Deming 1983). This publication caused a media frenzy worldwide. However, it was what we would call today "fake news". Another American researcher, Jonathan Trent, identified all the flaws in this publication, but his rebuttal letter, also published in *Nature*, received much less attention (Trent et al. 1984). This episode, however, highlighted the issue of the stability of biological molecules at high temperatures. Specifically, the American biochemist Robert White published an article in 1984, again in *Nature*, which ended the controversy. Under conditions supposedly favorable to super-hyperthermophiles in black smokers – 300 atmospheres and 250°C – the

typical molecules of living organisms rapidly degraded, with the ATP molecule hydrolyzing in a few seconds and proteins in a few minutes (White 1984).

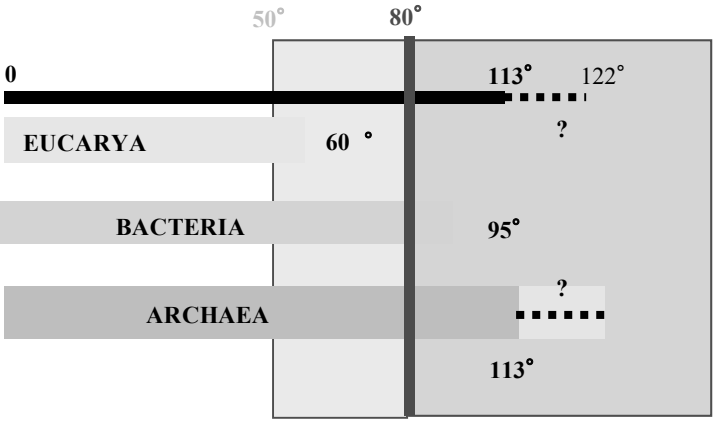


Figure 1.7. *Temperature ranges for organisms in the three domains of life. Hyperthermophiles are defined as organisms whose optimal growth temperature exceeds 80°C. The dotted lines indicate unconfirmed data*

While the upper temperature limit for life on Earth remains to be defined, it is already remarkable that this limit varies significantly across the three domains of life (Figure 1.7). Currently, no known eukaryote can divide above 60°C (a few fungi reach this limit). This is not for lack of trying. Karl Stetter himself, toward the end of his career, attempted to isolate hyperthermophilic eukaryotes. He only succeeded in isolating thermophilic protists capable of reproducing up to 55°C (De Jonckheere et al. 2011). Only archaea and bacteria seem capable of dividing above 60°C, and only archaea have surpassed the 100°C barrier. The difference in the upper viable temperature limits for archaea and bacteria (Figure 1.7) is likely due to differences in the composition of their membranes (see Chapter 2). The absence of hyperthermophilic eukaryotes could be explained by the lack of coupling between transcription and translation in eukaryotes, as messenger RNA is particularly fragile at high temperatures (Forterre 1995).

1.5.3. *The discovery of viruses in hyperthermophilic archaea*

While Karl Stetter specialized in searching for new hyperthermophilic archaea by investigating new metabolic types, Wolfram Zillig focused on the search for viruses infecting these microorganisms. Zillig aimed to isolate viruses to use as

models for studying the molecular biology of archaea, like how pioneers of molecular biology in the 1950s–1970s used viruses infecting bacteria (bacteriophages or bacterioviruses). Zillig expected to find archaeal "phages" producing head-tail-like virions resembling those of bacteriophages. Indeed, the first archaeal virus discovered in the 1970s, even before Carl Woese's development of the concept of archaebacteria, produced classic head-tail virions. This virus infected a halophilic archaeon (Torsvik and Dundas 1974).

To his surprise, Zillig and his students managed to isolate several original viruses in Icelandic and Japanese hot springs that infected anaerobic hyperthermophilic archaea, such as *Thermoproteus* (Figure 1.5(e)), or aerobic thermoacidophiles, such as *Sulfolobus* (Figure 1.5d). Some of these viruses produced virions in the form of long rigid filaments (rudiviruses, or rigid viruses) or flexible filaments (lipothrixviruses, lipid filaments, as their virions contain lipids) (Zillig et al. 1996). These virions resembled those produced by some eukaryotic RNA viruses, such as the tobacco mosaic virus, but were DNA viruses. One of the new viruses isolated in the late 1980s, SSV1 (*Sulfolobus solfataricus* virus 1), produced lemon- or spindle-shaped virions (now classified in the *Fuselloviridae* family), a morphology never previously observed in viruses infecting bacteria or eukaryotes (Schleper et al. 1992) (Figure 1.5(d)). Subsequently, Mike Dyall-Smith in Australia and Claire Geslin in France discovered other lemon-shaped viruses that infected halophilic archaea and anaerobic hyperthermophilic archaea, respectively (Bath and Dyall-Smith 1998; Geslin et al. 2003). These atypical viruses are therefore present throughout the domain of archaea (see Chapter 5).

During one of these Icelandic expeditions in 1995, Wolfram was accompanied by a Georgian researcher, David Prangishvili, who had joined his team in 1992, following the fall of the USSR. David Prangishvili would go on to become the undisputed world specialist in archaeal viruses in the 2000s, discovering viruses producing virions with increasingly astonishing shapes: some resembling bottles or springs, others capable of changing shape after leaving the cell, and still others ending in claws used to grip the pili of the archaea they infect (see Chapter 2). The discovery of different viruses in archaea, distinct from those infecting bacteria and eukaryotes, supported Carl Woese's vision of a living world divided into three major domains, each with its specific set of viruses. David Prangishvili continued his studies on archaeal viruses in the unit I led at the Pasteur Institute of Paris from 2004 to 2019 (Prangishvili 2013; Prangishvili et al. 2017). His work is now being continued by one of our former postdoctoral researchers, Mart Krupovic, who heads a research unit on archaeal virology at this institute (Forterre 2022a).

1.6. Non-extremophilic archaea discovered by molecular ecology: a new vision of the third domain

The discovery of halophilic, thermo-acidophilic and then hyperthermophilic archaea had led most biologists to consider archaea as essentially extremophilic organisms. Methanogens could also, to some extent, be considered "extremophilic" due to their very strict anaerobiosis, with many of them being thermophilic or even hyperthermophilic, like *M. kandleri.* Thus, mesophilic archaea seemed to be an exception. The surprise was all the greater when the first molecular ecology studies, based on the identification of 16S rRNAs present in the environment, revealed new lineages of archaea present in large numbers in cold environments, the ocean, lakes and terrestrial soils (DeLong 1992; Furhman et al. 1992). This work was partly initiated in the early 1990s by the teams of Norman Pace (a former student of Carl Woese) and Edouard DeLong in the United States.

Although not culturable at the time, the sequence of their 16S rRNAs, their "phylotype", allowed the placement of these new archaeal lineages in a phylogenetic tree. A large number of them corresponded to new *Euryarchaeota*, but an equally large number seemed to correspond to *Crenarchaeota*, a phylum for which all cultivated organisms known at the time were either thermo-acidophilic or hyperthermophilic. This observation triggered a new "hunt for archaea", which remained unsuccessful for a long time. Analysis of genomic fragments from these organisms showed that they must be capable of oxidizing ammonia (Treusch et al. 2004). In 2005, after 15 years of unsuccessful trials in many laboratories, this observation enabled David Stahl's team in the United States to cultivate, for the first time, one of these new oceanic mesophilic archaea. It was a very small archaea that they named *Nitrosopumilus maritimus* (*pumilus* means "dwarf" in Latin) (Könneke et al. 2005). Curiously, this archaeon was not isolated from an oceanic water sample but from a sample taken from the Seattle Aquarium!

Subsequently, researchers demonstrated that these cold-dwelling archaea play a major role in the nitrogen cycle, a role previously attributed solely to bacteria. Today, thanks to genome sequencing, we know that *N. maritimus* and other mesophilic "*Crenarchaea*" are, in fact, very different from thermophilic *Crenarchaea*. They were eventually classified, along with some thermophiles, into a new phylum called *Thaumarchaea* (Brochier-Armanet et al. 2008). Additionally, *Thaumarchaea* were discovered in hot environments (see Chapter 2). One of the leading research teams studying *Thaumarchaea* is led by Christa Schleper, a former

student of Wolfram Zillig, highlighting once again the importance of mentorship in scientific research[4].

1.7. Conclusion

The history of the discovery of archaea can be considered a triumph of the so-called "reductionist" approach in science (Forterre 2022a, 2022b). By using a single gene as an evolutionary marker to determine the relationships among microorganisms, Carl Woese was able to identify within "prokaryotes", a subgroup of microorganisms that constitute one of the three cellular lineages currently inhabiting our planet. This discovery was subsequently validated by genomics, but comprehensive approaches using hundreds and then thousands of genes did not provide fundamentally new insights compared to the concept proposed by Carl Woese. On the contrary, it was this concept that allowed scientists to make sense of the deluge of genomic and then metagenomic data which inundated the field of biology.

The concept of archaea faced significant resistance within the scientific community. Carl Woese encountered considerable opposition, particularly from American evolutionists such as Lynn Margulis and Ernst Mayr (Margulis and Guerrero 1991; Mayr 1998; Woese 1998; Quammen 2018). His exceptional contribution to 20th-century biology was only belatedly recognized with the prestigious Craaford Prize, awarded to him by the King of Sweden in 2003 (Figure 1.2(b)). Despite the considerable impact of his idea of characterizing microorganisms through molecular approaches, Carl Woese was never awarded the Nobel Prize, even though his work had profound implications not only in fundamental biology but also in medicine.

Even today, the concept of archaea is often ignored, or its importance underestimated by many biologists. Research continues to focus heavily on bacteria and eukaryotes; in the last 10 years, approximately 15,000 papers have been published on archaea, compared to 910,000 on bacteria and more than 7 million on eukaryotes.

However, history teaches us those researchers who ventured off the beaten path and took an early interest in archaea often had brilliant careers. This is a lesson for the younger generations, especially in an era where the pursuit of high-impact

4 An example worth mentioning is the discovery of the Asgard archaea (see Chapter 2) by Anja Spang, a former doctoral student of Christa Schleper, within the team of Thijs Ettema, a former doctoral student of Rolf Bernander, a pioneer in the study of the cell cycle in archaea.

factors and the influence of trends play an increasingly significant role in shaping research agendas and securing funding.

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