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Visions & Reflections

Microbial Life at high temperature, the challenges, the strategies

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Introduction

Ever since the discovery of microbial life in hot environments some 35 years ago, the scientific community has been intrigued by the diversity of extremophilic life forms and much interested, for reasons of fundamental interest, biotechnological applications and economic benefit, in the molecular adaptations that make life at high temperature possible. In the last decade much effort has been invested in unraveling the molecular bases of thermophily and the underlying physicochemical properties of biological materials.

Thermophily is such a vast domain that providing a complete overview of the state of the art of all aspects linked to life at high temperature is certainly far beyond the scope of this paper. Here we pinpoint particular problems linked to life in hot environments, describe mechanisms that make life at high temperature possible or, at least appear to be correlated with thermophily, and draw attention to particular challenges and bottlenecks in the analysis of thermophilic microorganisms and processes evolving at high temperature. We give an overview of strategies and molecular mechanisms that may play a crucial role in the thermostabilization of biological structures, different classes of macromolecules, and small thermolabile metabolites and coenzymes. It should be stressed that some molecular adaptations will not only confer increased resistance to heat inactivation, but lead to chemical structures that are more resistant to various kinds of harsh conditions. As a consequence, the evolutionary origin of presumed 'thermophilic traits' is not always clearly established and is still under debate. As developed below, this is particularly striking for membrane composition. Moreover, despite the deep branching of thermophiles in the phylogenetic tree of life, it is at present not unambiguously determined whether LUCA, the last universal common ancestor of all life on earth, was a thermophile. Clearly, many aspects linked to thermophily are still a matter of hot debate.

High temperature: the most challenging physical parameter?

Only prokaryotes, Bacteria and Archaea, can cope with very high temperatures

Even though in nature there is a continuum for thermal preference, microorganisms have been classified in relation to their temperature optimum, that is the temperature at which growth is most rapid, into four major groups: psychrophiles, mesophiles, thermophiles and hyperther-

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mophiles. By definition, thermophiles have their optimal growth temperature above 45 °C and under pressure, this can even exceed 100 °C. The more thermophilic organisms are further subdivided in extreme thermophiles, which have their optimum at or above 70 °C but below 80 °C, and the hyperthermophiles, which have their optimum at or above 80 °C. Hyperthermophiles are found in restricted and extremely hot terrestrial or submarine habitats, generally associated with geothermal and volcanic activity, such as geysers, hot springs, oil reservoirs, solfatara, shallow and deep sea hydrothermal vents, such as the astonishing black and white smokers and undersea volcanoes known as sea mounts. Many hyperthermophiles have been isolated from the deep sea, in the immediate surroundings and the chimney walls of smokers at depths of several kilometers, but none of these organisms is an obligate piezophile.

Since Tom Brock's historical discovery in the late 1960s of the very first real thermophilic microorganisms, the extreme thermophilic bacterium *Thermus aquaticus* (T_{opt} 70 °C) soon followed by the hyperthermophilic archaeon *Sulfolobus acidocaldarius* (T_{opt} 80 °C), both isolated from Yellowstone National Park [1, 2], it has become increasingly clear that certain prokaryotic life forms do not only withstand but rather require temperatures exceeding 80 or even 100 °C for optimal growth. Since these organisms do not grow below 60 °C, it is evident that thermophily has to be clearly distinguished from thermotolerance.

Eukaryotic life, at least as discovered so far, stops at 62 °C, though it is not clear why the eukaryal life style would be incompatible with high temperature. Only two bacterial genera (*Thermotoga* and *Aquifex*) have their optimum at or above 80 °C, but they do not grow above 95 °C. At present only Archaea have been found thriving above 95 °C [3]. High temperature might, therefore, be the most challenging parameter, the one that imposes the strongest limitations on the diversity of cellular life forms. In contrast, other equally bewildering biotopes (low temperature, low or high pH, high salt concentration or high pressure) are populated by microorganisms belonging to the three domains of life [4]. As only hyperthermophilic archaea grow optimally above 95 °C, it is in these organisms that one might expect to find the best-suited strategies and far-reaching molecular adaptations for promoting life at high temperature.

Why is high temperature so challenging? What is the upper limit compatible with microbial life?

Microorganisms can maintain a steep pH or salt gradient across the membrane, but they cannot insulate themselves from the hot, aqueous environment. Therefore, hyperthermophiles have to be adapted at all levels of cellular development. Each and every superstructure and molecular machinery has to be stable and active at high temperature, and all types of macromolecules have to be stabilized, intrinsically or extrinsically [5]. Small molecules, unstable substrates, and highly reactive and potentially harmful reaction intermediates have to be protected and shielded from the hot, agressive cytoplasmic fluids. Moreover, hyperthermophiles must possess efficient DNA-repair mechanisms as their genetic patrimony is subject to increased levels of damage [6].

Despite these strong limitations there exists a great variety of extreme and hyperthermophilic species, aerobes and anaerobes (mostly), including various primary producers and decomposers of organic material, exhibiting a broad range of energy-yielding reactions [7]. Hyperthermophily also comprises the recently discovered archaeal nanosized symbionts [8].

What is the maximal environmental temperature compatible with active cellular life? There is at present no clear answer to this question, but the most hyperthermophilic cultivatable microorganisms known today are the nitrate-reducing chemolithoautotrophic crenarchaeote *Pyrolobus fumarii* and Strain 121. These divide fastest at 105 °C and have their upper limit of growth at 113 °C or possibly above [9, 10]. Vegetative cells of these organisms can even survive autoclaving.

As yet uncultivable organisms, the Korarchaeota, have been sampled from environments at or near 125 °C, but their optimal and maximal growth temperatures are not known [11]. As sulphur plays an important role in the energetic metabolism of many hyperthermophiles, either as an electron donor in chemolithotrophic metabolism or as an electron acceptor in anaerobic respiration, the melting temperature of elemental sulphur (119 °C) might be an important determinant in setting the upper limit for those organisms. Considering the stability of predominant chemical bonds such as the peptide bond, and of major building blocks, heat-sensitive amino acids such as glutamine, asparagine, cysteine, histidine, methionine, tryptophan and tyrosine, and precursors of purine and pyrimidine nucleotides, it would be surprising to uncover any active life on earth above 150 °C.

Thermostability and activity of biological structures and macromolecules

Adaptations of bacterial and archaeal membranes to life at high temperatures

Primitive life can be defined as a well-delimited ensemble of organic material capable of perpetuating itself. To do so it must metabolize, duplicate and evolve.

A microbial cell is surrounded by a membrane and in most cases by a cell wall that delimit the cellular compartment. The membrane assures communication with the environment (active transport) and plays a crucial role in energy-transducing processes. The membrane lipids play a key role in the fluidity of membranes, which

is critical in these processes. It is well established that the fluidity and permeability of the membrane increases with temperature. Consequently, at high temperature the thermophilic membrane should be in the liquid-crystalline state to be functional, but should also be sufficiently impermeable, even to small molecules and ions, to allow the generation of a proton motive force and pH homeostasis. Adaptations to extreme temperatures involve modifications of phospholipids that maintain both membrane integrity and fluidity. Many organisms can adapt the degree of saturation of their membranes as a response to changes in environmental temperature [12–14]. In thermophilic Bacteria, major adaptations of membrane lipids to life at high temperature include an increase in acyl chain length and degree of saturation of fatty acids, branching and cyclization. These adaptations must limit the temperature-dependent increase in membrane permeability occasioned by increased diffusion.

The situation is somewhat different in thermophilic Archaea. Indeed, it is commonly argued that the composition of the archaeal membrane is one of the most remarkable biochemical differences that distinguishes the Archaea from the Bacteria [15–17]. However, it should be stressed that some hyperthermophilic Bacteria such as *Thermodesulfobacterium*, *Aquifex*, *Ammonifex* and *Thermotoga* also possess ether lipids (see [18] for an overview). Archaeal membrane phospholipids consist of saturated isoprenoid chains linked to the glycerol backbone by the chemically more resistant ether linkage instead of the ester linkage, which connects the glycerol moieties and fatty acids in most bacterial and eukaryal membranes. The most common form present in non-thermophilic Archaea are the monomeric diphytanylglycerol ethers (archaeols). In contrast, membrane-spanning dimeric dibiphytanyldiglycerol tetraethers (caldarchaeols) forming a monolayer structure and dibiphytanyl glycerol nonitol tetraethers (nonitolcaldarchaeols), providing a high degree of rigidity, are typically found in thermophilic Archaea [19] (fig. 1). In some hyperthermoacidophilic Archaea such as *Sulfolobus* the C_{40} biphytanyl chains may undergo further modifications such as internal cyclization (cyclopentane rings) as a response to increasing temperature [20]. To further limit the detrimental effects of increased proton permeability of membranes at high temperature, some thermophiles make use of a non-stoichiometric proton-sodium antiport system to generate a secondary sodium gradient, whose maintenance is less temperature-dependent [21].

As all Archaea, including mesophilic ones have ether linked phospholipids, this trait appears at first sight to be phylogenetic rather than thermophilic. However, on the assumption that Archaea emerged by thermoreduction from a protoeukaryotic and non-hyperthermophilic last common ancestor endowed with glycerol ester lipids, as some propose [18], the occurrence of glycerol ether lipids may still be directly related to thermophily. This proposal appears to be further supported by the occurrence of ether linkages in hyperthermophilic Bacteria, as mentioned above.

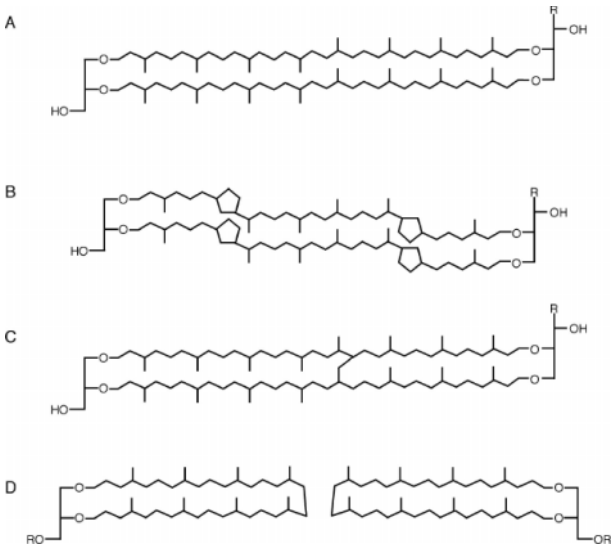

Figure 1. Architecture of some major ether-linked membrane lipid structures of thermophilic Archaea. (*A*) Membrane-spanning dibiphytanyl diglycerol tetraethers, (*B*) internal cyclization of dibiphytanyl diglycerol tetraethers, (*C*) internal covalent cross-linking, (*D*) cyclic diphytanyl glycerol diether. R = H (caldarchaeols). R = (CH_2OH)-$(CHOH)_3$-$CH(OH)_2$ (nonitolcaldarchaeols).

Thermostability of nucleic acids, DNA damage and repair

How do thermophiles maintain structure and functional integrity of nucleic acids, avoid denaturation of duplexes and cope with increased levels of DNA damage? Which modifications and processes ensure the functional integrity of DNA and RNA as substrates in the major cellular processes of DNA replication and partitioning, transcription and translation? What ensures the strict control and fidelity of these reactions and their correct insertion in the stream of cellular processes that guarantee the perpetuation of the species?

The maintenance of the secondary structure of the DNA duplex at high temperature appears to be no major problem. Linear DNA easily denatures by strand separation, but circular molecules are much more resistant to heat denaturation. Moreover, either positive or negative supercoiling could further increase the melting temperature (Tm) [5, 22]. In principle, an increase in the G-C ratio could also lead to thermostabilization of the duplex. However, this strategy is not used by thermophiles; there is no correlation between the overall G+C content of a microbial genome and the growth temperature of the organism. Only in restricted areas, coding for stable RNAs, could such a correlation be observed [23; see below]. This is comprehensible since ribosomal RNA (rRNA)

and transfer RNA (tRNA) should maintain a long-range structural and functional integrity at high temperature, unlike messenger RNAs (mRNAs), which, in prokaryotes, are rather short-lived [24]. In vitro, duplex DNA can be stabilized by the addition of salt, and yet some but not all hyperthermophiles contain high intracellular concentrations of ions [25, 26]. Molar concentrations of potassium di-inositol-1,1'-phosphate and tripotassium cyclic-2,3-diphosphoglycerate have been measured in *Pyrococcus woesei* and thermophilic methanogens, and in vitro these substances proved to be stabilizing agents for nucleic acids and protein [25, 26]. A greater diversity of polycations could also be correlated with hyperthermophily, and some polyamines apparently only occur in hyperthermophiles [27, 28]. In vitro, the longer linear polyamines found in thermophiles proved to be more efficient in thermostabilization of double-stranded DNA, whereas branched polyamines were more effective on single-stranded DNA and tRNA [28]. Interestingly, all hyperthermophiles, both Archaea and Bacteria, possess an enzyme – reverse gyrase – that is exclusively found in hyperthermophiles. Reverse gyrase displays helicase and type I topoisomerase activities and introduces positive superturns at the expense of ATP hydrolysis. Hyperthermophilic Archaea have relaxed to slightly positively supercoiled DNA, whereas Eukarya, Bacteria and mesophilic Archaea have negatively supercoiled DNA. The unique topological state of hyperthermophilic archaeal DNA is due to the combined action of reverse gyrase and the presence of type II topoisomerase activity that can remove but not introduce negative supercoils [29, 30]. The hyperthermophilic bacterium *Thermotoga* has both reverse gyrase and gyrase activities and has negatively supercoiled DNA [31]. Although the presence of reverse gyrase constitutes a hyperthermophilic signature, recent studies have indicated that the presence of reverse gyrase is not an absolute requirement for life at high temperatures. Therefore, it is at present unclear why reverse gyrase is so tightly associated with life at high temperatures. Table 1 gives a list of the main features that contribute to the maintenance of the functional integrity of nucleic acids at high temperature.

Controlling the local, transient strand separation and subsequent reannealing of DNA strands required in dynamic processes such as transcription, replication, recombination and repair of DNA may be more of a problem. How this is assured is not yet clear, but in this context it is interesting to notice that the binding of abundant small basic proteins such as the Sul7d family of chromatin proteins and Alba in *Sulfolobus* substantially increases the Tm of the DNA in vitro [32, 33]. Hyperthermophilic euryarchaeotes have true homologues of the eukaryal histone $(H3\text{-}H4)_2$ heterotetramer which exhibit a similar fold; their binding equally increases the melting temperature of the duplex [34, 35].

Table 1. List of main features which contribute to the maintenance of the functional integrity of nucleic acids (DNA and stable RNAs) at high temperature and/or appear to be correlated with thermophily.

DNA	RNA (tRNA, rRNA)
– circularization – supercoiling – nucleoid-associated proteins – reverse gyrase – high salt concentration – unusual, long linear polyamines – efficient, novel repair systems – multiple copies of the chromosome	– increased G+C content – unusual, branched polyamines – increased affinity of ribosomal proteins – increased levels of ribose and base methylation – abundant posttranscriptional modifications

Interestingly, DNA topology was shown to vary in hyperthermophiles during thermal stress: the linking number increases during heat shock and decreases during cold shock [36]. Such transient changes in DNA topology may have profound consequences on transcription, and it was suggested that a decrease in linking number could help to maintain transcription under cold-shock conditions [37]. Indeed, mesophiles appear to need the energy of negative supercoiling, generated either by the action of gyrase or by histone wrapping, for strand opening to allow the initiation of vital processes as DNA replication and transcription.

The maintenance of the primary structure of nucleic acids at high temperature is likely most problematic. The thermophilic chromosome is subject to increased rates of damage: deamination (especially of cytosine and 5-methyl cytosine), depurination, single- and double-strand breaks, and damage occasioned by oxidative stress are all expected to increase substantially with temperature and in geothermally active environments [6, 22]. At temperatures exceeding 80 °C these rates would appear to be incongruent with the requirements of accurate genome duplication. Yet hyperthermophiles are capable of maintaining the integrity of their hereditary material at temperatures that are detrimental for the integrity of mesophilic genomes. Therefore, and since mutation rates in hyperthermophiles are not significantly different from those observed in mesophiles [38], thermophiles must possess efficient repair systems. Homologues of bacterial/eukaryal DNA repair systems and enzymes such as photoreactivation, dark repair, RecA-RAD51, uracil *N*-glycosylase and O^6-alkylguanine-DNA transferase have been identified in thermophiles, and several enzymes of DNA metabolism of thermophiles exhibit unusual properties that might be relevant for DNA repair at high temperature [39–41]. Interestingly, a hypothetic and previously undetected DNA repair system specific for thermophilic Archaea and Bacteria was predicted

by genome context analysis [42]. Moreover, it is not excluded that novel (archaeal) hyperthermophilic DNA repair systems have not yet been identified because they might be too dissimilar from known systems, mostly of mesophilic bacterial and eukaryal origin, to be easily picked up in in silico screenings. Finally, it is noteworthy that exponentially growing hyperthermophilic Archaea contain several copies of the chromosome [43]. This might be particularly important for the repair of double-strand breaks, induced by heat or high-energy radiation, by direct end-joining and recombination.

Analogously to DNA, salt concentration seems to play an important role in the stabilization of rRNA and tRNA. Indeed, under physiological salt conditions, most of the secondary structures of RNA remain stable at the optimal growth temperature of the organism [44]. In contrast to genomic DNA, the G+C content of rRNA correlates linearly with the growth temperature of the organism [23]. This correlation is, however, restricted to the double-stranded stem regions of the RNA molecule [45]. Another important contribution to the thermal stability of the rRNA might be the observed increased affinity of ribosomal proteins for the rRNA [46–49]. Furthermore, posttranscriptional modifications, which are common in many types of RNA, are particularly abundant in the rRNA of the hyperthermophile *Sulfolobus solfataricus* [50], especially for ribose methylation at the 2'-hydroxyl position. Although the level of modification in rRNA is relatively high in *S. solfataricus*, its tRNA contains four to five times more modified nucleosides [51]. This quantitative difference is also observed for *Escherichia coli* (same reference). Posttranscriptional modifications in tRNA can dramatically influence codon specificity [52] and aminoacyl-tRNA synthetase recognition [53]. Does nucleoside modification also play a role in the stabilization of tRNA at elevated temperatures? It was already reported in the 1970s that a link exists between the growth temperature of the bacterial species *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus;* T_{opt} 57 °C) and *Thermus thermophilus* (T_{opt} 70 °C) and levels of tRNA modification [54, 55]. Indeed, the content of 2-thio-ribothymidine (s^2T) in unfractionated *T. thermophilus* increased when grown at higher temperatures [55]. The presence of a thiol group on ribothymidine leads to an increase of the melting temperature of *T. thermophilus* $tRNA^{Ile}_1$ [56]. Similar experiments showed an increase in the modified nucleosides 2'-*O*-methylguanosine and 1-methyladenosine (m^1A) as a function of temperature [57]. Base methylation at adenosine 58 of the T-loop of tRNA in *T. thermophilus* is also important for tRNA thermostabilization. Indeed, inactivation of the gene coding for the corresponding methyltransferase (m^1A58) results in a *T. thermophilus* strain with a growth defect at 80 °C [58]. The study of post-transcriptional modifications of archaeal tRNA from mesophilic and (hyper)thermophilic *Methanococcales* showed not only the presence of typically archaeal linked modifications but also two ribose-methylated species – N^2, 2'-*O*-dimethylguanosine and N^2,N^2, 2'-*O*-trimethylguanosine – characteristic of archaeal hyperthermophiles [59]. As for rRNA, tRNA ribomethylations seem to confer an increased stability to the molecule. It has indeed been reported that a 2'-*O*-methylated nucleoside confers conformational rigidity by favoring the C3'-endo ribose form, which contributes to the thermostability of the tRNA molecule [60]. The molecular mechanisms of thermostability of tRNA have been the subject of several in vitro studies. However, in vivo data are rare. A systematic analysis of inactivation of genes encoding modification enzymes in *Thermus thermophilus* is currently under way. But unfortunately such a study is still not applicable for archaeal organisms.

Thermostability of aminoacyl-tRNAs

Aminoacyl-tRNAs (AA-tRNAs) are essential elements in protein synthesis and are particularly sensitive towards high temperatures. The stability of these AA-tRNAs depends highly on the amino acid linked to the 3'-terminal adenosine of the tRNA [61]. What are the strategies that thermophilic organisms use to overcome such a possible translational difficulty? The involvement of noncanonical pathways or enzymes in certain thermophilic organisms could be a possible solution, as has been reported in the review of Ibba and Söll [62]. AA-tRNA thermolability could also be overcome by increasing the rate of polypeptide synthesis on the ribosome. However, studies on the rate of polypeptide chain elongation in thermophilic organisms are really scarce. A quick turnover together with increased AA-tRNA synthetase activity could possibly compensate for the faster decay of AA-tRNAs observed at high temperatures [63].

Thermostability and activity of enzymes and proteins

In recent years, more and more genome sequences of extreme and hyperthermophiles have been completed, leading to an overwhelming quantity of comparative studies on protein thermostability. The majority of these different proteins are intrinsically thermostable, but others require extrinsic factors [64]. Thermal tolerance is the result of a subtle balance of non-covalent interactions. Although no general rule could be proposed for thermostabilization, several recurrent themes, general tendencies (see table 2), were observed in a large-scale comparative analysis of proteins from closely related mesophilic and thermophilic *Methanococcus* species [65]. Proteins from thermophilic organisms contain an increased proportion of bulky hydrophobic and charged residues (in particular arginine) stabilizing the core and the periphery (ion networks) of the protein, respectively, and less non-polar residues such as Ser, Thr, Asn and Gln [64]. Other commonly observed strategies include the

Table 2. List of main features, recurrent themes and general tendences which contribute to increased intrinsic or extrinsic thermostabilization of proteins.

Intrinsic	Extrinsic
– ion networks	– compatible solutes
– higher residue volume	– higher oligomeric forms
– higher residue hydrophobicity	– enzyme-enzyme interactions
– more charged residues	– disulphide bridges
– fewer charged polar residues	– substrate binding
– improved packing of hydrophobic core	
– decreased solvent interactions	
– increased helix-dipole stabilization	
– inclusion of a metal binding site	
– increased level of proline residues in β-turns	

occurrence of more proline residues in β-turns, the introduction of a negatively charged amino acid residue at the N-terminus of an α-helix and the introduction of a metal binding site. Thermostabilization can also be obtained by shortening of external loops and by intermolecular interactions such as oligomerization, enzyme-enzyme interactions, substrate binding and interactions with compatible solutes [5, 66]. In recent years, disulphide bridges have been shown to contribute to the thermal stability of intracellular proteins from hyperthermophilic organisms [67, 68]. Interestingly, the homotetrameric methyltransferase TrmI from *Pyrococcus abyssi* is stabilized by intersubunit disulphide bridges [69].

Enzymes have to be sufficiently stable but, importantly, also sufficiently flexible for catalysis. Therefore, many thermophilic enzymes are only marginally stable at the optimal growth temperature of the organism. Remarkably, in vivo selections have demonstrated that one or a few amino acid substitutions are sufficient to turn a hyperthermophilic enzyme into a low-temperature-adapted derivative, and vice vers [70, 71]. Generally (though not always), a gain in activity at low temperatures is correlated with a substantial loss in thermostability.

Protection of small unstable and reactive molecules

Most biosynthetic pathways and molecular machineries of hyperthermophiles are equivalent to the mesophilic counterparts. Yet the corresponding enzymes utilize/produce many low molecular weight metabolites and coenzymes, such as NAD, NADP, acetyl phosphate, carbamoyl phosphate (CP), ATP and ADP, that are very unstable at 90 °C or above. Their half-life may be influenced by local environmental conditions such as pH, ion concentration, molecular crowding etc. Some of these compounds will thermally decompose into potentially toxic products. This is the case for CP, a precursor common to the biosynthesis of arginine and pyrimidines, which has a half-life of less than 2 s at 100 °C. Its major degradation product, cyanate, is a highly toxic non-selective carbamoylating agent. Evidently, hyperthermophiles can cope with major problems inherent to life at high temperature, and different strategies have been proposed to circumvent/minimize the risks linked to the occurrence of small unstable molecules [5]. These strategies can be grouped in four major categories that are not mutually exclusive: (i) utilization of an alternative pathway or of an alternative, more thermostable precursor/intermediate, (ii) increased turnover, (iii) local protection by the microenvironment and (iv) channeling. Thus a correlation was noticed between hyperthermophily and the use of the more thermostable non-haem iron proteins rather than NAD(P) in Bacteria and Archaea [72, 73]. Possibly, the presence of a non-phosphorylated Entner-Doudoroff pathway in several thermophiles might also be correlated with the higher thermostability of non-phosphorylated intermediates.

Though the idea of substrate channeling by physical contact between soluble enzymes catalyzing successive reactions was proposed long ago, there is still little experimental evidence for the existence of transient metabolons and their biological relevance. Recently, our colleagues Massant and Glansdorff gathered convincing evidence for efficient channeling of CP in *Pyrococcus furiosus* by physical interaction of the CP-producing carbamate kinase and ornithine carbamoyltransferase or aspartate carbamoyltransferase, which use CP as a substrate in the synthesis of arginine and pyrimidines, respectively [74]. Affinity electrophoresis, co-immunoprecipitation, isothermal titration calorimetry, Hummel-Dreyer equilibrium gel filtration and the yeast two-hybrid technique provided concrete evidence for the establishment of temperature-induced, rather weak and dynamic protein-protein interactions [75, 76].

It is noteworthy that in *E. coli* (which like most organisms, but unlike *Pyrococcus,* uses a carbamoylphosphate synthetase to produce CP) the highly reactive intermediates in the synthesis of CP (ammonia, carboxyphosphate and carbamate) are transferred from one catalytic site to the next in the enzyme through a long internal tunnel (about 96 Å, the longest tunnel characterized) [77, 78]. Thus, they are shielded from the cytoplasmic fluids. Protection of unstable and highly reactive intermediates and substrates by tunneling, channeling and metabolon formation is, therefore, not restricted to hyperthermophiles. These mechanisms also exist in mesophiles, but the problems are more acute and the processes probably more efficient and indispensable at high temperature.

What is next? Where are the challenges, virgin areas and major bottlenecks?

The discovery of life at high temperatures and the apparent deep branching of these organisms in the tree of life has greatly stimulated the debate concerning many fundamental questions and aspects of living organisms and organic materials. Among these, the origin of life, the probability of finding life forms on other planets and even the possibility of panspermia remain certainly among the most speculative and controversial ones. Nobody can deny that the study of thermophiles has led to a revived interest in many matters and provided new insights in our understanding of the behavior of small and macromolecules, biochemical and physiological processes, and has considerably broadened our vision of the living world. After all, the search for life at high temperature has revealed the existence of a new, third domain of life. What on earth could be more exciting? And yet much remains to be discovered.

The way has been paved for further detailed studies on the in vitro thermostability/activity of macromolecules and molecular machineries. In contrast, the thermal stabilization of small molecules is still a somewhat neglected aspect; especially the kinetics and thermodynamics of the transient protein-protein contacts involved in channeling remain to be thoroughly documented.

At present in vivo studies are rare; the lack of genetics and molecular biology tools for hyperthermophiles is certainly a major cause. Some areas will progress faster than others, in part because of economic interests, and therefore of financing. Interest dictated by economic profit may, however, change rapidly; a significant reduction in the cost of primary materials can be a sufficient argument to inspire a complete reorientation of the investigations, not an ideal situation for the development of fundamental research. Moreover, applications based on the utilization of thermophilic enzymes have not completely fulfilled expectations, and the initial burst of interest has decreased somewhat in recent years. Since today many fundamental questions remain unanswered, and novel aspects of thermophily have yet to be brought to light, it is imperative that governments and institutions develop long-term views and continue to provide sufficient funding for fundamental research. We should not forget that most of the greatest technological breakthroughs could not be foreseen and result from fundamental studies, sometimes made long before. One example perfectly illustrates this unpredictable state of affairs: the observation that the development of bacteriophages is restricted in certain host strains led decades later to the discovery of the restriction enzymes and their widespread use in recombinant DNA technology.

High-throughput approaches and in silico analyses of full genome sequences and their potential predicted products, have led to a major acceleration of investigations and discovery in most areas of life sciences, also of thermophiles. The great power of these approaches is their comparative and predictive character, which also makes them extremely valuable for elaborating general models. However, we should remain sufficiently alert and be cautious not to take the results of such theoretical and comparative studies as proven fact. Overgeneralization on the basis of insufficiently analyzed data could be misleading. Experimental verification is still required in many domains, especially considering that our approach is quite reductionistic; natural systems are far more complex and entangled than we like to believe. What is needed is the right, balanced combination of in-depth studies of single molecules, isolated enzymatic processes, genome-wide approaches and physiology, as well as the 'social' behavior of these bewildering organisms living in community in their natural hot environment.

Urgent need for genetics and molecular biology tools

In this post-genomic area we can amplify genes from environmental DNA and screen for genes encoding products of potential economic (industrial or medical) interest and use these pools and gene banks as starting material for the generation of even more robust thermostable and thermoactive variants. We have also accumulated quite a lot of data on the in vitro behavior of individual components, especially proteins (mostly overexpressed in *E. coli*), nucleic acids and lipids. We are starting to uncover general strategies of intrinsic and extrinsic thermostabilization, and we can, in part, reconstitute certain systems in vitro. However, in vivo data are scarce, and we know nearly nothing about the physiology of hyperthermophiles. How do they perform and control major cellular activities such as replication and repair of their genetic patrimony, cell division, gene expression, motility etc? If we want to unravel the molecular details of these aspects of life at high temperature and go beyond the stage of mere exploitation of these organisms as a primary source for industrially interesting compounds, if we want to better understand the behavior of extreme and hyperthermophiles and their evolution, it is imperative to develop the genetics of these organisms. With the exception of *Thermus thermophilus,* which is amenable to genetic analysis, at present this aspect is at most in its infancy for thermophiles and particularly distressing for hyperthermophilic Archaea (for a recent survey of the state of the art see [79]). There is an urgent need for routinely applicable genetic and molecular biology tools to generate knockouts, reporter gene fusions and partial heterodiploids to learn more about protein, gene and network functions in the natural physiological context.

Resident plasmids and viruses of hyperthermophilic Archaea are well known [80], and recently many new and diverse viruses have been isolated from hot environments

[81]. Moreover, conjugation and self-transmissible plasmids have been identified in *Sulfolobales* [82, 83]. Therefore, there is an important pool of naturally occurring vehicles and processes of genetic exchange that should be exploited more intensely as the raw starting material for the construction of shuttle vectors for thermophilic archaea. A handful of first-generation cloning vectors have been developed for the model eury- and crenarchaeotes *P. furiosus* and *S. solfataricus*, respectively, and a few gene disruptions and genomic reporter gene fusions have been reported [84–88]. But these are still isolated examples. Generally speaking, these techniques are only mastered by very few specialized groups and are not yet routinely applicable. We are still far from having hyperthermophilic equivalents of the bacteriophages lamba and P1, F-episome and the pBR322 plasmid systems which have contributed so much to the development of mesophilic bacterial genetics and the unraveling of cellular processes. Despite international efforts, these developments are still in an embryonic stage for hyperthermophiles. It is now time for a major (financial) effort and scientific breakthrough in order to unlock this strategic bottleneck in the analysis of life at high temperature.

Cell-to-cell signaling at high temperature: a virgin area

In their natural environment, microorganisms never live as isolated individuals nor in a purely insulated culture as they are mostly grown in laboratory conditions. Instead, they are part of complex microbial communities, form biofilms and mats, live in symbiosis, have to cope with competitors and the ever-changing environmental conditions of open systems, and, moreover, are constantly attacked by predators, parasites and viruses. This is no different for thermophiles, and therefore intercellular communication by means of small signal molecules must play a major role in the response of hot ecosystems to environmental changes.

Cell-to-cell signaling that leads to coordinated behavior of the population has been coined quorum sensing [89]. Mesophilic Gram-negative bacteria make use of *N*-acyl-homoserine lactones (AHLs) and a LuxR family member as signal molecules and signal receptors, respectively, whereas Gram-positive bacteria use oligopeptides as the most important quorum-sensing molecules [90–92]. Data on quorum sensing at high temperature are extremely scarce, but recently Kelly and his collaborators demonstrated cell density-dependent peptide signaling for the hyperthermophilic Gram-negative bacterium *Thermotoga maritima* [93, 94]. Small peptides are generally very thermostable and therefore appear better suited as signal molecules at high temperature than the AHLs typical of mesophilic Gram-negative bacteria. To the best of our knowledge, quorum sensing has not yet been investigated in thermophilic Archaea. Yet, intra- and interspecies microbial signaling is likely much more common and much more important in the establishment of microbial communities in their natural environment, including hot niches, than is currently realized. The reduced complexity of microbial communities thriving in hot environments and the lack of eukaryotic predators in such niches might even constitute a certain advantage to begin to reveal the complexity of microbial interactions. 'Avis aux amateurs'!

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