Insights into the evolution of mesophily from the bacterial phylum Thermotogae

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Running Title: Temperature adaptation in Thermotogae

Abstract: Thermophiles are extremophiles that grow optimally at temperatures > 45°C. In order to survive and maintain function of their biological molecules, they have a suite of characteristics not found in organisms that grow at moderate temperature (mesophiles). At the cellular level, thermophiles have mechanisms for maintaining their membranes, nucleic acids and other cellular structures. At the protein level, each of their proteins remains stable and retains activity at temperatures that would denature their mesophilic homologs. Conversely, cellular structures and proteins from thermophiles may not function optimally at moderate temperatures. These differences between thermophiles and mesophiles presumably present a barrier to evolutionary transitioning between the two lifestyles. Therefore, studying closely related thermophiles and mesophiles can help us determine how such lifestyle transitions may happen. The bacterial phylum Thermotogae contains hyperthermophiles, thermophiles, mesophiles and organisms with temperature ranges wide enough to span both thermophilic and mesophilic temperatures. Genomic, proteomic and physiological differences noted between other bacterial thermophiles and mesophiles are evident within the Thermotogae. We argue that the Thermotogae is an ideal group of organisms for understanding both the response to fluctuating temperature as well as long-term evolutionary adaptation to a different growth temperature range.

Key Words: lateral gene transfer, Kosmotoga, Mesotoga, thermostability, stress response

Introduction

Extremophiles are organisms that thrive under extreme environmental conditions unsuitable for survival of most other organisms. As such, they are of great interest for delineating the limits of conditions that permit life's existence, a key insight needed to advance efforts in the search for life on Earth and other planets (Pikuta *et al.* 2007; Rothschild and Mancinelli 2001). Additionally, due to their intrinsically "extreme" nature, these organisms are also desirable sources of enzymes and other biomolecules that function under conditions that render other organisms and their enzymes inactive. Such biomolecules may have a wide range of biotechnological and industrial applications from clean energy to bioremediation and carbon sequestration.

When examining temperature as a parameter that can either permit or exclude life, there are mesophiles, the organisms that grow optimally at moderate temperatures, and two types of extremophiles: psychrophiles, which grow optimally at temperatures below 15°C, and thermophiles, which grow optimally at temperatures above 45°C (Kimura *et al.* 2013). Within thermophiles, organisms growing optimally at > 80°C are commonly referred to as hyperthermophiles. Thermophiles are of particular interest due to their ability to withstand the denaturing effect of higher temperatures on biological molecules such as proteins and DNA (Li *et al.* 2005).

The phylogenetic position of the hyperthermophile-containing bacterial lineages Thermotogae, Thermodesulfobacteria and Aquificae at, or close to, the base of the 16S rRNA tree of life (Fig. 1), has been used as support for the hypothesis that the ancestor of the bacterial domain was a hyperthermophile (Achenbach-Richter *et al.* 1987). Similarly, thermophilic Archaea are also found at the base of the Archaeal domain (Fig. 1). Together with the proposed high temperature conditions of early Earth this led to the hypothesis that the last universal

common ancestor (LUCA) was a hyperthermophile (Pace 1991). A (hyper)thermophilic LUCA is also supported by experimental evidence from resurrection of ancestral nucleoside diphosphate kinases and characterizing their properties (Akanuma *et al.* 2013). Other lines of evidence, however, suggest that the LUCA may have been either a mesophile or a thermophile growing optimally below 80°C (Boussau *et al.* 2008; Brochier-Armanet and Forterre 2006). Whether the LUCA lived at the time of life's origin or much later remains debatable as well (Zhaxybayeva and Gogarten 2004).

Regardless of the optimal growth temperature of the LUCA, the ancestors of present day bacterial and archaeal lineages have had to modify their cellular structures and protein compositions to transition between mesophilic and thermophilic lifestyles (Boussau *et al.* 2008). Given the distribution of mesophiles and thermophiles on the Tree of Life (Fig. 1), we infer that such transitions likely happened independently multiple times. This same inference has been made based on multivariate analyses of the amino acid compositions of 279 prokaryotes (Puigbò *et al.* 2008) and from the different mechanisms of DNA supercoiling and the phylogeny of the involved genes (López-García 1999). This conjecture is also supported by reconstruction and synthesis of ancestral versions of enzymes and examining the optimal temperature at which they function. For example, examination of LeuB enzymes (3-isopropylmalate dehydrogenase) in the *Bacillus* genus suggests multiple transitions between thermophilic and mesophilic temperature optima when going forward in evolutionary time from the *Bacillus* ancestor (Hobbs *et al.* 2012). Therefore, thermophily has been lost and gained throughout the evolutionary history of the genus *Bacillus*. Similarly, analysis of extant and reconstructed ancestral *myo*-inositol-3-phosphate synthase enzymes from *Thermotoga* and Thermococcales suggests higher optimal growth temperatures of the ancestors (Butzin *et al.* 2013), indicating fluctuations of the tolerated

temperature ranges of these organisms throughout their evolutionary history. Together these studies imply that temperature adaptations may not be too difficult, and the growth temperature range may change rapidly and frequently in many lineages.

Temperature adaptation can be defined either as a response of an individual cell to changes in temperature, or as an evolutionary adaptation of an organismal lineage (such as 'species') to growth within a certain temperature range. To distinguish between the two, we will refer to temperature *response* for the former and temperature *adaptation* for the latter. These two phenomena are related, as selection acting on temperature *responses* may eventually lead to temperature *adaptations*. In this review we focus on organismal responses and lineage adaptations to moderate and high temperatures. For a review of adaptation to very low growth temperatures see Siddiqui *et al.* (2013). Specifically, we will discuss properties of thermophiles, and how these properties may relate to a transition between thermophily and mesophily, with a particular emphasis on the bacterial phylum Thermotogae.

The Thermotogae

Bacteria belonging to the Thermotogae phylum were first isolated by Karl Stetter and colleagues in 1986 from geothermally heated sea floors (Huber *et al.* 1986). Their name derives from the unique outer sheath-like structure that balloons over each end of the cell, known as the "toga" (Fig. 2) (Huber *et al.* 1986). There are 12 described genera in this phylum, most of which are thermophiles (Fig. 3). In the accepted taxonomy, these genera are all grouped in a single order, Thermotogales, and one family, Thermotogaceae. However, a reclassification of these bacteria into separate orders is overdue, and a division into three orders and four families has been

110 recently proposed (Bhandari and Gupta (2014); Fig. 3). While the new classification is based on
111 conserved indels, it is consistent with the 16S rRNA phylogeny (Fig. 3).
112 Thermotogae are anaerobes and organotrophs, capable of growing on a wide range of
113 complex substrates (Conners *et al.* 2006). They are found in hot ecosystems all over the world
114 including thermal springs, hydrothermal vents, and petroleum reservoirs (Huber and Hannig
115 2006; Ollivier and Cayol 2005), with some members growing at temperatures up to 90°C.
116 Although it was long thought that the Thermotogae only harbored thermophiles and
117 hyperthermophiles (11 of 12 genera are entirely composed of thermophiles or
118 hyperthermophiles) (Fig. 3), mesophilic Thermotogae from the genus *Mesotoga* have recently
119 been detected and isolated from cool hydrocarbon-impacted sites such as oil reservoirs and
120 polluted sediments (Ben Hania *et al.* 2011; Ben Hania *et al.* 2013; Nesbø *et al.* 2006b; Nesbø *et*
121 *al.* 2010; Nesbø *et al.* 2012). Interestingly, the closest relative of *Mesotoga*, *Kosmotoga olearia*,
122 has an unusually wide growth temperature range, which may have been important in *Mesotoga*'s
123 adaptation to low temperature (DiPippo *et al.* 2009; Nesbø *et al.* 2012).
124 As of May 2015, over 80 completed and ongoing Thermotogae genome projects
125 comprise 10 of the 12 described Thermotogae genera, with no genome projects for *Geotoga* nor
126 *Oceanotoga* (Benson *et al.* 2014; Reddy *et al.* 2014). The maximum divergence in the 16S rRNA
127 genes of these cultivated Thermotogae is ~25%, similar to what is observed for other bacterial
128 phyla (Konstantinidis and Tiedje 2005). For protein coding genes pairwise average amino acid
129 identity (AAI; Konstantinidis and Tiedje 2005) between genera ranges from 45 to 69% (average
130 49%). Phylogenetic analysis of environmental 16S rRNA gene sequences shows several novel
131 Thermotogae lineages without any cultivated members, and based on the nucleotide identity they
132 would be classified as new genera (Nesbø *et al.* 2010). Thus, as with most microbial lineages,

there is a large unknown diversity of Thermotogae. At least four of these new lineages have only been detected in low temperature environments (as low as 9.5°C), suggesting that Thermotogae might be common in mesothermic environments. Interestingly, on the phylogenetic tree these likely mesophilic lineages fall within multiple thermophilic clades (Nesbø *et al.* 2010), suggesting several independent adaptations to lower temperatures.

With mesophilic Thermotogae only recently discovered, the functional characterization of this phylum has focused on thermophiles, mainly the hyperthermophilic organisms *Thermotoga maritima* and *Thermotoga neapolitana*. Protein crystal structures have also been experimentally determined for a large portion of the *T. maritima* proteome (DiDonato *et al.* 2004; Lesley *et al.* 2002), and the protein structures of its central metabolic networks were modeled by Zhang *et al.* (2009). Complimented with models of high temperature hydrogen and sulfur metabolism (Cappelletti *et al.* 2014; Schut *et al.* 2012), this wealth of functional information makes the Thermotogae a promising microbial lineage for industrial and biotechnological applications. For example, most Thermotogae produce hydrogen that may be harvested (e.g., Nguyen *et al.* (2008) and Maru *et al.* (2012)). The hydrogen production of *T. maritima* can be boosted via metabolic engineering, as was demonstrated by an *in silico* re-design of its metabolism (Nogales *et al.* 2012). Additionally, while the degradation of sugars by many Thermotogae results in the production of CO_2 and acetate, *T. neapolitana* has been shown to convert these by-products to lactic acid when grown in a CO_2 atmosphere, a process suggested to have potential in carbon capture (D'Ippolito *et al.* 2014).

Carbohydrate utilization by *T. maritima* has been examined by studying the substrate specificities and affinities of its carbohydrate transporters (Boucher and Noll 2011; Cuneo *et al.* 2009; Ghimire-Rijal *et al.* 2014; Nanavati *et al.* 2005; Nanavati *et al.* 2006) and their

transcriptional regulation in response to growth on different saccharides (Frock *et al.* 2012). Information about substrate specificities, enzymatic activities and catalytic mechanisms of many of *T. maritima*'s glycoside hydrolases are also available (Arti *et al.* 2012; Comfort *et al.* 2007; Kleine and Liebl 2006), which has been used, for instance, to engineer an alpha-galactosidase from *T. maritima* into an efficient alpha-galactosynthase (Cobucci-Ponzano *et al.* 2011). The transcriptional regulation of glycoside hydrolases and other carbohydrate metabolism-related genes in response to growth on various carbohydrates highlights the differences in carbohydrate utilization, even between closely related Thermotogae lineages (Chhabra *et al.* 2002; Chhabra *et al.* 2003; Frock *et al.* 2012). Moreover, interconnections exist between sugar regulons in *T. maritima*'s carbohydrate utilization network, suggesting coordinated regulatory responses to particular types of complex carbohydrates (Rodionov *et al.* 2013). This rich knowledge base will be very useful in comparative studies of thermophilic and mesophilic Thermotogae lineages and, ultimately, will lead to understanding processes leading to shifts in an organism's growth temperature range.

General cellular adaptations to thermophily

Regardless of whether cells are responding to transient temperature increases within their growth range or evolving to an alternate growth range, changes in temperature require major modifications across the cell to optimize cell function and growth. The following sections discuss some of these temperature responses and adaptations in microbial cells.

The effect of temperature on cellular membranes: maintaining a fluid envelope

178 The cell membrane is critical to cell function since it maintains and separates the interior cell
179 environment from the exterior environment. In order to serve its function, a lipid membrane must
180 be impermeable to most solutes and maintain a liquid crystalline phase, even under stress (de
181 Mendoza 2014). As the temperature increases, membrane integrity and impermeability become
182 compromised, which eventually results in cell death (Chang 1994). Therefore, thermophiles must
183 maintain their membranes under conditions that could inactivate those of mesophiles. Bacteria
184 and Archaea handle this challenge differently due to the dissimilar structures of their membrane
185 lipids (reviewed in detail by Oger and Cario (2013), Koga and Morii (2005), Koga (2012), and
186 Mansilla *et al.* (2004)). We will only focus on bacterial lipids here. For a review on archaeal lipids
187 see Oger and Cario (2013).

188 Bacterial polar membrane lipids consist mainly of straight-chain fatty acids that are bound
189 to the polar head group predominantly by ester linkages (Koga and Morii 2005). Bacteria respond
190 to various temperatures by altering the composition (length, degree of branching and degree of
191 unsaturation) of their fatty acid chains to maintain membrane fluidity (Mansilla *et al.* 2004; Zhang
192 and Rock 2008). The types of fatty acids bacteria are able to produce will therefore influence the
193 temperature range within which they can grow. For example, hyperthermophilic Thermotogae
194 have unusual membrane-spanning diabolic fatty acids in their membrane, which are thought to be
195 an adaptation to high temperature growth (Carballeira *et al.* 1997; Damsté *et al.* 2007). In
196 agreement with this hypothesis, these diabolic fatty acids are not found in the membranes of the
197 mesophilic *Mesotoga prima* (Nesbø *et al.* 2012). Moreover, *M. prima* (grown at 35°C) contained
198 branched, mono-unsaturated and saturated fatty acids, while *K. olearia* (grown at 55°C) contained
199 only saturated fatty acids (Nesbø *et al.* 2012). Fatty acid composition is also part of the immediate
200 cold-shock response with genes involved in production of, for instance, branched fatty-acids

being up-regulated in the thermophile *Thermoanaerobacter tengcongensis* when grown at sub-optimal temperatures (Liu *et al.* 2014). Increase of branched fatty acids is a common response to lower temperatures (Suutari and Laakso 1994), and in *Listeria monocytogenes* this is due to temperature-dependent substrate selectivity of FabH, the enzyme responsible for the first condensation reaction in fatty acid biosynthesis (Singh *et al.* 2009). Interestingly, in *Bacillus* a transmembrane two-component response regulator, which controls the desaturase that introduces double bonds in preexisting fatty acids, senses changes in membrane fluidity and not the actual temperature changes (de Mendoza 2014).

In addition to the lipid structure of cell membranes, integral membrane proteins affect the temperature tolerance of an organism (Thompkins *et al.* 2008). Therefore, while the lipid composition of the membrane is crucial for its function, integral membrane proteins may also play a significant role, particularly with respect to the temperature limit of an organism's growth range. For example, mutations of integral membrane proteins of the DedA family cause temperature sensitivity and cell division defects in *Escherichia coli* (Thompkins *et al.* 2008). Interestingly, proteins from the DedA family have been shown to be essential in at least two bacterial species (*E. coli* and *Borrelia burgdorferi*), but their homologs are not detected in several thermophilic and hyperthermophilic Thermotogae genomes (Doerrler *et al.* 2013). This suggests that the function provided by DedA is either not needed by these organisms, or is being provided by analogous integral membrane proteins, or that their DedA homologs are too divergent to be detected by sequence similarity searches.

Nucleic acids: a challenge to keep the strands together

High temperatures denature double stranded DNA and secondary structures of RNA. This presents a problem for thermophiles, and for hyperthermophiles in particular. These organisms must maintain their chromosomes in an orderly state for both efficient packaging as well as coordinated gene expression. Therefore, to survive the damaging effects of high temperature thermophiles need to either continuously repair their damaged DNA or protect it from damage in the first place. For example, the archaeon *Pyrococcus abyssi* has a highly efficient DNA repair system that continuously repairs temperature-induced DNA damage (Jolivet *et al.* 2003). Very high levels of homologous recombination are observed in hyperthermophilic *Thermotoga* spp. where the ratio of nucleotide changes introduced by recombination relative to point mutation (*r/m*) is in the range 24-100 for genomes originating from geographically distant sites (Nesbø *et al.* 2006a; Nesbø *et al.* 2014). This in the upper range of values reported in a comparison of *r/m* across a large sample of mostly mesophilic Bacteria and Archaea (0.02 – 64), where values above 10 were interpreted as very high (Vos and Didelot 2009). The high level of recombination may be explained by the need for DNA repair in thermophiles (Johnston *et al.* 2014). This hypothesis is supported by observations of high levels of recombination and repair in other hyperthermophilic microorganisms, such as *Pyrococcus furiosus* (DiRuggiero *et al.* 1997), *Sulfolobus islandicus* (Whitaker *et al.* 2005), and *Persephonella* (Mino *et al.* 2013).

Protection of DNA is known to occur via multiple unrelated mechanisms. Primarily, thermophiles safeguard their DNA with thermostable proteins analogous to eukaryotic histones. For example, in the archaeon *Thermococcus kodakaraensis* HpkA and HpkB dramatically increase the melting temperature of a given DNA sequence upon binding, with HpkB being able to raise the melting temperature of poly(dA-dT) DNA by > 20°C (Higashibata *et al.* 1999), suggesting that these proteins play a major role in the stabilization of *Thermococcus*

246 *kodakaraensis* chromosomes. In the bacterium *T. maritima* the histone-like protein HU stabilizes
247 and protects the DNA (Mukherjee *et al.* 2008).

248 Thermophiles can also use polyamine compounds to stabilize their DNA and RNA, as
249 well as many other cellular components. Multivalent polyamine compounds such as putrescine,
250 spermidine, and spermine, or their acetylated forms, compact histone-bound DNA in
251 *Thermococcus kodakaraensis*, stabilizing it at temperatures as high as 90°C (Higashibata *et al.*
252 2000). In *Thermotoga* species the polyamines caldopentamine and caldohexamine increase in
253 concentration with increased temperature, suggesting a role in thermal response and thermal
254 adaptation (Zellner and Kneifel 1993). Indeed caldopentamine and caldohexamine, as well as five
255 other long linear polyamines found in *Thermus thermophilus*, have been shown to stabilize
256 double-stranded DNA at high temperature, with a greater stabilizing effect by polyamines with a
257 larger number of amino nitrogen atoms (Terui *et al.* 2005).

258 Thirdly, unique RNA modifications can confer thermostability in thermophiles
259 (McCloskey *et al.* 2001). For example, modifications from adenosine to 2'-*O*-methyladenosine or
260 from guanosine to N^2,2'-*O*-dimethylguanosine in the tRNAs are often growth temperature-
261 specific, even among closely related lineages (McCloskey *et al.* 2001).

262 Lastly, thermal adaptation may be achieved via reverse gyrase-mediated DNA
263 supercoiling. Reverse gyrase is a protein found almost exclusively in hyperthermophiles and,
264 importantly, it is a gene carried by all known hyperthermophiles (Brochier-Armanet and Forterre
265 2006; Forterre 2002; Lulchev and Klostermeier 2014). While deletion of the reverse gyrase gene
266 from *Thermococcus kodakaraensis* results in slower growth at high temperatures (90°C), it does
267 not abolish its growth, suggesting that this enzyme is not essential for hyperthermophilic growth
268 as was once thought (Atomi *et al.* 2004). However, since the *T. kodakaraensis* mutant lacking

reverse gyrase grew poorly at 90°C, and unlike the wild-type strain, could not grow above 90°C (Atomi *et al.* 2004), this enzyme is still considered to be a critical adaptation for *optimal* growth at high temperatures (Brochier-Armanet and Forterre 2006). Although reverse gyrase catalyzes ATP-dependent positive supercoiling of DNA *in vitro*, its function *in vivo* remains unknown. The increased heat protection provided by this enzyme may be linked to a role in the DNA damage response, possibly through recruitment to lesions (Lulchev and Klostermeier 2014; Perugino *et al.* 2009). Interestingly, cultivated hyperthermophilic species from both the Thermotogae and the Aquificae have acquired their reverse gyrase genes from Archaea by lateral gene transfer (LGT), suggesting that hyperthermophily may have been acquired by Bacteria *from* Archaea (Brochier-Armanet and Forterre 2006; Forterre *et al.* 2000).

While some of these adaptations for nucleic acid stabilization have only been found in thermophiles (e.g., reverse gyrase (Forterre 2002), certain RNA modifications (McCloskey *et al.* 2001) and thermostable histones (Higashibata *et al.* 1999)), others are found in mesophiles as well. For instance, the same polyamines found in *Thermotoga* are also found in mesophilic microalgae (Nishibori *et al.* 2009). Hence, transition between thermophily and mesophily may only require a re-purposing of certain cellular constituents, rather than removing or acquiring them.

In addition to cellular components interacting with nucleic acids for stabilization, the composition of some nucleic acids appears adapted to the thermophilic lifestyle of the host organism. The extra hydrogen bond in G:C nucleotide pairs was long thought to play a part in optimal growth temperature. While genome-wide G+C content does not correlate with optimal growth temperature (Galtier and Lobry 1997; Hurst and Merchant 2001; Zeldovich *et al.* 2007), the G+C content of some structural RNA encoding genes does. For example, the G+C content of secondary structures of rRNA and tRNA molecules, specifically in the stem structures, increases

with optimal growth temperature (Galtier and Lobry 1997; Kimura *et al.* 2013; Zhaxybayeva *et al.* 2009). As a result, the GC content variation of the 16S rRNA gene can be used as a proxy for studying temperature adaptation within the Thermotogae. For example, the temperature optimum for uncultured members of the phylum was predicted by establishing a correlation between the 16S rRNA gene distances and optimal growth temperature of 33 Thermotogae isolates (Dahle *et al.* 2011). Additionally, inference of the ancestral states of the 16S rRNA gene that gave rise to 30 Thermotogae isolates allowed Green *et al.* (2013) to hypothesize that the thermotolerant Thermotogae lineages are under directional selection and that transition from high to low optimal growth temperature is easier to achieve.

Compatible solutes: the power of redundancy

Compatible solutes are organic compounds that are accumulated by cells under stressful conditions such as osmotic stress and heat stress (Santos *et al.* 2011). These compounds, particularly polyamines, are known to stabilize nucleic acids in thermophilic cells (see above). Moreover, in the bacterium *Calderobacterium hydrogenophilum* polyamine compounds stabilize the 70S initiation complex of ribosomes (Mikulik and Anderova 1994). Many temperature studies in the Thermotogae have focused on the accumulation of these organic compounds and polyamines and the elucidation of their biosynthetic pathways in *T. maritima* and the more moderate thermophile *Petrotoga miotherma* (Jorge *et al.* 2007; Oshima *et al.* 2011; Rodionova *et al.* 2013; Rodrigues *et al.* 2009; Zellner and Kneifel 1993). Several compatible solutes have so far only been found in thermophiles including di-*myo*-inositol phosphate, mannosyl-di-*myo*-inositol phosphate, mannosylglyceramide, and diglycerol phosphate (Borges *et al.* 2010; Gonçalves *et al.* 2012) and novel thermophilic solutes continue to be identified (Jorge *et al.* 2007; Rodrigues *et al.*

2009). However, while these compounds are thermophile-specific and may represent thermophile-specific adaptations, they are not the only compatible solutes used to deal with heat stress. When the ability to synthesize di-*myo*-inositol phosphate was removed from *Thermococcus kodakarensis* by deleting a key synthesis gene, the growth of this archaeon was unaffected, and aspartate accumulated as an alternative compatible solute (Borges *et al.* 2010). In the Thermotogae multiple solutes accumulate under stress conditions (Jorge *et al.* 2007; Rodrigues *et al.* 2009). This suggests that although the role compatible solutes play in thermal protection is not fully understood, there is functional redundancy among the solutes.

Protein dynamics and turnover; assistance from chaperones and proteases

Chaperones are large protein complexes that assist the proper folding and re-folding of proteins. The chaperonins represent an extensively studied subclass of chaperones with a stacked double-ring structure (Large *et al.* 2009). Distribution of the chaperone families varies across Bacteria and Archaea, and some chaperones are considered indispensable (Large *et al.* 2009). For example, some chaperonins help fold new polypeptides, as well as re-fold and rescue proteins that have been inactivated due to stress (Techtmann and Robb 2010). A major stressor that triggers chaperone-mediated protein repair is heat shock, which has resulted in many chaperones being named heat shock proteins (HSP) (Large *et al.* 2009). By preventing inactivation and aggregation of proteins at high temperatures, this ubiquitous system is thought to be especially important in thermophiles, which employ chaperones in both unstressed and heat-stressed states (Pysz *et al.* 2004). Thus, while these proteins are part of high temperature *response* in mesophiles, their constitutive expression in thermophiles may be part of their temperature adaptation. For example, the predicted chaperone TM1083 in *T. maritima* is thought to stabilize the DNA gyrase enzyme at temperatures near optimal

338 growth (Canaves 2004). Moreover, the molecular chaperone trigger factor (TM0694) from *T.*
339 *maritima* strongly binds model proteins and decreases their folding rate, while these activities are
340 much weaker in the homologous trigger factor from the psychrophile *Pseudoalteromonas*
341 *haloplanktis*, which instead shows increased prolyl isomerization (Godin-Roulling *et al.* 2014).
342 However, it should be noted that chaperones, although always highly expressed in thermophiles, are
343 part of their high temperature *response* as well. For instance, examination of the *T. maritima*
344 proteome at four temperatures spanning its growth range revealed higher relative abundance of
345 chaperones at supra-optimal temperatures (Wang *et al.* 2012).
346 Proteases are also part of the heat shock response in mesophilic organisms (Richter *et al.*
347 2010). A key distinction between well-studied bacterial mesophiles and the hyperthermophile *T.*
348 *maritima* is the lack of regulation in *T. maritima* of most of its proteases in response to
349 temperature stress (Conners *et al.* 2006). This may be explained by an absence of major
350 regulators of the mesophilic proteolytic response (i.e., rpoH or ctsR homologs) in the *T. maritima*
351 genome (Conners *et al.* 2006; Pysz *et al.* 2004). Perhaps this bacterium gains a survival
352 advantage from constitutive expression of most proteases. A similarity search revealed an
353 absence of detectable rpoH and ctsR homologs in 38 Thermotogae, including the thermophilic *K.*
354 *olearia* and the mesophilic *M. prima*, suggesting that any regulation of protease expression in the
355 Thermotogae involves different genes than those used by other Bacteria and Archaea.

356

357 **Thermal adaptation at the protein level**

358 Although chaperones aid in proper folding and maintenance of proteins under high temperature
359 conditions, proteins from thermophilic organisms are themselves adapted to high temperature.
360 This adaptation is required to maintain activity at temperatures that would denature mesophilic

361 homologs and is found at all levels of protein structure, from primary through quaternary. Protein
362 thermostability is also not uniform across the proteome and depends on its functional role:
363 proteins either having catalytic activity or regulating other catalytic proteins appear to be under
364 greater selection to be temperature adapted than proteins involved in, for example, core
365 transcriptional or translational processes (Gu and Hilser 2009).

366 While there are many examples of specific thermostabilizing characteristics and
367 interactions at each of the four levels of globular protein structure (reviewed by Imanaka (2011)
368 and Li *et al.* (2005)), there is no universal property that confers thermostability. Rather, it is the
369 combination of factors at all levels of structure that grants high temperature activity in globular
370 proteins. Increased thermostability is often due to slight differences in sequence and structure, and
371 thermophilic and mesophilic counterparts are typically very similar proteins (Taylor and Vaisman
372 2010). Below we briefly overview known pathways to temperature adaptation in globular proteins.

373 Protein primary structure is the amino acid sequence of the polypeptide chain. Ultimately,
374 the properties and sequence of the amino acids determine the final higher level structures of the
375 protein. One characteristic associated with thermostable proteins is enrichment of amino acids that
376 contribute to a strong hydrophobic core. Larger aliphatic amino acids with more branches are
377 favored at positions that fill cavities, which may ultimately strengthen the protein through
378 increased hydrophobic interactions (Clark *et al.* 2004). Taylor and Vaisman (2010), however,
379 found that it is only a moderately good indicator of protein thermostability.

380 Comparisons of amino acid composition of thermophilic and mesophilic proteins have
381 revealed several trends at the global proteome level. The observed excess of charged (D,E,K,R)
382 versus polar (N,Q,S,T) amino acids in soluble proteins from hyperthermophiles, known as the
383 CvP bias (Cambillau and Claverie 2000; Gao and Wang 2012; Holder *et al.* 2013; Suhre and

Claverie 2003), may reflect larger importance of ionic interactions between charged amino acids over hydrogen-bond interactions for retaining protein structure as temperature increases (Cambillau and Claverie 2000). Additionally, a systematic evaluation of all possible subsets of amino acids revealed that the total fraction of the amino acids IVYWREL in a proteome most strongly correlates with optimal growth temperature (Zeldovich *et al.* 2007).

The CvP and IVYWREL biases have been explored thoroughly in the Thermotogae where both indices show strong linear correlations with optimal growth temperature (Zhaxybayeva *et al.* 2009). Specifically, the distribution of CvP values was unimodal for each of the Thermotogae proteomes, arguing against the hypothesis that thermophily is a recently acquired trait of the Thermotogae. Moreover, calculation of CvP values from estimated ancestral Thermotogae sequences suggested that the ancestral Thermotogae proteome belonged to organisms with an optimal growth temperature of ≈84.5°C, higher than that of any characterized extant Thermotogae bacterium (Zhaxybayeva *et al.* 2009). While the average CvP value for most of the thermophilic Thermotogae lineages was above 10.62, the mesophilic *M. prima* proteome has an average CvP value of 8.96 (Zhaxybayeva *et al.* 2012). Also this genome has a unimodal CvP distribution, suggesting it has maintained a mesophilic lifestyle for a long time. An exception to the trend is observed in the *P. lettingae* genome, which has an average CvP value of 8.42 (Zhaxybayeva *et al.* 2009), but an optimal growth temperature of 65°C. However, *P. lettingae*-like 16S rRNA genes and genomic DNA have been recovered from environments with temperatures < 65°C (e.g., 40-50°C, (Nesbø *et al.* 2010; Nobu *et al.* 2014)), suggesting that these bacteria often live at temperatures below the optimal growth temperature of the cultivated isolate.

Protein secondary structure describes the local folding of polypeptide sequences. This includes regular structures like α-helices and β-sheets, or irregular structures like β-turns, coils

and loops. These are formed primarily by hydrogen bond interactions between the backbone and side chain elements of the amino acids. In addition to having secondary structures that facilitate tighter packing and rigidity at the tertiary level, thermophilic proteins tend to have secondary structures that are more stabilized than their mesophilic counterparts (Facchiano *et al.* 1998; Koga *et al.* 2008; Prakash and Jaiswal 2010). For example, thermostable proteins have been reported to have a larger fraction of their amino acid residues arranged in α-helices than mesophilic proteins do (Prakash and Jaiswal 2010).

Protein tertiary structure is the arrangement of a folded polypeptide chain in three-dimensional space. This is achieved by disulfide bridges, electrostatic interactions within the polypeptide chain, and hydrophobic interactions and hydrogen bonding within the chain as well as between the peptides and solvent. Thermophilic proteins tend to have conformations that are more rigid and more tightly packed, with reduced entropy of unfolding and conformational strain compared to their mesophilic counterparts (Li *et al.* 2005). The strongest contributors to thermostability are increased ion pairs on the protein surface combined with a more strongly hydrophobic interior (Taylor and Vaisman 2010). In agreement with this, additional salt bridges on the surface of the enzyme diguanylate cyclase from *T. maritima* accounted for its greater thermostability compared to the same enzyme found in the mesophiles *Pseudomonas aeruginosa*, *Marinobacter aquaeolei* and *Geobacter sulfurreducens* (Deepthi *et al.* 2014). Additionally, the glutamate dehydrogenase enzymes of the hyperthermophilic bacterium *T. maritima* and hyperthermophilic archaeon *P. furiosus* have smaller hydrophobic accessible surface area (ASA) and greater charged ASA than the glutamate dehydrogenase from the mesophilic bacterium *Clostridium symbiosum* (Knapp *et al.* 1997). Since few other structural differences were found

between the thermophilic and mesophilic enzymes, this tighter packing is thought to contribute to the thermal stability of the proteins.

Protein quaternary structure is the arrangement of multiple folded polypeptide chains into a multimeric complex. In globular proteins this level of structure is formed and maintained by many of the same forces that contribute to the tertiary structure of a protein, but *between* polypeptide chains rather than *within* them. These forces include disulfide bridges, electrostatic interactions, hydrophobic interactions and hydrogen bonding. In thermostable proteins, greater numbers of these interactions, or stronger interactions over weaker ones, are favored (Li *et al.* 2005).

One additional way of achieving greater protein stability is to increase the number of subunits. For example, the malate dehydrogenase (MDH) enzyme, which is usually a dimer in mesophiles, is a tetramer in the thermophilic bacterium *Chloroflexus aurantiacus* (Bjørk *et al.* 2003). The additional dimer-dimer interface of the tetrameric MDH is hypothesized to provide thermal stability due to the higher number of inter-polypeptide interactions compared to the mesophilic dimers. To test this hypothesis, Bjørk *et al.* (2003) introduced a disulfide bridge that would strengthen dimer-dimer interaction further, and found that the new enzyme had a melting temperature 15°C higher than the wild-type enzyme. In addition, removing excess negative charge at the dimer-dimer interface by replacing a glutamate residue with either glutamine or lysine resulted in an increase of apparent melting temperature by ~ 24°C (Bjørk *et al.* 2004).

Tolerating new temperatures: Is it possible to modify just a few proteins?

As discussed above, adaptation to a high optimal growth temperature is achieved differently by Bacteria and Archaea, by one species than another, and even by one protein than another within

452 the same organism. Given that all of these factors combine in unique ways to permit growth
453 within a specific temperature range, how could a shift in permissive temperature range be
454 accomplished? While some of these strategies are universal to thermophiles and mesophiles, such
455 as utilization of chaperones and compatible solutes, others, like shifting of membrane properties,
456 would have to be radically altered to accommodate large changes in temperature range.
457 Changing a few key proteins may have global stabilizing effects on the whole cell. For
458 instance, some of the proteins whose stability appears most affected by thermal adaptation are
459 involved in production of compatible solutes that stabilize other proteins (Gu and Hilser 2009).
460 Such changes would reduce the need to modify the stability of *all* components of the proteome. It
461 may also be possible to lower the maximal growth temperature of an organism through changes to
462 a single protein (Endo *et al.* 2006). By replacing the chromosomal copy of *groEL* chaperonin in
463 *Bacillus subtilis* 168 (growth range from 11 to 52°C) with a psychrophilic *groEL* from
464 *Pseudoalteromonas* sp. PS1M3 (growth range from 4 to 30°C), Endo and colleagues noted a 2°C
465 reduction in the maximal growth temperature of the newly constructed *B. subtilis* strain. Similarly,
466 the heterologous expression of a small heat shock protein from *Caenorhabditis elegans*, enabled *E.*
467 *coli* cells to grow at temperatures up to 50°C (and survive heat shock at 58°C for 1/2h) extending
468 its growth range by 3.5°C (Ezemaduka *et al.* 2014). While these changes do not constitute true
469 shifts in growth temperature range or changes to optimal growth temperature, these studies
470 suggest that changes to a single key protein (involved both in temperature adaptation and
471 response) could extend or narrow the temperature range at which an organism is able to grow by a
472 few degrees. Accumulation of several such mutations could eventually lead to a more substantial
473 shift in growth range. Some of these mutations may be advantageous at lower temperatures, while
474 others may be loss-of-function mutations, where abilities to function at higher temperatures are

lost for proteins in individuals living in an environment with temperatures at the lower end of their original growth range. Under the latter scenario, change in the growth temperature range might not be a result of selection, but rather a product of random genetic drift or genetic hitchhiking with another, unrelated trait selected for in the new environment.

Role of Lateral Gene Transfer in Temperature Adaptation: Acquisition of Already 'Adapted' Genes

Lateral gene transfer (LGT) is a major force in prokaryotic evolution, allowing rapid adaptation to changes in the environment by acquiring clusters of genes or single genes that confer a selective advantage (Boucher *et al.* 2003; Zhaxybayeva and Doolittle 2011) and LGT has been implicated in adaptation to extreme environments including high temperatures (see for example Omelchenko *et al.* (2005)). Genes encoding proteins linked to adaptation to higher or lower growth temperatures have been laterally exchanged (reviewed in Boucher *et al.* 2003). Reverse gyrase is a classic example of lateral transfer of a single gene that is thought to have been crucial for evolutionary adaptation to high temperatures by hyperthermophilic Bacteria (Brochier-Armanet and Forterre 2006; Forterre 2002). Phylogenetic analyses suggest two ancient acquisitions of this gene by bacterial lineages from Archaea, followed by secondary transfer events among Bacteria (Brochier-Armanet and Forterre 2006).

Similarly, the compatible solute di-*myo*-inositol phosphate is thought to be important for heat tolerance in thermophiles and hyperthermophiles (Borges *et al.* 2010). Two key genes involved in the synthesis of this compound (inositol-1-phosphate cytidylyltransferase and di-*myo*-inositol phosphate phosphate synthase) are suggested to have been laterally transferred from an archaeal lineage to hyperthermophilic marine *Thermotoga* species, while in other lineages the two

genes are predicted to have fused before being exchanged among several bacterial and archaeal lineages (Gonçalves *et al.* 2012).

Reverse gyrase and the *myo*-inositol pathway genes are just two examples of a large number of genes transferred into the Thermotogae. When the genome of *T. maritima* MSB8 was first sequenced (Nelson *et al.* 1999), 24% of its open reading frames (ORFs) showed greatest similarity to sequences from Archaea, suggesting that these genes have been acquired from these distantly related organisms that inhabit the same environment. Comparative genomic analyses of additional Thermotogae genomes have confirmed an influx of genes from Archaea (albeit the total number dropped to 10-11% of the ORFs, due to increased number of bacterial homologs in GenBank) and an even larger fraction of Firmicutes genes in these genomes (Mongodin *et al.* 2005; Nesbø *et al.* 2009; Zhaxybayeva *et al.* 2009; Zhaxybayeva *et al.* 2012). Phylogenetic analysis of all the ORFs in the *M. prima* genome suggests this lineage has undergone extensive gene exchange with diverse mesophilic lineages, and that LGT has aided its transition from a thermophilic to a mesophilic lifestyle (Zhaxybayeva *et al.* 2012). Thus, as a major force that has shaped the genomes of the Thermotogae, LGT may have also been important for the acquisition and development of the temperature ranges of the various Thermotogae lineages. Most of the acquired genes in Thermotogae (including *M. prima*) are involved in carbohydrate metabolism (Mongodin *et al.* 2005; Nesbø *et al.* 2009; Zhaxybayeva *et al.* 2009; Zhaxybayeva *et al.* 2012). However, *M. prima* has additionally acquired genes involved in signal transduction mechanisms, secondary metabolite biosynthesis, and amino acid transport and metabolism (Zhaxybayeva *et al.* 2012), suggesting the potential importance of genes from these functional categories for life at lower temperatures.

Transition to mesophily in *Kosmotoga* and *Mesotoga*

The discovery of the mesophilic Thermotogae lineage (*Mesotoga*) raised the possibility that (hyper)thermophily was not ancestral to the phylum. However, as discussed above, the amino acid composition (CvP bias and IVYWREL amino acids frequency) of the reconstructed ancestral Thermotogae proteome suggests that the ancestral Thermotogae was a hyperthermophile (Zhaxybayeva *et al.* 2009), and that the transition to mesophily in the Thermotogae phylum is secondary. Moreover, ancestral sequence reconstruction of *myo*-inositol-3-phosphate synthase enzymes in the *Thermotoga* genus also suggests that the ancestor of this hyperthermophilic lineage grew optimally at temperatures higher than those of extant species (Butzin *et al.* 2013). The G+C content of ribosomal RNA, which correlates with optimal growth temperature, also suggests that the reconstructed 16S rRNA of the ancestor of all Thermotogae belonged to a thermophile (Green *et al.* 2013; Zhaxybayeva *et al.* 2009).

So far, the genus *Mesotoga* is the only strictly mesophilic Thermotogae, with optimal growth occurring between 37 and 45°C (Ben Hania *et al.* 2013; Nesbø *et al.* 2012). Initially *Mesotoga* spp. were only detected using molecular tools such as community 16S rRNA PCR and metagenome analyses (Nesbø *et al.* 2006b). *Mesotoga prima* was the first described isolate of the genus (Nesbø *et al.* 2012), which now includes another validated species, *Mesotoga infera*, (Ben Hania *et al.* 2013), one yet to be validated, *Mesotoga sp.* PhosAc3 (Ben Hania *et al.* 2011), and several isolates with ongoing genome sequencing projects (Benson *et al.* 2014; Reddy *et al.* 2014). The 2.97 Mb genome of *M. prima* is considerably larger than any previously sequenced Thermotogae genome, which range between 1.86 and 2.30 Mb (Zhaxybayeva *et al.* 2012). This larger size is due to both higher numbers of protein-coding genes and larger intergenic regions. A unimodal distribution of CvP values of *M. prima*'s proteome, with a mean value in the

mesophilic range, indicate that native *M. prima* proteins have also changed in response to its evolved mesophilic lifestyle (Zhaxybayeva *et al.* 2012).

Analysis of additional Thermotogae shows that the variation in size may be related to optimal growth temperature: thermophiles have more streamlined genomes, with little intergenic space and a higher number of genes per transcription unit, while mesophiles have larger intergenic spaces and higher gene redundancy (Latif *et al.* 2013; Zhaxybayeva *et al.* 2012). This finding holds true for lineages outside of the Thermotogae, as examination of 1155 prokaryotes demonstrates (Sabath *et al.* 2013). However, the observed correlation in Thermotogae needs to be untangled from effects of phylogenetic history (Zhaxybayeva *et al.* 2012).

The closest relative of the *Mesotoga* lineage is the thermophilic lineage *Kosmotoga* (Fig. 3). Members of this genus have been found in hydrothermal sediments (L'Haridon *et al.* 2014; Nunoura *et al.* 2010) and oil production fluids (DiPippo *et al.* 2009; Feng *et al.* 2010). Like other thermophilic Thermotogae, the *Kosmotoga* are anaerobic chemoorganotrophs able to ferment carbohydrates and peptides (Nunoura *et al.* 2010) and to produce molecular hydrogen (DiPippo *et al.* 2009; Feng *et al.* 2010). The first isolated bacterium of this genus was *Kosmotoga olearia* (DiPippo *et al.* 2009). *K. olearia* grows optimally at 65°C and has a reported growth range of 20-80°C (DiPippo *et al.* 2009). Not only is this bacterium capable of growing at an unusually low temperature for a thermophile, but to our knowledge it represents the widest reported bacterial temperature growth range to date.

The ability of *Kosmotoga* to grow at such an extraordinary gamut of temperatures is intriguing for two reasons. First, it must maintain protein activity and membrane integrity. Every living organism has adapted to do this at a certain temperature range, but how these requirements can be maintained over a 60°C range is unknown. What evolutionary mechanisms would *maintain*

a 60°C growth interval in *Kosmotoga*? Perhaps this lineage continues to experience environments with more variable temperatures or, alternatively, the wide growth range may be a result of selection on another trait. Second, as discussed above, this ability of tolerating a wide range of temperature conditions, may have facilitated the transition of *Mesotoga* from thermophily to mesophily, because the capacity to grow at lower temperatures presumably already existed in *Mesotoga*'s ancestors.

As a result *Kosmotoga* and *Mesotoga* offer a unique model system for studying both immediate temperature responses and long-term temperature adaptation. Specifically, *K. olearia'*s exceptionally wide growth range allows examination of temperature responses under both mesothermic and thermic conditions in the same cell-line. For example, analysis of *K. olearia'*s transcriptome at different growth temperatures promises to shed light into the role of specific processes, functions, genes or proteins in thermoadaptation. Since *K. olearia'*s closest relative is a mesophile with a narrower growth range, comparative genomic, transcriptomic and proteomic analyses promise to reveal how *Kosmotoga*'s temperature responses may eventually lead to temperature adaptation. If we assume that *Mesotoga* and *Kosmotoga*'s common ancestor was a thermophile, possibly with a wide growth range, then the *Mesotoga* lineage lost its ability to grow at high temperatures, while *Kosmotoga* has either kept or expanded its growth range. For *Mesotoga* we have speculated that reduction of its growth temperature range may have happened as the lineage got 'trapped' in an oil reservoir that cooled down (Nesbø *et al.* 2006b; Zhaxybayeva *et al.* 2012) and therefore may be a result of loss-of-function mutations and genetic drift.

The existence of several additional Thermotogae lineages likely thriving in mesothermic environments (Nesbø *et al.* 2010) opens opportunities to study the evolutionary processes in lineages that have adapted to lower temperatures independently. These novel lineages can be

590 accessed through metagenomic studies or through further cultivation efforts. Taken together,
591 future genomic, transcriptomic and proteomic studies of temperature responses and adaptations
592 in *Kosmotoga, Mesotoga,* and other Thermotogae will help decipher how shifts in temperature
593 range and optimum are accomplished.
594

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Figure Legends:

Fig. 1. Distribution of organismal growth temperature adaptation across the three domains of life. Only major lineages with cultivated members (phyla for Bacteria and Archaea and supergroups for Eukarya) are shown. Most lineages contain organisms thriving at different temperature optima, suggesting that adaptation to temperature has happened multiple times independently. Given the uncertainty associated with the relationships among the shown taxonomic groups, their branching order is shown as unresolved, except for archaeal superphyla (Williams and Embley 2014) and several deep-branching bacterial lineages (after SSU rRNA-based "The All-Species Living Tree", November 2014 release; (Munoz *et al.* 2011)). Eukaryotic supergroups are after Adl *et al.* (2012). The root of the tree is placed on a branch leading to bacterial phyla after Gogarten *et al.* (1989) and Iwabe *et al.* (1989), although an alternative location of the root between Archaea and Bacteria remains plausible (Dagan *et al.* 2010). Data on optimal growth temperature were obtained from the Integrated Microbial Genomes system (Markowitz *et al.* 2014) and this figure does not represent an exhaustive overview of known lineages.

Fig. 2. Cells of *Mesotoga prima* MesG1.Ag.4.2. The toga can be seen ballooning out from the cell poles. The scale bar in the lower left corner corresponds to 0.5 µm. Cells of *M. prima* MesG1.Ag.4.2 were grown to exponential phase and samples prepared for microscopy as described by Spurr (1969). Images were acquired using a Philips Morgagni 268 transmission electron microscope (Philips-FEI, Hillsboro, Oregon, USA) operating at 80 kV with Gatan Orius CCD camera.

Fig. 3. Phylogenetic relationships among representative Thermotogae genera. 16S rRNA gene sequences were aligned using the NAST aligner in MOTHUR (Schloss *et al.* 2009) to the SILVA reference alignment (Yilmaz *et al.* 2014). Alignment sites with gaps were removed (resulting in a 1093 nt alignment), and the maximum likelihood tree was reconstructed in RAxML (Stamatakis 2014) under the GTR+Γ substitution model. The newly proposed Thermotogae classification into three orders and four families is shown to the right of the tree (Bhandari and Gupta 2014). Note that based on the 16S rRNA phylogeny, *Mesoaciditoga lauensis* should be have its own order (Mesoaciditogales) and family (Mesoaciditogaceae). Published optimal growth temperatures for each genus are shown. Taxonomic names of hyperthermophiles, thermophiles and mesophiles are depicted in bold black, black, and grey fonts, respectively. Bootstrap support values (out of 100 replicates) are shown at the nodes only for values above 70. The tree was rooted with the following taxa as an outgroup (collapsed into a wedge): *Alkalliphilus auruminator* (AB037677), *Marinithermus hydrothermalis* (AB079382), *Persephonella marina* (AF188332), *Aquifex pyrophilus* (AQF16SRRN), *Aquifex aeolicus* (AE000751), *Clostridium thermocopriae* (CLORG16SAA), *Clostridium botulinum* (NC_009495), *Flexibacter flexilis* (FBCRRB), *Thermus thermophilus* (X07998) and *Dictyoglomus thermophilum* (X69194).