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Original Paper

The stability of thermophilic proteins: a study based on comprehensive genome comparison

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Abstract. We address the question of the thermal stability of proteins in thermophiles through comprehensive genome comparison, focussing on the occurrence of salt bridges. We compared a set of 12 genomes (from four thermophilic archaeons, one eukaryote, six mesophilic eubacteria, and one thermophilic eubacteria). Our results showed that thermophiles have a greater content of charged residues than mesophiles, both at the overall genomic level and in alpha helices. Furthermore, we found that in thermophiles the charged residues in helices tend to be preferentially arranged with a 1-4 helical spacing and oriented so that intra-helical charge pairs agree with the helix dipole. Collectively, these results imply that intra-helical salt bridges are more prevalent in thermophiles than mesophiles and thus suggest that they are an important factor stabilizing thermophilic proteins. We also found that the proteins in thermophiles appear to be somewhat shorter than those in mesophiles. However, this later observation may have more to do with evolutionary relationships than with physically stabilizing factors. In all our statistics we were careful to controls for various biases. These could have, for instance, arisen due to repetitive or duplicated sequences. In particular, we repeated our calculation using a variety of random and directed sampling schemes. One of these involved making a "stratified sample," a representative cross-section of the genomes derived from a set of 52 orthologous proteins present roughly once in each genome. For another sample, we focused on the subset of the 52 orthologs that had a known 3D structure. This allowed us to determine the frequency of tertiary as well as main-chain salt bridges. Our statistical controls supported our overall conclusion about the prevalence of salt bridges in thermophiles in comparison to mesophiles.

Key words. Thermophiles - Mesophiles - Genomes - Protein stability - Salt bridges

Introduction

What are thermophiles and how do their proteins achieve stability?

The archaea and a few eubacteria, commonly known as thermophiles, thrive in high temperatures. They live in places such as hot springs and deep-sea hydrothermal vents under extreme conditions. It is not well understood how thermophiles stabilize proteins at the elevated temperatures that denature normal-temperature (10-45°C) mesophilic proteins. So far, several biophysical studies have been performed to determine the stability factors. These studies suggest about 15 different physicochemical factors for thermostability, such as hydrogen bonding, hydrophobic internal packing, helix dipole stabilization, and salt bridge optimization among others. The factors have been reviewed by several authors (Colacino and Crichton *1997*; Gupta *1995*; Jaenicke and Bohm *1998*; Querol et al. *1996*; Russel and Taylor *1995*; Scandurra et al. *1998*; Vieille et al. *1996*; Vogt and Argos *1997*; Vogt et al. *1997*).

The salt bridge as a major factor

Since the study of thermostability of bacterial ferredoxins by Perutz (Perutz and Raidt *1975*), a large number of 3D structures of thermophilic proteins have been determined. These structures, as well as structural information obtained through homology modelling revealed that there is a strong correlation between the number of salt bridges and protein thermal stability (Auerbach et al. *1998*; Hennig et al. *1995*, *1997*; Knapp et al. *1997*; Korndorfer et al. *1995*; Russell et al. *1997*; Salminen et al. *1996*; Spassov et al. *1995*; Szilagyi and Zavodszky *1995*; Wallon et al. *1997*; Xiao and Honig *1999*; Yip et al. *1995*). A theoretical study by Elcock and McCammon (*1997*) further supported this correlation, and showed that since the hydration free energies of the charged groups become less favorable at high temperatures, the unfavorable desolvation penalty incurred on forming the salt bridges is reduced in magnitude. Therefore, using the logic of solvation, the study suggested that the salt bridge becomes more stable at elevated temperatures.

Studies of protein structures have shown that there are several ways in which salt bridges can stabilize proteins. Ion pair networks, helix-stabilizing salt bridges, salt bridges buried in a hydrophobic core and surface salt bridges between two subunits are among the most frequently encountered types (Hennig et al.*1995*; Korndorfer et al. *1995*; Lebbink et al. *1998*; Petukhov et al. *1997*; Salminen et al. *1996*; Sindelar et al. *1998*; Vetriani et al. *1998*; Yip et al. *1995*)). All these salt bridges can be divided into two main classes:

1. Intra-helical or local: This class arises out of side-chain interaction between the charged residues in a helix. Biophysical studies have revealed that intra-helical EK, ER, DK, DR salt bridges paired with a separation of 3 and 4 stabilize helices (Huyghues-Despointes et al. *1993*; Scholtz et al. *1993*).

2. Tertiary: This class occurs as a result of interaction between non-local charged residues of proteins. The class includes a large variety of salt bridges, such as inter-helical salt bridges, helix-sheet salt bridges and inter-subunit salt bridges.

Figure 1A illustrates local and tertiary salt bridges. We studied the contribution of both types of salt bridges to protein thermostability. In addition to salt bridges, other electrostatic interactions, such as charge-helix dipole interactions, can also stabilize proteins. Previous research has shown that negatively charged residues can interact with the positive side of the helix dipole and thereby stabilize thermophilic proteins (Aqvist et al. *1991*; Nicholson et al. *1991*; Tidor et al. *1991*). In our study we also analyzed the results to see whether such interactions are important for protein thermostability.

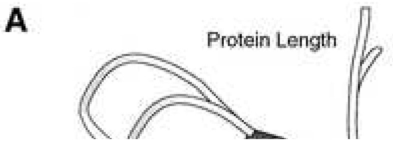

Fig. 1. **A** Three factors in a protein that are studied for their contribution to protein thermostability: (1) local salt bridges, (2) tertiary salt bridges, (3) protein length. **B** Determining the position of local (intra-helical) and tertiary salt bridges. The box in the figure represents a protein sequence with known structure, and each ± combination connected with dotted lines represents a salt bridge pair as observed in the structure. Since the EK pair involves an interaction between the charged residues in two separate secondary structural elements, it is defined as a tertiary salt bridge. Similarly the DK pair occurring within a helix is termed a local salt bridge. Using this definition we calculated the LOD values for the intra-helical amino acid pair as follows: the odds ratio *R* for any particular pair, say XY, at a separation *i* is defined by: $R\left[XY\left(i\right)\right]=\frac{Observed\ number\ of\ occurrences\ for\ XY\ pair\ separated\ by\ i}{Expected\ number\ of\ occurrences\ for\ XY\ pair\ separated\ by\ i}$The LOD value is the log (base 10) of the ratio. The observed number of occurrences for any salt bridge pair is the simple count for that pair in a genome. The expected number of occurrences for that pair is calculated as follows: given the frequencies of two amino acids X and Y in the helices as P(X) and P(Y), the probability of an XY pair occurring, assuming the occurrence of X and Y is completely uncorrelated, is: $P(X,Y)=P(X)\ P(Y)$.If the total number of all amino acid pairs is N, the expected number of occurrences of the XY pair is calculated as: $N(XY)=N\ P(X)\ P(Y)$. **C.** Method of stratified resampling based on orthologous relationship. This diagram illustrates our strategy of stratified resampling. A bar in the figure represents an ORF where positive-negative pairs indicate salt bridges that may be present in the protein. The first column in the figure shows various gene families. Second and third columns represent the ORFs in thermophilic and mesophilic genomes. Notice that some of the ORFs are grouped into families of paralogs. The ortholog column shows the idea of stratified sampling, where we extract one representative member from each gene family for every organism. The final column indicates whether a structure of a homologous protein is available for the family. The dashed lines (-) in the figure show the sequences that are missing for any orthologous group and are thus discarded from our calculation. More specifically, to identify orthologs we followed a five-step procedure:1. We started with the COGs classification at the NCBI (Tatusov et al. 1997). This currently contains 864 orthologous groups that are present in varying degrees in eight of the first genomes sequenced (a subset of the 12 genomes used in this study).2. We initially examined the 110 COGs present in all eight genomes.3. We then dealt with the issue of those COGs represented by multiple proteins in certain genomes (i.e. paralogs). To compensate for this effect, we chose only those COGs that had a maximum of ten sequences in total. In the few cases when we had paralogs, to pick a best representative, we consulted the dendograms on the COGs website.4. To enlarge a COGs cluster to the 12 genomes used here, we performed pairwise sequence comparison using the FASTA program (version 2.0) (Lipman and Pearson *1985*) where the COGs sequences were used as queries against the four additional genomes not part of the original COGs study (i.e. AA, OT, AF, and MT). We used an "e-value" threshold of 0.01 in these comparisons. The e-value describes the number of errors per query expected in a single database scan, so a value of 0.01 means that about 1 in 100 cluster linkages will be in error.5. Finally, we kept only those COGs that had easy-to-find members in the extra four genomes.Application of the whole procedure resulted in the list of 52 COGs that we used in our study. A subset of 18 of these had homologs in the PDB structure databank and was used for the tertiary salt bridge study. These are indicated by the rhombus in the last column of the figure. **D** Determination of tertiary salt bridges by an indirect method of structure mapping. To determine the positions of the salt bridges in a protein of unknown structure, where possible, we mapped the protein sequence onto a homologous protein of known structure in the PDB. All the salt bridges in the protein with known structure were determined by a program that takes coordinates of a protein and lists hydrogen bonds occurring in it (Gerstein *1992*). The list of hydrogen bonds considered here involved only side-chain/side-chain and side-chain/main-chain interactions between amino acids, as the main-chain/main-chain hydrogen bonds are mostly involved in forming secondary structural elements. Next we aligned all 12 sequences in each orthologous group with the corresponding PDB sequence by multiple sequence alignment using CLUSTALW (Higgins et al. *1996*). Then for every salt bridge pair in the PDB protein, a corresponding amino acid pair was determined in the similar position in other proteins. It has been observed that in some proteins, the amino acid pair corresponding to a salt bridge is conserved,

whereas in others it is replaced either by a non-ionic pair or by a complementary salt bridge pair

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A genome-wide comprehensive study: our goals and strategy

Most of the studies about protein thermostability referred to above involved analysis of only a few proteins. With the advent of fast DNA sequencing technology, complete genome sequences of several organisms are now available (Devine and Wolfe *1995*). As a result, it is now possible to comprehensively study all the proteins in an organism and compare the results with those of other organisms. We have done a number of similar analyses, comparing various aspects of protein structure, such as secondary structural composition and fold usage in several recently sequenced genomes (Gerstein *1997*, *1998a*, *b*; Hegyi and Gerstein *1999*). Similar studies have also been carried out by other investigators (Amano et al *1997*; Fetrow et al. *1998*; Frishman and Mewes *1997*; Kyrpides and Ouzounis *1999*; Sanchez and Sali *1998*; Wolf et al. *1999*).

In this investigation our aim is to study the effect of salt bridges, as well as other stability factors such as deamidation and protein length, in thermophiles and mesophiles by comprehensive analysis of all protein sequences in their genomes. All the factors that are studied here are shown in Fig. 1A. We have analyzed the genomes of 12 organisms, of which four archaeons and one hyper-thermophilic eubacteria are grouped together as thermophiles. The rest, one eukarya and six eubacteria, are grouped together as mesophiles. These organisms are listed in Table 1.

Table 1. The 12 organisms whose sequences are used in calculations. Column 3 shows the two-letter abbreviations for the genomes of the organisms listed in the first column. The fourth column lists the number of open reading frames found in the genome. The last column shows the physiological temperatures of thermophiles. For mesophiles we referred to "mesophilic temperatures" which range from 10 to 45°C. Data-files of predicted proteins were taken from the websites referred to in the papers above, with the exception of OT, for which predicted proteins were from the analysis of Suckow et al. (*1998*)

Organism	**Category**	**Genome ID**	**No. of Proteins**	**Physiological condition**
Pyrococcus horikoshii (Strain OT3) (Kawarabayasi et al. 1998)	Archaea	OT	2061	98°C, anaerobe
Aquifex aeolicus (Deckert et al. 1998)	Eubacteria, gram negative	AA	1522	95°C
Methanococcus janaschii (Bult et al. 1996)	Archaea	MJ	1735	85°C, anaerobe
Archaeoglobus fulgidus (Klenk et al. 1997)	Archaea	AF	2409	83°C, anaerobe
Methanobacterium thermoautotrophicum (Smith et al. 1997)	Archaea	MT	1869	65°C, anaerobe
Haemophilus influenzae (Fleischmann et al. 1995)	Eubacteria, gram negative	HI	1680	Mesophilic temp.
Mycoplasma genitalium (Fraser et al. 1995)	Eubacteria, gram positive	MG	470	Mesophilic temp.
Mycoplasma pneumoniae (Himmelreich et al. 1996)	Eubacteria, gram positive	MP	677	Mesophilic temp.
Helicobactor pylori (Tomb et al. 1997)	Eubacteria, gram negative	HP	1590	Mesophilic temp.
Escherichia coli (Blattner et al. 1997)	Eubacteria, gram negative	EC	4288	Mesophilic temp.
Synechocystis sp. (Kaneko et al. 1996)	Cyanobacteria	SS	3168	Mesophilic temp.
Saccharomyces cerevisiae (Goffeau et al. 1997)	Eukaryote, fungus	SC	6218	Mesophilic temp.

Our overall strategy of salt bridge analysis is shown in Fig. 1B-D. In the first step of our study we calculated the amino acid composition of all 12 genomes.

In the second step we focused on the intra-helical salt bridges, calculating the frequency of putative salt bridge pairs in the helices of proteins, and analyzed the data. Since it is possible that the frequency of intra-helical salt bridges in a genome may be biased due to sequence repeats and multiple paralogs specific to the organism, we analyzed a small set of orthologous proteins that are present in all the organisms and performed a similar calculation on this set.

In the third step we looked into the frequency of tertiary salt bridges in proteins in all the genomes. Within the above orthologous set, we calculated this frequency in only those proteins whose structures are known.

Results and discussion

Study of local salt bridges

Amino acid composition in the entire genome and in protein helices

We estimated amino acid composition both in the entire genome and protein helices. These are shown in Fig. 2A, B.

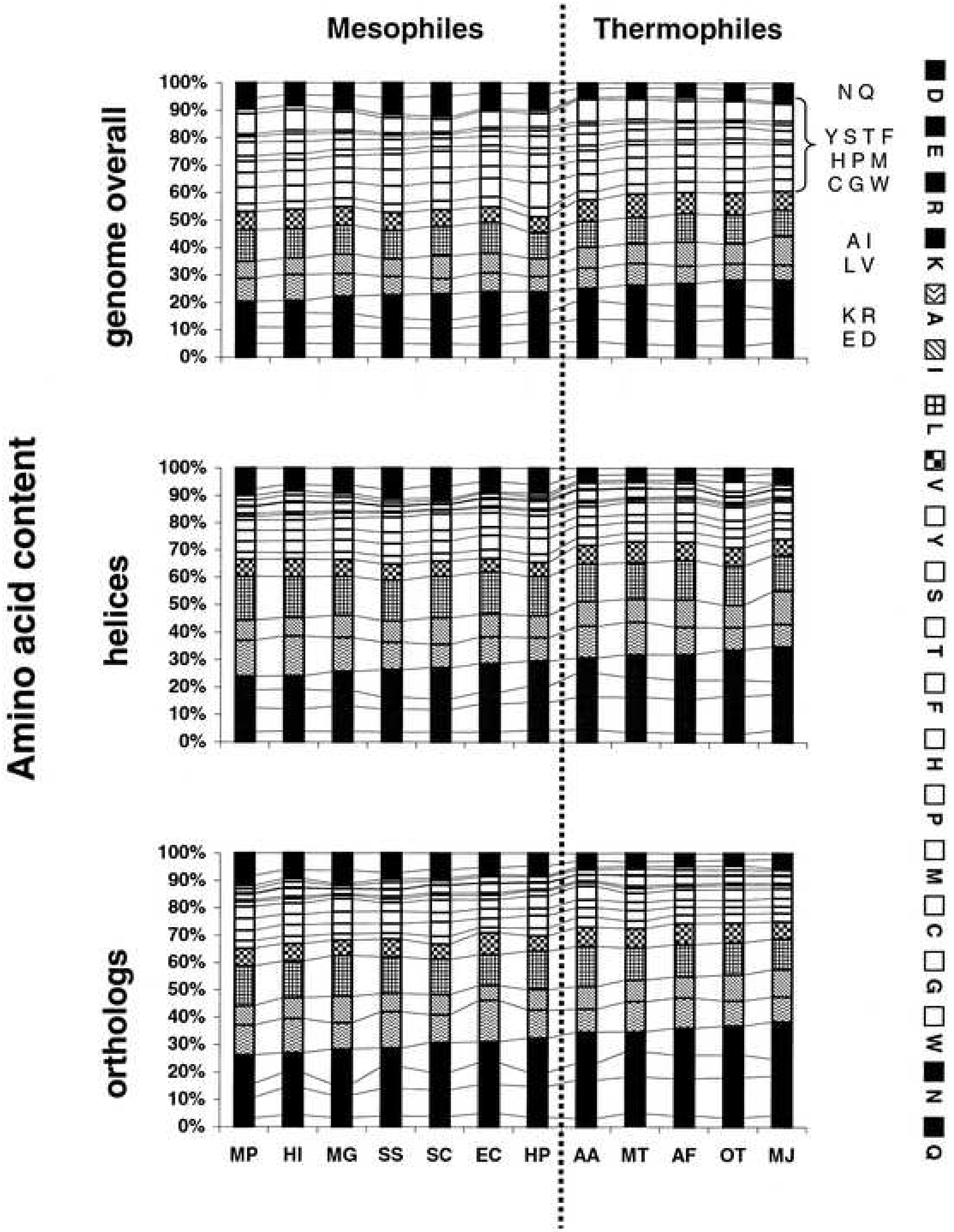

Fig. 2. Amino acid composition in genome, helix and 52 orthologous proteins. Amino acid composition in genome-overall, helix and for orthologous proteins are shown by the additive bar graphs in A, B and C respectively. The blackened areas in the figures represent the portion of charged residues E, D, K and R. This area increases from mesophiles to thermophiles and the trend is followed at all three levels. Conversely, the amounts of amine residues N and Q decrease in thermophilic helices. Also note that

among hydrophobic groups (AILV) there is an increase in the contents of L and V in thermophiles

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Since secondary structures of most proteins are unknown, we predicted them in order to calculate the amino acid composition of helices. Secondary structure prediction was performed using the GOR(IV) program. (Garnier et al. *1996*, *1978*; Gibrat et al. *1987*). This is a well-established and commonly used method. It is statistically based, so that the prediction for a particular residue to be in a given state (e.g., Ala to be in a helix) is based directly on the frequency of the residue's occurrence in that state in a database of solved structures (taking into account neighbors at ±1, ±2 etc.). The GOR method uses only single sequence information compared to current "state-of-the-art" methods that incorporate multiple sequence information (King and Sternberg *1996*; Rost *1996*; Rost et al. *1996*). While single sequence predictions are slightly less accurate than multiple sequence methods (65% versus 71%), we felt that using single sequence methods avoids various bias problems that can plague multiple sequence methods - i.e. we can only get multiple sequence information for a biased sample from each genome. Furthermore, we felt that the difference in accuracy between single and multiple sequence methods was not so vital in the overall context of our study, given our focus on bulk-averaged results.

It is observed that both at the overall genomic level and in the helices, the amounts of glutamate, lysine and arginine (E, K, R) are higher in thermophilic proteins than in mesophilic proteins. This increase in charged residues suggests that in general we can expect to see more salt bridges in thermophiles than in mesophiles. Analysis of amino acid composition has shown that the amount of negatively charged aspartate residue remained almost the same in all the organisms.

Use of log odds calculation: abundance of local salt bridges

As a result of the high content of charged residues in helices, we can generally expect to see a greater number of intra-helical salt bridges in thermophiles than in mesophiles. In order to see whether salt bridges are even more numerous than this elevated a priori "baseline", we calculated an odds ratio for all possible 400 amino acid pairs in helices. As described more fully in the caption to Fig. 1B, this is essentially the observed number of occurrences of a given pair, divided by its expected number if there was no correlation, e.g. the frequency of EK(3) divided by the product of the individual E and K frequencies. Note that here the notation EK(3) implies the EK salt bridge pair with a separation of 3; we use similar notations throughout the text. We then took the logarithm of this odds ratio, arriving at a log of odds (LOD) value. LOD values represent a measure of relative abundance for each pair in helices. Therefore, a higher LOD value for a particular pair would mean a higher frequency of that pair than of other pairs in the genome. We calculated the LOD values for 400 amino acid pairs with a separation of 1 to 6. It is observed that for any salt bridge pair, the LOD values peak at a separation of either 3 or 4, indicating that these pairs probably represent intra-helical salt bridges, as suggested by the previous biophysical studies. As an illustration of this general result we plotted the LOD values of EK pairs at various separations in helices (see Fig. 3). Note that the LOD values of EK pairs peak at a separation of 3 for all the organisms.

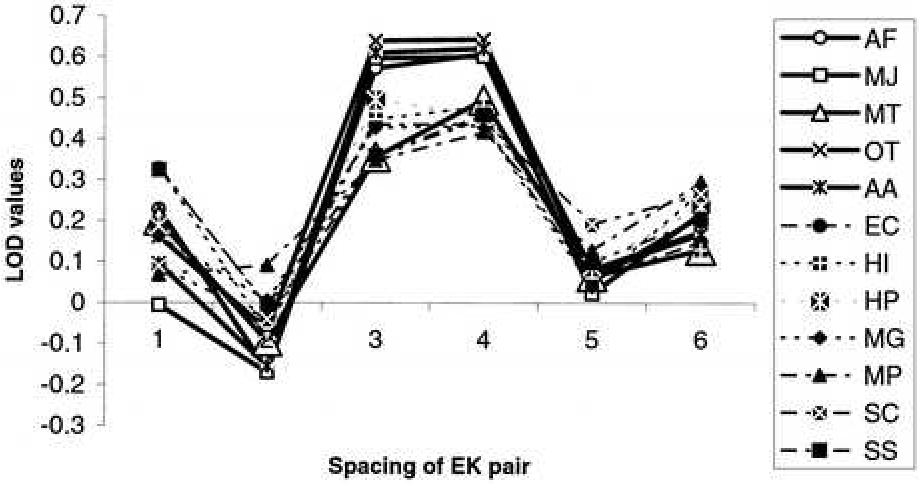

Fig. 3. LOD values of the EK pair in helix as a function of separation. LOD values for EK pair peak at a separation of either 3 or 4, suggesting that the pair at these positions represents a salt bridge pair

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Results of LOD value calculations show that the LOD values for salt bridge pairs EK, ER and DR with a separation of 3 and 4 are generally higher in helices of thermophilic proteins than in mesophilic ones. In order to see whether the charged residues in the strand part of the protein sequences were correlated in this fashion, we performed a similar LOD-value calculation on the strands and compared the result with that for helices. Similarly, we calculated genome-wide LOD values for the pairs by performing calculations on entire protein sequences. Comparison of the results shows that the LOD values for salt bridge pairs are higher in helices than in other secondary structural elements and that this is true to a greater degree in thermophilic organisms. Our results thus imply that the charged amino acid residues are not only more numerous in thermophiles than mesophiles, but are also more highly correlated in helices of thermophilic proteins with a salt bridge separation of 3 and 4.

Correlation between temperature and salt bridge frequency

We computed the LOD values for EK pairs with a separation of 3 and 4, and the result is shown in Fig. 4A. The figure shows that the LOD values are higher for thermophiles than for mesophiles. Also in the thermophilic region, LOD values increase from MT to OT commensurate with the steady increase in physiological temperatures from MT (65°C) to OT (98°C). This correlation of physiological temperatures with the intra-helical salt bridge frequency suggests that higher temperatures require a greater number of salt bridges to stabilize the helices in proteins.

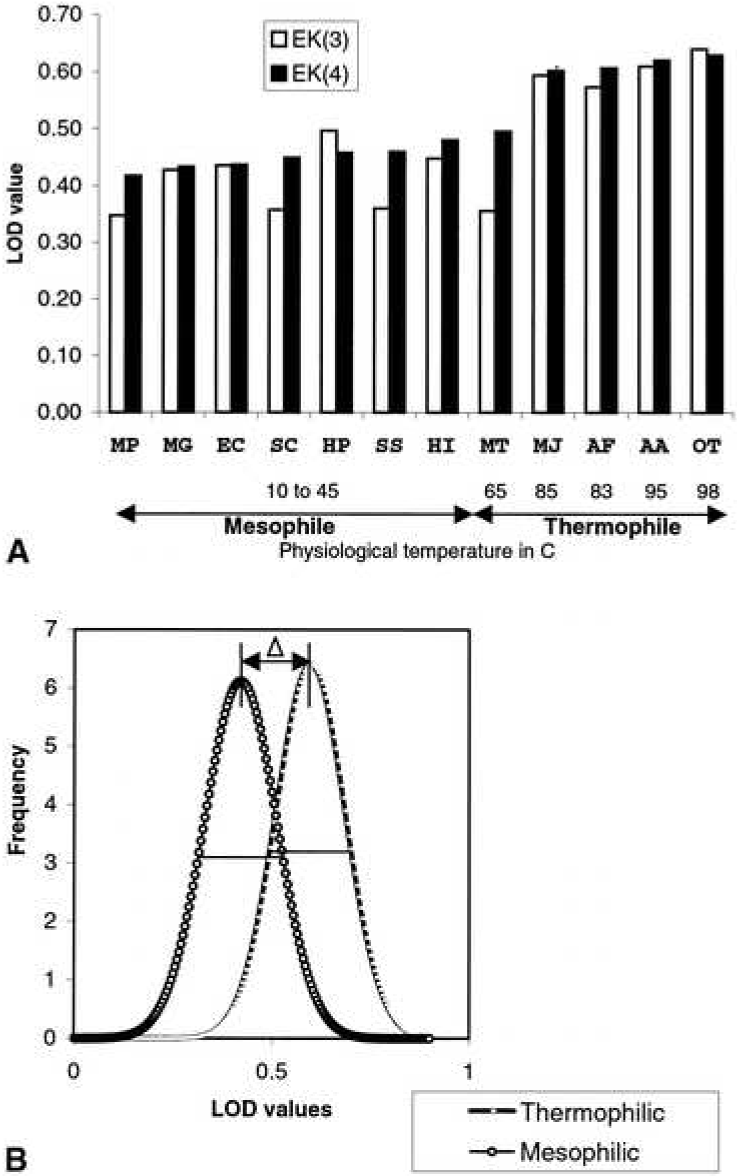

Fig. 4. A LOD values of EK salt bridge pairs with separation of 3 and 4. LOD values increases with the increase in physiological temperatures shown along the horizontal axis. For mesophiles, they are indicated by a range of 10-45°C. **B** Distribution of LOD values of EK(3) for randomly generated mesophilic and thermophilic genomes. The distribution curves of EK(3) LOD values for randomly generated thermophilic and mesophilic genomes are shown. The difference of the two means of the distribution is $|\Delta|=0.18$. The sample variance for thermophiles is $s_x^2=0.0038$, and for mesophiles, $s_y^2=0.0059$. We performed a standard double-blind experiment to test the significance of the difference of means. We calculated the Z score as follows: $Z = \frac{\langle X\rangle - \langle Y\rangle}{\sigma}$, where X and Y are the means of thermophilic and mesophilic distributions, respectively, $\sigma^2=s^2_x/n_x+s^2_y/n_y$, and n_x and n_y are the number of observations for each distribution (500 here). Results show that the probability that the two distributions will have same mean is less than 5%

Helix-dipole stabilization

In our LOD calculation in helices we found that the values for EK(3) and EK(4) pairs are always higher than the corresponding values for KE pairs (data not shown). This variation of LOD values on the orientation of the charged pair is significant in terms of charge-helix dipole interaction. Since negatively charged glutamate residue can stabilize a helix by interacting with the positive amino end of a helix dipole (Aqvist et al. *1991*; Eijsink et al. *1992*; Nicholson et al. *1991*; Tidor and Karplus *1991*), this observation indicates that the thermophilic proteins gain stability from helix-dipole stabilization.

Analyses to control for biases in the statistics

The problem of bias in our comprehensive genome-wide statistics

While doing genome-wide surveys, one has to be careful to assess the degree to which the calculated statistics could be biased. There are a number of specific issues relevant here. Firstly, sequence repeats, e.g. repetitive charged sequences in a set of thermophilic proteins, could skew the results. Secondly, unique protein sequences enriched in salt bridges could be highly duplicated in the thermophile genomes (forming large paralogous families), and this could also influence our results (e.g. see Fig. 1C). A similar situation may arise involving only the sequences unique to mesophiles. We therefore need to test the significance of LOD results and verify our conclusions with statistical controls and alternative procedures.

Rank statistics

One technique to test the significance of our results is rank statistics. Here the idea is that if we arrange the LOD values of all 400 pairs for each separation in an ordered list and observe that a particular pair - EK(3), for example - is at the top of the list, then we could infer that this pair is among the most over-represented in the helices of the proteins for that organism. Table 2 summarizes the rank statistics for salt bridge pairs that ranked in the top 20 of a possible 400. The results show that while the ranks of salt bridge pairs vary greatly among all 12 genomes, the ranks of EK(3) pairs are generally higher for thermophiles compared to mesophiles in helices. MT is an exception to this general trend. In contrast, when the non-helical regions are considered, this distinction lessens.

Table 2. Rank statistics of salt bridge pairs. The ranks of other salt bridge pairs [ER(3), EK(4) and DR(3)] were not markedly different between thermophiles and mesophiles. A similar study on the predicted strand sequence did not show any significant ranking for salt bridge pairs (results are not shown)

		HELIX											
		Thermophile					Mesophile						
Sep	Pair	AF	MJ	MT	OT	AA	EC	HI	HP	MG	MP	SS	SC
3	EK	4	5	-	4	4	9	7	7	13	-	13	**19**
3	ER	10	-	13	18	-	12	-	14	-	-	-	-
3	DR	13	-	-	13	12	8	10	-	-	-	10	-
4	EK	5	9	12	9	7	12	9	13	11	15	10	10
4	ER	11	14	-	14	-	-	-	-	-	-	-	-
4	DR	-	-	13	13	-	9	10	11	9	7	11	18
3	DK	9	13	8	8	16	2	5	6	11	10	4	9
4	DK	10	-	16	19	-	6	11	17		13	15	9
		GENOME											
		Thermophile					Mesophile						
Sep	Pair	AF	MJ	MT	OT	AA	EC	HI	HP	MG	MP	SS	SC
3	EK	4	5	9	3	3	3	5	3	9	-	7	-
3	ER	6	-	4	11	11	4	7	9	-	-	9	-
4	EK	4	8	11	9	6	12	10	6	-	-	-	-
4	ER	9	-	7	14	15	3	-	-	-	-	-	-

Random resampling

We directly addressed the problem of sequence repeats by a random resampling procedure. We simulated thermophilic and mesophilic genomes by randomly drawing proteins from two large pools of thermophilic and mesophilic sequences. From these simulated genomes we calculated the LOD values for charged amino acid pairs in helices. Figure 4B shows the distribution of these values for the EK(3) pair. Note the distinct difference in the distributions. Statistical tests were performed to estimate the degree of significance of this difference, and it was found that given the width of the distribution, the chance that any mesophile could have a LOD value similar to a thermophile is less than 5% (for EK(3) and EK(4)). This implies that our LOD calculation results are statistically significant. (These calculations are described in more detail in the figure caption.)

Stratified resampling using orthologs

Another way of removing biases is to use stratified sampling procedures (Anderson and Finn *1996*). This can most easily be described in terms of a demographic comparison of a particular characteristic between populations such as height in northern versus southern populations. It is possible that the overall population could be further divided using another parameter potentially linked to height, e.g. age (old vs. young). Our initial analysis of salt bridge statistics was analogous to computing average height over an entire population irrespective of age. However, the possibility that one population has more of a certain age group than another could potentially skew the statistics (e.g. Northerners are older and taller). To compensate for such bias in the sample we could take a representative sample from every age group and calculate the average height for that strata. This is what we did in stratified sampling to study the salt bridge abundance.

Our strata were sets of orthologous proteins present in each of the 12 genomes. Orthologous proteins evolved from a common ancestral gene and usually share the same structure and function (Fitch *1970*). Statistics obtained from sets of orthologous proteins can be considered to be relatively free of bias arising from sequence repeats or large paralogous families. In our study we selected 52 sets of orthologous proteins (listed on our website). Our ortholog selection strategy is explained in detail in Fig. 1C. It was derived using the cluster-of-orthologous groups (COGs) approach (Tatusov et al. *1997*). We used only COGs for which we could determine a single best representative for each genome, and we extended the initial COGs assignments (currently eight genomes) to include all 12 genomes in our study.

We performed similar analyses on our set of 52 orthologous proteins to those performed on the entire genome. Composition analysis showed a similar trend of increasing amounts of charged residues from mesophile to thermophile, as observed in the overall genome analysis (Fig. 2C). Note that the hyperthermophilic eubacteria *Aquifex aeolicus* has moved closer in position to the other eubacteria, perhaps indicating that some exclusively archaeal paralogous family is heavily weighted with charged residues. Likewise, we calculated LOD values for our set of 52 orthologs. The results for important salt bridge pairs are shown in Table 3 (C). Although the LOD values for EK(3) had decreased for both thermophiles and mesophiles, thermophiles still maintained higher average LOD values for EK(3), EK(4), DR(3) and ER(4). This result is important: despite using only 52 groups of proteins, the stratified resampling comparisons showed that putative salt bridge frequency was clearly higher in thermophiles than mesophiles.

Table 3. Parts A and B show LOD values (%) of salt bridge pairs in helix and genome. Since in helices salt bridge pairs at a separation of 3 and 4 are known to stabilize proteins, we have listed their LOD values separately. LOD values of the salt bridge pairs in strands are not shown here, as they are obvious from the whole genome results. Part C lists the LOD values of the ion pairs for 52 orthologous proteins. Note that LOD values for the salt bridge pairs remained high even in the small set of 52 orthologous proteins

			LOD values (%)											
			Thermophile					Mesophile						
	Spacing	Pair	AF	MJ	MT	OT	AA	EC	HI	HP	MG	MP	SC	SS
A. Helix	3	EK	57	59	36	64	61	44	45	50	43	35	36	36
		ER	48	25	42	39	36	38	27	36	27	37	25	32
		DR	45	33	53	48	48	48	40	32	-3	20	30	40
	4	EK	61	60	50	63	62	44	48	46	43	42	45	46
		ER	47	27	48	46	43	36	31	23	33	25	27	33
		DR	38	38	48	44	44	44	47	48	56	60	36	42
B. Genome	3	EK	43	47	23	14	48	27	27	38	27	21	22	22
		ER	37	17	30	22	25	26	22	27	15	25	14	21
		DR	16	8	18	1	10	18	12	9	2	4	-1	11
		EK	40	40	28	14	40	21	24	31	19	25	23	24
	4	ER	31	17	31	-7	27	24	19	12	17	14	12	21
		DR	9	4	12	24	5	16	17	22	12	13	4	13
C. 52 COG proteins	3	EK	48	57	55	38	61	38	36	45	40	23	41	26
		ER	38	-27	14	24	36	30	43	17	-5	51	-5	21
		DR	33	65	37	31	45	47	29	29	6	20	37	35
	4	EK	58	60	57	26	62	41	65	35	46	24	62	33
		ER	29	15	39	34	43	37	38	9	-2	12	38	5
		DR	54	37	76	23	32	55	52	44	60	70	52	73

Study of tertiary salt bridges

So far, our study of salt bridges has focussed only on intra-helical salt bridges. Moreover, these statistics depend on accuracy in the prediction of protein secondary structures. Therefore, to complement our conclusions on intra-helical salt bridge abundance, we studied the tertiary salt bridges in thermophilic and mesophilic proteins of known structure. Here, we followed a procedure similar to that performed by Schueler and Margalit (*1995*). Since any such study of tertiary salt bridges requires the knowledge of detailed 3D protein structure, which is unknown for most proteins in the genome, we tried the following strategy, schematized in Fig. 1D. Where possible, we mapped the sequence of a protein with a known 3D structure onto a corresponding orthologous group of sequences to identify the putative tertiary salt bridges in the new sequences. This approach rests on the idea that since orthologous proteins conserve their structures, knowledge of one protein structure can be extended to others in the same group. More specifically, we took query sequences from each of our 52 orthologous groups of proteins and compared them with the Protein Data Bank (PDB) structural database by pairwise sequence comparison (Lipman and Pearson *1985*; Sussman et al. *1998*). This resulted in a list of 18 PDB structures that map onto corresponding orthologous groups. As listed in Table 4, we classified these 18 orthologous groups of known structure into three categories: (1) ribosomal proteins, (2) amino-acyl tRNA synthetases, and (3) other proteins (including proteins with various functions).

Table 4. Summary of the results of tertiary salt bridge counts. First column one shows the COG identifiers for the orthologous groups that are selected. Second column gives the functional class for each of this group and the fourth column lists the PDB identifiers for homologous proteins with known structures. Third column represents our category. For every protein, we calculated the average number of salt bridges present in thermophiles and in mesophiles as shown in columns 5 and 6. Column 7 shows the difference between the two. Based on this difference we set up a scoring scheme that qualitatively describes the relative abundance of tertiary salt bridges. If the difference is >1.0, a positive (+) sign is assigned showing a predominance of salt bridges in thermophiles; if the difference is <-1.0, a negative (-) sign is assigned showing a predominance of salt bridges in mesophiles; for any other value of difference, no sign is assigned to either thermophiles or mesophiles, thus showing no bias for salt bridges. Note that in the two main categories (ribosomal proteins and tRNA synthetases thermophiles), thermophiles have a higher amount of tertiary salt bridges than mesophiles

COG ID	Class	Category	PDB ID	Therm. average of salt bridge	Meso. average of salt bridge	Difference	Score	
49	J	Ribosomal	1rss	5.6	3.1	2.5	1	+
80	J	Ribosomal	1aci	0.8	0.7	0.1	0	
81	J	Ribosomal	1ad2	6.4	4.3	2.1	1	+
91	J	Ribosomal	1bxe	1.8	0.9	0.9	0	
93	J	Ribosomal	1whi	3	1.9	1.1	1	+
96	J	Ribosomal	1sei	2	2.1	-0.1	0	
98	J	Ribosomal	1pkp	0.6	1.7	-1.1	-1	-
184	J	Ribosomal	1a32	1.8	1.9	-0.1	0	
186	J	Ribosomal	1rip	0.4	0.9	-0.5	0	
16	J	Synthetase	1pys	7.6	2.6	5	1	+
124	J	Synthetase	1ady	9.6	6.1	3.5	1	+
162	J	Synthetase	2ts1	3.8	3.3	0.5	0	
30	J	Other	1yub	5	5.3	-0.3	0	
125	F	Other	1tmk	0.8	0.4	0.4	0	
149	C	Other	1btm	3	4.3	-1.3	-1	-
541	N	Other	1fts	3.6	3.4	0.2	0	
112	E	Other	1cj0	6.2	4.6	1.6	1	+
552	N	Other	1ffh	4.2	4.6	-0.4	0	

Using the strategy outlined in Fig. 1D, we obtained rough estimates of the number of salt bridges for each protein in the 18 orthologous groups of known structure. Table 4 shows some summary statistics based on these numbers. It shows that for two categories, ribosomal proteins and tRNA-synthetases, thermophiles have somewhat more tertiary salt bridges than mesophiles. For proteins in the "other" category, there is less of a difference between thermophiles and mesophiles.

Other stabilizing factors

So far we have discussed the role of salt bridge interactions in thermophilic proteins. One should note here that since structures for most of the proteins are unknown, it was not possible for us to study the contribution of other factors, such as the effect of hydrophobic internal packing on protein thermal stability. However, in addition to salt bridges, we also studied the effect of two other factors on thermostability of proteins and compared their results with that of the salt bridges: (1) deamidation (2) protein length.

Deamidation

Studies showed that glutamine and asparagine undergo a deamidation reaction, leading to instability in a protein (Catanzano et al. *1997*). Therefore reduction in the amounts of these two amino acids can stabilize proteins. In our amino acid composition study, we observed that the amounts of glutamine (N) and asparagine (Q) have decreased in thermophiles compared with mesophiles. Furthermore, we noticed that among hydrophobic amino acids the amounts of valine and isoleucine have increased in thermophiles. Note in this context that the amount of proline which is believed to contribute thermal stability in proteins (Hardy et al. *1993*; Matthews et al. *1987*; Wallon et al. *1997*) did not exhibit any bias and remained almost the same both in thermophiles and mesophiles.

Protein length and thermal stability: the contradictory position of *Aquifex aeolicus*

It has been argued that shorter protein length increases the compactness of the protein and reduces its flexibility. A biophysical study by Nagi and Regan (*1997*) suggested that there is an inverse correlation between loop length and protein stability. In a recent study, Thompson and Eisenberg (*1999*) put forward a thermodynamic argument supporting this correlation, and showed that the thermophilic proteins have a higher tendency towards shorter loops than their mesophilic counterparts, by comparing homologous proteins from the genomes of a large number of organisms. In our study, we analyzed the sequence length distribution of proteins for all organisms to understand how protein length is related to thermostability. Our results, shown in Fig. 5A, indicate that the length distributions for thermophiles do indeed fall off more rapidly than those for mesophiles. Furthermore, when we fitted curves to the length distribution of just thermophilic or just mesophilic proteins, we found that the median (and mode) length was less in the thermophiles than in the mesophiles (Fig. 5B). This result was true for both the genome overall and for just our sample of 52 orthologs. Therefore, our "first pass" results on protein length appear to support the notion that the proteins in thermophiles are shorter than those in mesophiles.

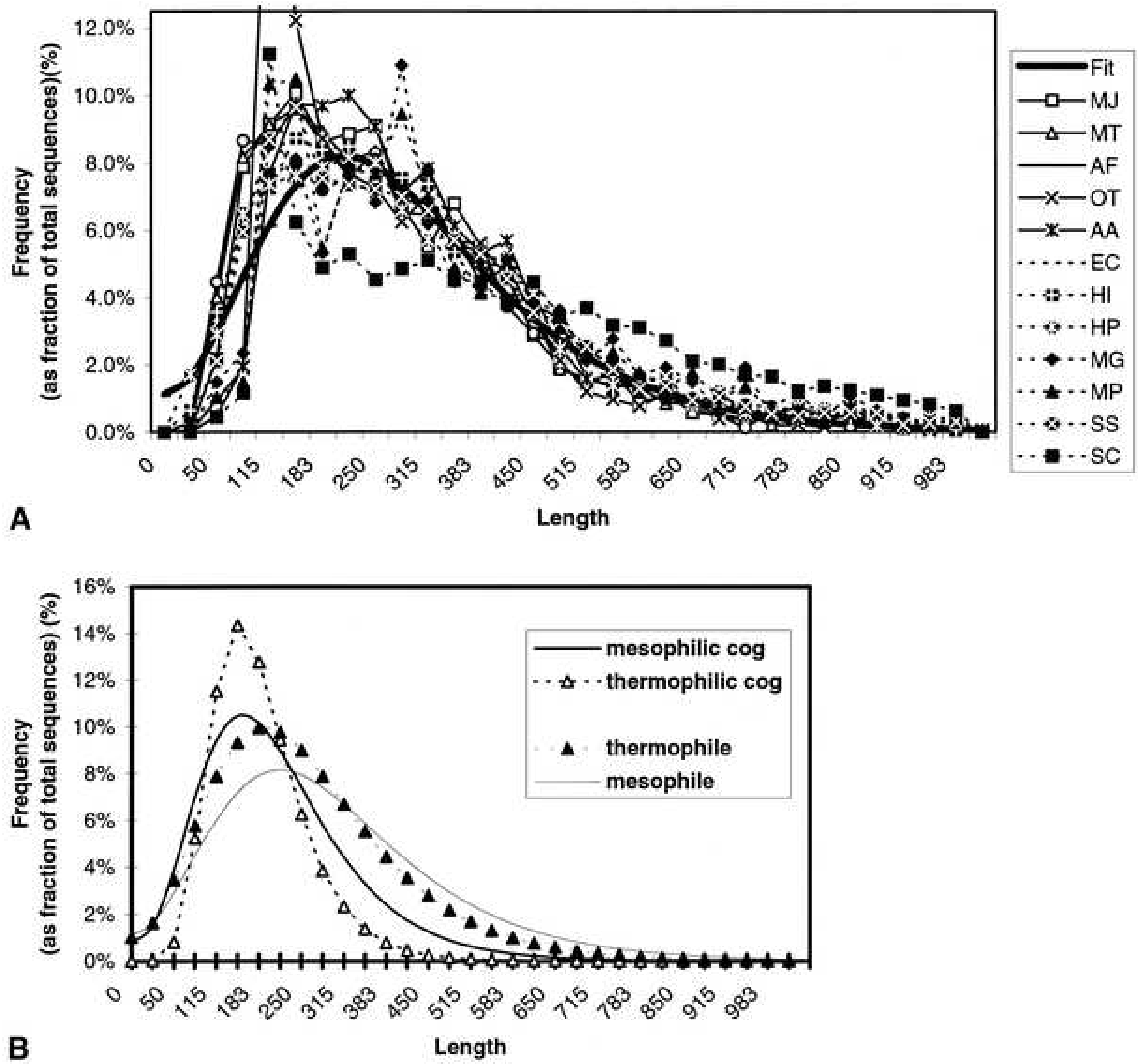

Fig. 5. **A** Length distribution of proteins in 12 organisms. We used an extreme value distribution for the fit curve shown by the bold line. Frequency at any protein length x is given by: $y=\exp[c-b(x-a)-\exp(-b(x-a)]$ where $a=211.0$, $b=0.007142$, and $c=0.2277$. Note that some sequences longer than 983 amino acids are not shown in the graph. Two letter abbreviations are defined in Table 1. At shorter protein lengths thermophiles exceed the fit curve while mesophiles are below it, but at longer protein lengths mesophiles exceed the fit curve and thermophiles are below it. **B** Comparison of thermophilic and mesophilic fit curves for length distribution both for overall genome sequences and orthologous proteins. We used the same extreme value distribution for the fit curve as in Fig. 6. Only the fit curves are shown here

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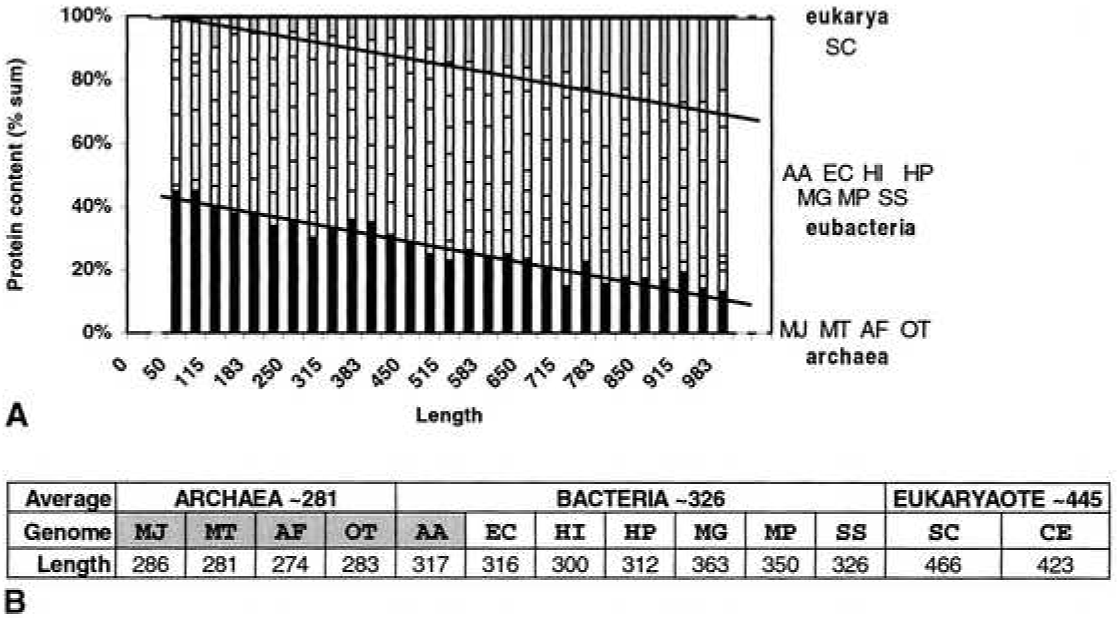
However, when we looked at the sequence lengths in further detail, we found that the story was more complicated. The distribution of protein lengths for *Aquifex aeolicus*, a hyperthermophilic eubacteria, is more similar to those of the mesophilic eubacteria than to the other thermophilic organisms, which are all archaeas. Furthermore, yeast appears to have distinctly longer proteins than those in either of the prokaryotic kingdoms. The distribution of protein lengths therefore appears to be related more to kingdom than to environment, reflecting historical contingency rather than chemical necessity. This result is illustrated in Fig. 6A, which shows how "phylogenetic composition" of proteins with a given length becomes progressively less archaeal and more eukaryotic as one moves to longer proteins. This result is further borne out in the table of Fig. 6B, where it can be seen how average protein lengths are correlated with kingdom. In this table we included average protein length for *C. elegans*, the other known eukaryotic genome, to illustrate that long sequences are characteristic of other eukaryotes beside yeast.



Average	ARCHAEA ~281				BACTERIA ~326							EUKARYAOTE ~445	
Genome	MJ	MT	AF	OT	AA	EC	HI	HP	MG	MP	SS	SC	CE
Length	286	281	274	283	317	316	300	312	363	350	326	466	423

Fig. 6. A Length distribution of proteins in terms of overall percentage composition at various length. Amount of protein at various protein lengths in different genomes. The vertical axis represents fraction of total amount of proteins present at various protein length for all the 12 organisms. Percentage content of proteins with longer protein length increases in yeast and decreases in archaea. **B.** Average protein length in 12 organisms. In the eukaryote category we included average protein length in the *C. elegans* genome (CESC 1998). Shaded genomes represent the thermophiles. Overall averages for each category are given on the top of every category-column. Note that the average protein length for archaea is shorter than that for either of the other forms of life

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Conclusion

From the comparison of our results on amino-acid composition and LOD statistics, we argue that the occurrence of excess intra-helical salt bridges in thermophiles may originate in two factors. Firstly, the thermophiles have a higher content of charged amino acids than the mesophiles. Secondly, these charged residues are more preferentially arranged with a 1,4 salt bridge spacing in thermophilic helices than in mesophilic ones. Since the results of our calculations on orthologous groups of proteins were similar to our genome-wide results, we infer that the sequence repeats or paralogous sequence families do not skew the observed abundance of intra-helical salt bridges in thermophiles. Our results also showed that the thermophilic proteins have more tertiary salt bridges than the mesophilic proteins. Thus we conclude that the salt bridge interactions play a vital role in stabilizing thermophilic proteins. Our study also showed that, in addition to salt bridges, there are other factors that can contribute to protein thermal stability. Reduction of deamidation by decreasing the amounts of glutamine and asparagine in proteins confers stability to thermophilic proteins. We examined the contribution of protein sequence lengths, but we found that they are only loosely connected with protein thermostability. Therefore, among the three factors that we studied here, we found that while the extent of contribution to thermostability varies with each factor, salt bridge contribution varies most consistently with increasing physiological temperatures and is one of the most important factors for protein thermostability.

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