Archaeal cold-adapted proteins: structural and evolutionary analysis of the elongation factor 2 proteins from psychrophilic, mesophilic and thermophilic methanogens

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Abstract To identify structural features important for low temperature activity in archaeal proteins, elongation factor 2 (EF-2) genes (*aef2*) were sequenced from psychrophilic, mesophilic and thermophilic methanogens. Scatter plots were used to compare evolutionary distances for EF-2 amino acid sequences vs. 16S-rRNA sequences from methanogens growing at diverse temperatures. The absence of a temperature bias for the rate of protein vs. nucleic acid evolution demonstrated the importance of comparing closely related proteins in order to identify changes indicative of thermal adaptation. Three-dimensional modelling of the new EF-2 sequences enabled the identification of amino acid residues that may be important for conferring low temperature activity and included greater structural flexibility produced by fewer salt bridges, less packed hydrophobic cores and the reduction of proline residues in loop structures.

Key words: Archaea; Psychrophile; Elongation factor 2; Structure prediction; Protein evolution

1. Introduction

Archaea are capable of growth from 113°C [1] to below 0°C [2], however, knowledge about hyperthermophiles and their proteins [1,3] vastly outweighs information about low temperature adapted (psychrophilic) archaea and their associated proteins [4]. Archaea are found in many low temperature aquatic and soil environments and their prevalence has recently been extended to members of the Crenarchaeota (a kingdom thought to be composed exclusively of hyperthermophiles), which indicates archaea may be as prevalent in mesophilic and low temperature environments as bacteria [5–7]. Studies have been initiated on a novel symbiotic Crenarchaeum which have shown that its DNA polymerase is well adapted to function at low temperatures [8]. Three free-living members of the Euryarchaeota (a kingdom that includes mainly methanogens and halophiles) have been isolated from Antarctica and presently represent the only low temperature adapted archaea that are available in pure culture; two methanogens, *Methanococcoides burtonii* (T_{min}: −2.5°C; T_{opt}: 23°C) [9] and *Methanogenium frigidum* (T_{min}: −10°C; T_{opt}: 15°C) [10] and one halophile, *Halorubrum lacusprofundii* (T_{min}: 2°C; T_{opt}: 33°C) [11]. Both of the methanogens were recovered from the bottom of Ace Lake where the water is saturated in methane and remains at a constant 1–2°C [9,10] thus highlighting their adaptation to near freezing temperatures.

Organisms growing at low temperature encounter a number of growth limiting constraints: enzyme reaction rates decrease, the affinity of uptake and transport systems decreases, membranes become less fluid and nucleic acid structures become more stable (reviewed in [12,13]). In response, microorganisms have evolved various ways to adapt. Structures of cold-active proteins have evolved to produce structurally flexible and catalytically efficient proteins at cold temperatures [14]. To date, the structural features that lead to changes in flexibility have been determined from comparative studies of mesophilic and thermophilic proteins based on homology modeling, and by analysis of three recently determined crystal structures of psychrophilic bacterial proteins [15–17]. These reports from bacteria and fish indicate that structural features of cold active proteins may include less disulfide bonds, a lower net charge in helix-dipole structures, less salt bridges, more solvent interaction, a decrease in the number of hydrogen bonds at domain interfaces and a general decrease in the number of hydrophobic interactions within the core of the protein (reviewed in [12,18,19]).

As part of our global objective to examine the molecular mechanisms of low temperature adaptation in archaea, we are studying the structural features that confer low temperature activity to archaeal proteins. EF-2 was chosen as a model protein due to its essential cellular function and ubiquity in all known life, and the fact that the X-ray crystal structure of the *Thermus thermophilus* EF-G and EF-Tu have been resolved [20–22], thus enabling three dimensional modelling. A further advantage for future work is that EF-2 is a GTPase, thus enabling the use of GTP-binding and GTP-hydrolysis assays to assess activity as a function of temperature. Ribosomes and associated factors (e.g. initiation and elongation factors) from psychrophilic bacteria (*Pseudomonas* and *Bacillus* spp.) have also been shown to be adapted to activity at low temperature in comparison to mesophilic counterparts (reviewed in [23]), indicating that the stability and activity of the machinery of the translation apparatus is thermally adapted to the growth temperature of the organism. In addition, the temperature for half inactivation after 10 min exposure for the EF-2 from the hyperthermophilic archaeon, *Sulfolobus solfataricus*, is 95°C, and the optimal growth temperature for *S. solfataricus* is 75–85°C [24], thus indicating that the thermal properties of archaeal EF-2 proteins reflect the growth temperature of the organism.

This paper reports the sequencing of the archaeal elongation factor *2* (*aef2*) genes from psychrophilic (*Methanococ-*

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The nucleotide sequence data reported in this paper will appear in the GenBank Nucleotide Sequence Databases under accession numbers AF003869, AF022779 and AF026165 for *aef2* and flanking regions of *M. burtonii*, *M. methylutens* and *M. thermophila*, respectively.

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coides burtonii; T_{opt}: 23°C), mesophilic (*Methanococcoides methylutens*; T_{opt}: 35°C) and thermophilic (*Methanosarcina thermophila*; T_{opt}: 50°C) methanogens. We construct scatter plots from distance matrices of archaeal EF-2 amino acid and 16S-rRNA nucleotide sequences, and determine if growth temperature has a demonstrable effect on the rate of protein evolution. Using protein homology modelling we are able to separate changes into two sets: a predominant set that reflects species divergence only, and a smaller set that is likely to include changes necessary for thermal activities.

2. Materials and methods

2.1. Isolation and sequencing of the aef2 genes

M. burtonii (DSM 6242), *M. methylutens* (DSM 2567^T) and *M. thermophila* (DSM 1825) were grown in liquid methanogen growth media (MGM) under anaerobic conditions as described by Franzmann et al. [9]. Genomic DNA was prepared by the method of Tillet et al. (submitted).

An *aef2*-gene specific probe was generated by the polymerase chain reaction (PCR) using degenerate primers constructed from the known gene sequences of seven archaeal elongation factor 2 proteins. The sequences were 5′-CCNGGNCATGTNGATTT-3′ for the forward primer (EF2F) and 5′-TTATGCTTATTNGGNGA-3′ for the reverse primer (EF2R). Following PCR amplification from *M. burtonii* genomic DNA, a unique 1.2-kb fragment was generated and subsequently non-radioactive labelled with a random-priming DIG-labelling kit (Boehringer-Mannheim). This probe was used to screen an *M. burtonii* genomic library, which was constructed in a Stratagene λ-Zap Express system. Positive clones were physically mapped using restriction enzymes and specific fragments were subcloned in pGem-7Zf$^+$ prior to DNA sequencing (see below).

The *aef2* genes of *M. methylutens* and *M. thermophila* were obtained using a protocol of Siebert et al. [25] and a modification by Tillet and Neilan (submitted). Briefly, fragments of the *aef2* gene from the two organisms were generated using PCR and the archaeal specific, degenerate primers, EF2F and EF2R. The resulting 1.2-kb fragments were sequenced (see below) with the two degenerate primers. From this sequencing data, two specific primers were constructed which were complementary to the 5′ and 3′ strands. Genomic DNA was digested with a variety of blunt-end producing restriction enzymes and the resulting fragments were ligated with adapter oligonucleotides (Siebert et al., 1995). The adapter-fragment chimeras were purified and used in a PCR reaction with primers that were complementary to one of the adapter oligonucleotides and primers that were specific for the *aef2* sequence. The resulting PCR reaction allows amplification of the region from within the *aef2* gene to a site upstream or downstream of the gene (defined by the position of the blunt-end restriction enzyme site).

The inserts of the plasmid constructs based on pBK-CMV and pGem-7Zf$^+$ and the PCR generated fragments were sequenced by thermal cycling reactions with a Dye-terminator kit (Perkin Elmer) and the reactions analysed on an Applied Biosystems 337 DNA Sequencing System. The sequences for the *aef2* genes and flanking regions were confirmed by completely sequencing both strands of DNA.

2.2. Phylogenetic analyses

The EF-2 amino acid sequences and the 16S-rRNA sequences of *M. burtonii*, *M. methylutens* and *M. thermophila* were aligned to seven other members of the Archaea and two bacterial organisms (as an outgroup) using the programs Pileup and ClustalW (GCG-software package). Gaps and ambiguous positions were manually deleted and distances were calculated using Kimura [26] and PAM-Dayhoff [27] matrices in the program PROTDIST and DNADIST. Distance matrix trees were constructed using the neighbourhood joining method in the program NEIGHBOR. Parsimony and bootstrap analysis were performed using PROTPARS, PROTDIST, DNAPARS and DNADIST. Distance values of the Kimura matrix were used to plot the phylogenetic distances of the organisms based on the EF-2 amino-acid tree against the one based on the 16S-rRNA tree (scatter plot) and the relationship was statistically analysed using the SPSS program package. A similar approach was used by Springer et al. [28] to construct a linear least-squares fit of methyl-coenzyme M reductase (*mcrI*) genetic distance data to 16S-rRNA distance data, to confirm the assignments of species to genera, and genera to family within the *Methanosarcinaceae* family.

2.3. Three-dimensional modelling

Sections of the derived EF-2 amino-acid sequences which showed a similarity of greater than 25% over at least 40% of the sequence to the EF-G or EF-Tu sequence of *Thermus thermophilus*, were submitted to the modelling program of the SwissModel server [29,30]. This automated modelling software uses a knowledge-based homology modelling approach where crystal structures with sufficiently high primary amino acid sequence similarity to the sequence submitted are superimposed using a dynamic sequence alignment algorithm SIM [31], resulting in a three-dimensional framework for the new protein structure. Missing loops in the new model are generated by sequence based comparison to known loop structures, and based on the position of the α-carbon atoms in the loops, the α-carbon backbone is completed. Regions lacking side chains are modelled using a library of 'allowed' chain rotamers, which are sorted by the increasing frequency of occurrence in known three-dimensional structures. The generated model is ultimately analysed for misfolded areas by the method of Lüthy and Bowie [32] and by measuring the packing density of the atomic model. The generated atomic models were visualised, analysed and superimposed using the SwissPdbViewer, SETOR and PROCHECK programs.

3. Results and discussion

3.1. The effect of growth temperature on the relative rates of EF-2 evolution

To our knowledge it has not been established whether the evolution of proteins from organisms growing at different temperature ranges is accelerated relative to those that evolved in environments of the same temperature. That is, whether the evolutionary rate of proteins (e.g. EF-2) is more sensitive to the selective pressures of environmental temperature than conserved forms of nucleic acid (e.g. 16S-rRNA). Clearly proteins have evolved to function at different temperatures and there is a positive correlation between growth temperature and the temperature profile of enzyme activities. This is illustrated by the lactate dehydrogenase enzymes from psychrophilic, mesophilic and thermophilic bacilli [33]. In contrast, there is no correlation between growth temperature and nucleic acid composition. For example, *M. burtonii, M.*

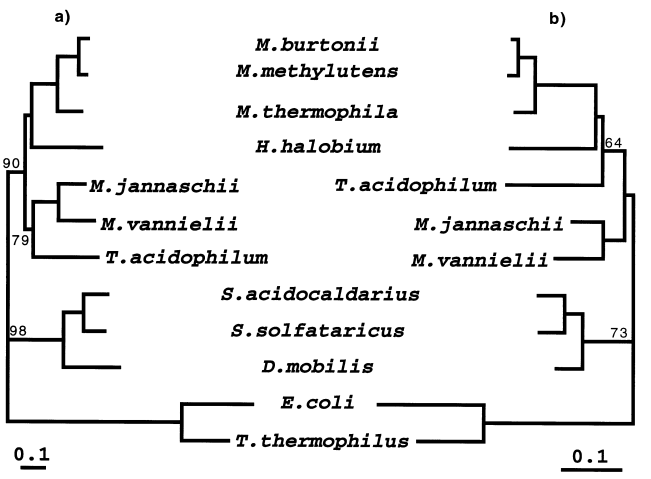

Fig. 1. Distance-matrix trees calculated by the method of Kimura [26] for EF-2 amino acid (a) and 16S-rRNA nucleotide (b) sequences. Scale bar = 1 substitution for every 10 base pairs or amino acids. Numbers on branches indicate boot-strap values for 100 repeats (unless otherwise stated, all values are 100).

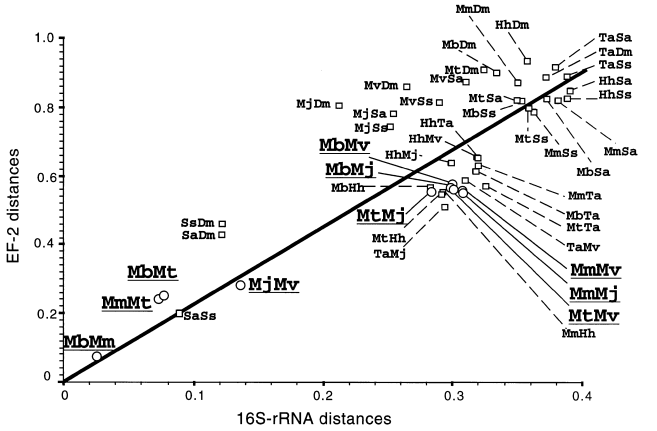

Fig. 2. Scatter plot of pairwise comparisons of phylogenetic distances calculated with the Kimura-distance method for the EF-2 amino acid sequences against the 16S-rRNA sequences. The solid black line shows the least-square linear regression curve forced through the origin ($r^2 = 0.97$). Methanogen pairs are indicated as open circles, and highlighted with underlined name abbreviations and solid lines, and all other pairs are indicated with open squares and dotted lines. Abbreviations: Mb = *Methanococcoides burtonii*, Mm = *Methanococcoides methylutens*, Mt = *Methanosarcina thermophila*, Mj = *Methanococcus jannaschii*, Mv = *Methanococcus vannielii*, Sa = *Sulfolobus acidophilus*, Ss = *Sulfolobus solfataricus*, Dm = *Desulforococcus mobilis*, Hh = *Halobacterium halobium*, Ta = *Thermoplasma acidophilum*.

methylutens and *M. thermophila* have a T_{opt} between 23 and 50°C but a constant G+C mol% of 40–42% for the whole genome [9,34] and 54–56% for their 16S-rRNA sequences, and similar trends are observed for (hyper-)thermophiles [34]. Consequently, if the changes caused by protein adaptation to different temperatures were marked, it would be expected that the differences in rate of amino acid changes for EF-2 relative to nucleotide changes in 16S-rRNA for organisms growing at different temperatures (e.g. psychrophiles vs. thermophiles) would be larger than for organisms growing at similar temperatures (e.g. between mesophiles).

The EF-2 and 16S-rRNA distance-matrix phylogenetic trees for the set of archaeal organisms showed identical topologies, with the exception of the position of *Thermoplasma acidophilum* which was clustered with *Methanococcus jannaschii* and *Methanococcus vannielii* in the EF-2 tree (Fig. 1a) and with *Halobacterium halobium* in the 16S-rRNA tree (Fig. 1b). High boot-strap values were obtained for these distance-matrix trees. In addition, maximum parsimony phylogenetic trees were found to be identical to the distance-matrix 16S-rRNA tree (data not shown), thus indicating that the trees generated had robust construction.

The 16S-rRNA distances (i.e. the number of differences per nucleotide site) were compared with the EF-2 distances (the number of changes per amino acid residue) for each pair of organisms and used to generate a linear regression of least-squares fit (Fig. 2). The correlation coefficient (r^2) was 0.97 when forced through the origin. The difference in divergence may be attributed to different rates of EF-2 evolution (i.e. number of changes within a certain time period) when compared to the rate of 16S-rRNA evolution, or by the same rate of evolution but over a different time span (i.e. the last common EF-2 ancestor was older than the last common 16S-rRNA ancestor).

To determine if there is a correlation between rates of protein evolution and temperature we examined the position of the methanogenic archaeal pairs (*M. burtonii*, *M. methylutens*, *M. thermophila, M. jannaschii* and *M. vannielii)* on the scatter-plot (highlighted in Fig. 2), and avoided comparisons of all other archaeal species that differed not only with respect to their growth temperature, but also in salt-concentration, pH-optimum or oxygen requirements. The scatter plot shows that the methanogen pairs are not consistently located above or below the line. Furthermore, the relative positions of methanogen pairs with similar optimum growth temperatures (e.g. between *M. vannielii* (T_{opt}: 37°C) and *M. methylutens* (T_{opt}: 35°C)) were not significantly different to methanogen pairs with different growth temperatures (e.g. between *M. burtonii* (T_{opt}: 23°C) and *M. thermophila* (T_{opt}: 50°C) or *M. vannielii* (T_{opt}: 35°C) and *M. jannaschii* (T_{opt}: 85°C)). A significant relationship between the methanogen pairs is indicated by a Spearman correlation coefficient of 0.014. These data show that while the evolutionary distances for the EF-2 sequences are about double of those for 16S-rRNA, there is a uniform and direct correlation between EF-2 and 16S-rRNA divergence between pairs of organisms, including all combinations of methanogen pairs. This provides evidence that the amino acid changes that are important for thermal activity are hidden amidst the changes produced by genetic drift and all other effectors of natural selection, and highlights the need to choose a set of closely related proteins (e.g. EF-2 amino acid identity $>77\%$) from phylogenetically coherent organisms such as *M. burtonii*, *M. methylutens* and *M. thermophila* (16-rRNA identity $>90\%$), in order to identify changes indicative of thermal adaptation.

3.2. The EF-2 amino acid sequences of *M. burtonii*, *M. methylutens* and *M. thermophila*

The predicted EF-2 amino acids sequences for *M. burtonii*, *M. methylutens* and *M. thermophila* contain 730 residues each. The gross physico-chemical properties (M_r 80.5, 80.5 and 80.6 kDa and p*I* 5.3, 5.0 and 5.6 for *M. burtonii*, *M. methylutens* and *M. thermophila*, respectively) are predicted to be similar.

Table 1
Structural differences predicted from homology models of *M. burtonii* (M.b.), *M. methylutens* (M.m) and *M. thermophila* (M.t.) EF-2 proteins

Amino acid position	*M. burtonii*	*M. methylutens*	*M. thermophila*	Predicted effect on structure
63/81	Ala/His	Glu/His	Glu/His	Salt bridge missing for M.b.
289/351	Lys/Ala	Thr/Lys	Asp/Lys	Salt bridge missing for M.b. and M.m.
322/332	Glu/Thr	Glu/Pro	Glu/Lys	Salt bridge missing for M.b. and M.m.
565/569	Thr/Lys	Val/Lys	Glu/Arg	Salt bridge missing for M.b. and M.m.
292/318/335	Val/Leu/Iso	Leu/Leu/Iso	Leu/Phe/Val	Decrease in packing density
543/561/585	Thr/Iso/Leu	Thr/Iso/Leu	Phe/Leu/Leu	Decrease in packing density
630/644/675	Iso/Iso/Phe	Iso/Iso/Phe	Val/Leu/Phe	Decrease in packing density
268	Gly	Gly	Pro	More backbone rigidity in loop for M.t.
332	Thr	Pro	Lys	More backbone rigidity in loop for M.m.; salt bridge for M.t.

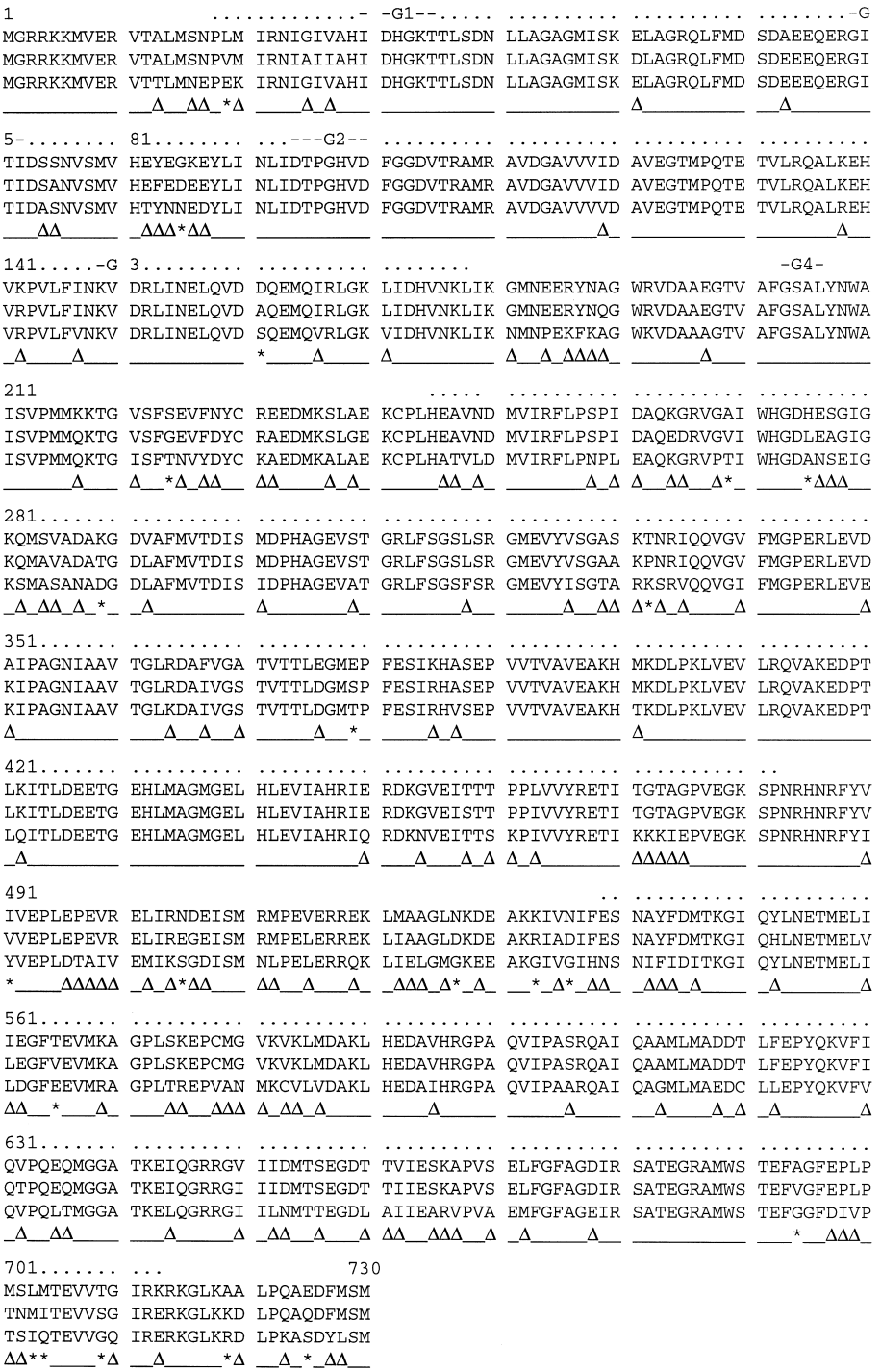

Fig. 3. Alignment of the EF-2 amino acid sequences from *M. burtonii*, *M. methylutens* and *M. thermophila*. Triangles below the amino acid single letter codes indicate unique residues for one sequence, asterisks highlight amino acids that are different in all three sequences, and lines indicate invariant residues. The conserved motifs of the G-domain (domain 1) are marked above the sequence, and dots above the sequence indicate the regions which have been used for three-dimensional protein modeling.

The ratio of arginine+aspartate+asparagine/glutamate+lysine+glutamine (R/D/N to E/K/Q) was 0.76, 0.82 and 0.84 and hydrophobic indices −144, −140 and −122 (predicted using the method of Kyte and Doolittle [36]) for the EF-2 proteins for *M. burtonii*, *M. methylutens* and *M. thermophila*, respectively. The moderate increase in the R/D/N to E/K/Q ratio and decrease in the overall hydrophobicity in the archaeal EF-2 is similar to trends that have been observed for the lactate dehydrogenase proteins of *Bacillus* species [35]. Other biases in amino acid composition that have been associated with thermal activity were not observed between the three species [12,19,35].

Based on pairwise alignments, the amino acid identity of the EF-2 sequences is 91.6% for *M. burtonii* and *M. methylutens*, 77.2% for *M. burtonii* and *M. thermophila*, and 78.4% for *M. methylutens* and *M. thermophila*. The multiple se-

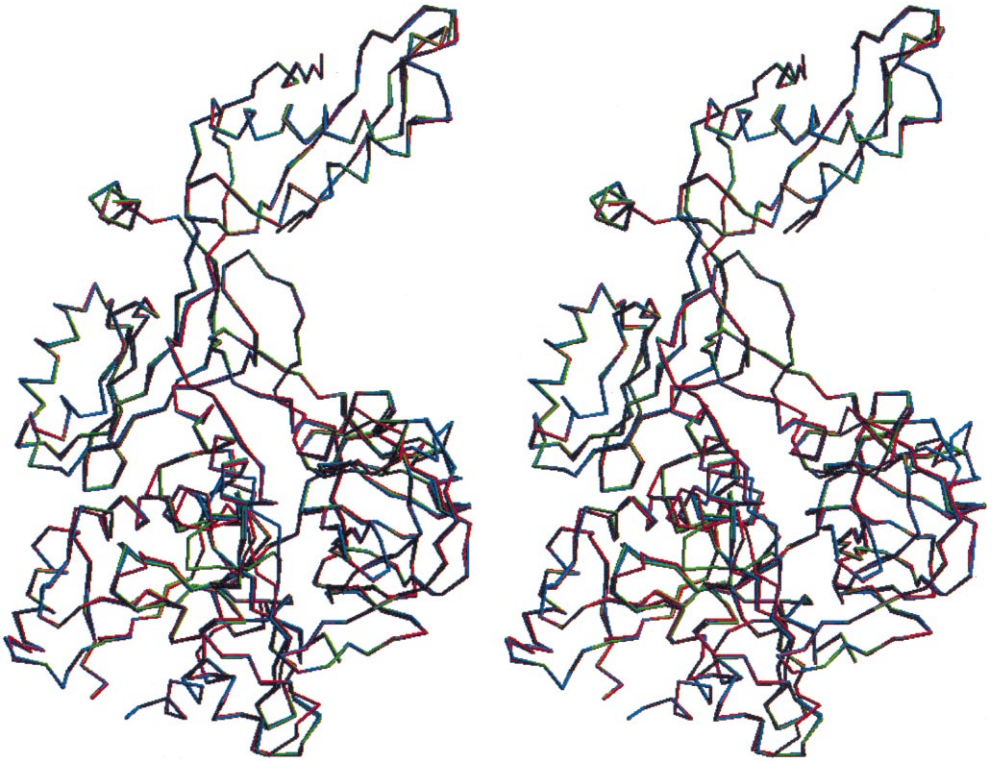

Fig. 4. Stereoimage of the superimposition of the full atomic models of EF-2 proteins from *M. burtonii*, *M. methylutens* and *M. thermophila*. The blue, green and red lines represent the backbone atoms for *M. burtonii*, *M. methylutens* and *M. thermophila*, respectively.

quence alignment showed 41, 37 and 144 unique amino acid residues for *M. burtonii*, *M. methylutens* and *M. thermophila*, respectively, with 22 positions altered in all three sequences (Fig. 3). No gaps were present in the alignment. No changes were observed in the five conserved motifs of the G-domain (G1: 30–36; G2: 94–100; G3: 148–151; G4: 203–206; G5: 68–72); regions involved in GTP/GDP binding and hydrolysis [37].

3.3. Three-dimensional modelling of EF-2

The EF-G of *Thermus thermophilus* consists of 5 domains [21,22] that map in the methanogen EF-2 proteins to approximately positions 7–259 (domain 1), 260–378 (domain 2), 379–464 (domain 3), 465–623 and 692–707 (domain 4) and 624–691 (domain 5). The domain positions were calculated based on the structure based alignments of Ævarrson [38] and alignments performed using the BESTFIT program. Models were generated for all three EF-2 sequences for the regions spanning amino acids 19–176, 245–482 and 538–713 covering 78% of the total protein (Fig. 4). The major gap in the models between amino acids 176 and 245 contains the G″-subdomain, which is not found in bacterial elongation factors [38]. The full atomic models for the archaeal EF-2 proteins were superimposed (Fig. 4). The spatial deviation (RMS values) of the models were 0.29, 0.39 and 0.42 Å for the pairs *M. burtonii*/*M. methylutens*, *M. burtonii*/*M. thermophila* and *M. methylutens*/*M. thermophila*, respectively, and 0.63 Å for 489 α-carbon atoms of the *M. burtonii* model and the crystal structure of EF-G without bound nucleotide [21]. The spatial deviation in these model pairs is similar to that observed for the structural comparison (RMS value of 0.59 Å) of triose-phosphate isomerase from *Vibrio marinus* (psychrophile) with *Escherichia coli* [16]. Furthermore, the homology modelling resulted in predicted three-dimensional models for which $>98\%$ of residues (excluding Gly and Pro) had phi-psi angles in most favoured or allowed regions in a Ramachandran plot. Moreover, side chain and main chain parameters were all within acceptable limits as assessed by PROCHECK [39].

Every position that was altered in any of the three EF-2 sequences was analysed for potential interaction with other residues or the solvent, as well as for possible effects on the backbone (Table 1). There is a successive decrease in the number of salt bridges from the thermophilic to the psychrophilic protein which may enhance flexibility in the psychrophilic protein. In the three hydrophobic core regions identified in domains 2, 4 and 5, the EF-2 from *M. burtonii* exhibits smaller residues than the two other proteins. This may correspond to a decrease in the packing density and, as a consequence, to a destabilisation of this region [40]. The psychrophilic protein is lacking proline residues in loop structures in domains 2 and 3, which are likely to affect the backbone flexibility.

Nine positions were found to have methionine substitutions in at least one of the sequences and there is a higher methionine composition in the psychrophilic (41 residues) compared to the thermophilic (34 residues) EF-2. The high entropy (degrees of freedom) and lack of interacting groups (branches, charge or dipoles) associated with the methionine residues may produce a more fluid protein to enable function at low temperature. Analysis of the methionine substitutions in the models revealed that they are outside directed (surface exposed) for *M. burtonii* and *M. methylutens* (positions 301, 546, 579, 586, 703, 701 and 704) while those in the *M. thermophila* EF-2 are buried inside the protein (positions 581 and

586). In the thermostable citrate synthase from the hyperthermophilic archaeon, *Pyrococcus furiosus,* the methionine content is reduced compared to a pig citrate synthase and the authors suggest this may be due to the tendency for methionine residues to undergo oxidation at high temperature [4]. Furthermore, they indicate that the methionine residues in *P. furiosus* are buried in the interior where they may be less susceptible to covalent modification. The precise roles of methionine residues in thermal adaptation are not clear at this stage and await biochemical analyses of mutant proteins from both psychrophiles and hyper/thermophiles.

Differences in the charge of residues exposed to the surface were observed in a number of cases (positions 82, 84, 85, 86, 87, 246, 275, 276, 278, 287, 471, 472, 473, 475, 538, 539, 584 and 635) and found to be clustered spatially. However, no trend towards an increased solvent interaction that has been described in other proteins, was observed [12].

Our comparative studies represent the first steps towards identifying the structural features important for the low temperature activity of archaeal proteins and demonstrate that differences in general amino acid composition and specific residue changes can be identified in the archaeal psychrophilic EF-2, and these changes appear to be similar to those identified in cold-active enzymes from bacteria and eucaryotes. Furthermore, the structural features noted for increasing protein flexibility appear to be largely the converse of those that lead to increased rigidity in thermophilic proteins. Our work is now focusing on the assessment of temperature dependent differences between the psychrophilic, mesophilic and thermophilic proteins, as well as between wild-type and mutant proteins (residues identified in this study), using activity and stability assays with purified EF-2.

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References

[1] Stetter, K.O. (1996) FEMS Microbiol. Rev. 18, 149–158.
[2] Franzmann, P.D. (1996) Biodiversity Conservation 5, 1295–1305.
[3] Völkl, P., Markiewicz, P., Stetter, K.O. and Miller, J.H. (1994) Protein Sci. 3, 1329–1340.
[4] Danson, M.J. and Hough, D.W. (1998) Trends Microbiol. 6, 307–314.
[5] Fuhrman, J.A., McCallum, K. and Davis, A.A. (1992) Nature 356, 148.
[6] DeLong, E.F., Wu, K.Y., Prezelin, B.B. and Jovine, R.V.M. (1994) Nature 371, 695–697.
[7] DeLong, E.F. (1997) Trends Biotechnol. 15, 203–207.
[8] Schleper, C., Swanson, R.V., Mathur, E.R. and DeLong, E.F. (1997) J. Bacteriol. 179, 7803–7811.
[9] Franzmann, P.D., Springer, N., Ludwig, W., De, M.E.C. and Rohde, M. (1992) Syst. Appl. Microbiol. 15, 573–581.
[10] Franzmann, P.D., Liu, Y., Balkwill, D.L., Aldrich, H.C., Conway de Macario, E. and Boone, D.R. (1997) Syst. Appl. Microbiol. 47, 1068–1072.
[11] Franzmann, P.D., Stackebrand, E., Sanderson, K., Volkman, J.K., Stevenson, P.L., McMeekin, T.A. and Burton, H.R. (1988) Syst. Appl. Microbiol. 11, 20–27.
[12] Feller, G., Narinx, E., Arpigny, J.L., Aittaleb, M., Baise, E., Genicot, S. and Gerday, C. (1996) FEMS Microbiol. Rev. 18, 189–202.
[13] Graumann, P. and Marahiel, M.A. (1996) Arch. Microbiol. 166, 293–300.
[14] Gerday, C., Aittaleb, M., Arpigny, J.L., Baise, E., Chessa, J.P., Garsoux, G. and Feller, G. (1997) Biochim. Biophys. Acta 1342, 119–131.
[15] Agahajari, N., Feller, G., Gerday, C. and Haser, R. (1998) Protein Sci. 7, 564–572.
[16] Alvarez, M., Zeelen, J.P., Mainfroid, V., Rentier-Delrue, F., Martial, J.A., Wyns, L., Wierenga, R.K. and Maes, D. (1998) J. Biol. Chem. 273, 2199–2206.
[17] Russell, R.J.M., Gerike, U., Danson, M.J., Hough, D.W. and Taylor, G.L. (1998) Structure 6, 351–361.
[18] Marshall, C.J. (1997) Trends Biotechnol. 15, 359–363.
[19] Russell, N.J. and Hamamoto, T. (1998) in: Extremophiles: Microbial Life in Extreme Environments (Horikoshi, K. and Grant, W.D., Eds.) pp. 25–45, Wiley-Liss, New York, NY.
[20] Kjeldgaard, M. and Nyborg, J. (1992) J. Mol. Biol. 223, 721–742.
[21] Ævarrson, A., Brazhinikov, E., Garber, M., Zheltonosova, J., Chirgadze, Y., Al-Karadaghi, S., Svensson, L.A. and Liljas, A. (1994) EMBO J. 13, 3669–3677.
[22] Czworkowski, J., Wang, J., Seitz, T.A. and Moore, P.R. (1994) EMBO J. 13, 3661–3668.
[23] Russell, N. (1990) Phil. Trans. R. Soc. Lond. B 326, 595–611.
[24] Vendittis, E., Amatruda, M.R., Raimo, G. and Bocchini, V. (1997) Biochimie 79, 303–308.
[25] Siebert, P.D., Chenchik, A., Kellog, D.E., Lukyanov, K.A. and Lukyanov, S.A. (1995) Nucleic Acids Res. 23, 1087–1088.
[26] Kimura, M. (1980) J. Mol. Evol. 16, 111–120.
[27] Dayhoff, M.O., Eck, R.V. and Park, D.M. (1972) Atlas of Protein Sequence and Structure, 5, Suppl. 3, National Biomedical Research Foundation, Washington, DC.
[28] Springer, E., Sachs, M.S., Woese, C.R. and Boone, D.R. (1995) Int. J. Syst. Bacteriol. 45, 554–559.
[29] Peitsch, M.C. (1995) Bio/Technology 13, 658–660.
[30] Peitsch, M.C. (1996) Biochem. Soc. Trans. 24, 274–279.
[31] Huang, X. and Miller, M. (1991) Adv. Appl. Math. 12, 337–367.
[32] Lüthy, R. and Bowie, J.U. (1992) Nature 356, 83–85.
[33] Sowers, K.R. (1995) in: Archaea: a Laboratory Manual (Methanogens) (Sowers, K.R. and Schreier, H.J., Eds.) pp. 3–13, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[34] Shuisong, N. and Boone, D.R. (1998) in: Extremophiles: Microbial Life in Extreme Environments (Horikoshi, K. and Grant, W.D., Eds.) pp. 211–233, Wiley-Liss, New York, NY.
[35] Zuber, H. (1988) Biophys. Chem. 29, 171–179.
[36] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[37] Chirgadze, Y.N. (1997) Mol. Biol. 30, 453–460.
[38] Ævarrson, A. (1995) J. Mol. Evol. 41, 1096–1104.
[39] Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) J. Appl. Crystallogr. 26, 283–291.
[40] Richards, F.M. (1997) Cell. Mol. Life Sci. 53, 790–802.