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Isolation and cloning of Ompα, a coiled-coil protein spanning the periplasmic space of the ancestral eubacterium *Thermotoga maritima*

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We have discovered a new oligomeric protein component associated with the outer membrane of the ancestral eubacterium *Thermotoga maritima*. In electron micrographs, the protein, Ompα, appears as a rod-shaped spacer that spans the periplasm, connecting the outer membrane to the inner cell body. Purification, biochemical characterization and sequencing of Ompα suggest that it is a homodimer composed of two subunits of 380 amino acids with a calculated M_r of 43 000 and a pI of 4.54. The sequence of the *ompα* gene indicates a tripartite organization of the protein with a globular NH_2-terminal domain of 64 residues followed by a putative coiled-coil segment of 300 residues and a COOH-terminal, membrane-spanning segment. The predicted length of the coiled-coil segment (45 nm) correlates closely with the spacing between the inner and outer membranes. Despite sequence similarity to a large number of coiled-coil proteins and high scores in a coiled-coil prediction algorithm, the sequence of the central rod-shaped domain of Ompα does not have the typical 3.5 periodicity of coiled-coil proteins but rather has a periodicity of 3.58 residues. Such a periodicity was also found in the central domain of staphylococcal M protein and β-giardin and might be indicative of a subclass of fibrous proteins with packing interactions that are distinct from the ones seen in other two-stranded coiled-coils.

Key words: coiled-coil protein/electron microscopy/inverse PCR/periplasmic space/*Thermotoga maritima*

Introduction

Thermotoga maritima is an extremely thermophilic organism representing the deepest known branch within the eubacteria; hence it may have retained characteristics ancestral to this kingdom. Because of its unique position in the phylogenetic tree, it has become the subject of various morphological, physiological and biochemical studies (see e.g. Londei *et al.*, 1988; Ludwig *et al.*, 1990; Wrba *et al.*, 1990; Tiboni *et al.*, 1991). The cell envelope of *T.maritima* has many unusual features, including a novel type of lipid (De Rosa *et al.*, 1988), an uncommon peptidoglycan structure (Huber *et al.*, 1986) and an outer membrane that adheres to the cylindrical part of the cell body but 'balloons' at the cell poles. Recently, we have shown that the outer membrane is basically a two-dimensional periodic array composed of porin trimers. Biochemical characterization of the purified protein and a low resolution three-dimensional map obtained by electron crystallography revealed striking similarities to the *Escherichia coli* porins (Rachel *et al.*, 1990; for a recent review on *E.coli* porins see Jap and Walian, 1990). Because its β-sheet structure is characteristic for porins, we propose to call this protein Ompβ. We also incorporated the isolated trimer into artificial membranes and performed conductance experiments, providing evidence that this protein indeed functions as a porin (Engel,A.M., Brunen,M. and Baumeister,W., manuscript in preparation).

We have now isolated a second major component of the *T.maritima* outer membrane which links the outer membrane to the cell body, and thus could be seen as a functional analogue of Braun's lipoprotein in *E.coli*. CD and IR spectroscopy of the purified protein indicate a predominantly α-helical structure; therefore we propose to call this protein Ompα. In this communication we describe the purification and biochemical and biophysical characterization of Ompα, the cloning and nucleotide sequence of the *ompα* gene, and electron microscopy studies revealing the shape of Ompα and its location in the cell envelope.

Fig. 1. Micrograph of a freeze-fractured cell of *Thermotoga maritima*, coated with 1 nm Pt/C. The outer membrane is maintained at a constant distance of ~50 nm from the cylindrical part of the cell body and it 'balloons' around the cell poles. At higher magnification, faint striations are visible in the periplasmic space (inset). Scale bar: 500 nm.

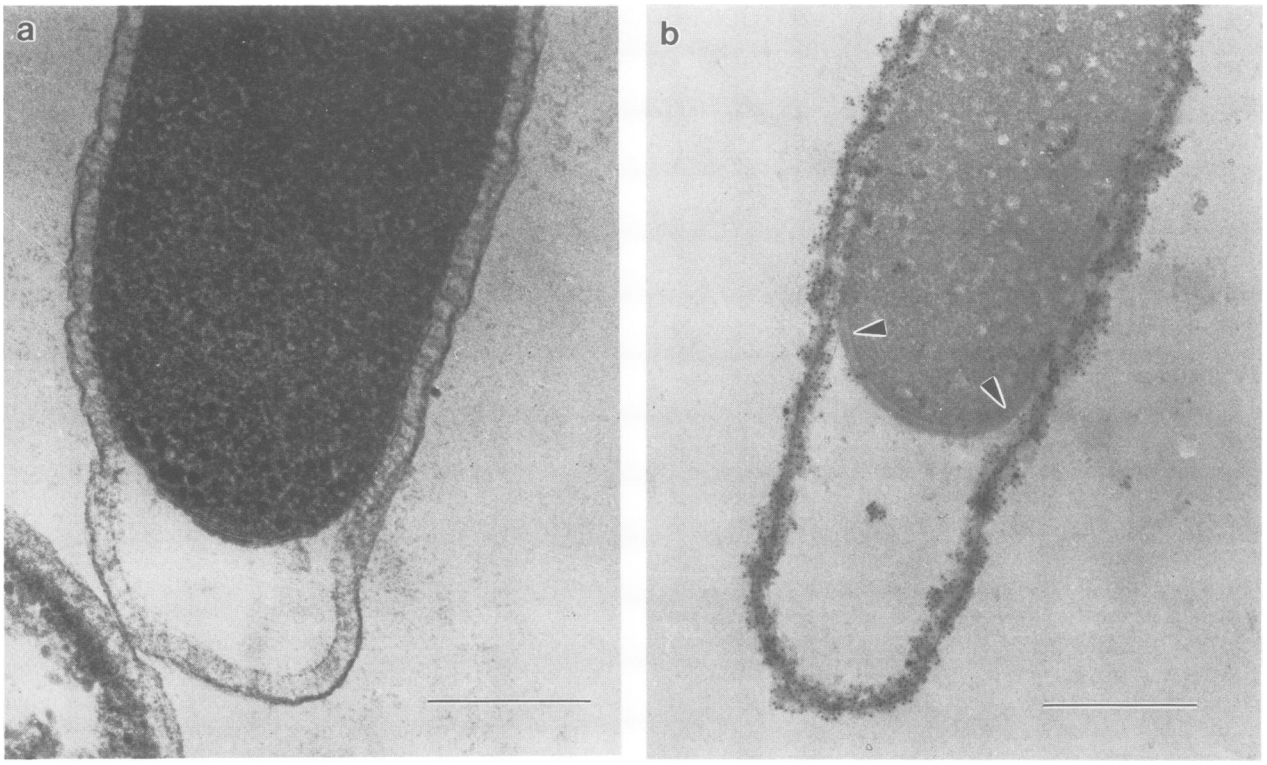

Fig. 2. (**a**) Micrograph of an OsO_4-fixed and Epon-embedded thin section of *Thermotoga maritima*. A seam of fibres spans the unusually wide periplasmic space connecting the outer membrane to the inner cell body. At the cell poles where the outer membrane has no contact with the cell body these fibres remain associated with the outer membrane. Scale bar: 300 nm. (**b**) Micrograph of an aldehyde-fixed and Lowicryl-embedded thin section of *T.maritima*. Immunogold-labelling was done firstly with anti-Ompα IgG and secondly with gold-labelled (5 nm) goat anti-rabbit IgG. The labelling is almost exclusively associated with the outer membrane. An as yet unidentified periodic structure is apposed to the inner surface of the cytoplasmic membrane in the polar regions where the outer membrane 'balloons' (arrows). Scale bar: 500 nm.

Results

Topology of the membrane system

Electron micrographs of *T.maritima* cells (Figures 1 and 2) show that the outer membrane adheres to the cylindrical part of the cell body but 'balloons' around the cell poles. Where it is in contact with the cell body, the outer membrane is maintained at a constant distance of ~50 nm by a fibrous structure oriented perpendicular to the membrane (Figures 1 and 2a). These fibres appear to be inserted at one end into the outer membrane. By analogy to the *E.coli* murein lipoprotein, one may be expect that the fibres are connected to the peptidoglycan at the other end. However, this is unlikely to be true for the polar regions where the outer membrane has separated from the cell body (Figure 2). Here the peptidoglycan appears to remain in contact with the cell body, while the fibres remain associated with the outer membrane: when incubated with lysozyme, the rod-shaped cell body rounds up to a sphere, while the outer membrane appears to remain largely unaffected (data not shown).

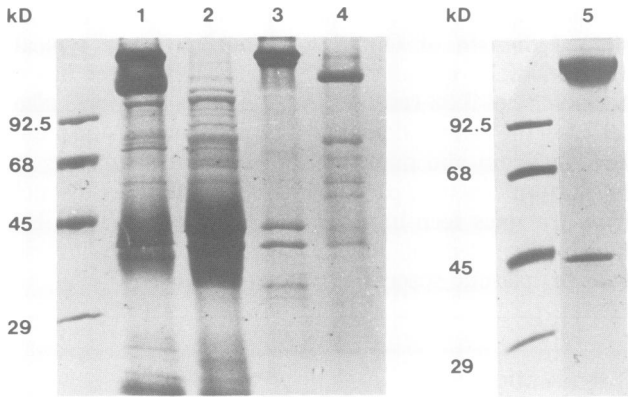

Fig. 3. SDS–PAGE of the isolated outer membrane of *Thermotoga maritima* solubilized in 2% SDS at 37°C (lane 1) and 100°C (lane 2). Fractionation of Ompα (lane 3) and Ompβ (lane 4) after octyl-POE extraction of the outer membrane and gel filtration on Sephacryl S-300 HR. Lane 5, purified Ompα after anion exchange chromatography on Mono-Q 5/5 HR.

Isolation of Ompα and Ompβ

SDS–PAGE analysis of the outer membrane shows that it is constituted mainly of almost equal amounts of two different oligomers: Ompα, with an M_r of 180 kDa, and Ompβ, with an M_r of 120 kDa (Figure 3, lane 1). Both oligomers dissociate into monomers (Ompα: M_r 43 kDa; Ompβ: M_r 40 kDa) when boiled in SDS prior to electrophoresis (lane 2). Separation of the oligomers was achieved by gel chromatography on Sephacryl S-300 HR. The 180 kDa oligomer of Ompα was eluted with the void volume together with some monomeric impurities of Ompβ and an additional 30 kDa protein (lane 3). Ompβ was eluted along with several minor proteins between 50 and 70 kDa, but without Ompα (lane 4).

Purification of Ompα to homogenity (lane 5) was achieved by anion exchange chromatography using a Mono-Q 5/5 HR anion exchange column. Under the conditions used, Ompβ

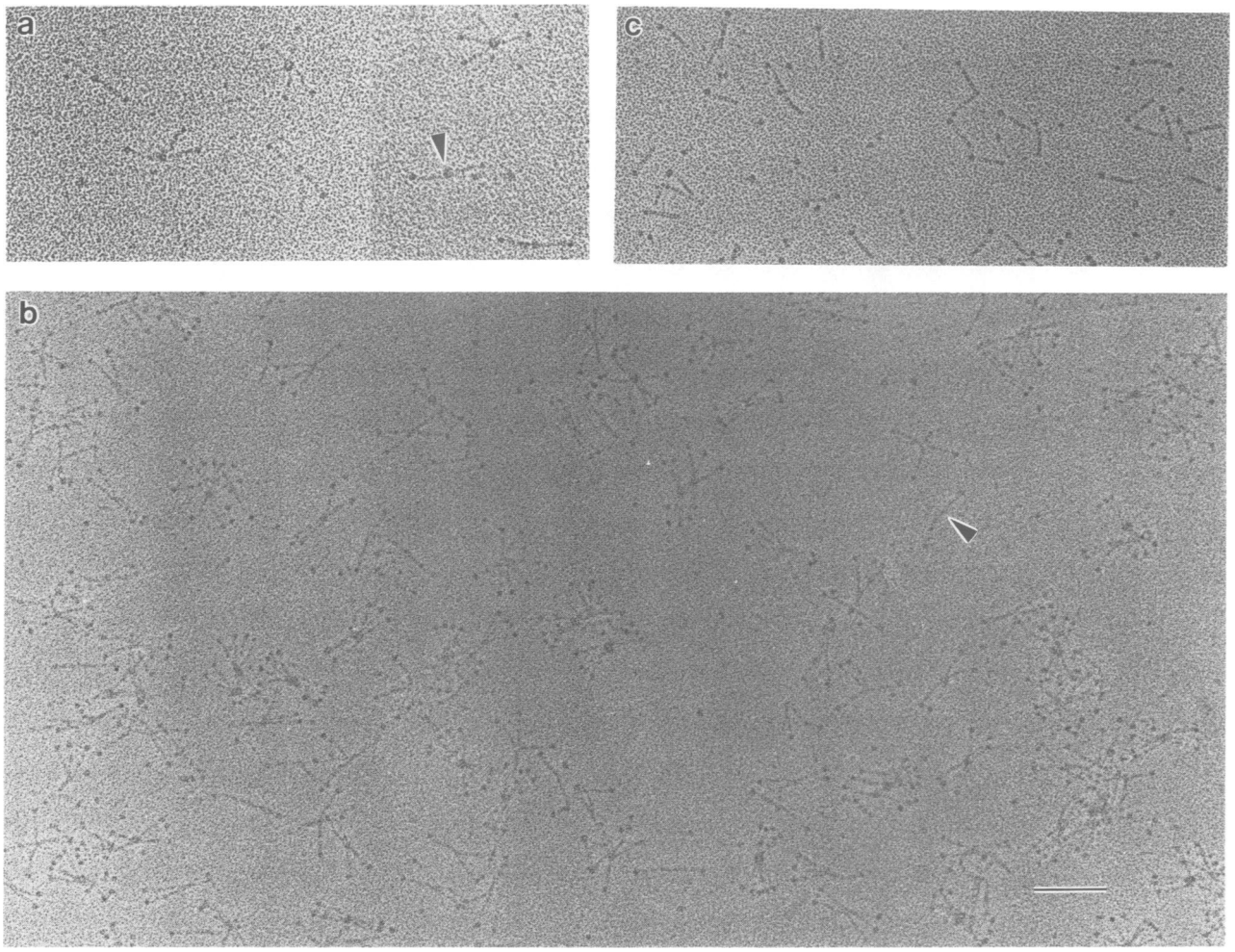

Fig. 4. Electron micrographs of low angle-rotary shadowed (Ta/W) Ompα oligomers. Scale bar: 100 nm. (**a**) Ompα fraction after gel filtration, solubilized in 1% octyl-POE. The smallest aggregates are dimers of the basic unit and are probably formed by two Ompα fibres associating via their hydrophobic COOH-termini (arrow). Various higher order aggregates ('rosettes') are also found. (**b**) Ompα fraction after purification by anion exchange chromatography, solubilized in 0.1% Zwittergent 3-14. Dimers of the basic unit (arrow) as well as higher aggregates ('rosettes') are observed as in (a). However, the globular mass at the centre of the dimers or rosettes is now missing. Since the protein is unchanged according to SDS–PAGE this indicates that the masses in (a) are mainly due to bound lipid and/or detergent. (**c**) In the course of labelling experiments with anti-Ompα IgG, the Ompα preparation was incubated in PBS, 0.05% Tween overnight. Under these conditions Ompα occurs predominantly in the form of the basic fibre unit.

did not bind to the column, while Ompα was eluted with 250 mM Na_2SO_4. Purified Ompα was then used to raise rabbit anti-Ompα sera; the anti-Ompα IgGs were purified via affinity chromatography. Immunolabelling of *T.maritima* thin sections with these antisera showed that the fibres associated with the outer membrane are formed by Ompα (Figure 2b).

Image analysis of isolated Ompα fibres

Isolated Ompα has a strong tendency to aggregate; simple adsorption of the sample onto the grid creates a disordered meshwork. Moreover, visualization of the oligomers by negative stain gave unsatisfactory results because of low contrast. A better way of preparation was to dilute the sample with an equal volume of glycerol and to spray it onto freshly cleaved mica followed by low angle rotary shadowing with either platinum/tungsten or tantalum/tungsten. Such preparations clearly revealed the fibrous structure of Ompα (Figure 4).

Depending on the stage of preparation, different kinds of Ompα oligomers were obtained: The Ompα fraction after gel filtration, solubilized in 1% octyl-POE (polyoxyethylene), is shown in Figure 4a. The smallest of the aggregates, probably dimers of the basic fibre unit, were most suitable for image processing. Figure 5a shows a gallery of 74 straightened and aligned oligomers. The resulting average shown below was used to estimate the linear dimensions: the oligomer has a symmetry axis perpendicular to its longitudinal extension and it resembles a double dumb-bell with an overall length of 95 nm. The two rods are connected by a globular mass with an apparent diameter of 12.8 nm and end in two smaller globular domains with an apparent diameter of 9.8 nm. Bound detergent and the low angle of incidence (10°) used for rotary shadowing must be expected to exaggerate grossly the dimensions of the globular features; this makes it difficult to deduce the lengths of the rod-shaped parts more accurately.

The Ompα fraction, purified by anion exchange chromatography and solubilized in 0.1% Zwittergent 3-14, has lost the large globular mass in the middle of the double

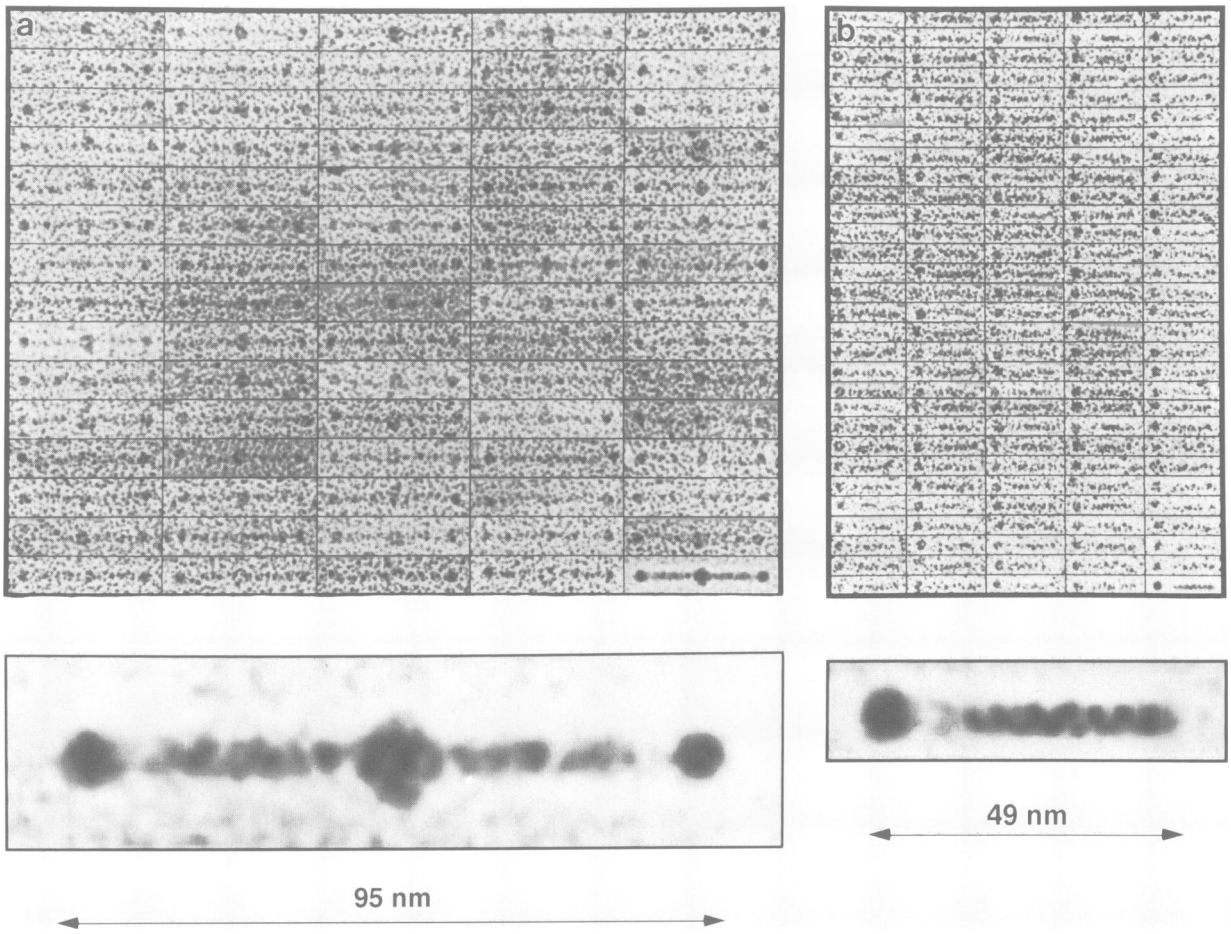

Fig. 5. Galleries of micrographs of straightened and aligned dimeric aggregates of Ompα containing the central mass (**a**) and basic fibre units (**b**). The corresponding averages which were used to derive the linear dimensions of the fibres are shown at the bottom right of each gallery and enlarged below. The size of the globular features is exaggerated due to the shallow shadowing angle.

fibre (Figure 4b). Since the protein is unchanged according to SDS–PAGE, this indicates that the central mass is mainly due to bound lipid and/or detergent. Following incubation in 10 vol PBS, 0.05% Tween overnight at room temperature, the double fibre splits into the basic fiber unit (Figure 4c). The corresponding average of 149 such independent fibres is shown in Figure 5b. The overall length of the fibre, i.e. including the globular domain, is 49 nm.

Nucleotide sequence analysis

We determined 32% of the total primary sequence of Ompα by Edman degradation of the NH_2-terminus and several cleavage peptides (bold letters in Figure 6). We were not able to clone the gene coding for Ompα in a single piece. This is reminiscent of earlier cloning attempts with other outer membrane proteins, e.g. the porin Omp32 of *Comamonas acidovorans* (Gerbl-Rieger *et al.*, 1991) or protein F of *Pseudomonas aeruginosa* (Duchene *et al.*, 1988) in high-copy plasmids. The expression of these proteins may be lethal to the host because of membrane lesions. Since in some cases this problem was overcome by using bacteriophage as cloning vehicles, we screened a λgt11 gene library of *T.maritima*—likewise without success. Therefore, we cloned the *ompα* gene in pieces. The missing parts were amplified by inverse PCR and directly sequenced (for details, see Materials and methods).

The nucleotide sequence we obtained (Figure 6) contains one open reading frame consisting of 1200 bp and encoding 400 amino acids (aa). The other two forward reading frames have 16 and 13 stop codons, respectively. A putative Shine–Dalgarno sequence, 5′-GGAGGT-3′, is situated 13 bp upstream of the translation start codon ATG; this sequence matches exactly the *T.maritima* 16S rRNA sequence 3′-CCUCCA-3′ (Achenbach-Richter *et al.*, 1987) and is also found at position −7 in the *T.maritima rpoC* gene (Schleper, 1989). Downstream of the translation termination signal, there is no hairpin structure typical for factor-independent regions.

The GC content of *ompα* is 46 mol %, the same value as was obtained for the whole genome (Huber *et al.*, 1986); the preference of G/C over A/T in codon position 3 is 60%. The codon usage (nine of 59 codons are not used) shows some remarkable features: of the six possible codons for Arg, only AGA and AGG are used (six and five times respectively). AAC is used preferentially for Asn (24 out of 28), TAC for Tyr (13 out of 14) and TTC for Phe (10 out of 11). For Gln, only CAG is used (nine times). For Leu, CTA and TTA are not used while CTC and CTT are preferred (40 out of 48). GTA is not used for Val; the other three codons occur with equal frequency (seven times each).

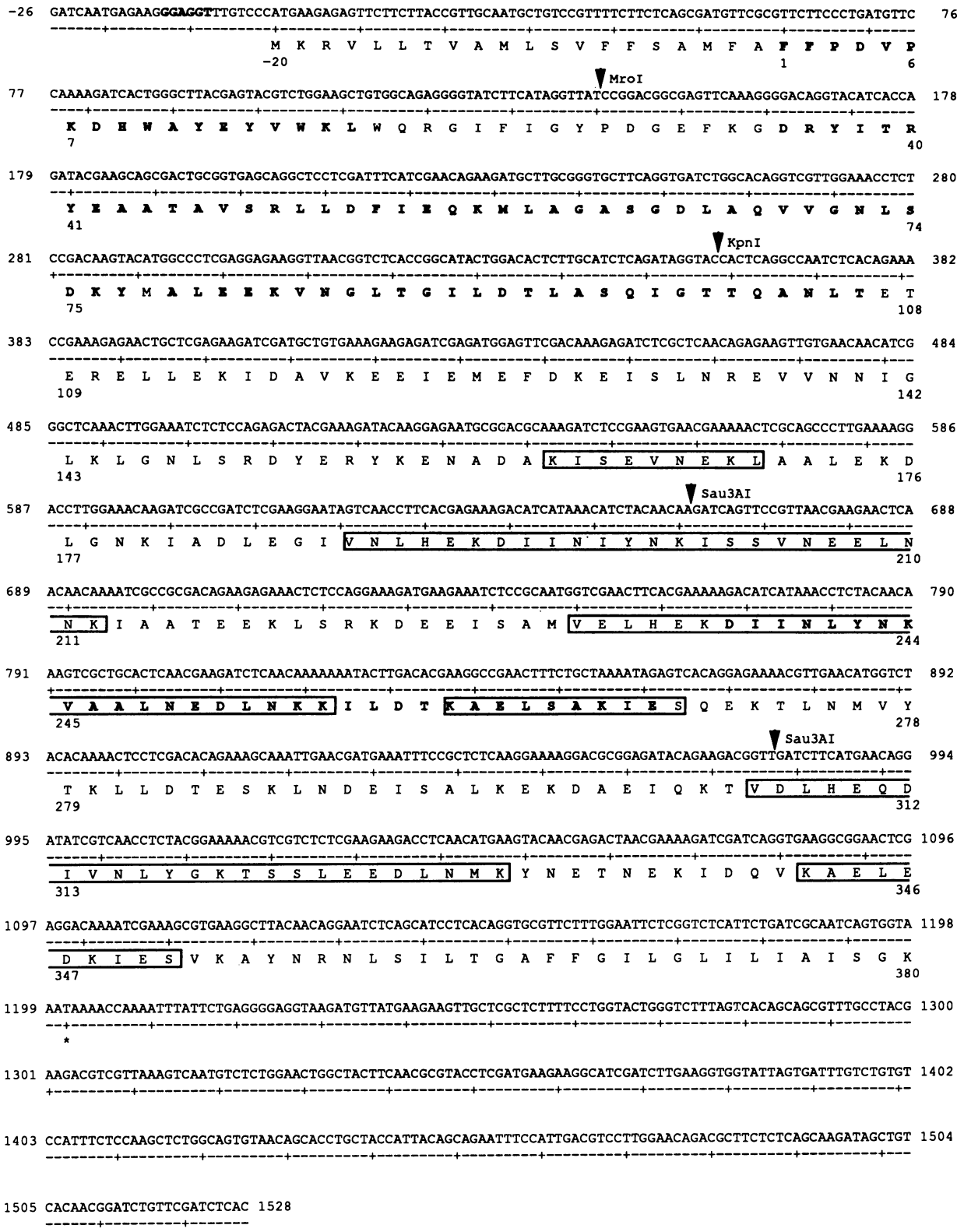

Fig. 6. Nucleotide sequence (upper line) and derived amino acid sequence (lower line) of Ompα of *Thermotoga maritima*. The putative ribosome-binding region (5′-GGAGGT-3′), marked bold, is located at nucleotide positions −13 to −8. Only the relevant restriction sites are shown; for details see Materials and methods. Protein sequences in bold letters were determined by Edman degradation. Amino acid residues −20 to −1 represents a typical bacterial signal sequence. Repeated regions, probably resulting from gene duplication events, are boxed. The translation stop codon is marked by an asterisk. The nucleotide sequence data reported here are available from the EMBL, GenBank and DDBJ databases under accession number X68276.

Amino acid sequence analysis

The NH_2-terminus of the mature protein, FFDVP, is preceded by a typical 20 aa leader peptide (Figure 6, aa −20 to −1) which shares characteristic features with many secretory proteins (Perlman and Halvorsen, 1983), including two positively charged residues followed by a hydrophobic

core of 12 aa, a Ser near the COOH-terminus of the core and the potential cleavage site AMFA.

The mature Ompα protein consists of 380 aa and has a molecular mass of 43.1 kDa, which is in agreement with the apparent molecular mass determined by SDS–PAGE (Figure 3). One-third of the amino acids are charged, giving Ompα a total charge of −25. The hydropathy profile (Kyte and Doolittle, 1982) of Ompα reveals an even distribution of hydrophilic residues throughout the molecule, with the exception of the COOH-terminus, which contains a hydrophobic tail of ~20 aa. These residues may represent a membrane anchor, analogous to the one found in the staphylococcal M proteins (Fischetti *et al.*, 1988).

The COOH-terminal half of the Ompα sequence has several repeated regions (boxed in Figure 6), which are probably the result of gene duplication events. A 25 residue segment is repeated three times (aa 188–212, 231–255 and 306–330); a shorter segment of nine residues (aa 162–170) shows strong homology to the first of the three repeats (aa 201–209). Another repeat of 10 residues is found twice (aa 260–269 and 342–351).

A FASTA alignment of Ompα against the PIR database (George *et al.*, 1986) yielded almost exclusively coiled-coil proteins in the top 20 scores, with the exception of the 125 kDa surface layer protein of *Bacillus sphaericus* (Bowditch *et al.*, 1989). The ALIGN program (Orcutt *et al.*, 1986) was then used for a more detailed comparison of Ompα with the four most similar proteins: the M6 protein (Fischetti *et al.*, 1988) gave the highest score (7.59 SD units), followed by intermediate filament protein B (Weber *et al.*, 1989; 6.10 SD units), paramyosin (Kagawa *et al.*, 1989; 5.73 SD units) and rabbit myosin heavy chain (Nagai *et al.*, 1988; 5.56 SD units). Alignment of the M6 protein to the same proteins yielded comparable scores. The aligned sequences showed an amino acid identity of ~25%, distributed randomly over the sequences and thus indicative of structural analogy rather than true homology.

Secondary structure analysis

As expected from the sequence alignment studies, Ompα is a predominally α-helical protein: IR spectroscopy of outer membrane fractions (Figure 7) yields a saddleback peak between 1630 and 1655 cm^{-1}, which combines amide I absorption bands typical of α-helices (~1650 cm^{-1}) and β-sheets (~1630 cm^{-1}). Purified Ompα shows almost exclusively the amide I absorbance peak characteristic for α-helices while the porin Ompβ shows the typical absorption spectrum of a β-sheet. From CD spectroscopy data (Figure 8) we estimated the α-helix content of Ompα to be ~75%, using the ellipticity at 222 nm (Staprans and Watanabe, 1970); by the same method, the M proteins were shown to be ~70% α-helical (Phillips *et al.*, 1981).

Predictive secondary structure analysis according to the algorithm of Garnier *et al.* (1978) was consistent with the CD results: the majority of Ompα exhibits strong α-helical potential with the exception of an NH_2-terminal domain of 41 residues and several small segments of β-sheet potential (aa 66–71, 89–95, 138–145 and 359–377). Analysis with the coiled-coil prediction algorithm of Lupas *et al.* (1991) yielded coiled-coil forming probabilities of >50% for amino acids 65–139 and 143–365, and >90% for amino acids 80–107, 144–187, 193–233, 261–290 and 319–363 (Figure 9). The program predicted periodic skips and stutters

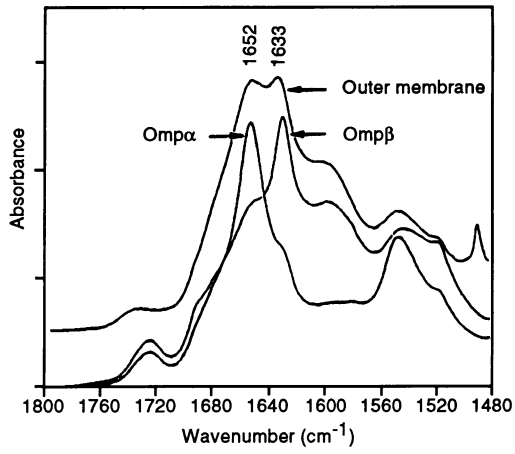

Fig. 7. Superimposition of the IR spectra of the outer membrane fraction, purified Ompα and purified Ompβ. The spectrum of the outer membrane indicates that Ompα and Ompβ occur in approximately similar amounts. The spectra were recorded with a Fourier transform infrared (FT-IR) spectrograph (Nicolet 740) using the total attenuated reflection technique (Kleffel *et al.*, 1985). Amide I bands at ~1650 cm^{-1} are characteristic for α-helices and those at ~1630 cm^{-1} for β-sheets.

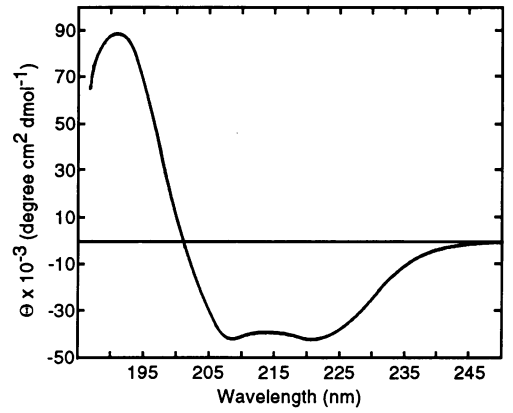

Fig. 8. CD spectrum of Ompα (concentration = 0.1548 mg/ml), recorded with a Jobin Yvon Dichograph Mark IV at 20°C. The absorption at 222 nm indicates an α-helical content of ~75%.

approximately every 50 residues. While such disruptions have been described for most known coiled-coil proteins (Cohen and Parry, 1990), their periodicity was surprising and reminiscent of the pattern described for the central domain of the M6 protein and in β-giardin, a cytoskeletal protein from the parasitic flagellate *Giardia lamblia* (Holbertson *et al.*, 1988).

Fourier analysis of Ompα, β-giardin and M6 with a program that was originally used to examine the packing of type I collagen (Hofmann *et al.*, 1978) illustrates the distinct sequence properties of these proteins: the strongest periodicity detected in canonical coiled coils is the 3.5 repeat of hydrophobic residues, F, I, L, M, V and Y (Cohen and Parry, 1990). This periodicity is also seen in repeat A (aa 12–108) and C (aa 234–362) of the M6 rod segment (3.52 and 3.51, respectively). In contrast, the periodicity of hydrophobic residues is 3.58 in Ompα (from aa 65 to the COOH-terminus), 3.62 in β-giardin, and 3.57 in repeat B of the M6 rod (aa 110–230).

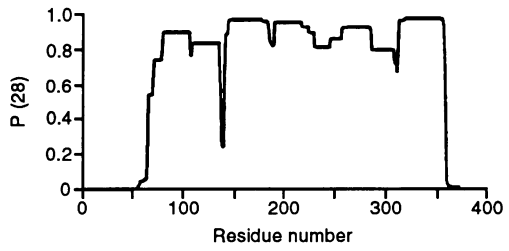

Fig. 9. Coiled-coil formation probabilities for the Ompα sequence as calculated using the prediction program of Lupas *et al.* (1991). The sequence was scanned with a 28 residue window and yielded high coiled-coil probabilities from aa 65 to aa 365.

Discussion

Localization and function of Ompα

On micrographs of freeze-etched cells and thin sections, single fibres, most probably the rod domain of Ompα, are visible connecting the outer membrane of the cell body (Figures 1 and 2a). By immunolabelling of thin sections of *T.maritima* with anti-Ompα IgGs we showed that the seam of fibres on the periplasmic side of the outer membrane is indeed formed by Ompα (Figure 2b). Although the orientation of Ompα fibres relative to the outer membrane cannot be deduced from electron micrographs, it is likely that the COOH-terminal hydrophobic tail is buried in the membrane since the protein remains attached to the membrane in the 'ballooning' regions and is isolated with the outer membrane fraction. Isolated fibres in solution tend to associate via their putative hydrophobic membrane anchor region, forming either dimeric units or higher aggregates (rosettes) as seen in Figure 4a and b.

On the other side, it is not clear how Ompα is connected to the peptidoglycan or the cytoplasmic membrane. The staphylococcal M proteins are fixed with a COOH-terminal membrane anchor in the cytoplasmic membrane, but face towards the exterior and help the parasite evade the host immune system (Fischetti *et al.*, 1988). While the structural organization and the size of Ompα are similar to that of the M proteins, its functional role may be closer to Braun's lipoprotein in *E.coli*: both proteins seem to act as spacers or 'yardsticks', with the much greater length of Ompα resulting in a distance of ~50 nm between the inner and outer membranes, as compared with the ~20 nm width of the periplasmic space of *E.coli* (Graham *et al.*, 1991). Braun's lipoprotein also forms a coiled-coil, but is much shorter (58 residues) and lacks a hydrophobic transmembrane sequence. It is covalently linked via Cys1 to three fatty acid molecules which anchor it in the outer membrane and via the ε-amino group of Lys58 to the carboxyl group of mesodiaminopimelic acid of the peptidoglycan (McLachlan, 1978). After isolating *T.maritima* murein we could not detect any covalently linked Ompα (data not shown).

Together with the porin Ompβ, Ompα is the most abundant protein in *T.maritima*; both proteins seem to occur in about the same copy number as can be estimated by SDS–PAGE (Figure 3, lane 1) and the IR spectrum (Figure 7) of the outer membrane. Several questions with regard to structure and function arise from the results presented here. For example, the packing geometry of Ompα within the two-dimensional periodic array of porin trimers will require further investigation. Also, the suggestion that Ompα serves merely as a passive spacer raises questions about whether an increased periplasmic space confers any advantage on the cell and whether this is related to life at high temperature. Other potential functions for Ompα may include an involvement in the peculiar temperature-dependent swimming behaviour of *T.maritima* (Gluch,M. and Baumeister,W., in preparation), perhaps as a sensory element, or an as yet unidentified enzymatic activity of the globular domain.

Structure of Ompα

For a straight rod with a helix rise of 1.485 Å (Fraser and McRae, 1973), the length of the rod domain of Ompα (aa 65–365) can be calculated to be 44.7 nm, which is in good agreement with the dimension of the average shown in Figure 5b as well as with the spacing between the inner and outer membranes (Figures 1 and 2). From the molecular weight of an individual fibre and by analogy with the lipoprotein, we conclude that each fibre is formed by a parallel two-stranded coiled coil of Ompα monomers, giving the image as shown in Figures 4c and 5b. Nevertheless, we cannot entirely exclude the possibility that the functional fibre is constituted by a four-helix bundle, although molecular weight considerations and lack of precedent for a bundle of this length make such a structure unlikely.

Structure of the rod domain of Ompα

Although all the methods that we applied to the analysis of Ompα, including CD spectroscopy, electron microscopy and sequence analysis, agree on assigning a coiled-coil structure to the protein, results from the coiled-coil prediction algorithm and Fourier transform analysis point to properties of the Ompα sequence which distinguish it from most coiled-coil proteins.

Canonical coiled coils are built from a repeated heptapeptide pattern $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, in which positions a and d are generally nonpolar residues forming a hydrophobic core at the helical interface (Crick, 1953; for a review of coiled-coil structure, see Cohen and Parry, 1990). Oppositely charged residues in positions e and g flank the core and stabilize the protein through ionic interactions. The outer positions, b, c and f, are not directly involved in the formation of the helical bundle and are free to interact with surrounding molecules. This sequence organization yields a continuous heptad repeat and a periodicity of 3.5 for hydrophobic residues in Fourier transform analysis. Many coiled-coil proteins contain one or more discontinuities in their rod segment, most commonly insertions of one residue (skips), which shift the hydrophobic core from a–d to b–e, and omissions of three residues (stutters), which shift the core from a–d to e–a. Rather than representing gradual shifts in the sequence, discontinuities generally cause a sharp transition in the location of the hydrophobic core (for a discussion of skip residues in myosin, see McLachlan and Karn, 1982).

Fourier analysis of Ompα shows that, despite sequence similarity to canonical coiled coils, this protein has a periodicity of hydrophobic residues close to 3.6. A helical net plot of the sequence shows that this is not due to periodic skip residues, as suggested by analysis with a coiled-coil prediction algorithm, but is the result of a continuous drift of the hydrophobic core across the helix surface (Figure 10). In the light of these analyses, it is likely that the

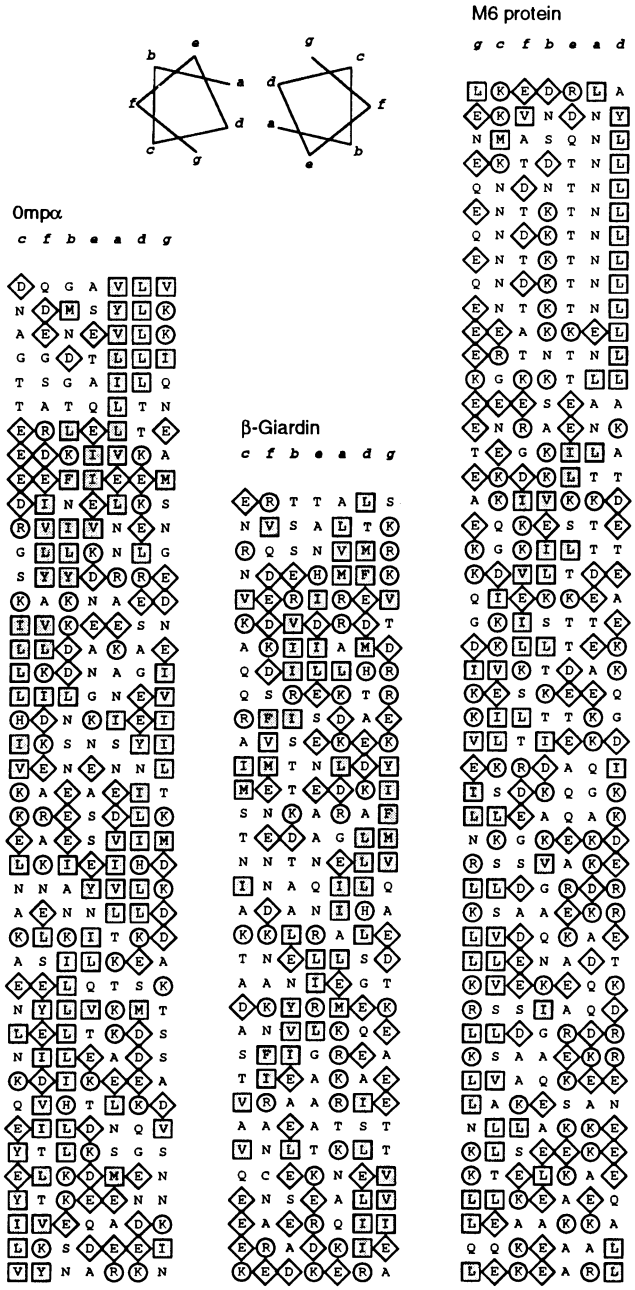

Fig. 10. Helical net diagrams of Ompα (aa 65−358), β-giardin (aa 7−237) and M6 protein (aa 12−361); a cross-section through an idealized coiled-coil is shown at the top of the figure. Columns are labelled by their position (a−g) in an idealized heptad repeat. Hydrophobic residues (F, I, L, M, V and Y) are shadowed, basic (H, K and R) circled and acidic (D and E) enclosed in diamonds. In the M6 protein and initial 14 heptads and the terminal 26 heptads (repeats A and C) display the typical 3.5 periodicity of coiled coils, as seen from the straight ridge of hydrophobic residues in the diagram. Ompα, β-giardin and repeat B of M6 protein have a periodicity close to 3.6, seen in the continuous drift of hydrophobic residues across the helical surface.

discontinuities predicted by the algorithm are a result of shoehorning the protein into a heptad repeat frame. Similar periodic discontinuities described in β-giardin and staphylococcal M6 protein may be the result of the same 'error', since the hydrophobic core in these proteins shows a continuous drift in the regions presumed to be discontinuous (Figure 10). This view is supported by Fourier analysis which assigns these regions periodicities close to 3.6. Thus, Ompα appears to be a representative of a subgroup of coiled-coil proteins whose periodicity is not compatible with the canonical coiled-coil packing proposed by Crick. For β-giardin, Holbertson *et al.* (1988) proposed that segments of four heptads with regular packing interactions would be staggered by skip residues to yield a zigzagging structure with local crossing angles of 20° but an overall crossing angle of 0°. While we do not find evidence for such a regular alternation of continuous and discontinuous packing in Ompα or β-giardin, we would concur that the topology of the hydrophobic core in Ompα indicates a packing angle close to 0°. Only extensive model building will be able to tell whether an alternative quasi-regular packing is possible at such low angles.

Because of the unique phylogenetic position of *T.maritima* in the eubacterial kingdom it is tempting to speculate that Ompα represents an ancestral type of coiled-coil protein. Such a proposition would be difficult to verify; the low level of sequence similarity between various members of the family and the extensive sequence constraints needed to assume a coiled-coil conformation make us believe that similarities within the family of coiled-coil proteins are not due to genetic relatedness, but rather to structural analogy. We presume that the similarity between M proteins and myosin observed by Fischetti *et al.* (1988) is due to the same reasons and does not reflect genetic relatedness.

Materials and methods

Protein purification

T.maritima MSB 8 was kindly provided as a frozen cell paste by K.O.Stetter, University of Regensburg and W.Zillig, Max-Planck-Institute of Biochemistry, Martinsried.

The preparation of the outer membrane by Percoll gradient centrifugation, solubilization of its protein components by octyl-POE (Bachem, Bubendorf, CH) and gel filtration on Sephacryl S-300 HR (LKB-Pharmacia, Uppsala, Sweden) were performed as described in Rachel *et al.* (1990). For further purification, fractions containing Ompα were dialysed against 5 mM Tris−H_2SO_4 pH 7.6, 0.1 mM EDTA, 0.1% Zwittergent 3-14 (Boehringer, Mannheim, Germany) and applied onto a Mono-Q 5/5 HR anion exchange column (Pharmacia) equilibrated in the same buffer. The column was eluted with a salt gradient of 0−0.5 M Na_2SO_4 in 30 ml and a flow rate of 0.5 ml/min. Fractions containing Ompα were concentrated and dialysed against the starting buffer supplemented with 2 mM PMSF and 1 mM azide. Protein was quantified using the BCA Protein Assay Kit (Sigma, Deisenhofen, Germany).

Prior to electrophoresis, the protein samples were precipitated to remove the nonionic detergents used above (Wessel and Flügge, 1984). PAGE was carried out using 8% tricine gels according to Schägger and Jagow (1987).

Peptide cleavage procedures and amino acid sequence analysis

Cyanogen bromide cleavage of 100 μg Ompα was carried out in 100 μl 70% formic acid with a protein:CNBr ratio of 1:1 (w/w). The reaction was stopped by freeze-drying. The peptides were separated in a 12% tricine gel and blotted onto glass fibre for protein sequencing (Eckerskorn *et al.*, 1988).

Prior to proteolytic cleavage, Ompα was denatured in 50 mM Tris−HCl pH 7.6, 0.1% SDS at 100°C for 5 min. AspN Proteinase (Boehringer) was added to an enzyme:protein ratio of 1:100 and the digest was done at 30°C for 20 h. The resulting peptides were separated on a LiChrospher RP-18 reversed phase column (Merck, Darmstadt, Germany) using a linear gradient of 0−60% acetonitrile in 0.1% TFA and a flow rate of 0.5 ml/min.

Edman degradation of the intact Ompα as well as the cleavage peptides was carried out by sequencing on a 477A sequencer equiped with an on-line 120A PTH amino acid analyser (both from Applied Biosystems, Foster City, CA, USA) according to the manufacturers instructions.

Preparation of antisera and isolation of immunoglobulin G

For immunization of a rabbit, 100 μg of Ompα were solubilized in 600 μl 10 mM sodium phosphate pH 7.2, 137 mM NaCl (PBS), 0.1% SDS and

mixed with 1 ml of Freund's complete adjuvant (Behring, Marburg, Germany). After four and six weeks, booster injections with the same amount of Ompα and incomplete adjuvant were performed. Blood was collected one week after the last injection, and the serum was obtained after centrifugation of the clotted blood. The IgGs against Ompα were purified by affinity chromatography: 400 μg of Ompα was coupled onto 0.3 g CNBr-activated Sepharose 4B-CL (Pharmacia) following the manufacturer's instructions and packed into a small column. 1 ml of the serum diluted 10-fold with PBS, was passed several times over the column. The column was intensively washed with PBS before the specific antibodies were eluted by 1 ml of 3 M KSCN in PBS, which were collected on ice and immediately dialysed against PBS.

DNA techniques

Chromosomal DNA of *T.maritima* was isolated by the method of Rodriguez and Tait (1983). Intensive proteinase treatment in the presence of 1% SDS overnight was necessary to set the DNA free. For restriction analysis, chromosomal digests by diverse endonucleases were separated on 0.8% agarose gels in TAE buffer. Degenerated oligonucleotides (usually 17mers) were derived from the amino acid sequences and synthesized by the phosphoamidite method (Beaucage and Caruthers, 1981). Southern hybridization with oligonucleotides, 5'-end labelled with [γ-^{32}P]ATP (Amersham, Braunschweig, Germany), were performed using the standard protocols of Sambrook *et al.* (1989). To minimize the number of clones for colony screening, chromosomal digests were fractionated on gels and only the fractions containing the DNA fragments which hybridized with the oligonucleotides were excised for cloning into pUC18 (Messing, 1983). Size fractions of chromosomal digests for ligation into dephosphorylated pUC18 (Pharmacia) were cut out of the gels and extracted with the Geneclean Kit (Bio101 Inc., La Jolla, CA, USA). The ligation products were used to transform *E.coli* DH5α (*supE*44 Δ*lac* U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) with the $CaCl_2$ method of Sambrook *et al.* (1989). Recombinant clones were screened on LB agar plates containing 50 μg/ml ampicillin, 0.1 mM IPTG and 0.004% X-Gal. Minipreparations of positive clones for subsequent DNA sequencing using a Sequenase™ Kit (US Biochemicals Corp., Cleveland, OH, USA) were done according to Birnboim and Doly (1979).

Cloning strategy

By Southern hybridization of chromosomal digests with four pools of degenerated oligonucleotides we derived the restriction pattern shown in Figure 11: two fragments, a 3.2 kb *Bam*HI fragment and a 4.5 kb *Hin*dIII fragment, were shown to contain the complete *ompα* gene. However, despite extensive colony screening, we were not able to clone the gene in a single piece. Next, we tried to clone the gene in separate pieces, taking advantage of a unique *Kpn*I site (Figures 6 and 11); the *Bam*HI fragments of ~3 kb were cleaved with *Kpn*I, partially digested with *Sau*3AI and ligated into *Bam*HI and *Kpn*I-digested pUC18. Out of 2×10^3 clones, three contained the 5' third of the gene (nucleotides −26 to 360 in Figure 6). The rest of the gene could not be identified even after screening 10^3 clones for the 2.2 kb *Kpn*I fragment (see restriction pattern in Figure 11). The 3 kb *Bam*HI fragments were therefore partially digested with *Sau*3AI and ligated into the *Bam*HI site of pUC18. Two of 1.5×10^3 clones contained nucleotides 663−982 of *ompα* (Figure 6). At this stage, nearly all protein sequences obtained by Edman degradation had been cloned. The missing parts between nucleotides 361 and 662, and at the 3' end of the gene were obtained through inverse PCR (Ochman *et al.*, 1988).

Inverse polymerase chain reaction

For circularization of the 3 kb *Bam*HI fragment, 100 ng of the appropriate restriction fragments were extracted from the gel, diluted in 100 μl ligation buffer and heated for 10 min at 65°C. After cooling down to 16°C, the ligation reaction for 16 h was initiated by adding 100 U of highly concentrated T4 DNA ligase (USB). After ethanol precipitation, the DNA was treated with *Mro*I to relinearize the 3 kb fragment. The DNA was precipitated again and used for the subsequent PCR in the presence of 1.25 mM dNTPs and 100 pmol of the following primers: 5'-GATCTTTTGGAACATCAGGG-3', which is complementary to nucleotides 66−85, and 5'-GCATCTCAGA-TAGGTACCAC-3', which corresponds to nucleotides 343−362 in Figure 6. We used 25 cycles of denaturation at 95°C for 1 min, primer annealing at 56°C for 40 s and extension by 3 U *Taq* polymerase (Amersham) at 72°C for 4 min in a Thermo Cycler (Perkin Elmer Cetus, Norwalk, CT, USA).

The 1.8 kb *Bam*HI−*Kpn*I portion of this PCR product was ligated into pUC18 and cloned so that the remainder of the *ompα* gene could be sequenced. On average, 250 nucleotides were obtained per DNA sequencing reaction. On the basis of these results, sequence-specific oligonucleotides were synthesized and used as primers for continued and reversed sequencing.

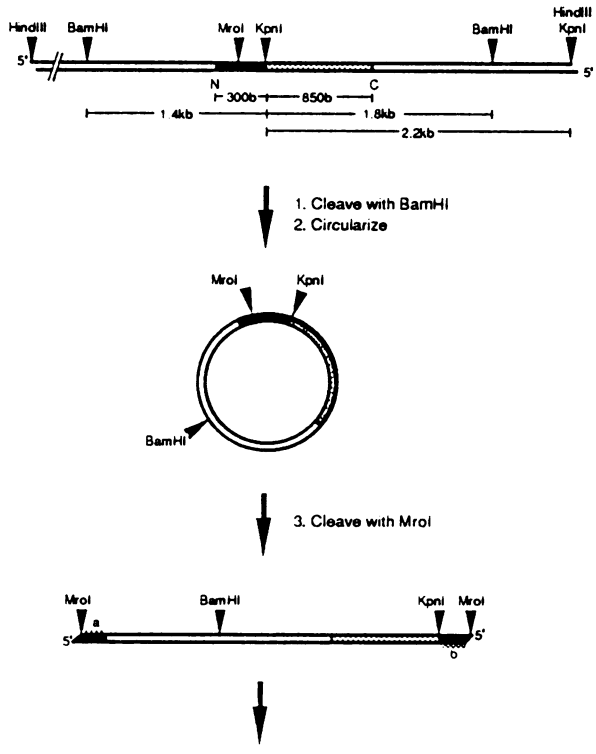

Fig. 11. Strategy for cloning of the *ompα* gene and the inverse PCR. Restriction analysis of Ompα revealed a 4.5 kb *Hin*dIII fragment and a 3.2 kb *Bam*HI fragment with a unique *Kpn*I site suitable for cloning. The inverse polymerase chain reaction was done as follows: The *Bam*HI fragment was circularized using T4 ligase and relinearized again with *Mro*I. In this way, a known sequence of the *ompα* gene (black boxed) was cut to flank the unknown part of the gene (grey boxed), so that either a symmetric PCR could be performed for subsequent cloning or suitable assymetric PCRs for direct sequencing of the product.

We also tried to sequence the PCR product directly to overcome the danger of having cloned a PCR product with misincorporated bases (Gyllensten and Erlich, 1988). Therefore, small segments of ~400 bp were asymmetrically amplified (35 cycles, with conditions as above) using the 3 kb *Bam*HI fragment as template and the oligonucleotides synthesized so far as primers. By adding one primer in excess (100 pmol) and keeping the other limiting (1 pmol), ssDNA is created which can be sequenced by the standard protocol for the Sequenase™ Kit after desalting and removing excess dNTPs and primers via Centricon 30 microconcentration columns (Amicon Corp., Danvers, MA, USA).

Electron microscopy and image processing

Cells used for freeze fracturing were sedimented by centrifugation and frozen in a Cryojet QFD 101 (Balzers, Liechtenstein) at −170°C in propane/liquid nitrogen slush. The cells were cleaved and freeze-etched for 30 s at −98°C in a Balzers BA360 apparatus. The exposed surface was shadowed unidirectionally with 1 nm Pt/C under a nominal angle of incidence of 45°C. The replica was stabilized with a carbon coat and floated on the surface of 75% H_2SO_4 for 24 h to remove the cell debris. Electron micrographs were recorded using a Philips CM12 at a nominal magnification of 21 000×.

For thin-sectioning of whole cells two different techniques were used. (i) The best preservation and visualization of the fibre structure (see Figure 2a) was obtained by fixation with OsO_4 and embedding in Epon. Overnight cultures of *T.maritima* were fixed for 2 h at room temperature by addition of 2% OsO_4 and 2% glutaraldehyde. The cells were pelleted and dehydrated in successive 10 min incubations in 30, 50, 70, 90, 95 and 100% ethanol on ice. The preparation was infiltrated in successive 1 h incubations with 2:1, 1:1 and 1:2 ethanol−Epon 812 mixtures (Luft, 1961) and finally with pure Epon (all at room temperature). The Epon was

polymerized at 45°C for 1 day and at 65°C for three further days. (ii) Immunolabelling (Figure 2b) was accomplished on thin sections embedded by the PLT technique (Kellenberger *et al.*, 1980). Cells were suspended in a fixation solution of 2% paraformaldehyde, 1% glutaraldehyde and 0.5% uranyl acetate at room temperature for 2 h. The cells were pelleted, resuspended in 2% low-gelling agarose Type VII (Sigma) at 37°C and again pelleted immediately as the agar solidified. The agar containing the cells was cut into cubes and dehydrated in successive 1 h incubations in 30, 50 and 70% ethanol at −10, −20 and −35°C and overnight in 100% ethanol at −35°C. The preparation was then infiltrated in successive 2 h incubations with 3:1, 1:1 and 1:3 ethanol−Lowicryl K4M mixtures (Lowi, Waldkraiburg, Germany) and overnight in 100% Lowicryl (all at −35°C). The infiltrated sample was polymerized under UV light for 3 days and thin sections were collected on nickel grids. Immunolabelling was done by incubation for 2 h with the anti-Ompα IgG, diluted 1:10 in PBS, 0.05% Tween, 2% milk powder ('incubation buffer'). Grids were washed several times in PBS, 0.05% Tween ('washing buffer') and subsequently incubated for 1 h with 5 nm gold-labelled goat anti-rabbit IgG (Amersham) diluted in the incubation buffer. The grids were washed again; for the last time in distilled water, before poststaining for 5 min in 1% uranyl acetate.

Solubilized and purified Ompα was adsorbed on carbon coated grids and negatively stained using 2% unbuffered uranylacetate or 2% ammonium molybdate pH 6.8. Alternatively, the same sample was mixed with an equal volume of glycerol, applied to freshly cleaved mica and visualized by low angle rotary shadowing with Pt/W or Ta/W. Electron micrographs were recorded using a Philips EM 420 at a nominal magnification of 30 000×.

For image processing, areas of 256 × 256 pixels from the micrographs were digitized with an Eikonix model 1412 camera system (Eikonix Corp., Bedford, MA, USA) at a pixel size of 15 μm, corresponding to 0.42 nm at the specimen level. For processing, the SEMPER system was used (Saxton *et al.*, 1979): well defined particles were selected and interactively prealigned using an arbitrarily selected centred reference. After the alignment it was possible to reduce the size of the images to 256 × 64 pixels and to straighten the particles using a fibre straightening algorithm. The final alignment was done by means of repeated translational and orientational cross-correlation with an average from the first alignment cycle as a reference. The resulting average of all aligned particles was used for the estimation of the molecular dimensions.

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References

Achenbach-Richter,L., Gupta,R., Stetter,K.-O. and Woese,C.R. (1987) *System. Appl. Microbiol.*, **9**, 34−39.

Beaucage,S.L. and Caruthers,M.H. (1981) Tetrahedron Lett., **22**, 1859−1862.

Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513−1522.

Bowditch,R.D., Baumann,P. and Yousten,A.A. (1989) *J. Bacteriol.*, **171**, 4178−4188.

Cohen,C. and Parry,D.A.D. (1990) *Proteins: Struct., Function, Genet.*, **7**, 1−15.

Crick,F.H.C. (1953) *Acta Crystallogr.*, **6**, 689−697.

DeRosa,M., Gambacorta,A., Huber,R., Lanzotti,V., Nicolaus,B., Stetter,K.O. and Trincone,A. (1988) *J. Chem. Soc. Chem. Commun.*, **13**, 1300−1301.

Duchene,M., Schweizer,A., Lottspeich,F., Krauss,G., Marget,M., Vogel,K., von Specht,B.-U. and Domdey,H. (1988) *J. Bacteriol.*, **170**, 155−162.

Eckerskorn,C., Mewes,W., Goretzki,H. and Lottspeich,F. (1988) *Eur. J. Biochem.*, **176**, 509−519.

Fischetti,V.A., Parry,D.A.D., Trus,B.L., Hollingshead,S.K., Scott,J.R. and Manjula,B.N. (1988) *Proteins: Struct., Function, Genet.*, **3**, 60−69.

Fraser,R.D.B. and MacRae,T.P. (1973) *Conformation in Fibrous Proteins.* Academic Press, New York.

Garnier,J., Osguthorpe,D.J. and Robson,B. (1978) *J. Mol. Biol.*, **120**, 97−120.

George,D.G., Baker,W.C. and Hunt,L.T. (1986) *Nucleic Acids Res.*, **14**, 11−15.

Gerbl-Rieger,S., Peters,J., Kellermann,J., Lottspeich,F. and Baumeister,W. (1991) *J. Bacteriol.*, **173**, 2196−2205.

Graham,L.L., Beveridge,T.J. and Nanninga,N. (1991) *Trends Biochem. Sci.*, **16**, 328−329.

Gyllensten,U.B. and Erlich,H.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7652−7656.

Hofmann,H., Fietzek,P.P. and Kühn,K. (1978) *J. Mol. Biol.*, **125**, 137−165.

Holbertson,D., Baker,D.A. and Marshall,J. (1988) *J. Mol. Biol.*, **204**, 789−795.

Huber,R., Langworthy,T.A., König,H., Thomm,M., Woese,C.R., Sleytr,U.B. and Stetter,K.-O. (1986) *Arch. Microbiol.*, **144**, 324−333.

Jap,B.K. and Walian,P.J. (1990) *Q. Rev. Biophys.*, **23**, 367−403.

Kagawa,H., Gengyo,K., McLachlan,A.D., Brenner,S. and Karn,J. (1989) *J. Mol. Biol.*, **207**, 311−333.

Kellenberger,E., Carlemalm,E., Villiger,W., Roth,J. and Garavito,R.M. (1980) *Low Denaturation Embedding for Electron Microscopy of Thin Sections.* Chem. Werke Lowi, Waldkraiburg, Germany.

Kleffel,B., Garavito,R.M., Baumeister,W. and Rosenbusch,J.P. (1985) *EMBO J.*, **4**, 1589−1592.

Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105−132.

Londei,P., Altamura,S., Huber,R., Stetter,K.O. and Cammarano,P. (1988) *J. Bacteriol.*, **170**, 4353−4360.

Ludwig,W., Weizenegger,M., Betzl,D., Leidel,E., Lenz,T., Ludvigsen,A., Möllenhoff,D., Wenzig,P. and Schleifer,K.H. (1990) *Arch. Microbiol.*, **153**, 241−247.

Luft,J.H. (1961) *J. Biophys. Biochem. Cytol.*, **9**, 409−414.

Lupas,A., van Dyke,M. and Stock,J. (1991) *Science*, **252**, 1162−1164.

McLachlan,A.D. (1978) *J. Mol. Biol.*, **122**, 493−506.

McLachlan,A.D. and Karn,J. (1982) *Nature*, **299**, 226−231.

Messing,J. (1983) *Methods Enzymol.*, **101**, 20−78.

Nagai,R., Larson,D.M and Periasamy,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1047−1051.

Ochman,H., Gerber,A.S. and Hartl,D.L. (1988) *Genetics*, **120**, 621−623.

Orcutt,B.C., Dayhoff,M.O., George,D.G. and Barker,W.C. (1986) *User's Guide to Software Systems of Protein Identification Resource, Alignment Score Programm.* National Biomedical Research Foundation, Washington, DC.

Perlman,D. and Halvorson,H.O. (1983) *J. Mol. Biol.*, **167**, 391−409.

Phillips,G.N., Flicker,P.F., Cohen,C., Manjula,B.N. and Fischetti,V.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4689−4693.

Rachel,R., Engel,A.M., Huber,R., Stetter,K.-O. and Baumeister,W. (1990) *FEBS Lett.*, **262**, 64−68.

Rodriguez,R.L. and Tait,R.C. (1983) *Recombinant DNA Techniques.* Addison-Wesley, Reading, MA, pp. 45−46.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molcular Cloning. A Laboratory Manual.* Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Saxton,W.O., Pitt,T.J. and Horner,M. (1979) *Ultramicroscopy*, **4**, 343−354.

Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368−379.

Schleper,C. (1989) Diploma thesis, University of Konstanz.

Staprans,I. and Watanabe,S. (1970) *J. Biol. Chem.*, **245**, 5962−5966.

Tiboni,O., Cantoni,R., Creti,R., Cammarano,P. and Sanangelantoni,A.M. (1991) *J. Mol. Evol.*, **33**, 142−151.

Weber,K., Plessman,U. and Ulrich,W. (1989) *EMBO J.*, **8**, 3221−3227.

Wessel,D. and Flügge,U.I. (1984) *Anal. Biochem.*, **138**, 141−143.

Wrba,A., Schweiger,A., Schultes,V., Jaenicke,R. and Zavodszky,P. (1990) *Biochemistry*, **29**, 7584−7592.

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