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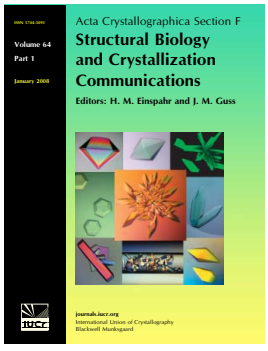

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Expression, crystallization and preliminary X-ray analysis of an outer membrane protein from *Thermus thermophilus* HB27

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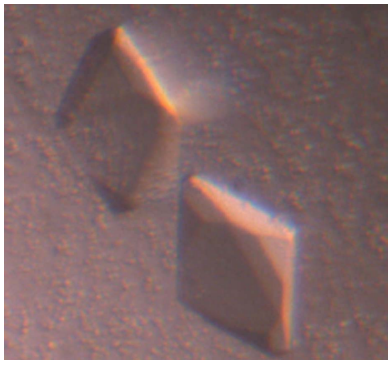
The cell envelope of the thermophilic bacterium *Thermus thermophilus* is multilayered and includes an outer membrane with integral outer membrane proteins that are not well characterized. The hypothetical protein TTC0834 from *T. thermophilus* HB27 was identified as a 22 kDa outer membrane protein containing eight predicted β-strands. TTC0834 was expressed with an N-terminal His tag in *T. thermophilus* HB8 and detected in the S-layer/outer membrane envelope fraction. His-TTC0834 was purified and crystallized under various conditions. Native data sets were collected to 3.2 Å resolution and the best diffracting crystals belonged to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 166.67$, $c = 97.53$ Å.

1. Introduction

The thermophilic bacterium *Thermus thermophilus* belongs to one of the oldest branches of bacterial evolution (Gupta, 2000). The cell envelope is multilayered and differs from those of modern Gram-negative bacteria. The inner membrane is surrounded by a thin peptidoglycan, to which a secondary cell-wall polysaccharide (SCWP) is covalently linked. Pyruvylated components of the SCWP interact with the SLH domain of the S-layer protein, attaching the S-layer protein/outer membrane (OM) layer to the SCWP (Cava *et al.*, 2004). The OM is poorly characterized and neither the precise lipid composition nor details of the OM proteins are known. The genome sequences of two *T. thermophilus* strains, HB8 and HB27, are available (Henne *et al.*, 2004).

As deduced from the genome sequence, both strains encode an Omp85-family protein, which we named TtOmp85. Proteins of the Omp85 family are integral OM proteins that are found in all Gram-negative bacteria sequenced to date as well as in mitochondria and chloroplasts. They are involved either in the translocation of proteins across the OM or in the insertion of β-barrel proteins into the OM (Schleiff & Soll, 2005). To date, we have characterized TtOmp85 *in vitro* as a monomeric stable protein that forms ion channels (Nesper *et al.*, 2008). In order to obtain evidence of whether TtOmp85 is involved in the biogenesis of OM proteins in *T. thermophilus*, we first had to identify putative β-barrel OM proteins. Two different approaches, searching the genome sequence of strain HB27 for potential β-barrel proteins and identifying proteins from OM preparations, identified the protein TTC0834 as a putative eight-stranded β-barrel OM protein (unpublished results). TTC0834 is annotated as a hypothetical protein and is encoded by strain HB27 but not by strain HB8. Apart from the S-layer protein, TTC0834 is the major protein of the OM/S-layer envelope.

Currently, no crystal structure of an OM protein from *T. thermophilus* is available. In this study, we report the homologous overexpression of N-terminally His-tagged TTC0834 in *T. thermophilus* HB8, its purification from detergent-solubilized membrane prepara-



tions, its crystallization and preliminary crystallographic characterization.

2. Materials and methods

2.1. Cloning, expression and purification

The TTC0834 gene was amplified together with its putative promoter region (Baetens *et al.*, 1998) from *T. thermophilus* HB27 genomic DNA using the Phusion DNA polymerase (Finnzymes) and the primers 1fwd, 5′-AA**CTGCAG**GCCCTTTACACCATTGACA-3′ (*Pst*I site in bold), and 2rev, 5′-T**GAATTC**ACCTCTTAGAACCG-ATAGGC-3′ (*Eco*RI site in bold). The PCR product was digested with *Pst*I and *EcoR*I and cloned into the *Pst*I- and *EcoR*I-digested plasmid pMK18 (Biotools) to give plasmid pMK18-TTC0834. The plasmid expressing His-tagged TTC0834, pMK18-HisTTC0834, was constructed such that an in-frame hexahistinine tag and two alanines were incorporated at position 2 of the mature sequence. Two PCR products were amplified from plasmid pMK18-TTC0834. One PCR product was amplified using the primers 3for, 5′-AGGCGATTAA-GTTGGGTAA-3′, and 4rev, 5′-GAACTTTGCAGCGTGGTGTG-GGTGGTGGTGCTGCGCCATAGCTAAGGTCA-3′, and the other using 5for, 5′-GCGCAGCACCACCACCACCACCACGCTGCAA-AGTTCTCTGTAGAGGCGGG-3′, and 6rev, 5′-TCACACAGGA-AACAGCTATGA-3′. These two PCR products were used as a template for PCR using the primers 1fwd and 2rev. The PCR product was digested and ligated into the *Pst*I-and *EcoR*I-digested plasmid pMK18. The resulting construct expresses N-terminal His-tagged TTC0834 from the TTC0834 promoter.

pMK18-HisTTC0834 was transformed into *T. thermophilus* HB8 (Koyama *et al.*, 1986). Transformed cells were grown overnight at 343 K in *Thermus* broth medium (Koyama *et al.*, 1986) containing 25 µg ml^{-1} kanamycin and cell pellets were frozen at 253 K.

Cells were thawed in buffer *A* (50 m*M* Tris pH 8.5, 500 m*M* NaCl) containing 1 m*M* MgCl and a small amount of DNaseI and lysed by passing the suspension through a French pressure cell. Cell envelopes were obtained as a pellet after centrifugation at 100 000*g* for 1 h. Proteins were solubilized in buffer *A* containing 2% *n*-octyltetraoxyethylene (C8E4, Bachem) for 1 h at 295 K followed by centrifugation at 100 000*g* for 1 h at 293 K. The supernatant was loaded onto a His-Trap column (GE Life Sciences) previously equilibrated with buffer *A* containing 0.35% C8E4 and the column was washed with 50 column volumes of the equilibration buffer. His-TTC0834 was eluted with a linear gradient of 0–100 m*M* imidazole in equilibration buffer and loaded onto a Superdex 200 prep-grade column (GE Life Sciences). Gel filtration was performed in a buffer containing 20 m*M* Tris pH 8.5, 200 m*M* NaCl and 0.35% C8E4.

2.2. Cell fractionation

Cell pellets of *T. thermophilus* HB8 pMK18-HisTTC0834 were resuspended in 50 m*M* Tris pH 8 and lysed by passing the suspensions through a French pressure cell. Cell envelopes were obtained as a pellet after centrifugation of the lysate at 100 000*g* for 1 h. Cell envelopes were washed once with 50 m*M* Tris pH 8, resuspended in 50 m*M* Tris pH 8 and loaded onto a step gradient of 30%, 40%, 50%, 55% and 65% sucrose as reported previously (Maier *et al.*, 2001). The gradient was centrifuged at 110 000*g* for 17 h at 293 K and subsequently fractionated into eight equal fractions with a gradient fractionator (Teledyne Isco).

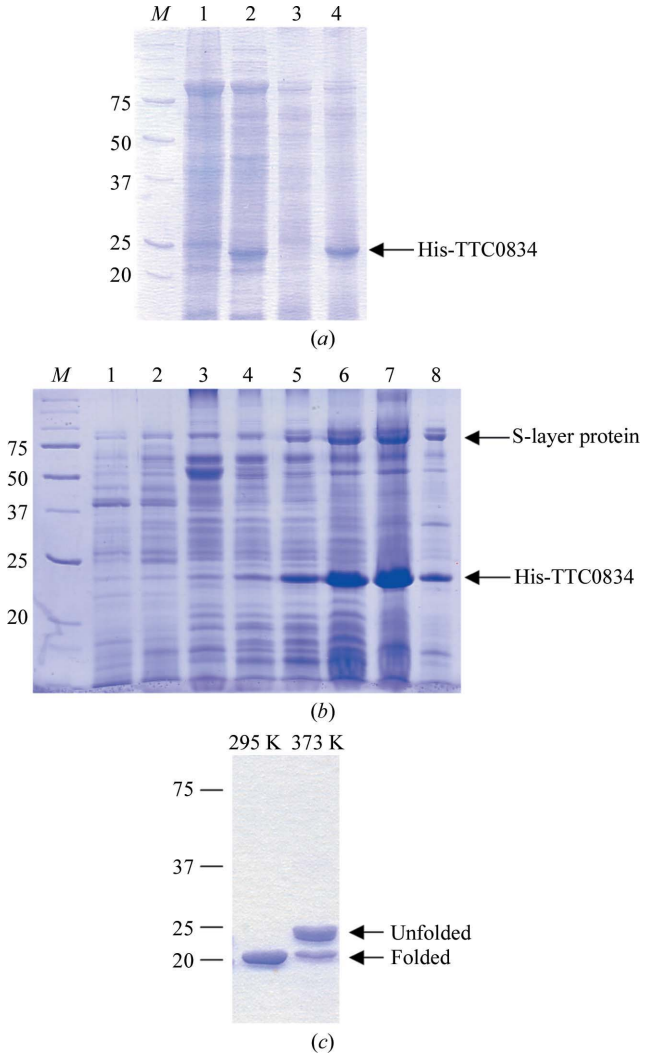


Figure 1
(*a*) Expression and cell-envelope localization of His-TTC0834 in *T. thermophilus* HB8. A Coomassie-stained 12% polyacrylamide gel is shown. Lanes 1 and 2, whole cell extracts of HB8 pMK18 (lane 1) and HB8 pMK18-HisTTC0834 (lane 2). Lanes 3 and 4, cell envelopes of HB8 pMK18 (lane 3) and HB8 pMK18-HisTTC0834 (lane 4). Lane *M* contains molecular-weight markers (kDa). (*b*) Fractionation of cell envelopes from HB8 expressing His-TTC0834 by centrifugation in sucrose-density gradients. A Coomassie-stained 12% polyacrylamide gel containing samples of fractions 1–8 (from the top to the bottom of the gradient) is shown. Lane *M* contains molecular-weight markers (kDa). (*c*) Heat stability of His-TTC0834. A Coomassie-stained 12% polyacrylamide gel with purified His-TTC0834, loaded either directly (295 K) or after boiling for 15 min (373 K), is shown. Note that His-TTC0834 could not be denatured completely by boiling for 15 min.

Table 1
Data-collection statistics.

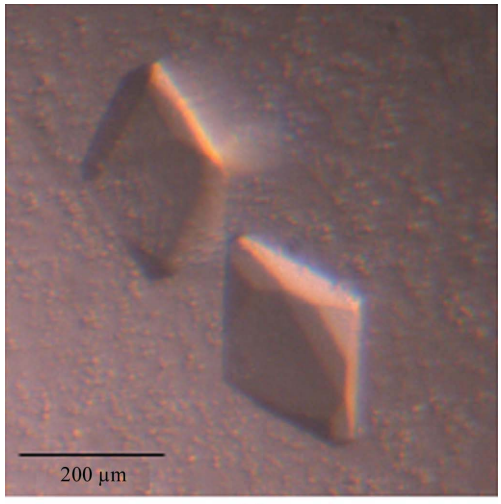
Values in parentheses are for the highest resolution shell.

Unit-cell parameters (Å, °)	$a = b = 166.67$, $c = 97.53$, $\alpha = \beta = 90.00$, $\gamma = 120.00$
Space group	$P3_121$ or $P3_221$
Temperature (K)	100
Resolution (Å)	40–3.2 (3.4–3.2)
Wavelength (Å)	1.072
Oscillation angle (°)	0.5
Unique reflections	26110 (4160)
Observed reflections	291482 (46654)
Completeness (%)	99.8 (99.6)
Mean $I/\sigma(I)$	17.38 (3.46)
R_{meas}† (%)	11.9 (78.6)
$R_{mrgd\text{-}F}$† (%)	9.4 (47.1)

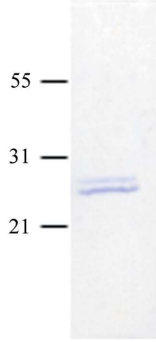
† As defined in Diederichs & Karplus (1997).

2.3. Crystallization and X-ray crystallographic analysis

His-TTC0834 in 20 m*M* Tris pH 8.5, 200 m*M* NaCl and 0.35% C8E4 was concentrated to 9 mg ml^{-1} by ultrafiltration (Vivaspin 50 000 Da, Vivascience). Initial crystallization conditions were identified using the sitting-drop vapour-diffusion method by applying Nextal Screening Suites (Quiagen) to 96-well microplates using various protein-to-buffer ratios. The temperature during crystal growth was set to 291 K. Crystals appeared overnight or within a few days under numerous conditions, with buffers containing 30% PEG 400 at neutral pH or >40% MPD at basic pH being the most promising. Crystallization conditions were refined using the hanging-drop vapour-diffusion method in 24-well microplates according to the initial hits, choosing a total volume of 2 µl for the crystallization drops. These refinements led to crystals with final dimensions of up to 100–300 µm. Crystals were flash-frozen in liquid nitrogen prior to data collection without the addition of further cryoprotectants. Native data sets were collected from crystals grown in 30% PEG 400, 0.1 *M* NaCl, 0.1 *M* MES pH 6.5 on beamline X06SA of the SLS (Swiss Light Source, Villigen, Switzerland), but determination of the space group and unit-cell parameters was not possible owing to the high degree of mosaicity and the anisotropic diffraction characteristics of the crystals, which diffracted to 5 Å resolution in the *a* and *b* directions and 9 Å in the *c* direction. Another set of crystals grown in 42–45% MPD and 0.2 *M* sodium malonate were tested on beamline ID23-1 of the ESRF (European Synchrotron Radiation Facility, Grenoble, France) and native data sets of sufficient quality were collected. The wavelength was 1.072 Å and diffraction was measured at 100 K. Data sets were processed (Table 1) using the program *XDS* (Kabsch, 1993).



(*a*)



(*b*)

Figure 2
(*a*) Native His-TTC0834 crystals grown in 44% MPD, 0.2 *M* sodium malonate. (*b*) Coomassie-stained PAA gel loaded with crystals dissolved in SDS sample buffer. The sample was boiled for 7 min prior to loading.

3. Results and discussion

We have cloned the OM protein TTC0834 from *T. thermophilus* HB27 with an N-terminal His tag for purification. His-tagged TTC0834 was expressed from its native promoter (Baetens *et al.*, 1998) from a plasmid in *T. thermophilus* HB8, which naturally lacks this protein (Fig. 1*a*, lane 2). His-TTC0834 could be detected in the cell-envelope fraction of HB8 harbouring this plasmid (Fig. 1*a*, lane 4) but not in HB8 (Fig. 1*a*, lane 3). The cell envelope of the His-TTC0834-expressing strain was further separated into inner and outer membranes by sucrose-gradient centrifugation (Fig. 1*b*). Fractions 2–5 were yellow, indicating inner membrane vesicles (Maier *et al.*, 2001), while white bands occurred, corresponding to OM vesicles (Maier *et al.*, 2001), in fractions 6 and 7. The S-layer protein and His-TTC0834 were found in fractions 5–8 (Fig. 1*b*). Having shown that His-TTC0834 is localized in the OM of strain HB8, we purified it from C8E4-solubilized membranes using Ni-affinity and gel-filtration chromatography. His-TTC0834 showed a similar heat-modifiable behaviour to that known for small OM proteins from *Escherichia coli* (Rosenbusch, 1974), indicating that it is properly folded. In SDS sample buffer His-TTC0834 runs faster on SDS–polyacrylamide gels when not heated (Fig. 1*c*, 295 K) compared with His-TTC0834 boiled at 373 K for 15 min.

His-TTC0834 crystallized under various conditions, producing crystals of different morphologies. Crystals grown in 0.1 *M* MES pH 6.5, 0.1 *M* NaCl and 30% PEG 400 were needle-like; their shape and size were comparable to those of the OmpW crystals reported by Albrecht *et al.* (2006). However, the diffraction of these crystals was limited to 5 Å resolution and they were too anisotropic to determine

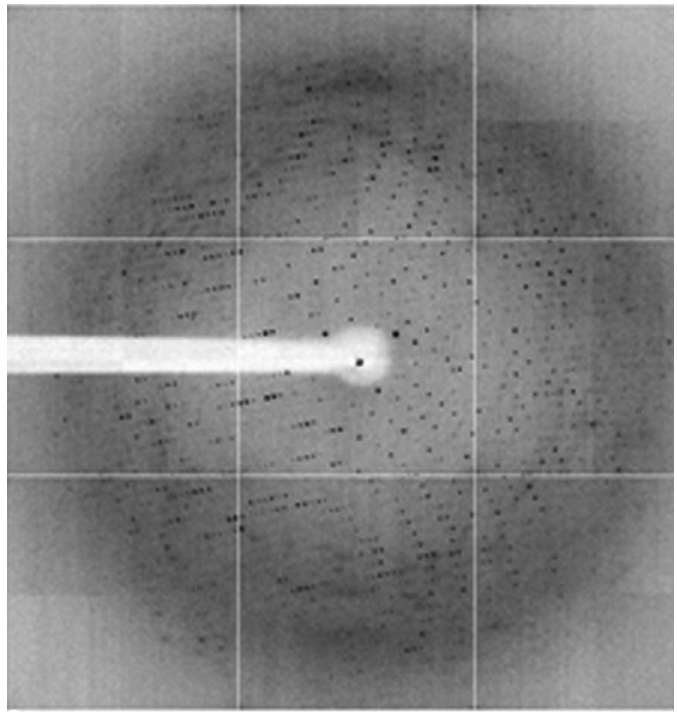


Figure 3
Diffraction pattern of a His-TTC0834 crystal as shown in Fig. 2 recorded on beamline ID23-1 at the ESRF.

the space group and unit-cell parameters (data not shown). Crystals grown in MPD at basic pH were diamond-shaped (Fig. 2*a*) and diffracted isotropically to 3.2 Å resolution (Fig. 3). To verify the presence of His-TTC0834 in these crystals, they were dissolved in sample buffer. SDS–PAGE analysis revealed the presence of His-TTC0834 in these crystals (Fig. 2*b*). Native data sets were collected and the space group was determined as $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 166.67$, $c = 97.53$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ (Table 1). Crystals remained stable during the entire data-collection process. Calculation of the Matthews coefficient (Matthews, 1968) showed that the possible number of His-TTC0834 monomers could be between three and eight per asymmetric unit, corresponding to a solvent content ranging from 77.7% to 40.6%.

To solve the structure, we are currently attempting to prepare SeMet-labelled protein and screening for derivatives using heavy-atom soaking of the crystals.

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