Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

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

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Received 18 April 2008
Accepted 7 May 2008

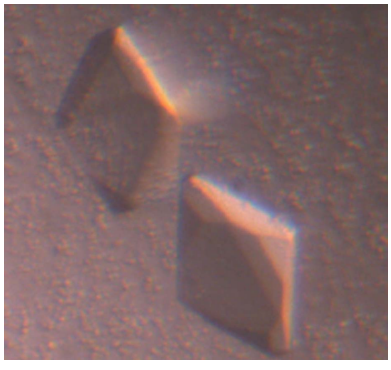
The cell envelope of the thermophilic bacterium *Thermus thermophilus* is multilayered and includes an outer membrane with integral outer membrane proteins that are not well characterized. The hypothetical protein TTC0834 from *T. thermophilus* HB27 was identified as a 22 kDa outer membrane protein containing 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-



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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 *Eco*RI and cloned into the *Pst*I- and *Eco*RI-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 *Eco*RI-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).

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).

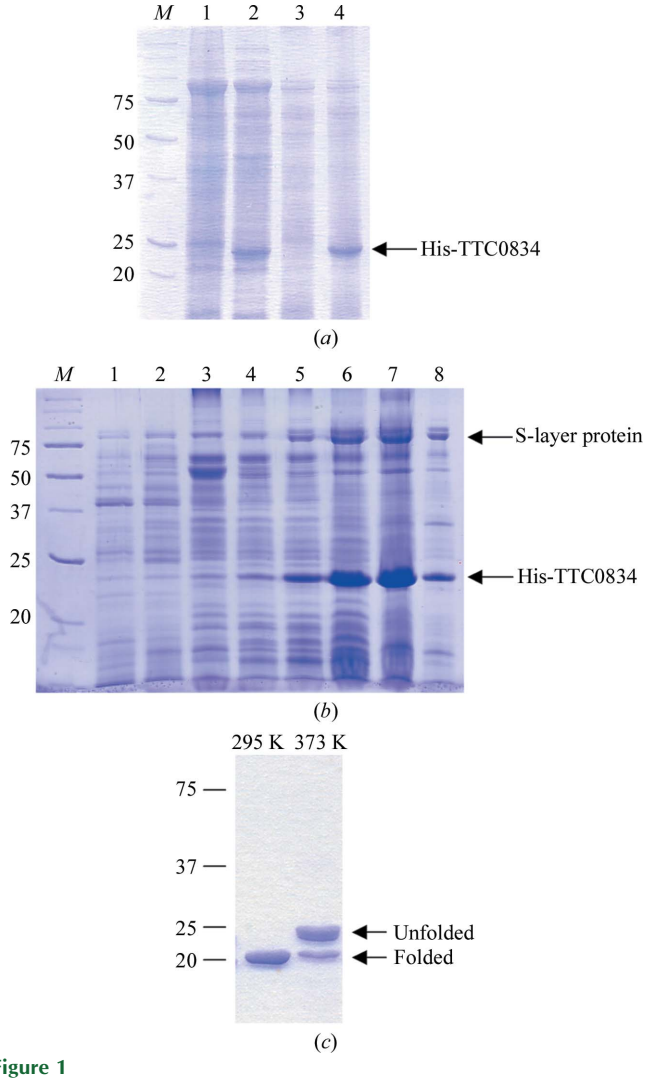
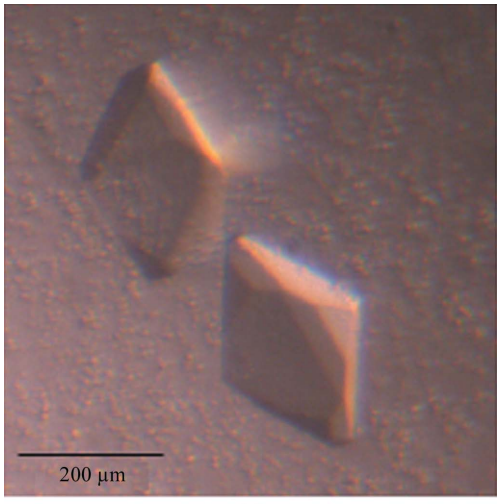


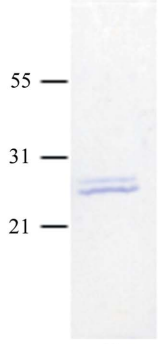
Figure 1
(*a*) Expression and cell-envelope localization of His-TTC0834 in *T. thermophilus* HB8. A Coomassie-stained 12% polyacrylamide gel is shown. Lanes 1 and 2, whole cell extracts of HB8 pMK18 (lane 1) and HB8 pMK18-HisTTC0834 (lane 2). Lanes 3 and 4, cell envelopes of HB8 pMK18 (lane 3) and HB8 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.

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

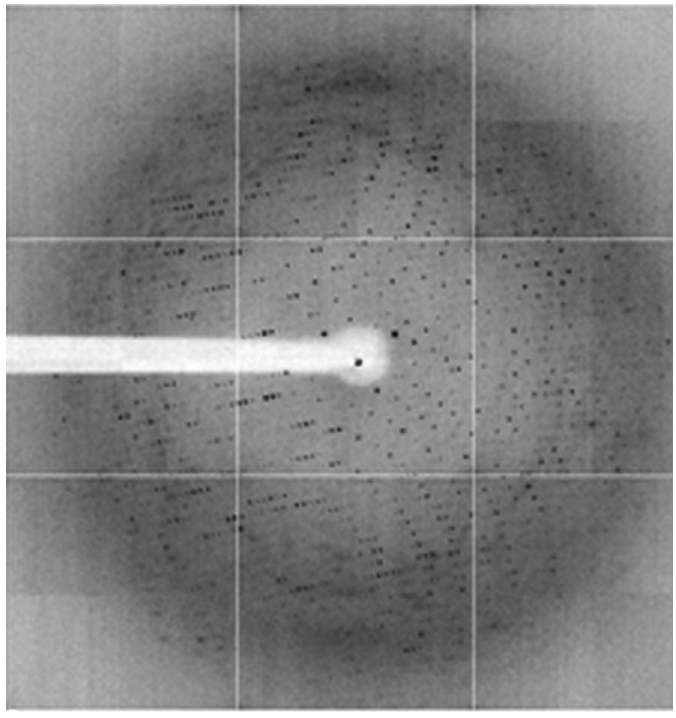


Figure 3
Diffraction pattern of a His-TTC0834 crystal as shown in Fig. 2 recorded on beamline ID23-1 at the ESRF.

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.

We thank the staff of the synchrotron beamlines ID23-1 at the ESRF and X06SA at the SLS for their technical assistance and beamline support. We also thank Professor Winfried Boos (University of Konstanz) for his support and critical reading of the manuscript.

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