Omp85<sub>Tt</sub> from *Thermus thermophilus* HB27: an Ancestral Type of the Omp85 Protein Family

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Received 13 March 2008/Accepted 18 April 2008

Proteins belonging to the Omp85 family are involved in the assembly of β-barrel outer membrane proteins or in the translocation of proteins across the outer membrane in bacteria, mitochondria, and chloroplasts. The cell envelope of the thermophilic bacterium *Thermus thermophilus* HB27 is multilayered, including an outer membrane that is not well characterized. Neither the precise lipid composition nor much about integral membrane proteins is known. The genome of HB27 encodes one Omp85-like protein, Omp85<sub>Tt</sub>, representing an ancestral type of this family. We overexpressed Omp85<sub>Tt</sub> in *T. thermophilus* and purified it from the native outer membranes. In the presence of detergent, purified Omp85<sub>Tt</sub> existed mainly as a monomer, composed of two stable protease-resistant modules. Circular dichroism spectroscopy indicated predominantly β-sheet secondary structure. Electron microscopy of negatively stained lipid-embedded Omp85<sub>Tt</sub> revealed ring-like structures with a central cavity of ~1.5 nm in diameter. Single-channel conductance recordings indicated that Omp85<sub>Tt</sub> forms ion channels with two different conducting states, characterized by conductances of ~0.4 nS and ~0.65 nS, respectively.

The thermophilic bacterium *Thermus thermophilus*, with a growth temperature ranging from 45° to 85°C, belongs to one of the oldest branches of bacterial evolution and forms a phylum together with the genus *Deinococcus*. It most likely represents an evolutionary intermediate between today’s gram-positive and -negative bacteria (18). The genome sequences of two *T. thermophilus* strains, HB8 and HB27, are available (20). The chromosomes are highly conserved with an identity of 94%, but variations are found, predominantly in cell envelope structures (6). The cell envelope of *T. thermophilus* is multilayered, comprising, as first identified in strain HB8, a thin murein layer (39), an outer membrane (OM) (7), an S-layer (8), and an outermost layer of amorphous material (9). The lipid composition of the OM is not precisely known. However, recently two glycolipids and one phosphoglycolipid were identified in whole-cell membranes of *T. thermophilus* strain SamuSA1, reflecting adaptation to heat (27). Almost no information is available about integral OM proteins (OMPs). In black lipid bilayer experiments done using OM preparations from HB8, one 185-kDa protein was identified as a putative porin that formed unusually large pores (31). For HB27, Rumszauer et al. described OMPs involved in pilus biogenesis (42). In addition, a recent phylogenetic sequence analysis of the Omp85 protein family revealed that the genome of HB8 codes for one Omp85-like protein (4).

Proteins of the Omp85 family are integral OMPs found in all gram-negative bacteria sequenced to date as well as in mitochondria and chloroplasts (46). In respect to the putative function, the Omp85 family of proteins can be grouped in two classes. One class is proposed to be involved in insertion of β-barrel proteins into the OM and the other in the transport of proteins across the OM (46). Examples of Omp85-like proteins involved in the translocation of proteins are Toc75 from chloroplasts (22), nOmp85 (formerly alr2269) from the cyanobacterium *Nostoc* sp. strain PCC7120 (4, 13), and the integral membrane components, called TpsB, of the two-partner secretion system in gram-negative bacteria. TpsB proteins secrete large, mostly β-helical proteins called TpsA proteins through the OM. A well-studied TpsB protein is FhAC of *Bordetella pertussis*, which secretes a filamentous hemagglutinin (23). A well-characterized eukaryotic Omp85-like protein suggested to be involved in the insertion of β-barrel proteins into the mitochondrial OM is Tob55 from *Saccharomyces cerevisiae* (37), also called Sam50 (25). Omp85 of *Neisseria meningitidis* was the first example shown to function in the biogenesis of OMPs in gram-negative bacteria (51). Later, the same properties were found for the *Escherichia coli* Omp85 homologue YaeT (53). Both, Omp85<sub>Nm</sub> and YaeT are essential for cell viability, and depletion resulted in reduced amounts of OMPs in *E. coli* (53) or in the accumulation of unfolded OMPs in *N. meningitidis* (51). Apart from the TpsB proteins, Omp85 proteins are often part of multiprotein complexes (46). Neither the mechanism of protein secretion nor the mechanism of insertion of β-barrel OMPs by Omp85 family proteins in the absence of an obvious energy source is understood in detail.

Based on the amino acid sequence and secondary structure predictions, the Omp85 family is currently defined by the presence of two modules: an N-terminal periplasmic module containing one to five polypeptide transport-associated (POTRA) domains and a C-terminal transmembrane domain composed of 16 predicted β-strands (15). The POTRA domains are proposed to mediate protein-protein interaction, while the mem-
brane-integrated C terminus forms a pore (44). Recently the structure of FhaC, a TpsB-type Omp85 family protein, was solved (11), showing two POTRA domains and 16 antiparallel β-strands. There is no complete structure of an Omp85 family protein involved in the assembly of β-barrel proteins, but the structure of the four N-terminal POTRA domains of YaeT has been solved (24).

In this paper we report the characterization of an Omp85 family protein from *T. thermophilus* HB27. We purified Omp85<sub>Tt</sub> in native form from *T. thermophilus* and demonstrated that it exists primarily as a monomer in detergent-containing solutions. Omp85<sub>Tt</sub> reconstituted into liposomes appeared as ring-like structures in the electron microscope (EM) and showed pore activity in lipid bilayer experiments.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *T. thermophilus* strains HB27 (DSM7039; obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and HB27:nar (35) as well as *E. coli* strain Mach1-T1 (Invitrogen) were used in this study. *T. thermophilus* was grown at 70°C in medium containing 8 g/liter Trypticase, 4 g/liter yeast extract, and 3 g/liter NaCl dissolved in distilled water (pH 7.5) (34). For transformation of *T. thermophilus* HB27:nar, Ca<sup>2+</sup> and Mg<sup>2+</sup>-rich medium containing 8 g/liter tryptone, 4 g/liter yeast extract, and 3 g/liter NaCl dissolved in Evian mineral water (pH 7.7) was used (5). Expression of proteins from the nar promoter of pMEK2 in *T. thermophilus* HB27:nar was performed as previously described (34). *E. coli* was grown in LB medium at 37°C. Kanamycin was used at 25 µg/ml for *T. thermophilus* and *E. coli*.

**Plasmid construction.** DNA work was carried out using standard procedures (43). Chromosomal DNA from *T. thermophilus* was prepared as described recently for *E. coli* (16). Phusion DNA polymerase (Finnzymes) was used for PCRs.

To obtain a plasmid overexpressing Omp85<sub>Tt</sub> in *T. thermophilus*, TTOC193 encoding Omp85<sub>Tt</sub> was amplified by PCR from chromosomal DNA of HB27 using the primers 5′-ACCATGGAGCGGCTTCTCGCCCTGGGC3′ (NcoI site underlined) and 5′-CTCGTAAGGGCCGGCTTGAACATGCGGCCGATGCG3′ (Ntt site underlined). The PCR product was cloned into TOPO vector pCR4 (Invitrogen), cut out by digestion with NcoI and NotI, and ligated into NcoI/NotI-digested pMEK2 (34). The insert was sequenced (GATC). Due to the introduction of the restriction site NcoI, the resulting plasmid, pMEK2-Omp85<sub>Tt</sub> expresses Omp85<sub>Tt</sub> with a modified signal sequence (MERLLALGLALTALA instead of MKRLLALGLALTALA).

**Protein analysis.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26) and stained with Coomassie brilliant blue R-250 (Serva). Protein concentration was measured by either the bichromatic acid method (49) or the method of Lowry (29).

**FFEM.** To obtain the low degree of protein reconstitution required for freeze-etch electron microscopy (FFEM), Omp85<sub>Tt</sub> (0.1 mg/ml) in 20 mM Tris (pH 8.5)–150 mM NaCl–0.05% Cymal-6 was mixed with either *E. coli* lipids or dimyristoylphosphatidylcholine (DMPC) (Avanti Polar Lipids) at a lipid-to-protein ratio (LPR) of 0.2 and incubated at 4°C for 2 days in the presence of Bio-Beads SM2 (Bio-Rad) to remove the detergent (40). *E. coli* lipids in 20 mM Tris (pH 8.5)–150 mM NaCl-0.05% Cymal-6 were similarly incubated and served as a control.

**Proteinase digestion.** Proteinase digestion was performed with purified Omp85<sub>Tt</sub> samples. Five micrograms of Omp85<sub>Tt</sub> was treated with either 5 µg of trypsin or proteinase K for 5 h at 56°C. The reaction was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride, and fragments were blotted onto a polyvinylidene difluoride membrane (Pall). The membrane was stained with Coomassie Blue G, and the two fragments were subjected to five steps of Edman degradation (Proteome Factory, Berlin, Germany).

**Cell fractionation.** Cell pellets of *T. thermophilus* were resuspended in 50 mM Tris (pH 8) and lysed by passing the suspensions through a French pressure cell. Unbroken cells were removed by centrifugation at 5,000 × g for 10 min, and cell envelopes were obtained as a pellet after centrifugation of the supernatant at 100,000 × g for 1 h. Cell envelopes were washed once with 50 mM Tris (pH 8) under the same conditions, resuspended in 50 mM Tris (pH 8), and loaded onto a step gradient of 30%, 40%, 50%, 55%, and 65% sucrose as previously reported (31). The gradient was centrifuged at 110,000 × g for 17 h at 20°C. Inner membrane fractions appeared as yellow and OM vesicles as white bands.

**Plurification of native Omp85<sub>Tt</sub>.** The OM fraction was isolated from the sucrose gradient (see “cell fractionation” above). The extract was diluted threefold with 50 mM Tris (pH 8), and OM vesicles were recovered after centrifugation at 100,000 × g for 1 h at 20°C. Proteins were solubilized in 20 mM Tris (pH 8.5) containing 0.5% Cymal-6 (Anagrade) for 1 h at 37°C, followed by centrifugation at 100,000 × g for 1 h. The supernatant was loaded on a MonoQ HR5/5 column (Amersham). After extensive washing with 20 mM Tris (pH 8.5) containing 0.5% Cymal-6, bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in 20 mM Tris (pH 8.5)–0.05% Cymal-6. Fractions containing Omp85<sub>Tt</sub> were further purified by gel filtration chromatography (HiLoad 26/60 Superdex 200 prep grade; Amersham) using 20 mM Tris (pH 8.5)–150 mM NaCl-0.05% Cymal-6. If necessary, the gradient was replaced by a 20 mM Tris (pH 8.5)–150 mM NaCl–0.05% Cymal-6 buffer; then the protein was eluted from the MonoQ HR5/5 column by dialysis against 20 mM Tris (pH 8.5).
RESULTS

Omp85\textsubscript{Tt} can be overexpressed in \textit{T. thermophilus} and purified in native form from OM preparations. In the genome sequence of \textit{T. thermophilus} HB27 (20), one open reading frame, TTC0193, encodes an Omp85 family protein, which we have named Omp85\textsubscript{Tt}. Attempts to inactivate TTC0193 by insertion of a thermostable bleomycin (5) or kanamycin (32) cassette failed, suggesting that Omp85\textsubscript{Tt} might be essential. It was not possible to overexpress Omp85\textsubscript{Tt} in native form in \textit{E. coli} (data not shown), and therefore we tested plasmid-mediated overexpression in \textit{T. thermophilus}. An inducible expression system based on the presence of nitrate and absence of oxygen was recently described (34). Using this system, Omp85\textsubscript{Tt}, containing a slightly modified signal sequence could be overexpressed in \textit{T. thermophilus} (Fig. 1A). Subsequent cell fractionation studies, including sucrose gradient centrifugation, showed that Omp85\textsubscript{Tt} was localized together with the S-layer protein in the OM fraction (Fig. 1B, lane 2). A multi-step purification protocol was established to purify Omp85\textsubscript{Tt} from the OM and resulted in a preparation of highly enriched Omp85\textsubscript{Tt} (Fig. 1B, lane 3). Solubilization of Omp85\textsubscript{Tt} from the OM was best when the detergent Cymal-6 was used. Cymal-6 was also used during purification but was often changed afterwards to DDM or C8E4 without causing any aggregation.

We examined the secondary structure of Omp85\textsubscript{Tt} by CD spectroscopy (Fig. 1C). The far-UV CD spectrum of Omp85\textsubscript{Tt} in PBS with 0.05% DDM was characterized by a broad minimum at \textasciitilde 216.5 nm and a zero-ellipticity crossover at 203 nm, indicating that Omp85\textsubscript{Tt} has predominantly \( \beta \)-sheet secondary structure. Secondary structure analysis with the CDSSTR deconvolution algorithm (12) suggested \(-55\% \) \( \beta \)-sheet, \(-16\% \) \( \alpha \)-helix, and \(29\% \) random-coil secondary structure in Omp85\textsubscript{Tt}.

This is consistent with the prediction of a periplasmic N-terminal module containing \( \alpha \)-helices and \( \beta \)-sheets and a C-terminal \( \beta \)-sheet-rich domain.

Mature Omp85\textsubscript{Tt} (88.3 kDa) is predicted to have five POTRA domains within the predicted 449-amicino-acid (aa) N terminus (49.5 kDa) and 16 transmembrane \( \beta \)-strands within the predicted 357-aa C terminus forming one \( \beta \)-barrel (38.8 kDa). This two-module model was verified by proteinase digestion (Fig. 1D). Omp85\textsubscript{Tt} was resistant to trypsin (data not shown), but treatment with proteinase K for 5 h at 56°C resulted in two stable fragments. The fragments were sequenced by Edman degradation. One fragment (Fig. 1D, lane 2, a) started with APLER, corresponding to the predicted N terminus of the mature protein, and the other (Fig. 1D, lane 2, b)
obtained when the detergent was changed to DDM or C8E4 (data not shown), indicating that the formation of oligomers is independent of the detergent.

The mass of Omp85<sub>Tt</sub> in the presence of DDM was also determined by STEM. Evaluation of the images yielded the histogram shown in Fig. 2B. Considering the micellar mass of the associated DDM detergent (57 kDa), the main peak at 166 ± 40 kDa indicated that around 69% of the 1,301 Omp85<sub>Tt</sub> (molecular mass from sequence, 88.3 kDa) particles investigated were monomeric. In addition, the peaks at higher mass suggested that 18% of the particles were dimers, 9% trimers, and 4% tetramers.

**Projection structure of Omp85<sub>Tt</sub> reconstituted into liposomes.** Omp85<sub>Tt</sub>, solubilized in 20 mM Tris (pH 8.5)–150 mM NaCl–0.05% Cymal-6 was reconstituted into liposomes formed by DMPC or E. coli lipids, negatively stained either with 2% uranyl acetate or 2% phosphotungstic acid, and examined in a TEM. In all cases the incorporated protein was visible as rings on the liposome surface (Fig. 3). The insets in Fig. 3A show the average structures of such top views. Omp85<sub>Tt</sub> had two different diameters, approximately 5.1 nm (Fig. 3A, upper inset) and 5.9 nm (Fig. 3A, lower inset), suggesting that the protein had inserted into the bilayer in both the “up” and “down” directions. The central stain-filled cavities indicated the presence of an ~1.5-nm-wide pore. Omp85<sub>Tt</sub> could also be discerned at the edges of some vesicles and protruded ~4 to 5 nm from the lipid bilayer (Fig. 3B and C). An uncharged lipid vesicle is shown in the inset of Fig. 3B for comparison.

Both to confirm this result and to demonstrate reconstitution when starting from another detergent system, FFEM was employed to examine a reconstitution made using Omp85<sub>Tt</sub> in 20 mM Tris (pH 8.5)–150 mM NaCl–0.35% C8E4 and E. coli lipids. A very high LPR of 40 was chosen for this experiment to ensure that individual protein entities would be visible on the liposome surface. A typical proteoliposome is shown in Fig. 4A. The reconstituted proteins appear as particles peppered across the liposome surface. Uncharged liposomes prepared as a control exhibited the expected “orange-skin” surface but lacked these larger particles (Fig. 4B). The shadowing/replacation step required by this microscopy technique makes their apparent diameter, 9 ± 1 nm (n = 31), considerably larger than that measured by negative-stain microscopy, but the value is still compatible with the presence of Omp85<sub>Tt</sub> monomers.

**Omp85<sub>Tt</sub> forms channels in lipid bilayers.** To date, all OMPs of the Omp85 family have been found to form ion channels, with single-channel conductances ranging from 0.4 nS for YaeT from E. coli to 2.1 nS for nOmp85 from Nostoc (4, 13, 33, 37, 41). We tested pore formation by Omp85<sub>Tt</sub> in planar lipid bilayers. Purified native Omp85<sub>Tt</sub> in 20 mM Tris (pH 8.5) containing 100 mM NaCl and 0.05% Cymal-6 was reconstituted into black lipid membranes of diPhPC. Conductance measurements revealed the opening and closing of pores (Fig. 5A and B). Of out of 640 recorded channel conductance events, ~46% displayed a conductance change ΔG of ~0.4 nS at 20 mV in 1 M KCl (Fig. 5C). In addition, 9 to 10% of the channel events displayed a change in conductance ΔG of ~0.65 nS (Fig. 5C). The conductance recordings indicated that the large conductance change ΔG of ~0.65 nS always preceded the small conductance change, leading to a total conductance change ΔG of ~1.1 nS (Fig. 5A and B).
DISCUSSION

In the present work, we have analyzed the first protein of the Omp85 family that originates from a thermophilic bacterium, Omp85<sub>Tt</sub>. We were able to overexpress and purify Omp85<sub>Tt</sub> as a native, stable protein from the OM of <i>T. thermophilus</i>. Omp85<sub>Tt</sub> showed a high content of ß-sheet secondary structure, similar to most OMPs of gram-negative bacteria, indicating that it forms a transmembrane ß-barrel. The composition of the secondary structure (~55% ß-sheet, ~16% α-helix, and 29% random coil) closely resembles that of FhaC (~49% ß-sheet and ~9.1% α-helix) (11). The significant amount of α-helix secondary structure detected in Omp85<sub>Tt</sub> agrees well with the predicted presence of five POTRA domains within the periplasmic N terminus. The structures of several POTRA

![Image of Omp85<sub>Tt</sub> reconstituted into lipid vesicles](image)

FIG. 3. Negative-stain TEM images of Omp85<sub>Tt</sub> reconstituted into lipid vesicles. (A) Omp85<sub>Tt</sub> reconstituted into DMPC liposomes and negatively stained with 2% uranyl acetate. The reconstituted protein is visible as rings on the liposome surface (arrows). The insets show averages of the two different types of Omp85<sub>Tt</sub> ring seen; the diameter of the upper averages approximately 5.1 nm, and that of the lower averages approximately 5.9 nm. (B) Omp85<sub>Tt</sub> reconstituted into DMPC liposomes and negatively stained with 2% phosphotungstic acid. Omp85<sub>Tt</sub> protrudes roughly 4 to 5 nm from the membrane, giving the edge of the liposomes a rather lacy appearance (arrows). The inset shows liposomes prepared in the absence of protein. (C) Omp85<sub>Tt</sub> reconstituted into E. coli liposomes and negatively stained with 2% uranyl acetate. End views (black arrow) and side views (white arrow) of the protein can be distinguished. Bars, 50 nm (insets in panel A, 5 nm).

![Image of Omp85<sub>Tt</sub> proteoliposome and control liposomes](image)

FIG. 4. FFEM of reconstituted Omp85<sub>Tt</sub>. (A) Proteoliposome formed from Omp85<sub>Tt</sub> and E. coli lipids. It is peppered with shadowed particles, revealing the distribution of the Omp85<sub>Tt</sub> protein on the lipidic surface. The individual particles have an average diameter of 9 nm, compatible with the insertion of monomeric Omp85<sub>Tt</sub> in the lipid bilayer. (B) Control liposomes formed by pure E. coli lipids. Note the orange-skin appearance of their surface and the absence of larger shadowed particles. Bar, 100 nm.

![Image of single-channel conductance recordings](image)

FIG. 5. Single-channel conductance recordings indicate that Omp85<sub>Tt</sub> forms ion channels in black lipid films. (A) Stepwise increase of the conductance (G) recorded as a function of time (t), showing that Omp85<sub>Tt</sub> exhibits two channel states. (B) Expanded time window of panel A, indicating frequent channel openings with a conductance change ΔG<sub>1</sub> of ~0.4 nS. These openings required a preceding conductance change ΔG<sub>2</sub> of ~0.65 nS. (C) Histogram of Omp85<sub>Tt</sub> channel conductance events, indicating that about half of all channel events show this conductance change ΔG of ~0.4 nS. Conductance events with ΔG<sub>1</sub> of ~0.65 were far less frequent. A total of 640 channel events were recorded and evaluated.
domains were recently solved (11, 24). Although the primary sequence similarity between different POTRA domains is very low, their structures are similar and comprise a three-stranded β-sheet and two α-helices. POTRA domains are known to mediate protein-protein interactions, and the presence of at least one is essential for the function of proteins of the Omp85 family (3, 11, 19). They are reported to interact with unfolded substrates (11, 19) and to mediate homo-oligomerization (e.g., nOmp85 trimerizes via the POTRA domains [13]) and hetero-oligomerization (e.g., YaeT interacts with the four lipoproteins NlpB, SmpA, Y10, and Y1gL via the POTRA domains [24]). Incorporated into liposomes, Omp85Tt1 was found to protrude ~4 to 5 nm from the membrane, giving a rough idea of the size of the N terminus with its five predicted POTRA domains.

Three different techniques, DLS, BN-PAGE, and STEM, indicated that native Omp85Tt1 is present mainly as a monomer in detergent solutions. On reconstitution into lipid bilayers, Omp85Tt1 also appeared as single particles scattered across liposomes by FFEM (E. coli lipids) or as monomeric, ring-like particles with a central cavity by negative-stain TEM (DMPC and E. coli lipids). In the presence of detergent, higher-molecular-mass forms of Omp85Tt1 were observed by BN-PAGE analysis and STEM, suggesting that Omp85Tt1 has the capability to form homo-oligomers. Compared to those obtained by the other techniques, the STEM data suggested a relatively high content of oligomeric particles (around 31%). However, STEM is a single-molecule rather than a bulk technique, and not every particle imaged could be measured due to the close proximity of neighbors, which would have influenced the statistics. Some purified Omp85 family proteins have been reported to exist as dimers. In native form the TpsB-type Omp85 protein HMW1B was found to be a dimer (28). The cyanobacterial Omp85 protein nOmp85 was reported to exist as homotrimers when reconstituted into artificial membranes (4). Purified, refolded YaeT was suggested to form tetramers (41). In contrast, FhaC was crystallized as a monomer, suggesting that it is also predominantly monomeric in solution (11). Omp85 family proteins are thus able to oligomerize, but there are clearly differences in the degree. At present it is not understood whether the protein-trafficking activity of Omp85 family proteins in vivo depends on this property. Further, oligomerization might also be dependent on the association of other proteins to form one active complex.

Omp85Tt1 showed channel activity when incorporated into planar lipid bilayers. The most frequent change in conductance at 1 M KCl found for Omp85Tt1 (ΔG2, ~0.4 nS) is similar to that published for Omp85 proteins involved in the insertion of β-barrel proteins, e.g., 0.4 nS (4) or 0.5 nS (41) for the YaeT of E. coli and 0.56 nS for the Sam50 of Drosophila melanogaster mitochondria (4). Larger channel conductances have been observed for Omp85 proteins proposed to be involved in the translocation of proteins, e.g., 2.1 nS for the nOmp85 of the cyanobacterium Nostoc, 2 nS for the ToeC75 of Pisum sativum chloroplasts (4), and 1.2 nS for FhaC of Bordetella pertussis (33).

The channel conductance of Omp85Tt1 always displayed a two-step increase across the lipid bilayer. The first conductance increase (ΔG1, ~0.65 nS) was about twice as large as the second (ΔG2, ~0.4 nS). Two different interpretations would explain this behavior. Omp85Tt1 could insert as a monomer and first partially open into a substrate before it opens fully. The conductance of the fully open state would then correspond to ~1.1 nS. This behavior might indicate gating, as was assumed for nOmp85 from Nostoc. Interestingly, the β-barrel domain of nOmp85 itself did not show any subconductance steps, but these were induced when the N-terminal module of nOmp85 was added (13). This suggests that the POTRA domains might be involved in gating of the pore formed by the β-barrel domain, at least in some Omp85 family members. Alternatively, Omp85Tt1 might not be active as a monomer in this assay. Rather, dimers or trimers might have to be formed in the diPhPC bilayer. Our results would then indicate that one Omp85Tt1 channel is mostly open, while another opens and closes more frequently. Channel activity of an oligomeric form was suggested for refolded YaeT. It was found that some subunit of the active YaeT tetramer forms a pore that can open and close independently (41).

A first indication that the channel-forming C termini of Omp85 family proteins might be dynamic came from work on FhaC (11). While the conductivity of 1.2 nS measured for FhaC suggests a channel of 8 to 10 Å in diameter (33), the structure of FhaC revealed a narrow channel that is just 3 Å wide because it is occluded by a loop and an α-helix (11). This channel would be too narrow to allow the transport of a protein, and indeed the available evidence suggests that the loop is flexible and opens the channel by moving outwards when the substrate filamentous hemagglutinin is present (17). Omp85Tt1 reconstituted into liposomes showed a stain-filled cavity, implying a pore with a maximum diameter of approximately 15 Å. We propose that the pore of Omp85Tt1 is also largely occluded, as observed for FhaC. In agreement with this idea, sequence analysis of FhaC and Omp85Tt1 showed that while the helix occluding the channel of FhaC is not conserved in Omp85Tt1, the loop is (data not shown).

Our preliminary data suggest that Omp85Tt1 is essential. Since we found that Omp85Tt1 fractionates with the OM/S-layer envelope, this would further suggest that the OM is essential. So far it is known only that the S-layer protein is not essential, although it attaches the OM to the secondary cell wall polymer, a layer covalently bound to the peptidoglycan (14). An S-layer mutant looses its shape and produces instead so-called multicellular bodies, spherical shells formed by the OM that contain a large number of cells (10).

The function of Omp885Tt1 is not known. In gram-negative bacteria, genes encoding Omp85 family proteins are often flanked by genes indicating their function. In T. thermophilus the DNA region surrounding TCC0193 (omp885Tt1) does not allow such speculation. Bioinformatics did not reveal homologues of other proteins involved in the biogenesis of OMPs in gram-negative bacteria (data not shown). Only the Sec system and the Omp85 are present, but neither the cognate chaperones nor the cognate lipoproteins (2) seem to be conserved. The presence of only one Omp85 family protein in T. thermophilus suggests that Omp885Tt1 might be involved in the insertion of β-barrel proteins. In line with this, the C-terminal end of Omp85Tt1 (IHERGIPMF) shows the typical OMP signature sequence known for gram-negative bacteria (41), with phenylalanine at the ultimate position and hydrophobic residues at positions 5, 7, and 9 (underlined) from the C terminus. The C-terminal signature sequence was recently found to in-
teract with YaeT (Omp85) of E. coli (41). Therefore, it is tempting to speculate that Omp85 need not itself in order to integrate into the OM. Using bioinformatics, we have identified two more potential β-barrel OM proteins harboring this C-terminal signature sequence (unpublished results). In further studies we will characterize these putative β-barrel proteins and evaluate them as potential substrates for Omp85.

ACKNOWLEDGMENTS

We are grateful to Hans-Jürgen Apell (University of Konstanz) for help with the conductance recordings and for useful discussions. We also thank Andreas Engel (University of Basel) for his support. The work in Konstanz was funded by the Deutsche Forschungsgemeinschaft, an SFB/TR 11, CD experiments and single-channel recordings performed by G.J.P. were supported by grant TP B3 to J.H.K. The microscopy in Basel was supported by Swiss National Foundation grant 501221 and by the Maurice E. Müller Foundation of Switzerland.

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