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Characterization of Pores Formed by YaeT (Omp85) from *Escherichia coli*

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Proteins of the Omp85 family play a major role in the biogenesis of the bacterial outer membrane, since they were shown to mediate insertion of outer membrane proteins. The *Escherichia coli* Omp85 homologue YaeT is essential for viability, but its exact mode of action is not yet elucidated. We could show that YaeT is composed of two distinct domains, an amino-terminal periplasmic and a carboxy-terminal membrane domain. The full length YaeT and the isolated membrane domain induce pores when reconstituted in planar lipid membranes. The pores exhibit a certain variability of conductance indicating a flexible structure, which could be an essential property of a lateral opening channel releasing proteins into the bacterial outer membrane. We could further show that the periplasmic domain proves to be essential for *in vivo* function of YaeT.

Key words: bacterial outer membrane, membrane biogenesis, Omp85, reconstitution in black lipid membranes.

The outer membrane (OM) is an essential component of the cell envelope of Gram-negative bacteria. It serves as an additional selective permeability barrier and provides the cells with an increased resistance to antibiotics, digestive enzymes, detergents and host-defense proteins (*1*, *2*). The outer membrane is an asymmetric lipid bilayer formed by phospholipids in the inner and lipopolysaccharides in the outer leaflet. It contains two major classes of proteins: β-barrel proteins, which are incorporated, and lipoproteins, which are anchored in the OM. The proteins of the outer membrane are synthesized in the cytoplasm and must traverse the inner membrane and the periplasmic space to reach their final location. The translocation of proteins across the inner membrane is well understood especially after solving the protein structure of the inner membrane translocation machinery (*3*). However, the pathway of lipoproteins and integral β-barrel proteins into the outer membrane is different. Lipoproteins are transported and inserted into the OM by the LolA and LolB proteins (*4*, *5*). The biogenesis of β-barrel proteins is more complex and has been extensively studied for years (for recent reviews see Refs. *6*–*8*). A range of proteins have been identified, which act as folding factors in the periplasmic space. These include molecular chaperones, such as Skp, which stabilize the non-native conformations of outer membrane proteins (OMPs) and facilitate their folding (*9*, *10*). The periplasmic protease DegP might act under some physiological conditions also as chaperone (*11*). Other folding factors are folding catalysts like protein disulphide isomerases or *cis-trans* peptidyl propyl isomerases, the former catalyzing the formation and reshuffling of disulfide bonds and the latter catalyzing the *cis-trans* isomerization of peptide bonds (*12*–*14*). The crucial step in the biogenesis of OMPs is the insertion into the OM. It is clear that this process occurs not spontaneously as observed *in vitro*. When reconstituted in artificial membranes OMPs insert preferentially with the periplasmic site first due to the large hydrophilic extracellular loops (*15*, *16*). Therefore, OMP insertion *in vivo* must be mediated by a special mechanism.

In 2003, the OMP Omp85 of *Neisseria meningitidis* was characterized to be involved in protein insertion into the OM (*17*). It belongs to a highly conserved family of proteins present in all domains of life, except archaea (*18*). Depletion of Omp85 in *N. meningitidis* as well as depletion of the orthologous protein YaeT from *Escherichia coli* leads to a defect in outer membrane integrity (*19*, *20*). The OMPs accumulate transiently in the periplasm as soluble intermediates, which imply that Omp85 and YaeT might assist insertion of soluble OMP intermediates. The failure to construct *yaeT* and *omp85* knock out strains without complementation shows that the proteins are essential for the cells (*17*, *19*, *20*). Recently, it is shown that YaeT forms a multi-protein-complex with three OM lipoproteins YfgL, YfiO, and NlpB (*21*). All three belong to the SigmaE regulon. YfiO is shown to be essential for viability, whereas NlpB and YfgL are non-essential. However, *nlpB* and *yfgL* knock out strains exhibit phenotypes, which suggest that the proteins are also important for maintenance of the integrity of the cell envelope (*22*).

In this multi-protein complex YaeT is the only protein, which is an integral OM protein. This suggests a central role in inserting OMPs into the membrane. In this study, we have investigated the functionality of YaeT *in vitro* and *in vivo*. Reconstituted in planar lipid bilayer membranes, we show that YaeT has pore-forming ability. Characterization of an amino-terminal deletion mutant of YaeT shows that the pore forming domain is located in the C-terminus and that the amino-terminal periplasmic domain is essential for function *in vivo*.

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MATERIALS AND METHODS

Bacterial Strains and Construction of Plasmids—All bacterial strains were grown in LB medium or on LB agar plates with appropriate antibiotics (Sigma). For *E. coli* strains Top10F′ (Invitrogen), AG100 (*23*) and BL21DE3Omp8 (*24*) 100 μg/ml ampicillin, 40 μg/ml kanamycin and 25 μg/ml chloramphenicol were used for selection.

The cloning vector pET12a (Novagen) was upgraded by insertion of a DNA cassette, adding a His10-Tag sequence directly after the OmpT leader sequence and a more variable multi cloning site (*Sma*I/*Bam*HI/*Nco*I/*Sac*I/*Xho*I/*Bsr*GI/*Bpu*1102I). The cassette was constructed of oligonucleotides JS2_up and JS2_down leading to *Sal*I and *Bpu*1102I overhangs and ligated into a *Sal*I/*Bpu*1102I digested pET12a vector using T4 DNA ligase resulting in p12JS2 (all oligonucleotides are listed in Table 1). Due to the tighter control of the araBAD promoter compared to the plac promoter of p12JS2, the araBAD promoter together with *araC* was amplified by PCR using araBADC_up/down as primers and pBADMyc/HisA (Invitrogen) as template. The plac promoter of p12JS2 was replaced by araBADC using *Bgl*II/*Nde*I restriction sites resulting in pARAJS2. *yaeT* was amplified by PCR using YaeT_up and YaeT_down and cloned into *Sac*I/*Bpu*1102I digested pARAJS2. The resulting pARAJS2yaeT vector was used for expression of N-terminal His-tagged YaeT (HisYaeT). pARAJS2yaeTC expressing HisYaeT^{C} was constructed using the primer pair YaeTQChalf_up/down for insertion of a stop codon followed by a *Sac*I restriction site by Quick Change PCR into the *yaeT* gene. The N-terminal sequence part was cut off by *Sac*I digestion and the remaining vector was re-ligated. The excised N-terminal *yaeT* fragment was inserted into a *Sac*I/CIAP digested pARAJS2 vector leading to pARAJS2yaeTN expressing HisYaeT^{N}. The ligation products were introduced into TOP10F′ and the correct plasmid recombinants were determined by colony PCR using the primer pair pBADseq_up and pETseq_down.

Table 1. **Sequences of oligonucleotides.**

JS2_up	TCGACGCATCATCACCATCACCATCACCATCACCACGGCGCCGAAGGCCGCCCCGGGATCCATGGAGCTCGAGTGTACAGC
JS2_down	AGTCGACATGTGAGCTCGAGGTACCTAGGGCCCCGCCGGAAGCCGCGGCACCACTACCACCACCACTACCACTACTACGC
araBADC_up	GCGCGAGGCAGCAGATCTATTCG
araBADC_down	CGAGCTCGGATCCATATGTAATTCCTCCTGTTAGCCC
YaeT_up	CCACCGTATACGGAGCTCAAGGGTTCG
YaeT_down	GTCAGCCGGCTCAGCAGAAGTTGC
YaeTQChalf_up	GGCTGGGGTGAGCTCTGGTCGATCAGGGTAAG
YaeTQChalf_down	CTTACCCTGATCGACCAGAGCTCACCCCAGCC
pBADseq_up	CCTGACGCTTTTTATCGC
pETseq_down	CCTCAAGACCCGTTTAGAG
YaeTHisC_up	GGAAGAACGCATAATACATATGGCGATGAAAAAGTTGC
YaeTHisC_down	CATTCCTTTGTGGAGAACGAATTCCAGGTTTTACCGATG
YaeTBam_down	CGGCGATCTTATATGGATCCCCTAAAGTCATCG
YaeT-KO_up	CTCTCGGTTATGAGAGTTAGTTAGGAAGAACGCATAATAACGATGATTGAACAAGATGGATTG
YaeT-KO_down	GTGAAGTCGTCCGTTTTGAAGCTGTTATCCTGATCAGAGGTGCTCCGAACCCCAGAGTCCCGCTCA
YaeT-FR_up	CGGGTAAATCCTTAGCGTTAG
YaeT-FR_down	CGGCAGTGATGTCTTTTACA
YaeT610_up	CCGTGGTGGAACGTGGTATGAGCTCGTAAATACCAG

For pARAyaeT *yaeT* was amplified using the primers YaeTHisC_up and YaeT_down and the PCR product was inserted into pARAJS2 *via Nde*I/*Bpu*1102I. For construction of pARA21yaeT expressing the C-terminal His-tagged YaeTHis, pET21a (Novagen) was supplied with the araBAD promoter together with araC as described above for pET12a leading to pARA21a. *yaeT* was inserted after amplification using YaeTHisC_up/down as primers and *Nde*I/*Eco*RI as restriction endonucleases. For the construction of pNByaeT the primers YaeTHisC_up and YaeTBam_down were used for *yaeT* amplification. *yaeT* was inserted into pNB (Stegmeier *et al.*, submitted) after *Nde*I/*Bam*HI digestion. All expression plasmids were confirmed by sequencing (Seqlab).

Protein Expression and Purification—For protein expression the respective expression plasmids were transformed in the porin deficient BL21DE3Omp8 *E. coli* strain. The cells were grown in LB medium under appropriate antibiotic selection at 37°C to an OD_{600} of 0.4–0.7. Then the cells were transferred to room temperature (RT) and expression was induced after 30 min of adaptation by adding 0.02% arabinose. After 4–5 h cells were collected by centrifugation at 5,000 × *g* for 10 min at 4°C and resuspended in 10 mM Tris pH 7.5. Protease inhibitor cocktail (Calbiochem) was added before disrupting the cells by a French pressure cell. Cell debris was collected by centrifugation at 5, 000 × *g* for 10 min at 4°C. The supernatant was ultra-centrifuged at 170,000 × *g* for 1 h at 4°C revealing a membrane pellet. For membrane protein extraction the pellet was resuspended in 10 mM Tris/0.5% LDAO pH 7.5 and protease inhibitor cocktail was added before incubating the suspension 30 min at 4°C or RT followed by ultra-centrifugation at 170,000 × *g* for 30 min at 4°C. The supernatant was applied to 50 μl NiNTA Sepharose resin/100 ml cell culture and incubated overnight at 4°C. The resin was washed extensively using 10 mM Tris/0.5% LDAO/100 mM imidazole pH 7.5 followed by washing with 10 mM Tris/0.5% LDAO/200 mM imidazole pH 7.5. For elution 10 mM Tris/0.5% LDAO/600 mM imidazole pH 7.5 was used.

Protein purification under denaturing conditions was performed resuspending the previously described membrane pellet in 8 M urea/100 mM NaH_2PO_4/10 mM Tris pH 8. Extracted membranes were collected by ultracentrifugation at 170,000 × *g* for 30 min at 4°C and the supernatant was applied to NiNTA spin columns (Qiagen). After extensive washing with 8 M urea/100 mM NaH_2PO_4/10 mM Tris pH 6.3 and 10 mM Tris/0.5% LDAO/100 mM imidazole pH 7.5 the protein was eluted in 10 mM Tris/0.5% LDAO/300 mM imidazole pH 7.5. HisYaeT and

HisYaeT^C eluates containing degraded or denatured proteins were loaded on a 10% SDS gel and the band of the non-degraded protein was cut off and eluted in 10 mM Tris/0.5% LDAO pH 7.5 overnight at 4°C. The eluates were filled into an Amicon Ultra-15 30000MWCO (Millipore) filter device and washed intensively with 10 mM NaH_2PO_4/0.5% LDAO pH 7.4 buffer. HisYaeT^N was purified from the periplasm by following the initial steps of the water lysis method (*25*). YaeT^N was purified via NiNTA Sepharose as described above.

Sucrose-Step Gradient and NADH-Oxidase Activity Test—For the sucrose-step gradient experiment cell membrane pellets were prepared from TOP10F′ cells containing the expression plasmids as described above. Membrane pellets of 200 ml initial culture were carefully homogenized in 1 ml 10 mM Tris pH 7.5 and loaded on a sucrose-step gradient containing 3 ml 30%, 5 ml 50%, and 2 ml 70% sucrose. The gradient was centrifuged at 114,000 × *g* at 4°C for 16 h using a Beckmann SW40Ti rotor. Afterwards fractions of 1 ml were collected and investigated by immunoblot. Fractions containing the outer membrane were allocated by the presence of the major outer membrane proteins OmpC/F, OmpA, fractions containing the inner membrane were allocated by NADH-oxidase activity test. NADH-oxidase activity was measured by detecting the decrease of absorbance at 340 nm as formerly described (*26*).

SDS-PAGE and Western Blotting—Prior to electrophoresis protein samples were mixed with sample loading buffer and if not otherwise notated incubated for 10 min at 100°C. SDS-PAGE was performed according to the Laemmli gel system (*27*). For immunodetection, a tank blot system (Amersham Biosciences) was used as described previously (*28*). The anti-His and the HRP-linked anti-mouse antibodies were purchased from Amersham Biosciences as well as the ECL Western Blotting Detection Reagents.

Electrophysiological Experiments—All protein samples were diluted 1:20 in 10mM NaH_2PO_4/2% LDAO pH 7.4 and stored at 4°C before being used in electrophysiological experiments. The methods used for black lipid bilayer experiments have been described previously (*29*). The experimental setup consisted of a Teflon cell with two compartments filled with electrolytes connected by a small circular hole. The hole had an area of about 0.4 mm^2. Membranes were formed across the hole from a 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids) in *n*-decane. The temperature was maintained at 20°C during all experiments. All salts were obtained from Merck or Applichem and buffered with 1–10 mM Tris pH 7.5 depending on the salt concentration. The electrical measurements were performed using Ag/AgCl electrodes connected in series to a voltage source and a home-made current-to-voltage converter. The amplified signal was recorded on a strip chart recorder. The single- and multi-channel experiments were performed applying a potential of 20 mV, negative on the side of protein addition. Multi-channel experiments were performed with stirring to allow equilibration of added substances. Increasing concentrations of 1 M Citrate pH 4 were added to both compartments after the current was stationary to study pH dependence. Zero-current membrane potentials were measured by establishing a 10-fold salt gradient (30 mM to 300 mM KCl) across membranes containing at least 100 channels (*30*).

yaeT *knock-Out*—The chromosomal *yaeT* knock-out in AG100 pNByaeT was performed basically as described before (*21*, *31*). The primer pair YaeT-KO_up/ YaeT-KO_down and pKD4 as template, were used for PCR to produce a *yaeT* knock-out fragment. The knock-out fragment contained solely the two *yaeT* flanking regions and the kanamycin resistance gene (*kanR*). AG100 pNByaeT pKD46 was grown at 30°C in SOC medium supplemented with 0.2% arabinose and 10 μM IPTG for 4–5 h before transformation of the knock-out fragment. Correct transformants, with the first 1,667 bases of the *yaeT* gene substituted by *kanR*, were selected on LB agar plates containing 1 μM IPTG. Deletion of *yaeT* was verified by colony-PCR using primers that bind up- and downstream of *yaeT*: YaeT-FR_up and YaeT-FR_down. The resulting strain was denoted AG100ΔyaeT.

AG100ΔyaeT pNByaeT was transformed with pARA-yaeT, pARAJS2yaeT and pARAJS2yaeTC, respectively. Correct transformants were selected on LB agar plates containing the appropriate antibiotics and 0.02% arabinose. To allow the cells to forfeit the pNByaeT plasmid cultures and subcultures were grown for 40 h at 37°C with 0.02% arabinose and ampicillin. After the cells were streaked out on LB agar plates containing ampicillin and 0.02% arabinose, single colonies were checked for the loss of chloramphenicol resistance by transferring on LB agar plates containing chloramphenicol and 0.02% arabinose. Chloramphenicol sensitive colonies were used for colony PCR to verify the result. The primer combinations pBADseq_up/pETseq_down, pNBseq_up/pNBseq_down and YaeT610_up/pETseq_down were used to check for pARA plasmids, pNBYaeT, and pARA plasmids containing full length *yaeT*, respectively.

RESULTS

YaeT C-Terminus Is Located in the OM—Topology predictions reveal that Omp85 proteins are composed of two domains of almost equal size (Fig. 1). The amino-terminal half is predicted to be located in the periplasm while the carboxy-terminal half forms a β-barrel like structure embedded in the outer membrane (*17*, *32*). We have cloned the full length and the amino- and carboxy-terminal halves in the pARAJS2 plasmid resulting in the arabinose inducible, secreted, and amino-terminal His-tagged HisYaeT, HisYaeT^N and HisYaeT^C protein. Membrane fractionation by sucrose-step gradient revealed that HisYaeT and HisYaeT^C are localized in the outer membrane (Fig. 2). This shows that folding and membrane localization of the carboxy-terminal half is independent on the amino-terminal half. The amino-terminal half of YaeT, HisYaeT^N, was not found in the membrane fractions, but could be purified as a stable protein from the periplasm (Fig. 3).

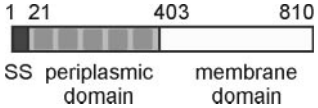

Fig. 1. **Schematic presentation of YaeT.** Residues marking the border of the domains are given. The five POTRA domains within the periplasmic domain are indicated by dark grey areas. SS stands for signal sequence.

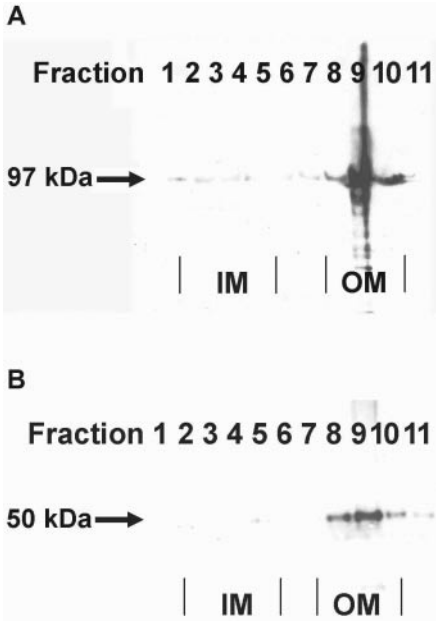

Fig. 2. **Immunoblot of Sucrose-step membrane gradient fractions of cells expressing HisYaeT (A) and HisYaeT^{C} (B).** For detection of HisYaeT and HisYaeT^{C} an anti-HIS antibody was used. The inner (IM) and outer membrane (OM) fractions were denoted after NADH-oxidase activity test and detection of characteristic outer membrane proteins.

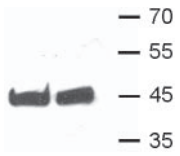

Fig. 3. **Mobility of HisYaeT^{N} purified by NiNTA affinity chromatography from the periplasm on SDS polyacrylamide gels after incubation at room temperature (left lane) and 100°C (right lane).** The protein was blotted to a nitrocellulose membrane and detected using an anti-His antibody. Positions of molecular mass markers (in kDa) are shown on the right.

Interestingly, a full length version of YaeT with a carboxy-terminal His-tag was also absent in the outer membrane fraction. This points towards an importance of the very carboxy-terminus of YaeT for proper assembly in the outer membrane as observed before for other outer membrane proteins (*33*). Taken together, our data support the prediction that the carboxy-terminal half anchors YaeT in the outer membrane, whereas the amino-terminal half is located in the periplasm.

YaeT Is Susceptible to Proteases—For purification, the His-tagged YaeT constructs were expressed in *E. coli*. After French pressure and ultracentrifugation HisYaeT^{N} was in the supernatant, whereas HisYaeT and HisYaeT^{C} were in the pelletized membrane fraction and were solubilized by detergent extraction. Proteins were bound to NiNTA affinity columns and eluted by imidazole. HisYaeT^{N} could be eluted as pure protein with a molecular weight of about 45 kDa. Although low temperature during isolation and addition of protease inhibitor several protein bands with lower molecular weight were visible on SDS PAGE of eluted HisYaeT and HisYaeT^{C}. As shown for HisYaeT, the immunoblot with Anti-His antibodies identified the smaller proteins as N-terminal fragments (Fig. 4). Furthermore, amino-terminal sequencing confirmed that the prominent 45 kDa band is the amino-terminal domain of HisYaeT. Various bands with molecular weight higher than 45 kDa imply that membrane inserted C-terminal half of the YaeT protein is highly protease accessible. The same observations were made with HisYaeT^{C} (Fig. 4, lane 3). When purified HisYaeT and HisYaeT^{C} were loaded unboiled on a SDS gel, a 97 kDa (HisYaeT) and 50 kDa band (HisYaeT^{C}) shifted to 75 kDa and 37 kDa, respectively (Fig. 5). This heat-modifiable mobility in SDS PAGE is characteristic for β-barrel OMPs (*34*, *35*). In contrast, the amino-terminal half of YaeT showed no heat-modifiable mobility (Fig. 3).

Taken together we could show that the two halves of YaeT form independent domains. The periplasmic domain forms a stable, protease inaccessible structure, whereas the membrane domain shows on the one hand heat-modifiable mobility, on the other hand protease accessibility.

YaeT Forms Pores in Planar Lipid Bilayer Membranes—For reconstitution in black lipid bilayer HisYaeT was further purified by gel elution. The protein sample obtained after NiNTA affinity chromatography was loaded unboiled on a SDS PAGE and the 75 kDa band corresponding to the undigested form of the protein was cut out. Protein were pure, devoid of protease digestion products and retained heat-modifiable mobility assuming that purification has no influence on protein conformation (Fig. 5A).

Purified HisYaeT was added to the aqueous compartment of a black lipid bilayer apparatus. The protein induced a stepwise increase of membrane conductance meaning that it forms water filled pores in black lipid membranes (Fig. 6A). The size of the steps was not homogeneous varying from, *e.g.* 20 to 100 pS in 100 mM (Fig. 7A) and most insertion events lead to a significant increase of the conductance fluctuation. Variation of the purification protocol, potential, pH, ions, or ionic strength, or addition of divalent cations failed to reveal more homogeneous single channel conductance. Rarely, we observed very large conductance steps of, *e.g.* over 6 nS in 1 M KCl. Most of these highly variable conductance steps had a very short lifetime (Fig. 6A) indicating that this state is not stable. To exclude that insertion events originate from other co-purified pore forming proteins, HisYaeT was purified extensively by repeated binding on NiNTA columns under denaturing conditions (8 M urea) eliminating attached proteins. Gel-eluted and refolded HisYaeT revealed the same heat-modifiable mobility in SDS PAGE. Added to the lipid bilayer it induced the same conductance steps as purified under native conditions (data not shown).

Single channel conductances were measured in different KCl concentrations. For each concentration a most frequent observed single channel conductance could be detected (Table 2). It should be noted that there is no linear dependence between single channel conductance and electrolyte concentrations, which means that point charges must exist in the water filled channel.

Varying the electrolyte showed that a significant decrease of the single channel conductance was observed when K^{+} was exchanged against Li^{+}. Exchange of Cl^{-} against acetate had no effect on the single channel conductance.

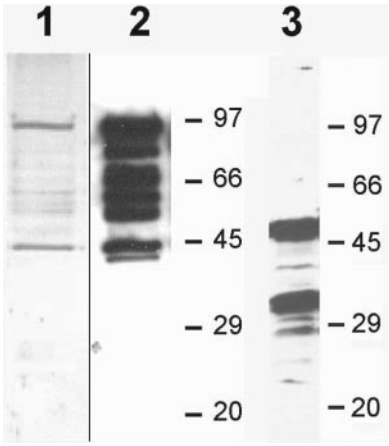

Fig. 4. **HisYaeT (lane 1 and 2) and HisYaeT^{C} (lane 3) after purification using NiNTA sepharose.** Lane 1: Detection of HisYaeT *via* silver nitrate staining. Lane 2 and 3: Immunoblot using an anti-His antibody. Positions of molecular mass markers (in kDa) are shown on the right. All samples were boiled for 10 min before SDS PAGE.

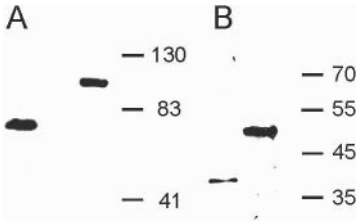

Fig. 5. **Mobility of HisYaeT (A), HisYaeT^{C} (B) isolated and purified by detergent extraction, gel-elution, and NiNTA-affinity chromatography on SDS polyacrylamide gels after incubation at room temperature (left lane) and 100°C (right lane).** The proteins were blotted to a nitrocellulose membrane and detected using an anti-His antibody. Positions of molecular mass markers (in kDa) are shown on the right.

This indicates that the YaeT pores are cation selective. The cation selectivity was confirmed by multi channel experiments. Under zero current conditions a 10-fold KCl gradient was established across a YaeT containing membrane. It resulted in a potential of 33.5 ± 7.0 mV, positive on the diluted side, which means that the pores are cation selective with a permeability ratio p*K*/pCl of 5.7.

The pH dependence of YaeT was also examined by multi channel experiments. HisYaeT was added to the Teflon cell containing 1 M KCl pH 8.0. After conductance reached a constant level 1 M Citrate pH 4.0 was added to both compartments. Lowering of pH down to 4.6 resulted in a decrease of conductance of about 70% (Fig. 8) showing a pH dependent closure of YaeT pores. Taken together, we show that YaeT forms cation selective pores with variable conductance states, which close under acidic conditions.

The Membrane Domain Is Responsible for Pore Formation—For reconstitution experiments in black lipid bilayers the carboxy-terminal membrane domain of YaeT was purified as described for the full length protein. It should be noted that the absence of the periplasmic domain has no influence on the heat-modifiable mobility of the membrane domain (Fig. 5B). Reconstitution of HisYaeT^{C} in lipid membranes results in the similar

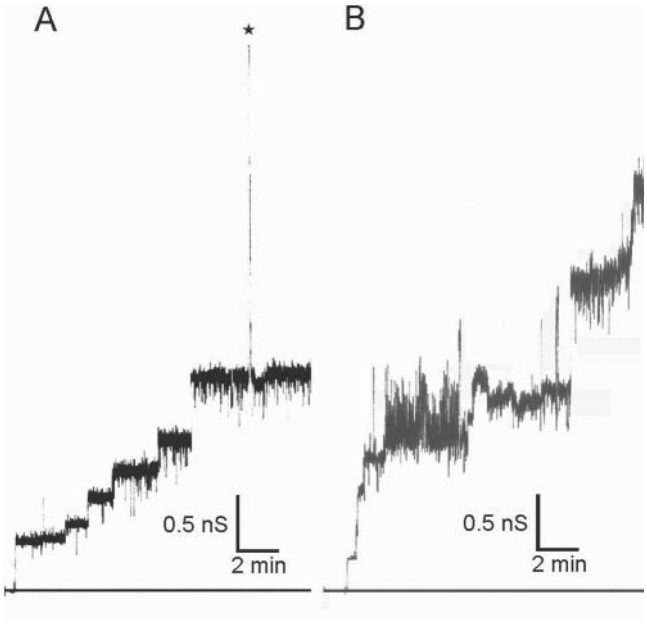

Fig. 6. **Single-channel recordings of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 5 ng/ml HisYaeT (A) or HisYaeT^{C} (B) protein.** The aqueous phase contained 1 M KCl. The applied voltage was 20 mV. Note that YaeT channels can adopt high conductance states (marked by an asterisk).

conductance steps and shows similar characteristics as observed for full length HisYaeT (Figs. 6B and 7B), with the exception that the fluctuations were more pronounced and the very high conductance steps were recorded less frequently. HisYaeT^{N}, however, had no pore forming activity in black lipid bilayer membranes (data not shown). The pore forming ability resides clearly in the carboxy-terminal half confirming that the membrane domain functions independent of the periplasmic domain.

The Periplasmic Domain of YaeT Is Essential for Function In Vivo—Since the C-terminus is sufficient for pore forming we studied its abilities *in vivo.* We have constructed the conditional *yaeT* knock out strain AG100ΔyaeT. The chromosomal *yaeT* gene was deleted as described before (*21*). To get mutants it was necessary to complement the strain by the plasmid pNB1yaeT encoding *yaeT* under the plac promoter. In order to exchange the leaky plac promoter with the tightly regulated pARA promotor and to study variants of YaeT, we transformed AG100ΔyaeT pNB1yaeT with either pARAyaeT encoding YaeT wild-type, pARAJS2yaeT encoding HisYaeT, or pARAJS2yaeTC encoding HisYaeT^{C}. After 40 h selecting just for pARA plasmids in the presence of arabinose, we checked for the loss of pNB1yaeT. 16 out of 34 (pARAyaeT) and 10 out of 34 (pARAJS2yaeT) had lost the pNB1yaeT plasmid as confirmed by PCR and by sensitivity for chloramphenicol. Growth experiments with AG100ΔyaeT pARAyaeT and AG100ΔyaeT pARAJS2yaeT show that growth was indistinguishable from AG100 wild-type when YaeT expression was induced, whereas without induction growth became reduced after several generations (data not shown). These observations are identical to the results reported recently by two independent groups

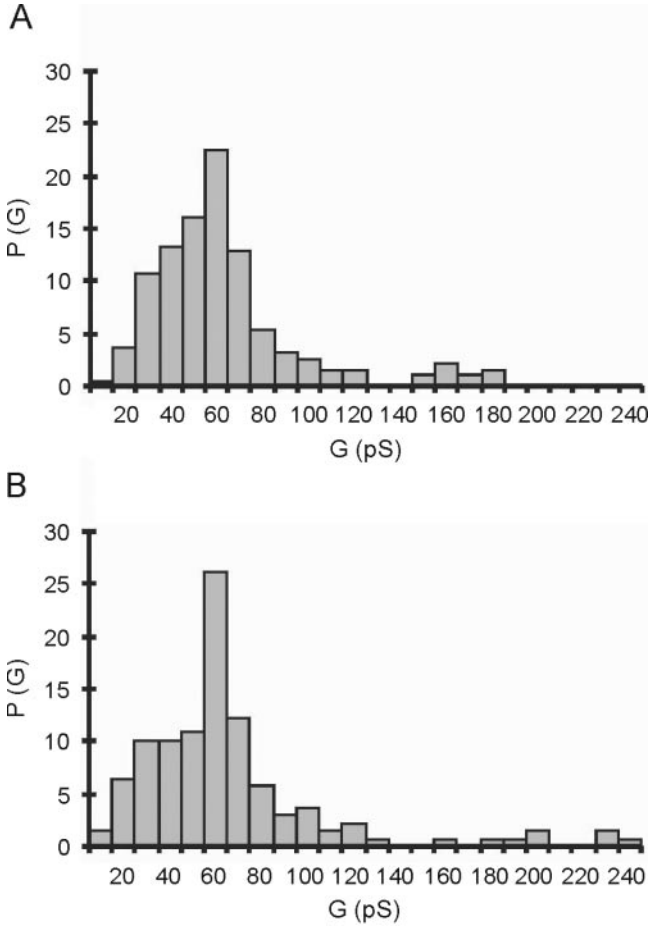

Fig. 7. **Histogram of the conductance steps observed with diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 5 ng/ml HisYaeT (A) and HisYaeT^{C} (B) protein.** The single channel conductance varied in a range between 20 and 80 pS with a main peak observed at 60 pS. P (G) is the probability that a given conductance increment G is observed in the single channel experiments. The aqueous phase contained 100 mM KCl, the applied voltage was 20 mV.

Table 2. **Single channel conductance of HisYaeT in different electrolytes and electrolyte concentrations.**

Electrolyte	Conductance peak (pS)	Conductance range (pS)
30 mM KCl	30	10–35
100 mM KCl	60	20–80
300 mM KCl	125	75–175
1 M KCl	400	100–600
3 M KCl	700	400–800
100 mM LiCl	20	5–40
100 mM KAc	60	20–80

(*19*, *20*). Our results show additionally that the amino-terminal His-tag did not disturb the functionality of YaeT.

Experiments trying to exchange the YaeT wild-type with the mutant HisYaeT^{C} were not successful. 7 out of 34 colonies had lost the chloramphenicol resistance gene as shown by chloramphenicol sensitivity and PCR, but all seven colonies had gained the *yaeT* wild-type gene. We could confirm by PCR that a gene transfer occurred from pNB1yaeT to pARAJS2yaeTC replacing the gene for the

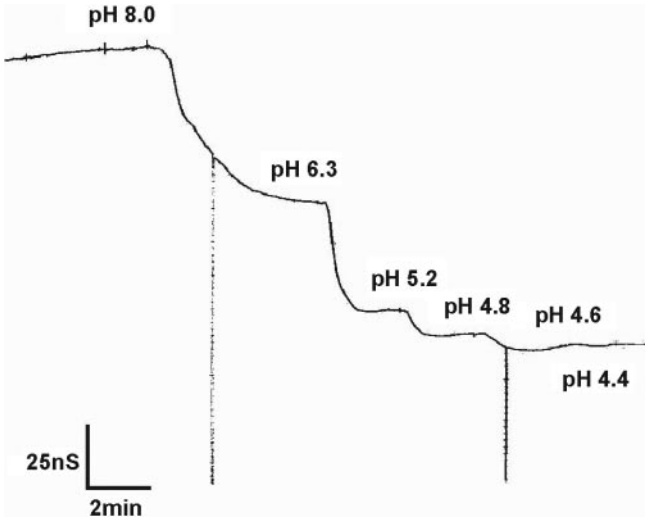

Fig. 8. **Influence of pH on membrane conductance induced by HisYaeT.** The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase on both sides of the membrane contained 1 M KCl pH 8.0. 2–5 μl protein sample (5 ng/ml) was added. A stable conductance was reached with approximately 400 inserted HisYaeT channels. Increasing amounts of 1 M Citrate pH 4 were added to both sides of the membrane. The lowering of pH was followed by a stepwise decrease of conductance. Decrease of pH from 8.0 to 4.6 resulted in a 70% closure of YaeT channels.

carboxy-terminal variant with the wild-type gene. This lets us conclude that the carboxy-terminal pore forming domain of YaeT is not sufficient to replace the wild-type YaeT.

DISCUSSION

In recent times, the family of Omp85 protein came into the focus of researchers investigating biogenesis of outer membranes of Gram-negative bacteria or organelles in eukaryotes (*8*, *32*). The bacterial Omp85 proteins are shown to be essential for insertion of membrane proteins into the permeability barrier. YaeT, the Omp85 ortholog from *E. coli*, forms a multi-protein complex together with three outer membrane lipoproteins (*21*). However, YaeT is the sole integral membrane protein in the OM multi-protein complex. Therefore, it might be the crucial component for membrane insertion of OMPs. In our study we could confirm the prediction that Omp85 proteins consist of two domains, an amino-terminal periplasmic domain and a carboxy-terminal membrane domain (*17*). We could show for YaeT of *E. coli* that the amino-terminal half can be expressed as a stable, soluble protein located in the periplasm, whereas the carboxy-terminal half is anchored in the OM and has the ability to form pores independently on the periplasmic domain. This means that YaeT indeed consists of two independent domains.

We have determined two features of the membrane domain, which seems to be contradictory. On the one hand, we could show that the membrane domain shows heat-modifiable mobility. Higher mobility in SDS PAGE is a feature often observed for outer membrane proteins as *e.g.* OmpA or OmpF trimers of *E. coli* (*34*, *35*). It could also be shown for the secretion component FhaC of the two partner secretion system of *Bordetella pertussis*, which

belongs to the Omp85 family (*36*). The higher mobility in SDS PAGE implies that the membrane domain adopts a compact, SDS stable structure. On the other hand, we observed that the membrane domain becomes degraded during purification. This seems to conflict with the image of a compact structure. Protease degradation is not typical for membrane domains of integral outer membrane proteins. Solved structures of diverse outer membrane proteins show that they form exclusively beta barrels (*37*). The compact folding of β-barrels protects proteins generally from protease degradation during purification. YaeT behaves differently. This might be explained by the fact that YaeT is natively part of a multiprotein complex and might be stabilized and protected by the interaction with the three corresponding lipoproteins (*21*). One can imagine that YaeT is affected by unbalanced expression of the proteins of the complex leading to a protease accessible conformation.

For reconstitution in black lipid membranes only non-degraded, extra-purified proteins were used, which show heat-modifiable mobility. When reconstituted in artificial membranes YaeT induces stepwise increase of conductance suggesting that it forms a hydrophilic pathway through the permeability barrier. The mutant protein HisYaeT^{C}, lacking the periplasmic domain, induces the same conductance steps, which also shows that the membrane domain functions independently of the periplasmic domain. The observed higher fluctuations of the pores induced by HisYaeT^{C} suggest that the periplasmic domain might stabilize the membrane domain, which could also be responsible for the more frequently observed high conductance states in recordings of the full length protein. It is clear that the temporarily observed high conductance states do not originate from a gating-like process with the periplasmic domain acting as a plug because they are also rarely observed for HisYaeT^{C}. Switches into the unstable high conductance state are therefore an intrinsic behaviour of the membrane domain.

Thus, this is the first report showing that the bacterial Omp85 proteins form pores in the outer membrane as suggested previously (*6*, *32*). The bacterial Omp85 proteins belong to a major superfamily separated in six clusters (*38*). In parts, sequence similarity is very low and is restricted partially to short regions close to the carboxy-terminal end (*39*). However, for a few representatives of three clusters pore formation was shown previously. Among these is the cluster comprising outer membrane components of the two-partner secretion (TPS) systems, which mediate translocation of large proteins across the outer membrane (*40*, *41*). Another cluster of pore forming Omp85 homologues comprises proteins in the mitochondrial outer membrane. In contrast to proteins of the TPS system, these proteins are not involved in the transfer of proteins across the membrane but are necessary for the insertion of proteins into the membrane (*42*). The third cluster comprises proteins found in the outer membrane of cyanobacteria or chloroplasts. For these proteins both functions, transport into and transport across the membrane, is reported (*43–45*). Interestingly, for almost all Omp85 homologous pore forming proteins transitions into higher conductance states are reported (*40*, *41*, *43*, *45*), which suggests that this is a general feature of this protein superfamily. It should be mentioned that the periplasmic domain of FhaC from the two partner secretion system of *B. pertussis* and of Toc75, the Omp85 protein from chloroplasts of *Pisum sativum*, was shown to affect also pore properties (*45*, *46*).

Compared to well characterized bacterial outer membrane porins, YaeT behave as a typical pore forming OMP concerning the pH dependent closure, which is suggested to be an immediate response to rapid pH changes of the environment protecting the cells (*15*, *47*, *48*). Concerning the single channel conductance, however, the YaeT pores behave differently. The conductance steps observed with reconstituted porins like OmpF, PhoE, LamB, etc. are uniform differing only about 10–20% (*49*). Higher variability of single channel conductance is only seen with porin mutants, having major structural elements deleted (*50*, *51*). In the case of YaeT, the single channel conductance varies. The reason for this might originate from the distinct function of YaeT. Contrary to porins, which transport substances *across* the OM, bacterial Omp85 proteins are predicted to transport proteins *into* the membrane.

There exist two distinct models about this process. One model suggests that YaeT forms a closed β-barrel, which assembles to homo-oligomers. The nascent OMP assembles in its centre and is released in the membrane plane by a gap between the YaeT protomers. Several reasons speak against this model. Until now, no oligomers are detected for proteins of the YaeT family. Furthermore, it is difficult to imagine, how the centre of such an oligomer remains free of lipids and forms a water filled channel. A water filled pathway in each YaeT protomer would have no physiological relevance. We favour a second model with single protein forming a functional transport unit (Fig. 9). However, this model makes a lateral opening towards the membrane plane necessary. The membrane domain is predicted to consist of 12 amphipatic β-strands (*17*). We suggest that the β-strands do not form a closed barrel. Breaking up the hydrogen bonds of the antiparallel β-strands within a β-barrel to provide a lateral opening would be energetically unfavourable. We assume that the β-strands within the

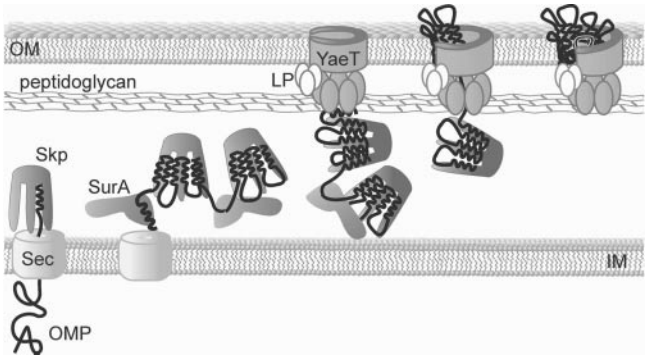

Fig. 9. **Schematic model of OMP biogenesis.** The outer membrane protein (OMP) is transported by the sec-system (Sec) across the inner membrane. The periplasmic chaperon Skp binds to the growing peptide chain keeping it in a partly unfolded state. Periplasmic folding factors such as SurA can act on the periplasmic intermediate. The OMP is thread into the periplasmic domain of YaeT, which might form a transition through the peptidoglycan layer. The β-barrel assembles within the YaeT pore and becomes laterally released into the membrane. Abbreviations: inner membrane (IM), outer membrane (OM), lipoproteins (LP).

C-terminal domain form a flexible barrel like assembly, which can open towards the membrane plane by a gap in the barrel. Thus, the variable conductance states might have physiological relevance and could be explained by the special architecture. The observed transient high conductance states might represent the unstable conformation of a YaeT pore with the maximal diameter. Nevertheless, in the resting state the protein adopts a compact structure as the OMP typical heat-modifiable mobility shows. The flexible structure would allow membrane insertion of β-barrels of different size varying between 8 and 22 β-strands. The hydrophilic interior of the YaeT pore could provide the pathway for extracellular loops of OMPs bypassing thus an energetically highly unfavourable direct transfer through the lipid phase.

As mentioned above, the periplasmic domain is not needed for pore formation. However, attempts to substitute full length YaeT by a variant lacking the amino-terminal domain failed, which shows that the periplasmic domain is essential for viability of the cells. It is composed of five repeated units, the so-called POTRA (polypeptide-transport-associated) domain (*32*, *52*). The size of the extramembraneous domain within the Omp85 superfamily is variable and the number of repeats ranges from one (TPS and mitochondrial Omp85 homologues) to five (bacterial Omp85 proteins). For TPS proteins it could be shown that the domain is important for folding of the substrates in their active form but not for secretion (*53*), whereas the extramembraneous domain of Omp85 homologues found in chloroplasts, functions as a substrate recognition and complex assembly unit (*45*). A similar chaperon like function could be postulated for the YaeT periplasmic domain. It is most likely that the YaeT periplasmic domain is the assembly site for the three lipoproteins YfgL, YfiO, and NplB to form the multi-protein complex. One can imagine that this periplasmic assembly serves as recognition site for OMPs, which are transferred from the inner membrane secretion apparatus bound to periplasmic chaperons. Released from the chaperons the partly folded OMPs become then thread into the YaeT pore assisted by the periplasmic domain. We could imagine an additional function of the periplasmic domain. A structure often neglected in models of OM biogenesis is the peptidoglycan layer. With a mesh size of about 2 nm it exhibits a major barrier for globular hydrophilic molecules of about 25 kDa (*54*, *55*). The periplasmic domain could provide a pathway for the OMP through the peptidoglycan layer on their way to the OM.

However, further experiments need to be done to elucidate the biogenesis of the outer membrane. In this study, we could show that YaeT can be reconstituted into artificial membranes investigating the electrophysiological properties of this essential OMP. In future, it will be possible to study mutant proteins and interaction with other components involved in the OMP membrane insertion. A detailed understanding of this vital process will be the basis to design antibacterial drugs targeting the Omp85 protein family.

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