Study of
Omp85 Family Proteins YaeT and YtfM
and
Multidrug Export Machineries in *Escherichia coli*

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PUBLICATIONS

Emir Berkane, Frank Orlik, Johannes F. Stegmeier, Alain Charbit, Matthias Winterhalter and Roland Benz (2006) Interaction of Bacteriophage Lambda with Its Cell Surface Receptor: An in Vitro Study of Binding of the Viral Tail Protein gpJ to LamB (Maltoporin) *Biochemistry*; 2006 Feb 28; 45(8): 2708-2720

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Johannes F. Stegmeier, Andreas Glück, Suja Sukumaran, Werner Mäntele and Christian Andersen (2006) Characterisation of YtfM, a second member of the Omp85 family of *E. coli* (submitted for publication)

CONTENTS

CHAPTER 1

1. Introduction

1.1. Gram-negative bacteria

The characteristic of Gram-negative bacteria is their cell envelope which is composed of two membranes enclosing the peptidoglycan-containing periplasm (Figure 1.1). The cytoplasm or inner membrane (IM) is a phospholipid bilayer, whereas the outer membrane (OM) is an asymmetrical bilayer containing phospholipids and lipopolysaccharides (LPS) in its inner and outer leaflet, respectively. The LPS molecules can be found exclusively in the outer leaflet of the OM and are composed of three parts (Huijbregts *et al.*, 2000): lipid A forms the hydrophobic membrane anchor, to which the core region is linked, which consists of a phosphorylated non-repeating oligosaccharide. The third component is the O-antigen, which contains repeating oligosaccharides. Attached to the core region it builds the outer part of a LPS molecule. Some Gram-negative bacteria lack the O-antigen (Raetz and Whitfield, 2002) and therefore their LPS are called rough instead of smooth. The periplasmic leaflet of the outer membrane contains, like the inner membrane, phospholipids. The phospholipids are the major lipid class in most Gram-negative bacteria and consist of a glycerol backbone with two fatty acid chains esterified at the 1 and 2 position. At position 3 a phosphate group is located, to which a hydrophilic group is esterified (Huijbregts *et al.*, 2000).

Both membranes include proteins that are inserted or anchored in the lipid bilayers. These proteins are all synthesized in the cytoplasm and are guided towards the cell envelope, where many of them have to cross at least the IM. After direction to their designated location, these proteins fulfill various functions concerning bacterial life. The lipoproteins are acylated proteins that are anchored in the IM or OM via an amino-terminal N-acyl-diacylglyceryl-cysteine (Ichihara *et al*., 1981). The major OM lipoprotein, Lpp (Braun´s lipoprotein), forms

the connection of the OM to the peptidoglycan layer via a peptide bond (Braun *et al*., 1969; Braun *et al*., 1970). The integral membrane proteins are inserted into the lipid bilayer and display characteristical structures. The membrane located parts of IM proteins are composed of α-helices, the ones of OM proteins of β-strands, forming a cylindrical β-barrel with hydrophobic residues at the outside (Nikaido and Vaara, 1985; Wimley, 2003).

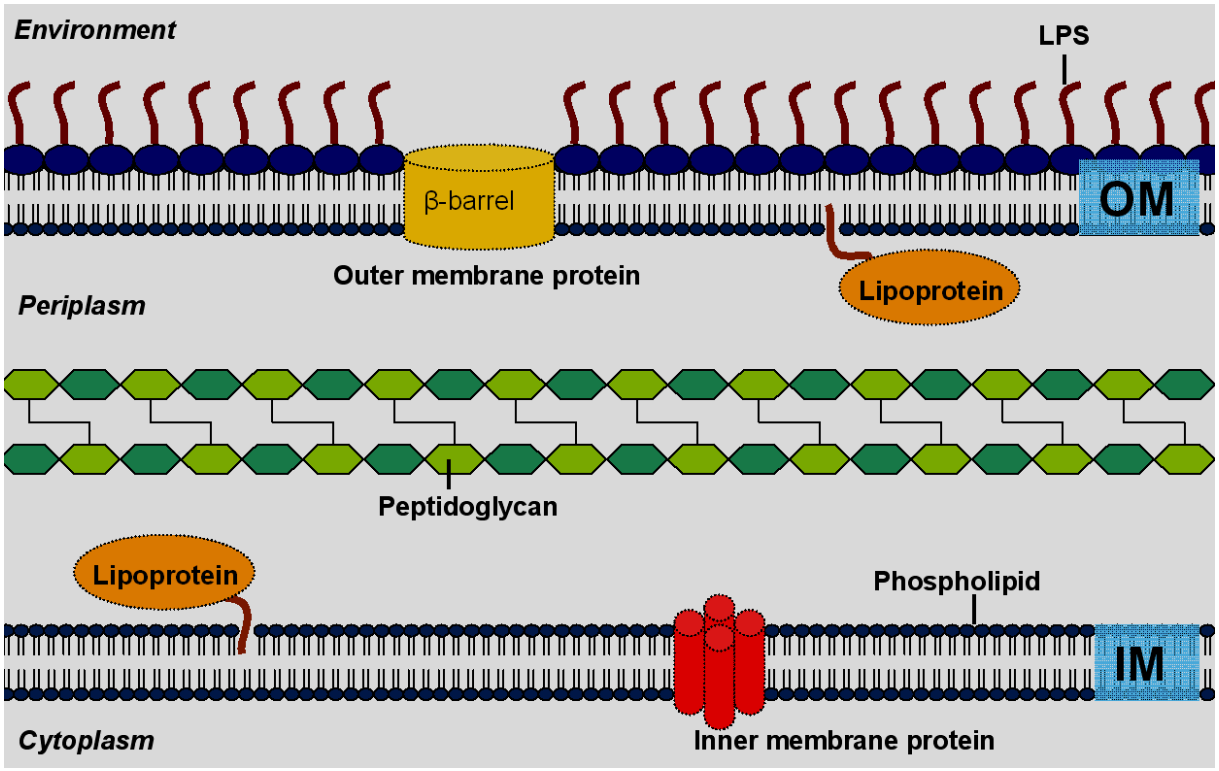


Figure 1.1: Model of the cell envelope of Gram-negative bacteria.

1.2. Biogenesis of the Gram-negative bacterial outer membrane

As mentioned above, the OM is an essential and exclusive 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-defence proteins (Nikaido, 1996; Beveridge, 1999). The composition and biogenesis of the OM is therefore subject for research since a long time.

1.2.1. Phospholipids

The major phospholipids of *Escherichia coli* are Phosphatidylethanolamin, Phosphatidylglycerol and Cardiolipin (Huijbregts *et al*., 2000). They are all synthesized of the precursor Diacylglycerol-3-phosphate at the cytoplasmic side of the IM by integral proteins or proteins that are attached to the IM (Shibuya, 1992; Cronan Jr. *et al*., 1996).

For transport to the OM the phospholipids have to traverse the IM using a "flip-flop" mechanism. It was speculated that a specific flippase is necessary for this process. However, the latest results suggest that the presence of typical α-helical membrane spanning regions of some IM proteins is sufficient for transport and no distinct transporter exists (Kol *et al*., 2003). The further transport of the phospholipids through the periplasm towards the OM and the insertion into the inner leaflet of the OM is still obscure. No components of these molecular mechanisms could yet be identified, but periplasmic transfer via proteins is a favored model (Huijbregts *et al*., 2000).

1.2.2. Lipopolysaccharides

The constituents of the LPS are also synthesized at the cytoplasmic leaflet of the IM. The O-antigen is transported by either of three different ways: the Wzy-, ABC-transporter- or synthase-dependent pathway, all denoted according to their key component (Raetz and Whitfield, 2002). In contrary, the transport of the lipid A-core moiety is mediated by the ABC-family transporter MsbA. The inactivation of MsbA in *E. coli* leads to a reduction of all major lipids in the OM of about 90% (Doerrler *et al*., 2001) and resulted in accumulation of lipids at the cytoplasmic side of the IM (Doerrler *et al*., 2004). However, the LPS transport cannot be the exclusive function of MsbA, since there are homologues in Gram-positive bacteria and a capacity to act as a multidrug transporter is reported (Reuter *et al.*, 2003). For *E. coli* it was suggested that MsbA is also one of the proteins assisting phospholipids to cross the IM, since the lack of MsbA additionally results in decreased amounts of these lipid molecules (Raetz and Whitfield, 2002). On the other hand, in *Neisseria meningitidis* a *msbA* mutant was still viable and built a double membrane, suggesting that MsbA is not required for phospholipids transport in this organism (Bos and Tommassen, 2004).

After transport across the IM the lipid A-core moiety is ligated to the O-antigen in a so far not well understood process. WaaL is the solely identified enzyme of this ligation. Probably WaaL is part of an enzyme complex which binds the lipid A-core moiety and the O-antigen, performs the linkage and releases a mature LPS molecule (Raetz and Whitfield, 2002). The

next unsolved issue is the passage of the LPS molecules through the periplasm. Currently two possibilities are favored, either a type I secretion system like pathway, or passage of the LPS to the OM guided by a soluble protein (Bos and Tommassen, 2004). The first mentioned way originates from the homology of MsbA to the ABC-transporter HlyB which is the IM component of the type I secretion apparatus HlyBD/TolC. The latter suggestion was supported recently by the finding of direct interaction between LPS and the periplasmic chaperone Skp (Walton and Sousa, 2004).

For insertion of LPS into the OM the protein Imp (increased membrane permeability)/OstA (organic solvent tolerance) is responsible. Imp is an essential protein in *E. coli* and a conditional mutant of this protein displayed altered membrane permeability due to a changed lipid: protein ratio of the OM (Abe *et al*., 2003). The exact role of Imp in OM biogenesis was shown in *N. meningitidis*. Since Imp is a non-essential protein in this bacterial strain, *imp* deletion mutants could be investigated. The mutants revealed that lack of Imp leads to a decrease of the amount of LPS to less than 10% compared to wild-type level. Furthermore, the synthesized LPS molecules were not accessible to LPS-modifying enzymes present in the OM or external medium (Bos *et al.*, 2004). Therefore, the role of Imp in LPS transport across the OM to the cell surface is obvious. The highly reduced amounts of LPS in an *imp* deficient mutant are most likely due to a feedback mechanism that inhibits further LPS synthesis. The role of Imp is emphasized by the fact that it is highly conserved among Gram-negative bacteria and absent in Gram-positives. A second candidate for LPS insertion into the OM was discussed earlier by Genevrois and colleagues (Genevrois *et al.*, 2003). The depletion of Omp85 in *N. meningitidis* resulted in changes of the lipid composition. Afterwards, the group of Tommassen has assigned Omp85 as the protein which is responsible for insertion of OM proteins into the OM. Therefore, the effect of Omp85 on membrane lipids is most likely indirect (Genevrois *et al.*, 2003), caused by the lack of Imp in the OM (Voulhoux *et al.*, 2003).

1.2.3. Lipoproteins

All lipoproteins are transported, like other OM proteins, across the IM via the Sec- (secretion) system (Bos and Tommassen, 2004). After translocation of the protein precursor, a cysteine residue at the amino-terminus is linked to the lipid component diacylglycerol by a transferase. After cleavage of the signal peptide turning the lipidated cysteine into the most amino-terminal residue of the mature protein, this cysteine is amino-acylated as a final step of

synthesis. The destination of the lipoproteins, whether they are attached to the inner or the outer bacterial membrane via their lipid anchor, is determined by the residue directly following the cysteine. If an aspartate is present at this position, the lipoprotein becomes anchored in the IM, whereas other residues lead to transport to the OM (Narita and Tokuda, 2006). The transport is performed by the Lol system, which is composed of five components. LolC, LolD and LolE form an ATP-binding cassette (ABC) transporter in the IM, which detaches lipoproteins at the expense of ATP. The lipoproteins are transferred to the periplasmic chaperone LolA and the formed LolA-lipoprotein complex moves through the periplasm towards the OM. In the OM, the complex interacts with the lipoprotein LolB which incorporates the delivered lipoproteins into the inner leaflet of the OM (Narita and Tokuda, 2006).

1.2.4. Integral outer membrane proteins (OMPs)

OMPs are also translocated across the IM using the well studied Sec-system (Thanassi and Hultgren, 2000; van den Berg *et al*., 2004). Further steps after cleavage of the signal peptide and release into the periplasm are not fully understood yet. OMPs all contain an even number of amphipatic β-strands that form a barrel like structure when inserted into the OM (Wimley, 2003). This distinguishes OMPs from integral IM proteins. Many denatured OMPs are shown to fold correctly in the presence of detergents *in vitro*, and spontaneous folding and insertion into liposomes has been reported (Kleinschmidt, 2003). However, the structural specificity and the very fast kinetics *in vivo* demand assistance for the transport of OMPs through the periplasm and their insertion into the OM. The hydrophobic surface at the outside of the barrel like structure makes a complete folding of OMPs in the periplasm unfavorable. Many periplasmic proteins involved in the interaction and folding of OMPs are described. The proteins DsbA and DsbC were shown to catalyze the formation of disulfide bonds which precedes the insertion of OMPs (Kadokura *et al*., 2003). The disulfide bonds suggest a partial folding of the OMPs during their passage through the periplasm (Bos and Tommassen, 2004). The peptidyl-prolyl *cis-trans* isomerase (PPIase) SurA was shown to act as periplasmic chaperone specifically for OMPs (Behrens *et al.*, 2001). The lack of SurA leads to a reduced amount of the major OMPs OmpC, OmpF, LamB and OmpA (Rouvière and Gross, 1996). Furthermore, it was shown to bind preferentially unfolded OMPs rather than folded OMPs or other unfolded proteins of similar size (Bitto and McKay, 2004). The crystal structure of SurA displays a potential peptide binding ability (Bitto and McKay, 2002) and confirmed that the

two PPIase domains are not necessary for chaperone activity (Behrens *et al.*, 2001), since the PPIase domains did not contribute to the potential peptide binding cleft of the protein. Most likely, SurA shares to some extent functions with another identified chaperone, Skp (seventeen kilodalton protein), since inactivation of both genes *surA* and *skp* resulted in a lethal phenotype (Rizzitello *et al*., 2001). The solely disappearance of Skp leads to a reduced level of OMPs in the OM and their accumulation in the periplasmic space (Schäfer *et al.*, 1999). Furthermore, in a Skp depleted strain, expression of the protease DegP increases whose function is to degrade unfolded proteins in the periplasm (Missiakas and Raina, 1997). Therefore, a role in prevention of premature folding and aggregation was suggested for Skp. Crystallography revealed a jelly fish shape of the trimeric Skp including α-helical tentacles protruding from a β-barrel body defining a central cavity (Walton and Souza, 2004). A model suggests binding of unfolded OMPs in the cavity between the flexible tentacles, directly after their release of the Sec-machinery at the IM followed by binding of LPS to a specific site at the outside of the tentacles (Harms *et al.*, 2001). Then the proteins are transported to the OM, completely folded and inserted. The ability of Skp to interact with LPS and membrane lipids might indicate a role of these molecules in the folding of OMPs. It was previously shown *in vitro* that LPS molecules stimulate the folding of OMPs (deCock *et al.*, 1999). On the other hand, Steeghs and colleagues (Steeghs *et al.*, 2001) have shown that a *N. meningitides* mutant that is completely devoid of LPS has OMPs assembled correctly in the OM. Thus, *in vivo*, LPS cannot play an obligatory role in OMP folding. The co-transport of LPS/lipids and the at least partially unfolded OMPs via Skp to the outer membrane could be explained by the last crucial step of OMP insertion. In recent times, members of the Omp85 protein family were identified to be essential for OMP membrane insertion. The first identified protein, Omp85 from *N. menigitidis*, was initially shown to effect the composition of lipids and LPS in the outer membrane (Genevrois *et al.*, 2003). Afterwards, Voulhoux and colleagues reported severe effects of Omp85 depletion on the OMP composition (Voulhoux *et al.*, 2003). Therefore, the effects observed by Genevrois and colleagues might be secondary, because Imp is an OMP itself and most likely affected by Omp85 depletion. However, one cannot exclude that Omp85 proteins might mediate LPS or lipid insertion. The effect of Omp85 on OMP insertion was studied in a *N. meningitidis* strain with a chromosomal deletion of *omp85* complemented by *omp85* on a plasmid under an inducible promoter. When Omp85 expression on the plasmid was not maintained and Omp85 was depleted, a decrease of integral OM proteins was observed. Furthermore, the porins PorA and PorB accumulated in an untypical monomeric state in the periplasm (Voulhoux *et al.*, 2003). The chromosomal knock-out

without compensation was lethal showing that *omp85* is an essential gene. The importance of Omp85 was confirmed by the Omp85 family member YaeT of *E. coli*. YaeT is also essential and leads to a reduction of inserted OMPs (Doerrler and Raetz, 2005). Effects on folding and OM insertion are described for the most important OMPs in Gram-negative bacteria, OmpF, OmpC, OmpA, TolC and LamB (Werner and Misra, 2005; Wu *et al.*, 2005). Co-purification of YaeT together with three other proteins leads to the suggestion that Omp85 proteins are part of a translocation-machinery (Wu *et al.*, 2005). The proteins which interact with YaeT are the OM lipoproteins YfgL, YfiO and NlpB. They are initially identified as suppressors of defects in the function of Imp (Ruiz *et al.*, 2005; Ruiz *et al.*, 2006). YfiO is also shown to be essential for viability. NlpB and YfgL are non-essential, but *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 (Onufryk *et al.*, 2005). However, since YaeT is the only integral OMP of this complex its major role in insertion of OMPs is reasonable.

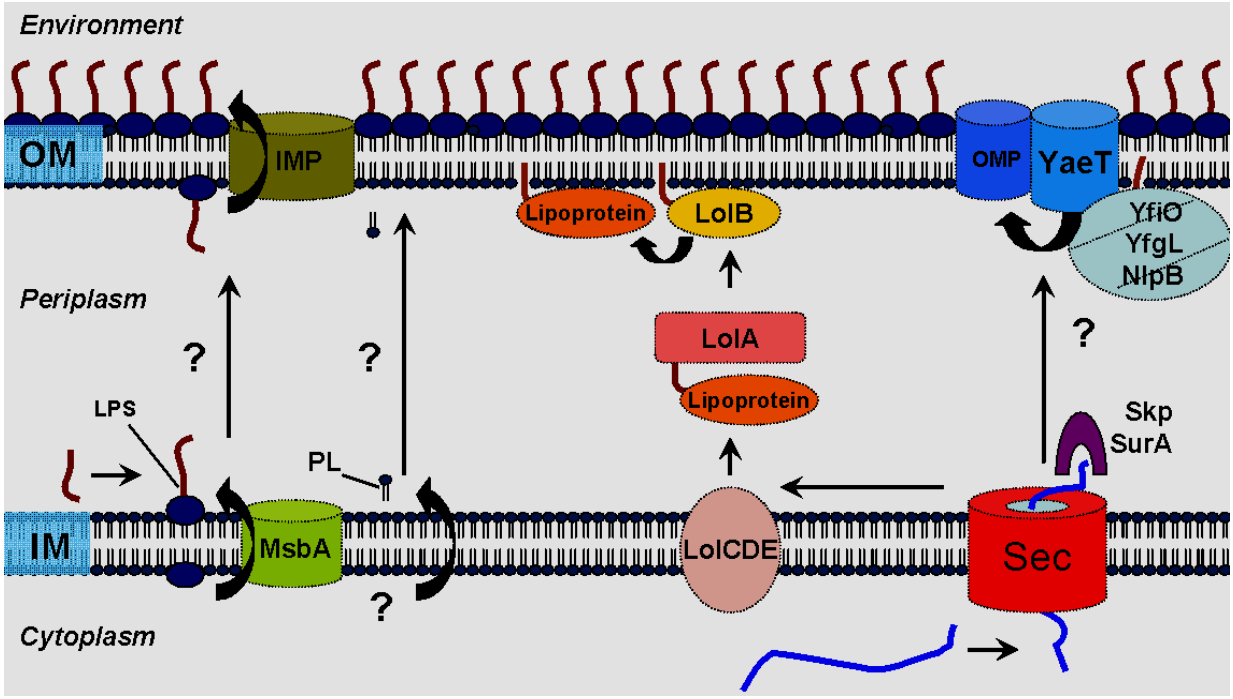


Figure 1.2: Model of the biogenesis of the bacterial OM. Not yet solved processes are marked by a question mark.

The interplay of processes in lipid and protein assembly during OM biogenesis is obvious considering the genetical background of some of the components mentioned above. The genes *yaeT*, respectively *omp85*, belong to a highly conserved cluster which contains the *skp* gene (Figure 1.3).

Furthermore, *fabZ* and *cdsA*, genes that are involved in the fatty acid and phosholipid synthesis are present (Raetz and Dowhan, 1990; Mohan *et al.*, 1994), as well as *lpxA*, *lpxB* and *lpxD* which code for important proteins in the lipid A biosynthesis (Raetz and Whitfield, 2002). YaeM is an enzyme in the isoprenoid synthesis and *yaeL* codes for an essential protease located in the IM (Takahashi *et al.*, 1998; Dartialongue *et al.*, 2001).

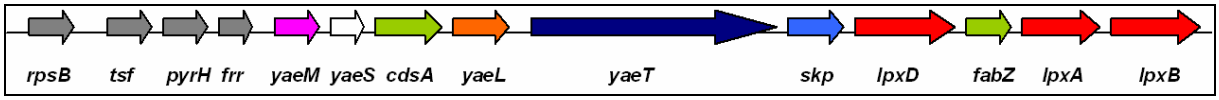


Figure 1.3: Schematic representation of *yaeT* gene cluster.

Furthermore, many of the above mentioned genes are part of the σ^E regulon including among others *yaeT*, *imp*, *skp*, *surA*, *yfiO*, etc. (Dartialongue *et al.*, 2001; Ruiz *et al.*, 2006). The genetical background and the overlapping results in biochemical studies clearly indicate an intimate connection of lipid and protein composition concerning the OM biogenesis. Whether this reflects a co-transport and co-insertion or indicates a cross-talk between separate lipid and protein pathways has to be elucidated in further studies.

1.2.5. The Omp85 protein family

Omp85/YaeT is an evolutionary conserved protein that is present in all Gram-negative genomes sequenced so far, as well as in evolutionary related mitochondria and chloroplasts (Bos and Tommassen, 2004). The mitochondrial Omp85 homologue, Sam50, was also shown to be essential for cell viability and sorting of OMPs into the mitochondrial OM (Schleiff and Soll, 2005). The Omp85 homolog Toc75 is present in chloroplasts and functions in the chloroplast protein-import machinery. Toc75 forms a channel for translocation of mitochondrial OMPs, whereas a function in OMP assembly and insertion is speculated, but not yet elucidated (Schleiff and Soll, 2005). There are also proteins in Gram-negative bacteria which belong to the Omp85 family transporting proteins across the OM. HMW1B in *Haemophilus influenzae*, FhaC in *Bordetella pertussis* and ShlB in *Serratia marcescens* transport specific substrates, adhesins and hemolysins, in an unfolded state across the OM. These systems are known as “two-partner-secretion” (TPS) systems (Surana *et al.*, 2004; Jacob-Dubuisson *et al.*, 2004). Considering the different functions, the Omp85 family proteins could be grouped in six clusters (Yen *et al.*, 2002). The proteins origin from a common ancestor and differences in structure and function developed during evolution. For

proteins that help to insert proteins into the OM, a more flexible structure is necessary, in contrast to proteins that act as a conduit for secretion. If Omp85 or YaeT form themselves a channel in the OM, the proteins should allow opening laterally for the release of OMPs into the lipid bilayer (Bos and Tommassen, 2004). The carboxy-terminal half of Omp85 is predicted to form a 12-stranded β-barrel with a long periplasmic extension. The carboxy-terminal domain was suggested to serve as a translocation pore, whereas the amino-terminal part could have chaperone like functions assisting the folding of OMPs in the periplasm (Voulhoux *et al.*, 2003). This amino-terminal half of the protein was denoted POTRA (polypeptide-transport-associated) domain (Sánchez-Pulido *et al*., 2003). It was shown to consist of repeating conserved sub-domains (Figure 1.4).

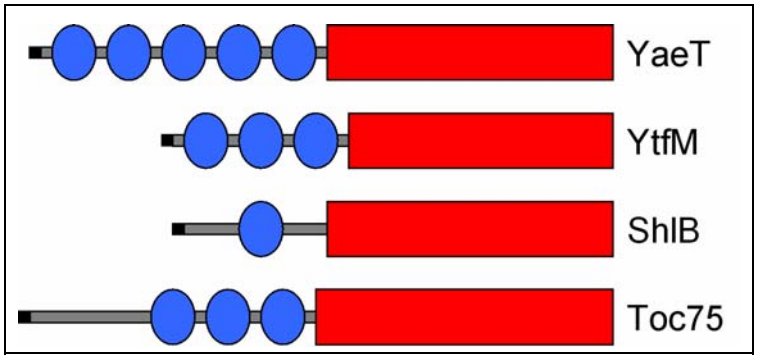


Figure 1.4: Schematic representation of the POTRA- (blue circles) and the carboxy-terminal (red squares) domain of different proteins of the Omp85 family: YaeT of *E. coli*, YtfM of *E. coli*, ShlB of *S. marcescens* and Toc75 of chloroplasts. The signal sequence is indicated as a black square.

Omp85/YaeT and close relatives contain five repeating sub-domains, whereas in other clusters proteins have just one or three (Figure 1.4). One cluster of the Omp85 family including YtfM of *E. coli* contains three repeating units in the POTRA-domain. The function of YtfM is completely unknown. Since YtfM is not able to compensate YaeT in a deletion mutant and the fact, that the genetical background of YtfM is not related to lipid- or protein-synthesis, a different function has to be considered.

In this study, the structure and function of the two Omp85 family members of *E. coli*, YaeT and YtfM were investigated, using the previous mentioned predictions as a basic. For the YaeT homologues Sam50 of mitochondria, Toc75 of chloroplasts and the TPS protein FhaC of *B. pertussis*, pore forming activity is reported (Paschen *et al.*, 2003; Moslavac *et al.*, 2005; Meli *et al.*, 2006), which suggests investigation of YaeT and YtfM in electrophysical experiments.

1.3. Multidrug resistance

Compared to Gram-positive bacteria, Gram-negative show much higher resistance levels for antibiotics and other harmful agents, due to their OM which is a second permeability barrier for hydrophilic substances. One of the most frequently observed mechanisms of drug resistance is mediated by multidrug efflux pumps, which expel intracellular noxious compounds keeping their concentration at a sub-toxic level. Together with the fact, that re-entry of those substances is slowed down by the less permeable OM, multidrug transporters can mediate high resistance levels (Andersen, 2003). Multidrug efflux pumps in Gram-negative bacteria are composed of three components including an OM channel protein, a periplasmic adaptor protein and an energized IM transporter. After assembly of the system, the substrates are recognized by the IM component and transported through the OM channel to the cell exterior, driven by a proton gradient or ATP hydrolysis. Typical examples for multidrug efflux pumps are AcrAB/TolC of *E. coli* and MexAB/OprM of *Pseudomonas aeruginosa*, whose transporters, AcrB and MexB, use a proton gradient as driving force (Andersen, 2003). The components of these two efflux pumps are highly homologue and will be further described in more detail (Wong and Hancock, 2000).

1.3.1. The IM transporter (AcrB/MexB)

Murakami and colleagues solved the structure of the RND-transporter AcrB (Murakami *et al.*, 2002). AcrB forms a homotrimer with a jellyfish shaped structure (Figure 1.5). It contains a transmembrane domain and a headpiece, protruding 70Å into the periplasm. The periplasmic headpiece is divided into the TolC-docking domain and the pore domain. A long hairpin structure protrudes from the TolC docking domain of one protomer into the next protomer, mediating a stable assembling of the headpiece. The TolC docking domain at top of the periplasmic domain opens like a funnel with a maximal diameter of about 30Å. The lower part of the funnel is connected to a central extra-membraneous pore, which is formed by three α-helices (Figure 1.5B). The pore is almost closed in the crystal structure, possibly due to the lack of substrate. Further studies revealed that the central pore is important for drug efflux and has to be opened for substrate transport (Murakami *et al.*, 2003). The pore connects the funnel with the central cavity of the headpiece. The cavity has lateral openings to the periplasm, the so-called vestibules. These vestibules are located between the protomers at the periplasmic

surface of the IM and serve most likely as entrances for AcrB substrates (Elkins and Nikaido, 2003).

The transmembrane domain is composed of 36 helices (12 of each monomer). It is about 50Å long and has a diameter of 100Å. The helices seem to be loosely packed and form a, probably phospholipid-filled, transmembrane pore with a diameter of approximately 30Å. Helices 4 and 10 of each monomer are surrounded by the remaining ten helices (Figure 1.5C). Helix 4 contains two aspartates and helix 10 one lysine residue, whose ion pairs are candidates for the proton translocation site. After disruption of the ion pairs the helices might undergo conformational changes and mediate an opening of the pore by a remote conformational coupling.

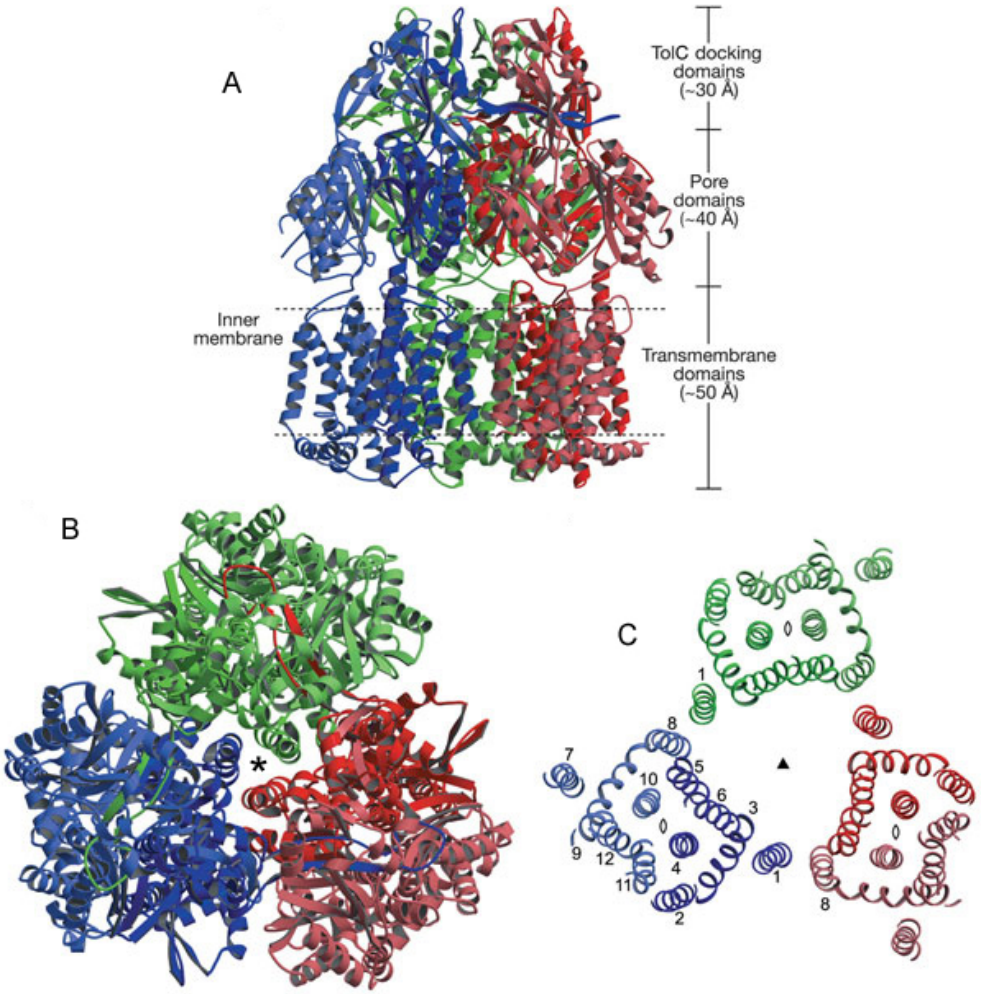


Figure 1.5: Structure of AcrB (Reprinted by permission from Macmillan Publishers Ltd: NATURE, Murakami *et al.*, 2002, Copyright ©2002). The three monomers are coloured green, red and blue, respectively.
A Side view of a ribbon representation. The periplasmic headpiece containing the TolC-docking and the pore domain is at the top. At the bottom is the transmembrane domain; the IM is indicated as dashed line.
B Top view of a ribbon representation. The pore of AcrB, formed by three α-helices, is indicated by an asterisk.
C Structure within a slab of the transmembrane domain parallel to the membrane plane close to the periplasmic surface. The numbers 1-12 indicate the 12 transmembrane helices per monomer.

While an AcrB monomer consists of 1049 amino acid residues, MexB consists of 1046 resulting in a molecular weight of about 113 kDa. Its exact structure is not solved yet, but topology models of MexB suggest a very similar shape. Overexpression and large scale purification facilitated first attempts of the structural characterisation of MexB. It was shown that MexB is also a homotrimer and therefore, models of MexB using AcrB as template could be confirmed (Mokhonov *et al.*, 2005).

1.3.2. The periplasmic adaptor protein (AcrA/MexA)

Whereas the IM and OM components could be crystallized in their native oligomerisation state, the two members of the adaptor protein family AcrA and MexA crystallized not in the native form of the protein (Akama *et al.*, 2004a; Mikolosko *et al.*, 2006). MexA crystallized as a 13-mer composed of two subunits containing six and seven MexA monomers, respectively, AcrA as a di-dimer. Since this is obviously not the native arrangement, the focus lies on a single monomer (Figure 1.6).The structure of the 360 amino acid protein MexA was solved in 2004 (Akama *et al.*, 2004a; Higgins *et al.*, 2004), except of its 28 amino-terminal and 101 carboxy-terminal residues. The MexA monomer forms an elongated structure with a length of 89Å and a maximum width of 35Å. The solved structure reveals three domains in a linear arrangement: a β-barrel, a lipoyl domain and an α-helical hairpin with a length of 47Å. The β-barrel domain consists of six antiparallel β-strands with a single α-helix at the entrance of the barrel. The second domain is, as predicted by Johnson and Church (1999), a structure of eight β-strands that is structurally homologous to lipoyl- and biotinyl-domains. Between β-strand 4 and 5 are 64 residues building the third domain, the α-helical hairpin, which contains residues that are conserved throughout the family of periplasmic adaptor proteins. The carboxy-terminal helix of the hairpin is straight, whereas the amino-terminal one shows a left-handed superhelical twist. The MexA hairpin has four heptadic repeats, which is one less than AcrA (Figure 1.6). The hairpin of AcrA consists of 14 additional residues mediating an additional length of 11Å (Mikolosko *et al.*, 2006). At both ends of the α-helical hairpin large hydrophilic residues are situated, which have the potential to form hydrogen bonds. Furthermore, there are small conserved residues at the exposed faces of the two helices (Higgins *et al.*, 2004). The lipoyl and the β-domains of AcrA are also highly similar to those of MexA, which is not surprising given the 62% identities and 72% similarities of both proteins, respectively. The AcrA crystal did also not reveal the complete structure of the

protein since 44 amino-terminal and 85 carboxy-terminal residues had to be removed (Mikolosko *et al.*, 2006).

It should also be mentioned that AcrA and MexA contain both a lipid modification at their amino-terminus anchoring the proteins in the IM. However, this lipid anchor was shown to be non-essential for function *in vivo* (Zgurskaya and Nikaido, 1999; Yoneyama *et al.*, 2000).

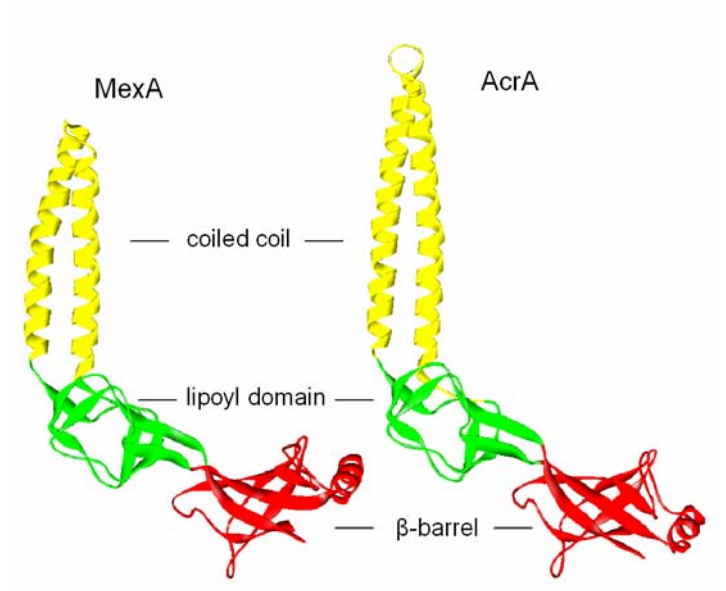


Figure 1.6: Ribbon representation of MexA (left) and AcrA (right) monomers (by Christian Andersen).
The hairpins are coloured yellow, lipoyl domains green, β-strand domains red. The comparison shows the difference in hairpin length.

1.3.3. The OM component (TolC/OprM)

The 3D structure of the 471 amino acid protein TolC was solved in 2000 by x-ray crystallography (Koronakis *et al.*, 2000). For crystallography the 43 carboxy-terminal residues had to be removed. TolC appeared as cannon-shaped cylindric homotrimer with a length of 140Å (Figure 1.7). The cylinder consists of a 40Å long β-barrel (channel domain) in the OM, a 100Å long α-helical region (tunnel-domain) protruding into the periplasm and a mixed α/β-domain (equatorial domain), which forms a "strap" around the midsection of the tunnel-domain. The extracellular end of TolC is open with an inner diameter of about 20Å (Figure 1.7B). This diameter is uniform for a length of about 100Å and then becomes smaller resulting in an almost closed periplasmic end. The interior of TolC is mostly solvent, filled with a volume of approximately 43 000 $Å^3$ (Koronakis *et al.*, 2000). Each TolC monomer is contributing four β-strands to the channel domain (S1,S2,S4,S5). The tunnel-domain consists of two long (H3,H7) and four short (H2,H4,H6,H8) α-helices, whereas the equatorial domain is comprised of small β-strand and α-helical structures (S3,S6,H1,H5,H9; Figure 1.7A).

The membrane anchoring channel domain consists of 12 antiparallel β-strands forming a right twisted barrel. The β-strands S1 and S2, as well as S4 and S5 are connected by loops of 8 and 21 residue, respectively, which are directed towards the cell exterior and allow interaction with the TLS bacteriophage and colicin E1 (German and Misra, 2001). The α-helices H2, H3, H6 and H7 of the tunnel-domain arrange also antiparallel and form the part of the tunnel with the uniform inner diameter of about 20Å (Calladine *et al.*, 2001). The axes of the helices are inclined approximately 20° relative to the molecule axis. The helices are bent in a way that they do not move off tangentially of the curved surface of the cylinder. To maintain this structure the helices must bend in a curve and untwist. The local packaging of the helices is similar to the conventional coiled-coil, the knobs-into-holes interaction (Crick, 1953). However, the helices do not wrap around each other, but form a barrel, meaning that one helix has two interfaces. The barrel structure finishes with the equatorial domain, where the small helices H2 and H6 end. Below the equatorial domain the helices H3 and H7 pair with helices H4 and H8, respectively, in a manner that H4 bends around the straight helix H3, whereas H7 and H8 form a conventional coiled coil. The coiled coil bends inwards and is responsible for tapering of the periplasmic end leading to an inner diameter at the periplasmic opening of just 3.9Å (Figure 1.7B). Electrophysiological studies reveal that this is a very stable conformation inducing a single channel conductance of 80pS in 1M KCl when inserted into a black lipid bilayer (Andersen *et al.*, 2002b).

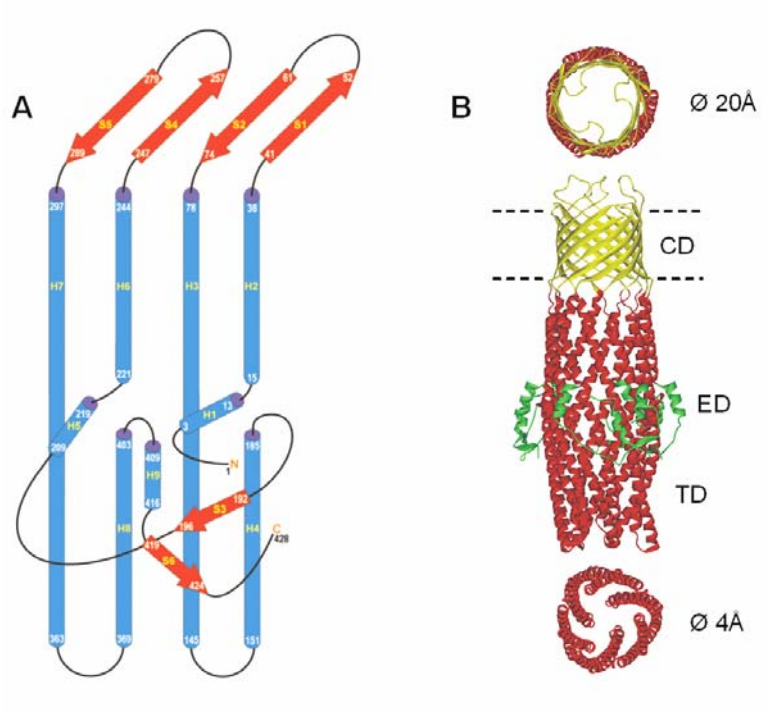


Figure 1.7: Structure of TolC (**A**, Reprinted by permission from Macmillan Publishers Ltd: NATURE Koronakis *et al.*, 2000, copyright ©2000; **B** by Christian Andersen).
A Topology model of TolC. β-strands (S1-S6) are coloured red, α-helices (H1-H9) blue. The amino acid residue numbers of which the secondary structure components consist are given.
B Overall structure of a TolC trimer and views of the part above (top) and below (bottom) the equatorial domain. The inner diameter of the channel domain (CD, yellow) is approximately 20Å; the tunnel-domain (TD, red) is tapering towards the periplasmic entrance leading to an inner diameter of about 4Å. The equatorial domain (ED) is colored green.

OprM was structurally elucidated via crystallography of 456 of its 468 amino acid residues in 2004 by Akama *et al*. (Akama *et al*., 2004b). An OprM monomer consists of four β-strands and eight α-helices forming an overall structure similar to the TolC channel tunnel (Figure 1.8). The OprM trimer has a length of 135Å and an inner diameter of about 25Å at its widest region (equatorial domain). The membrane anchoring β-barrel is constricted by three loops near the end of the barrel, leading to a pore diameter of 6-8 Å, whereas the periplasmic end is almost closed. The cavity inside the OprM trimer is also solvent filled with a volume of about 25000Å^3

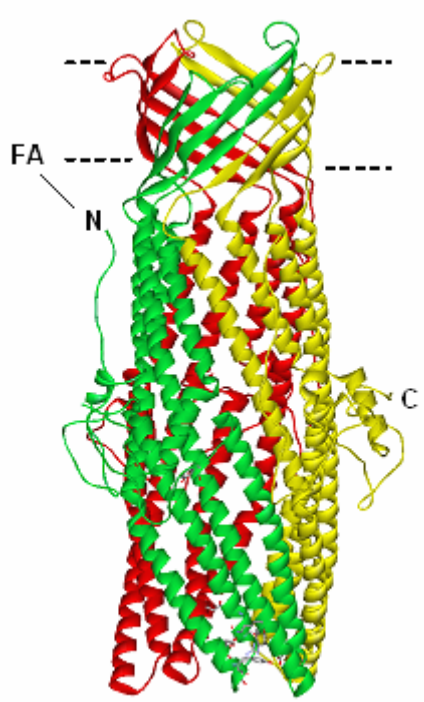


Figure 1.8: Structure of OprM (by Christian Andersen)
Ribbon representation of an OprM trimer. The protomers are colored in green, red and yellow, respectively. The amino-terminal (N) lipid modification of one protomer is indicated by FA (FA for fatty acid). The OM is schematized via dashed lines.

The 35Å long β-barrel is composed of 12 β-strands, four of each monomer, which arrange antiparallel (S1-4) like the ones of TolC. Interestingly, the β-barrel is not the only membrane anchor of OprM. OprM is a lipoprotein and the fatty acid at its amino-terminus is integrated in the inner leaflet of the OM (Naskajima *et al.*, 2000). The α-barrel is built by two long helices H3 and H7, as well as four short helices H2, H4, H6 and H8, of each monomer in the antiparallel configuration. The barrel is narrowed towards the periplasmic end and equal to TolC, H7/H8 are twisted inwards leading to the complete sealing of the periplasmic entrance of OprM.

However, TolC and OprM have many features in common, there are some differences concerning the two proteins. Besides the structural differences, like the already mentioned

amino-terminal fatty acid of OprM, the compatibility should be mentioned. While OprM transports only xenobiotic substances, TolC is also used for protein export (Andersen, 2003). OprM is the exclusive OM component of MexAB (Poole, 2001), whereas TolC can act as OM component of protein export systems and different drug efflux pumps (Andersen, 2003). The *mexAB/oprM* genes comprise a transcriptional unit in *P. aeuruginosa*. On the other hand, *tolC* belongs to the mar-sox regulon (Aono *et al.*, 1998) and is not part of an export operon.

1.3.4. Assembly and function of the drug efflux pumps

For modeling assemblies of efflux pumps known structures of the components are used. In the case of AcrB and TolC, AcrA is modeled using MexA as template (Figure 1.9), whereas AcrB substitutes MexB in a MexAB/OprM model.

The IM and OM components are trimeric proteins, whereas the oligomeric state of the periplasmic adaptor protein is still not known. In solution the adaptor is monomeric and oligomerization is suggested to be induced by interaction with the IM and/or the OM component. In the literature there exist different models proposing trimers, tri-dimers, nonamers and tri-decamers as oligomeric states of the adaptor protein (Fernandez-Recio *et al.*, 2003; Akama *et al.*, 2004a; Eswaran *et al.*, 2004).

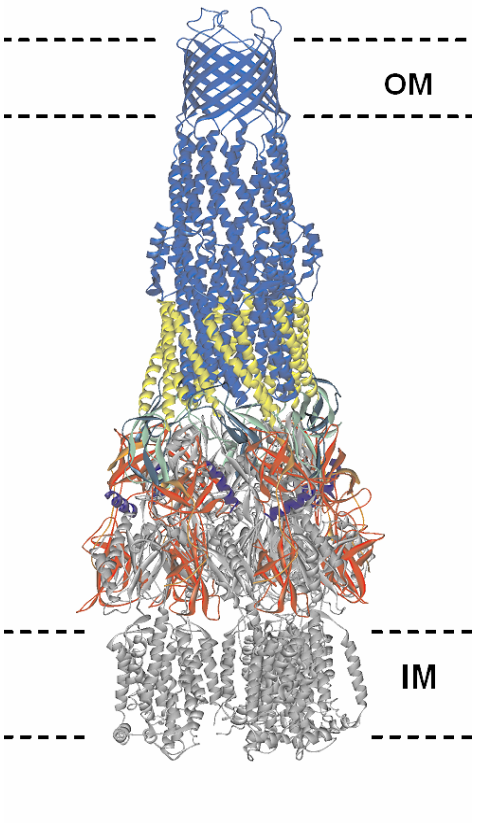


Figure 1.9: Model of the assembled multidrug efflux pump AcrAB/TolC (by Christian Andersen) Side view of AcrAB/TolC. The TolC trimer is colored blue, AcrB grey. AcrA monomers fitted to AcrB-TolC. This model contains six AcrA monomers, whose hairpins are colored yellow and the lipoyl domains green. The β-domain with its single α-helix (blue) is colored red, as well as the carboxy-terminus.

Since TolC could be cross-linked to AcrB showing that their large periplasmic domains are in close proximity (Touze *et al.*, 2004; Tamura *et al.*, 2005), it is obvious that there is direct contact between the channel tunnel and the RND transporter. In contrary, isothermal titration calorimetry detected no interaction between purified AcrB and TolC proteins, suggesting that the contact sites are not of large surface (Touze *et al.*, 2004). This is also supported by an unsuccessful co-purification of MexB and OprM in a MexA deficient strain (Mokhonov *et al.*, 2004). Therefore, the interaction of the IM and the OM component is not sufficient for a stable and functional efflux pump showing the importance of the adaptor protein *in vivo*.

In contrast to previous models assuming that the assembly of the outer membrane component with the inner membrane complex is transient, recent investigations show that the assembly appears to be constitutive (Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004), not depending on energy or substrates. This leads to the question how the assembly occurs resulting in conformational changes of the single components to form a functional efflux machinery. It is obvious that the periplasmic entrances of TolC and OprM have to reach an open state for substrate transport. The inner coiled coils (H7/H8) have to move outwards in an iris like manner to open the tunnel (Koronakis *et al.*, 2000; Akama *et al.*, 2004a). The closed state of the channel tunnels was shown to be very stable due to intra- and intermolecular hydrogen bonds and salt bridges formed by certain residues of the different coiled coils (Andersen *et al.*, 2002a). Interactions of the adaptor proteins, most likely of their hairpins, with the coiled coils of the channel tunnel could mediate the disruption of the hydrogen bonds. Access through the periplasmic entrance of the channel tunnel could be the key event in the function of efflux machineries, after several conformational changes induced after the recognition of substrates by the IM transporters (Eswaran *et al.*, 2004). A second possibility is a permanent open state of the channel tunnel after efflux pump assembly and drug release triggered by opening the pore of the RND transporters. However, the importance of the interaction of the channel tunnel with its dedicated adaptor protein indicates the fact, that MexAB cannot form a functional efflux pump together with TolC in *E. coli* (Tikhonova *et al.*, 2002), whereas on the other hand, OprM is not able to substitute TolC in a TolC-deficient strain. These results are surprising to some extent concerning the homology of the respective proteins.

In this study, the assembly and functionality of efflux pumps consisting of AcrB, TolC or OprM, respectively, and a chimeric adaptor protein were investigated to reveal the importance of the α-helical hairpin.

CHAPTER 2

2. Characterisation of pores formed by YaeT (Omp85) from *Escherichia coli*

2.1. Abstract

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. For *in vivo* function the amino-terminal domain is necessary, whereas for pore formation the carboxy-terminal domain is sufficient. Pores formed by YaeT exhibit a certain variability of conductance indicating a flexible structure. We also demonstrated that YaeT pores attract detergent molecules suggesting a hydrophobic area in the channel interior. The YaeT pore properties support the idea of a lateral channel opening and a release of outer membrane proteins into the bacterial outer membrane.

2.2. Introduction

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 (Nikaido, 1996; Beveridge, 1999). 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 (van den Berg *et al.*, 2004). 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 (Matsuyama *et al.*, 1997; Tokuda and Matsuyama, 2004). The biogenesis of β-barrel proteins is more complex and has been extensively studied for years (for recent reviews see: Bos and Tommassen, 2004; Mogensen and Otzen, 2005; Ruiz *et al.*, 2006). 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 (Schäfer *et al.*, 1999; Bulieris *et al.*, 2003). The periplasmic protease DegP might act under some physiological conditions also as chaperone (Spiess *et al.*, 1999). 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 (Lazar and Kolter, 1996; Dartialongue and Raina, 1998; Nakamoto and Bardwell, 2004). 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 (Andersen *et al.*, 2002c; Danelon *et al.*, 2003). Therefore, OMP insertion *in vivo* must be mediated by a special mechanism.

In 2003, the OMP Omp85 of *Neisseria meningitidis* was characterised to be involved in protein insertion into the OM (Voulhoux *et al.*, 2003). It belongs to a highly conserved family of proteins present in all domains of life, except archaea (Gentle *et al.*, 2004). Depletion of Omp85 in *N. meningitidis* as well as depletion of the orthologous protein YaeT from *E. coli* leads to a defect in outer membrane integrity (Werner and Misra, 2005; Doerrler and Raetz, 2005). 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 (Voulhoux *et al.*, 2003; Werner and Misra, 2005; Doerrler and Raetz, 2005). Recently, it is shown that YaeT forms a multi-protein-complex with three

OM lipoproteins YfgL, YfiO, and NlpB (Wu *et al.*, 2005). 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 (Onufryk *et al.*, 2005).

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*. Reconstituted in planar lipid bilayer membranes, we show that YaeT has pore-forming ability and attracts detergent molecules when inserted. Characterisation 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*.

2.3. Materials and Methods

2.3.1. 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 (Jellen-Ritter and Kern, 2001) and BL21DE3Omp8 (Prilipov *et al.*, 1998) 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 (SmaI/BamHI/NcoI/SacI/XhoI/BsrGI/Bpu1102I). The cassette was constructed of oligonucleotides JS2_up and JS2_down leading to SalI and Bpu1102I overhangs and ligated into a SalI/Bpu1102I digested pET12a vector using T4 DNA ligase resulting in p12JS2 (all oligonucleotides are listed in Table 2.1). 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 BglII/NdeI restriction sites resulting in pARAJS2. *yaeT* was amplified by PCR using YaeT_up and YaeT_down and cloned into SacI/Bpu1102I digested pARAJS2. The resulting pARAJS2yaeT vector was used for expression of N-terminal His-tagged YaeT (HisYaeT). pARAJS2yaeT^{C}

expressing HisYaeT^{C} was constructed using the primer pair YaeTQChalf_up/down for insertion of a stop codon followed by a SacI restriction site by Quick Change PCR into the *yaeT* gene. The N-terminal sequence part was cut off by SacI digestion and the remaining vector was re-ligated. The excised N-terminal *yaeT* fragment was inserted into a SacI/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.

For pARAyaeT *yaeT* was amplified using the primers YaeTHisC_up and YaeT_down and the PCR product was inserted into pARAJS2 via NdeI/Bpu1102I. 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 NdeI/EcoRI 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.*, 2006, submitted; see Chapter 4) after NdeI/BamHI digestion. All expression plasmids were confirmed by sequencing (Seqlab).

Table 2.1: Table of Oligonucleotides.

JS2_up	TCGACGCATCATCACCATCACCATCACCATCACCACGGCGCCGAAGGCCGCCCCGGGATCCATGG AGCTCGAGTGTACAGC
JS2_down	AGTCGACATGTGAGCTCGAGGTACCTAGGGCCCCGCCGGAAGCCGCGGCACCACTACCACCACCA CTACCACTACTACGC
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

2.3.2. 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 5000xg 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 5000xg for 10 min at 4°C. The supernatant was ultra-centrifuged at 170000xg for 1h at 4°C revealing a membrane pellet. $HisYaeT^N$ was purified from the supernatant as described later. For membrane protein extraction the membrane 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 170000xg for 30 min at 4°C. The supernatant was applied to 50µl NiNTA Sepharose resin/100ml cell culture and incubated overnight at 4°C. The resin was washed extensively using 10mM Tris/ 0.5% LDAO/ 100mM imidazole pH 7.5 followed by washing with 10mM Tris/ 0.5% LDAO/ 200mM imidazole pH 7.5. For elution 10mM 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 8M urea/ 100mM NaH_2PO_4/ 10mM Tris pH 8. Extracted membranes were collected by ultra-centrifugation at 170000xg for 30 min at 4°C and the supernatant was applied to NiNTA spin columns (Qiagen). After extensive washing with 8 M urea/ 100mM NaH_2PO_4/ 10mM Tris pH 6.3 and 10mM Tris/ 0.5% LDAO/ 100mM imidazole pH 7.5 the protein was eluted in 10mM Tris/ 0.5% LDAO/ 300mM 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 10mM 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 10mM NaH_2PO_4/ 0.5% LDAO pH 7.4 buffer.

2.3.3. 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 10mM 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 114000xg 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 (Mizuno and Kagayama, 1978).

2.3.4. 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 (Laemmli, 1970). For immunodetection, a tank blot system (Amersham Biosciences) was used as described previously (Towbin *et al.*, 1979). The anti-His and the HRP-linked anti-mouse antibodies were purchased from Amersham Biosciences as well as the ECL Western Blotting Detection Reagents.

2.3.5. 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 (Benz *et al.*, 1978). 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. Multi-channel experiments were performed in 100 mM KCl with stirring to allow equilibration of added substances. The YaeT samples were always added to the trans (negative) site of the Teflon cell, detergent molecules

(sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), dodecylmaltoside (DDM)) to both compartments after the current was stationary.
Zero-current membrane potentials were measured by establishing a 10-fold salt gradient (30mM to 300mM KCl) across membranes containing at least 100 channels (Benz *et al.*, 1979). Detergent molecules were added to both compartments and changes in the measured potential were recorded after they reached a constant value.

2.3.6. *yaeT* knock-out

The chromosomal *yaeT* knock-out in AG100 pNByaeT was performed basically as described before (Datsenko and Wanner, 2000; Wu *et al.*, 2005). 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 10µM IPTG for 4-5 hours before transformation of the knock-out fragment. Correct transformants, with the first 1667 bases of the *yaeT* gene substituted by *kanR*, were selected on LB agar plates containing 1µM 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 pARAyaeT, 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.

2.4. Results

2.4.1. YaeT C-terminus is located in the OM

Topology predictions reveal that Omp85 proteins are composed of two domains of almost equal size (Voulhoux *et al.*, 2003; Gentle *et al.*, 2005). 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. 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.

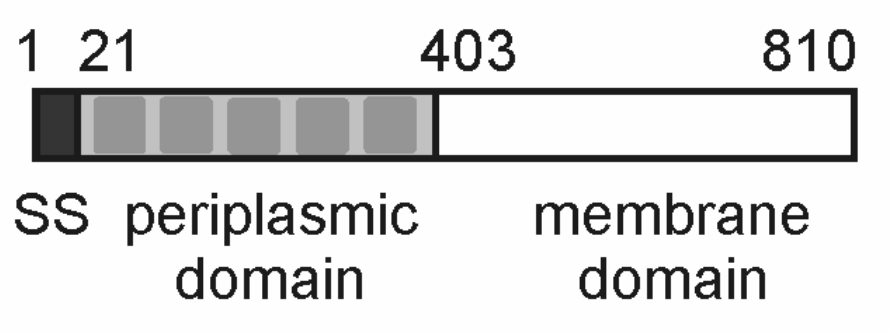


Figure 2.1: Schematic presentation of YaeT. Residues marking the border of the domains are given. The five POTRA domains within the periplasmic domain are indicated by dark grey areas. SS stands for signal sequence.

Membrane fractionation by sucrose-step gradient revealed that HisYaeT and HisYaeT^{C} are localized in the outer membrane (Figure 2.2). 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 (data not shown). The protein is soluble and due to its signal sequence it is most likely located in the periplasm. 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 (Struyve *et al.*, 1991).

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.

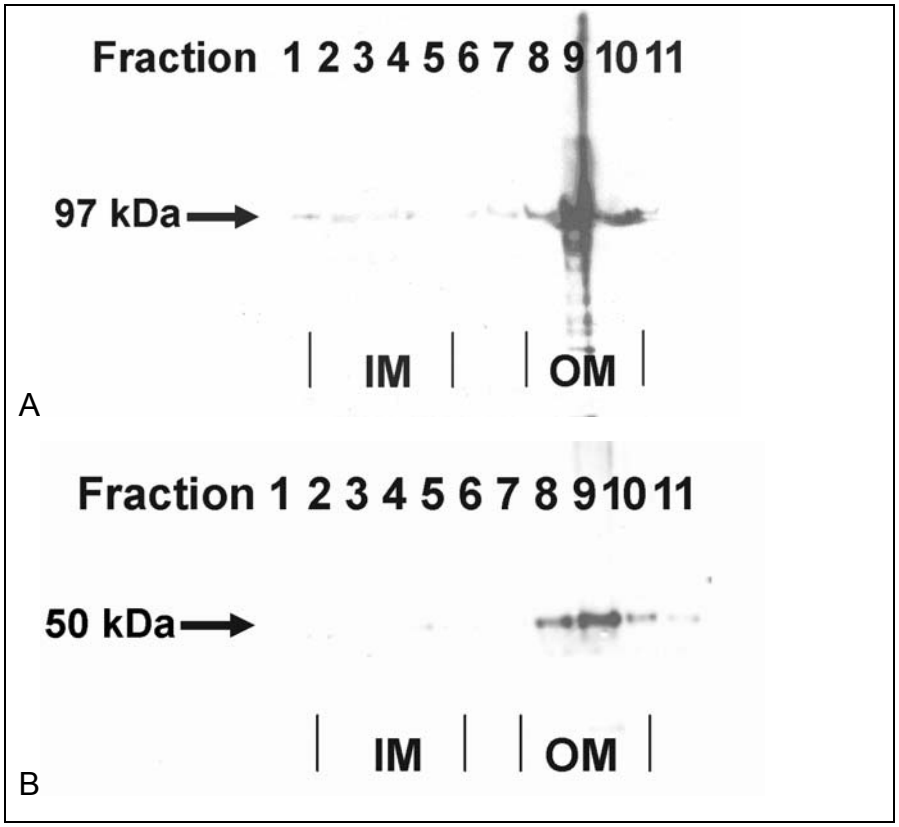


Figure 2.2: Immunoblot of Sucrose-step membrane gradient fractions of cells expressing HisYaeT (A) and HisYaeT^{C} (B). For detection of HisYaeT and HisYaeT^{C} an anti-HIS antibody was used. The inner (IM) and outer membrane (OM) fractions were denoted after NADH-oxidase activity test and detection of characteristic outer membrane proteins

2.4.2. 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 (Figure 2.3). 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} (Figure 2.3, lane 3).

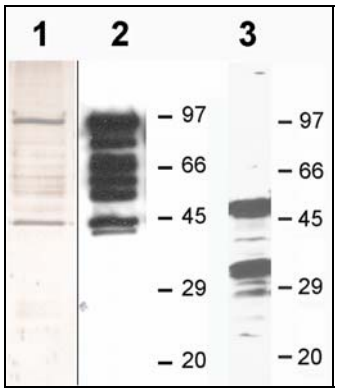


Figure 2.3: HisYaeT (lane 1 and 2) and HisYaeT^{C} (lane 3) after purification using NiNTA sepharose. Lane 1: Detection of HisYaeT via silver nitrate staining. Lane 2 and 3: Immunoblot using an anti-His antibody. Positions of molecular mass markers (in kDa) are shown on the right. All samples were boiled for 10 min before SDS-PAGE.

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 (Figure 2.4A,B). This heat-modifiable mobility in SDS-PAGE is characteristic for β-barrel OMPs (Beher *et al.*, 1980; Arcidiacono *et al.*, 2002). In contrast, the amino-terminal half of YaeT showed no heat-modifiable mobility (Figure 2.4C).

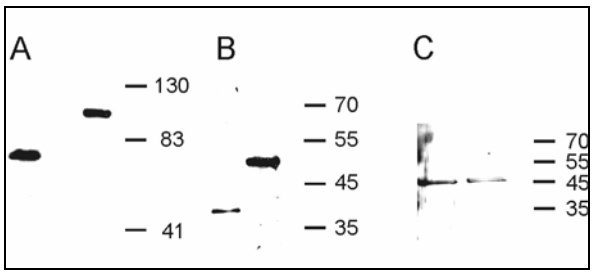


Figure 2.4: Mobility of purified HisYaeT (A), HisYaeT^{C} (B) after extraction and NiNTA purified HisYaeT^{N} (C) on SDS poly-acrylamid gels after incubation at room temperature (left lane) and 100°C (right lane). The proteins were blotted to a nitrocellulose membrane and detected using an anti-His antibody. Positions of molecular mass markers (in kDa) are shown on the right.

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.

2.4.3. YaeT forms pores in planar lipid bilayer membranes

For reconstitution in black lipid bilayer HisYaeT and HisYaeT^{C} were further purified by gel elution. Protein samples obtained after NiNTA affinity chromatography were loaded unboiled on a SDS-PAGE and a 75 kDa (HisYaeT) and 37 kDa (HisYaeT^{C}) band, respectively

corresponding to the undigested forms of the proteins were cut out. Protein were pure, devoid of protease digestion and retained heat-modifiable mobility assuming that purification has no influence on protein conformation (Figure 2.4A,B).

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 (Figure 2.5A-C). Thereby, most insertion events lead to a significant increase of the conductance fluctuation. Measured in different KCl concentrations, the size of the steps was not homogeneous varying from, e.g. 20 to 100 pS in 100 mM (Figure 2.6A). However, for every KCl concentration a most frequent observed single channel conductance could be detected (Table 2.2). Variation of the purification protocol, potential, pH, ions, or ionic strength, or addition of divalent cations failed to reveal more homogeneous single channel conductance. 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. This indicates that the YaeT pores are cation selective. Another important feature of the pores is 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.

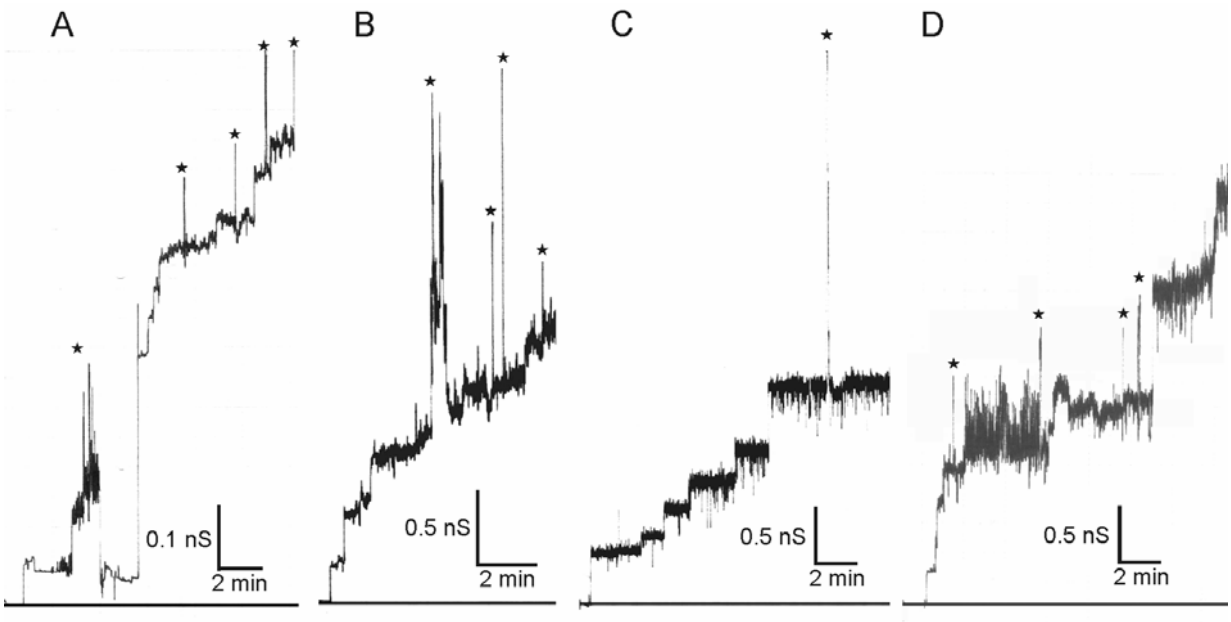


Figure 2.5: Single channel recordings of diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 5 ng/ml HisYaeT (A-C) or HisYaeT^C (D) protein. The aqueous phase contained 100 mM (A) and 1 M KCl (B-D), respectively. The applied voltage was 20 mV. Note that YaeT channels can adopt variable high conductance states of different lifetime (marked by asterisks).

Rarely, we observed very large conductance steps of, e.g. over 450 pS in 100 mM KCl. Most of these highly variable conductance steps had a very short lifetime (Figure 2.5A-C) 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 (8M 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).

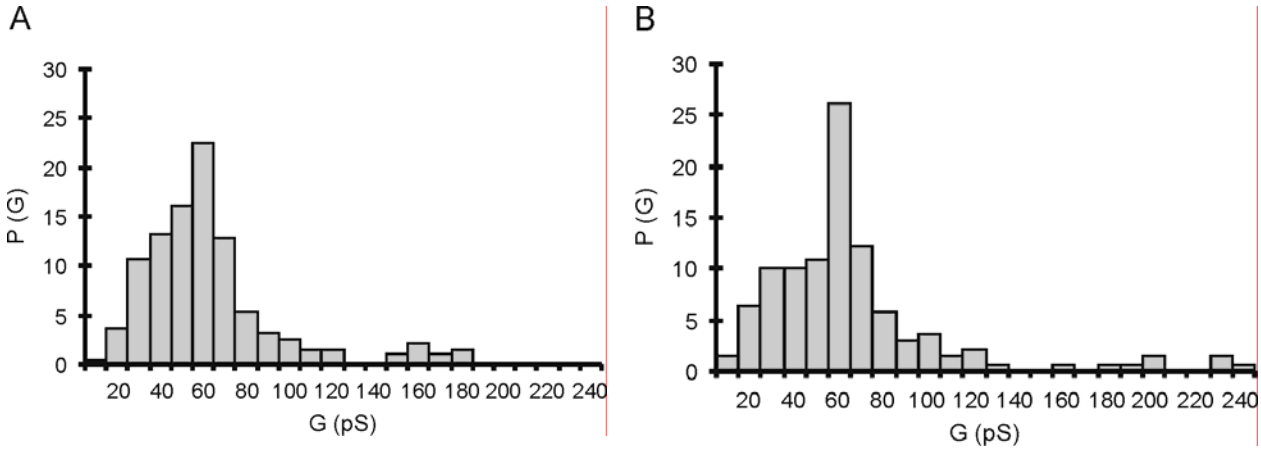


Figure 2.6: Histogram of the conductance steps observed with diphytanoyl phosphatidylcholine/*n*-decane membranes in the presence of 5 ng/ml HisYaeT (A) and HisYaeT^{C} (B) protein. The single channel conductance varied in a range between 20 and 80 pS with a main peak observed at 60 pS. P (G) is the probability that a given conductance increment G is observed in the single channel experiments. The aqueous phase contained 100mM KCl, the applied voltage was 20 mV.

Reconstitution of HisYaeT^{C} in lipid membranes results in the similar conductance steps and shows similar characteristics as observed for full length HisYaeT (Figure 2.5D, 2.6B), with the exception that very high conductance steps were recorded less frequently. In contrast, HisYaeT^{N} had no pore forming activity in black lipid bilayer membranes (data not shown). Taken together, we show that YaeT forms cation selective pores with variable conductance states. The pore forming ability resides clearly in the carboxy-terminal half confirming that the membrane domain functions independent of the periplasmic domain.

Table 2.2: Single channel conductance of HisYaeT in different electrolytes and electrolyte concentrations

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

2.4.4. Detergents change conductance and ion selectivity of YaeT pores

By chance, we observed that YaeT interacts with detergent molecules in a way that the electrophysiological properties of the pores changed. Purified HisYaeT protein was added to the trans (negative) compartment of the Teflon cell and after the conductance reached a constant level, detergents were added. Interestingly, we detected a rapid increase of the conductance after addition of SDS (Figure 2.7A). To exclude that the increase originates from newly inserted YaeT pores, we investigated the phenomenon with only a few channels inserted. After addition of SDS the conductance increased continuously and not stepwise as one would expect for insertion of new channels (Figure 2.8). This lets us conclude that SDS interacts with the inserted pores resulting in an increased conductance. It should be mentioned, that the effect was independent on the side of detergent addition, which means that detergent molecules can enter the pore from both sides. Further experiments were performed adding detergent molecules to both compartments.

Additional experiments support, that detergent molecules could influence conductance of YaeT. When cetyltrimethylammoniumbromid (CTAB) was added, the conductance decreased (Figure 2.7B). The decrease was not as rapid as the increase observed by addition of SDS. This can be explained by the much lower concentration of CTAB compared to SDS. Higher CTAB concentration could not be used because of membrane breakdown. Almost no effect had the addition of dodecyl-maltoside (DDM) (Figure 2.7C). Control experiments with *E. coli* porins OmpF and LamB showed that this behaviour is specific to YaeT. We exclude also that destabilization of the protein induce the effects because of the very low detergent concentrations used in the experiments and the specific reactions observed for different detergents. An experienced specialist also confirmed that this characteristic was not described

before for other pore forming proteins (R. Benz, personal communication). It is normal that detergent molecules bind to outer membrane proteins. However, this binding is generally restricted to the hydrophobic outside of the barrel. In the case of YaeT one has to claim that interactions of the detergent molecules occur inside the channel, otherwise the effects would not have been observed. It should be mentioned that YaeT pores are reconstituted in the lipid membranes with detergent molecules already present. Therefore, a potential detergent binding site might be occupied already by LDAO molecules and the effects of the second detergent needs to be discussed as competition between the two detergents for the binding site.

The fact that the tested detergents influence YaeT conductance differently, could be explained by the charge of the polar head group of the detergent molecules. The head group of SDS is negatively charged, whereas that of DDM and CTAB is neutral and positively charged, respectively. As mentioned above, YaeT is a cation selective channel, which means that negatively charged residues line the aqueous pathway through the pore. Interaction of SDS with the inside of the YaeT channel leads to additional negative charges, which attracts more cations resulting in a higher channel conductance. In the case of interaction with CTAB the negative net charge is reduced, which explains the decrease of the conductance. The neutral DDM has no influence of the charges making clear why it has almost no effect on conductance.

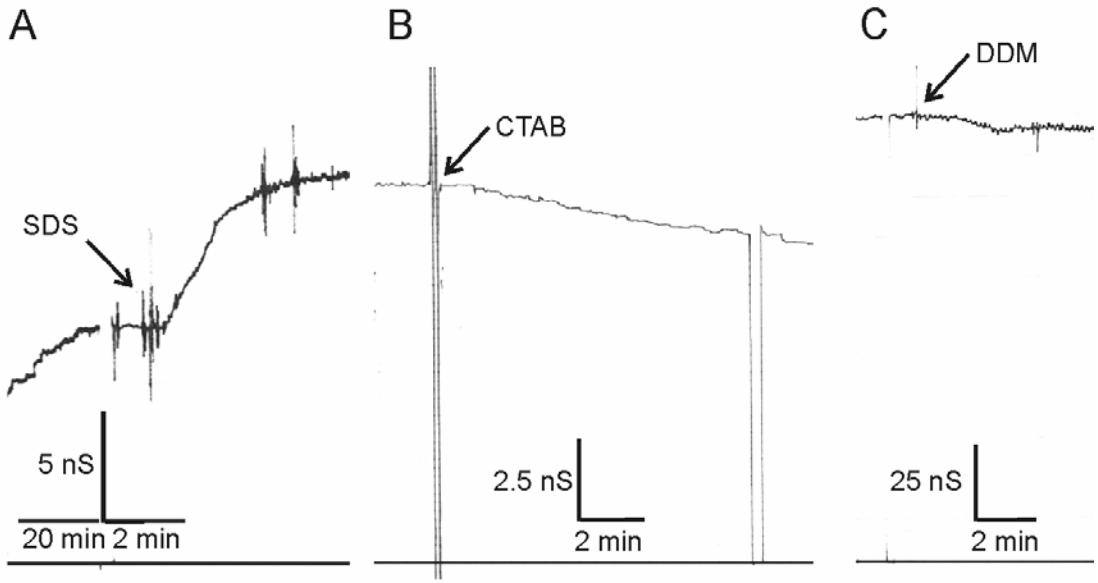


Figure 2.7: Influence of detergents on membrane conductance induced by HisYaeT. The membrane was formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase on both sides of the membrane contained 100 mM KCl. 2-5µl protein sample (5 ng/ml) were added to the trans site. A stable conductance was reached with approximately 200 (A), 200 (B), and 2700 (C) inserted HisYaeT channels. 0.7µM SDS (A), 0.01µM CTAB (B), and 0.4µM DDM (C) were added to both sides of the membrane. A relative conductance increase of about 60% (A) and decreases of 12% (B) and 3% (C) were recorded. The applied voltage was 20 mV.

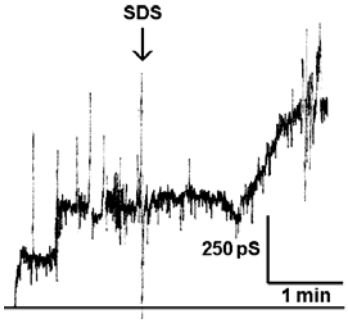


Figure 2.8: Influence of detergents on membrane conductance induced by HisYaeT on single channel level. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase on both sides of the membrane contained 100 mM KCl. 0.1 µl protein sample (5 ng/ml) were added to the trans site. About six single HisYaeT pores were reconstituted before 0.7µM SDS were added to both sides of the membrane. After a short lag time conductance increased steadily and came to a standstill after a relative increase of about 80%. The applied voltage was 20 mV.

To prove this explanation, we determined the ion selectivity of HisYaeT pores by applying an ion gradient across the membrane. Under zero current conditions a 10-fold KCl gradient revealed 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 pK/pCl of 5.7. Addition of SDS to both compartments increased the potential to 40.5 ± 3.5 mV (pK/pCl 9.2) indicating higher cation selectivity. As expected, CTAB had the opposite effect and caused a decrease of the potential to 29.0 ± 5.7 mV (pK/pCl 4.3). DDM had only a minor effect (32.3 ±7.6 mV, pK/pCl 5,3). The same effects concerning changes of conductance and cation selectivity could be observed for HisYaeT^{C} (data not shown).

Summarized, we show that YaeT interacts with detergent molecules influencing cation selectivity, which results in changes of the conductance. Hereby, the charge of the head group is the major determining factor. As far as we know, YaeT is the first channel forming protein, for which such interaction with detergent molecules is reported.

2.4.5. 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 (Wu *et al.*, 2005). 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 pARAJS2yaeT^{C} encoding HisYaeT^{C}. After 40 hours 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 (Werner and Misra, 2005; Doerrler and Raetz, 2005). 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 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.

2.5. 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 (Gentle *et al.*, 2005; Ruiz *et al.*, 2006). 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 (Wu *et al.*, 2005). 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. Omp85 proteins are predicted to consist of two domains, an amino-terminal periplasmic domain and a carboxy-terminal membrane domain (Voulhoux *et al.*, 2003). We confirm that the carboxy-terminal half anchors the protein in the OM.
The amino-terminal half can be expressed separately as a stable, protease resistant, soluble protein in the periplasm. 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* (Beher *et al.*, 1980; Arcidiacono *et al.*, 2002). 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 (Guedin *et al.*, 2000). 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 (Wimley, 2002). 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 (Wu *et al.*, 2005). 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. This experiment also excludes that the temporarily observed high conductance states originate from a gating-like process with the periplasmic domain acting as a plug. 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 (Bos and Tommassen, 2004; Gentle *et al.*, 2005). The bacterial Omp85 proteins belong to a major superfamily separated in six clusters (Yen *et al.*, 2002). In parts, sequence similarity is very low and is restricted partially to short regions close to the carboxy-terminal end (Moslavac *et al.*, 2005). 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 (Könninger *et al.*, 1999; Jacob-Dubuisson *et al.*, 1999). 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 (Paschen *et al.*, 2003). 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 (Hinnah *et al.*, 1997; Tu *et al.*, 2004; Ertel *et al.*, 2005). Interestingly, for almost all Omp85 homologous pore forming proteins transitions into higher conductance states are reported (Hinnah *et al.*, 1997; Könninger *et al.*, 1999; Jacob-Dubuisson *et al.*, 1999; Ertel *et al.*, 2005), which suggests that this is a general feature of this protein superfamily. It should be mentioned that in contrast to YaeT, 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 pore properties (Ertel *et al.*, 2005; Meli *et al.*, 2006).

Compared to well characterised bacterial outer membrane porins, YaeT pores behave differently. The conductance steps observed with reconstituted porins like OmpF, PhoE, LamB, etc. are uniform differing only about 10-20 % (Benz and Bauer, 1988). Higher variability of single channel conductance is only seen with porin mutants, having major structural elements deleted (Andersen *et al.*, 1999; Srikumar *et al.*, 1997). 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. However, this model makes a lateral opening towards the membrane plane necessary. The membrane domain is predicted to consist of 12 amphipatic β-strands (Voulhoux *et al.*, 2003). We believe 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 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, 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. OMPs consisting between 8 and 22 β-strands are found in the OM of Gram-negative bacteria. The hydrophilic interior of the barrel might provide the pathway for extracellular loops of OMPs bypassing thus an energetically highly unfavourable direct transfer through the lipid phase.

We observed that the YaeT channel interior has a high affinity to detergents. This means that there exist hydrophobic sites, which attract detergent molecules. This is in accordance with the function of YaeT, namely mediating transfer of OMPs into the OM. The outside of the β-barrels is covered by hydrophobic residues, which might bind transiently to the hydrophobic region inside the YaeT channel before released into the membrane.

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 (Sanchez-Pulido *et al.*, 2003; Gentle *et al.*, 2005). 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 (Yang and Braun, 2000), whereas the extramembraneous domain of Omp85 homologues found in chloroplasts, functions as a substrate recognition and complex assembly unit (Ertel *et al.*, 2005). 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 (Demchick and Koch, 1996; Pink *et al.*, 2000). 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.

CHAPTER 3

3. Characterisation of YtfM, a second member of the Omp85 family in *E. coli*

3.1. Abstract

The family of Omp85 proteins is an ubiquitous protein family found in all Gram-negative bacteria. Omp85 of *Neisseria meningitides* and YaeT of *Escherichia coli* are shown to be essential for outer membrane biogenesis. Interestingly, there exists a homologue to YaeT in *E. coli* and many proteobacteria, denoted YtfM, which function is not described yet. Like YaeT YtfM is predicted to consist of an amino-terminal periplasmic domain and a membrane located carboxy-terminal domain. In this study, we present a first characterisation of YtfM by comparison to YaeT concerning structural, biochemical and electrophysiological properties. Furthermore, a knock-out strain revealed that *ytfM* is a non-essential gene and lack of the protein had no effect on outer membrane composition and integrity. The only phenotype was a strongly reduced growth indicating an important role of YtfM *in vivo*.

3.2. Introduction

In recent times, the family of Omp85 proteins came into the focus of researchers investigating biogenesis of outer membranes of Gram-negative bacteria or organelles in eukaryotes (Gentle *et al.*, 2004). Omp85 of *N. meningitidis* was the first member, for which the role in membrane insertion of outer membrane proteins was described (Voulhoux *et al.*, 2003). This function could be confirmed by studies of the close homologue YaeT of *E. coli* (Werner and Misra, 2005; Doerrler and Raetz, 2005). The presence of Omp85 homologues in all Gram-negative

bacteria shows the importance of this protein family and the failure to knock-out the genes without complementation shows that they are essential (Werner and Misra, 2005; Doerrler and Raetz, 2005). Omp85 and YaeT belong to one out of six clusters, which were characterised by bioinformatical analysis (Yen *et al.*, 2000). Two clusters comprise eukaryotic proteins located in the outer membrane of mitochondria or chloroplasts and cyanobacterial proteins (Paschen *et al.*, 2003; Reumann *et al.*, 1999; Ertel *et al.*, 2005; Schleiff and Soll, 2005). For the mitochondrial Omp85 homologues a similar function is described as for the bacterial YaeT homologous proteins (Kozjak *et al.*, 2003; Paschen *et al.*, 2003), whereas homologues in chloroplast and cyanobacteria do not only transport proteins into but also across the membrane (Hinnah *et al.*, 1997; Tu *et al.*, 2004). The remaining four clusters comprise exclusively bacterial proteins. One of those four is a small cluster with exclusively Chlamydial proteins. The other three clusters include proteins found in almost all proteobacteria. Beside the already described cluster of YaeT homologous proteins, one cluster represents the outer membrane translocation component of two partner secretion systems (TPS) exemplified by the FhaC of *Bordetella pertussis* and ShlB of *Serratia marcescens* (Jacob-Dubuisson *et al.*, 1999; Könninger *et al.*, 1999). These outer membrane components mediate translocation of large proteins across the outer membrane. The protein studied in this work, YtfM, belongs to the last cluster, for which no function is described yet.

In our study, we represent an initial characterisation of YtfM in comparison to its homologue in *E. coli*, YaeT. First of all, the sequence homology as well as the proposed structural similarity were carefully investigated. Since, representatives of other clusters form pores, we tested YtfM for pore forming activity. Furthermore, an *ytfM* knock-out strain was constructed to get insight into the relevance of YtfM *in vivo*, revealing *ytfM* as non-essential but affecting growth when deleted.

3.3. Materials and Methods

3.3.1. 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 (Jellen-Ritter and Kern, 2001) and BL21DE3Omp8 (Prilipov *et al.*, 1998) 100µg/ml ampicillin, 40µg/ml kanamycin and 25µg/ml chloramphenicol were used for selection.

ytfM was amplified by PCR using YtfM_up and YtfM_down (for all oligonucleotides see Table 1) and cloned into pARAJS2 (Stegmeier and Andersen, 2006, submitted) after digestion with NcoI/Bsp1407I. The resulting pARAJS2ytfM vector was used for expression of N-terminal His-tagged YtfM (HisYtfM). pARAJS2YtfM^C (HisYtfM^C) was constructed using the primers YtfMCterm_up/YtfM_down for cloning the C-terminal *ytfM* into a SacI/Bsp1407I digested pARAJS2. The ligation products were introduced into TOF10F´ and the correct plasmid recombinants were determined by colony PCR using the primer pair pBADseq_up and pETseq_down.

For pARAytfM *ytfM* was amplified using the primers YtfMNdeI_up and YtfM_down and the PCR product was inserted into pARAJS2 via NdeI/Bsp1407. For the construction of pNBytfM *ytfM* was cut off pARAytfM using NdeI/Bsp1407 and inserted in a likewise prepared pNB2 (Stegmeier *et al.*, 2006, submitted) vector.

3.3.2. Protein expression and purification

For protein expression the respective expression plasmids were transformed in the porin deficient BL21DE3Omp8 *E. coli* strain (Prilipov *et al.*, 1998). 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 switched to room temperature (RT) and after 30min of adaption expression was induced by adding 0.02% arabinose. After 4-5h cells were collected by centrifugation at 5000xg for 10 min at 4°C. The pellet was resuspended in 10mM Tris pH7.5 and a protease inhibitor cocktail (Calbiochem) was added before disrupting the cells by a French pressure cell. Cell debris was collected by centrifugation at 5000xg for 10min at 4°C and then the supernatant was ultra-centrifuged at 170000xg for 1h at 4°C. For membrane protein extraction the membrane pellet was resuspended in 10mM Tris/ 0.5% SDS/ 1mM EDTA pH7.5 and protease inhibitor cocktail was added before incubating the suspension 30 min RT followed by ultra-centrifugation at 170000xg for 30 min at 4°C. The supernatant was applied to 50µl NiNTA Sepharose resin/100ml cell culture after adding 10mM $MgSO_4$ and incubated overnight at 4°C. For HisYtfM (HisYtfM^C), the resin was washed 8x using 10mM Tris/ 0.5% DDM/ 100mM imidazole pH7.5 followed by twice washing with 10mM Tris/ 0.5% DDM/ 200mM imidazole pH7.5. For elution 10mM Tris/ 0.5% DDM/ 600 mM imidazole pH7.5 was used. Buffers for HisYaeT (HisYaeT^C) purification contained 0.5% LDAO instead of 0.5% DDM.

For structural analysis and electrophysiological experiments HisYaeT, HisYtfM, HisYaeT^C and HisYtfM^C eluates were loaded on a 10% SDS gel and the band of the desired protein was

cut off and eluted in 10mM Tris/ 0.5% LDAO pH7.5 overnight at 4°C in the case of HisYaeT/ HisYaeT^C and 10mM Tris/ 0.5% DDM pH7.5 for HisYtfM/ HisYtfM^C. The eluates were filled into an Amicon Ultra-15 30000MWCO (Millipore) filter device and washed intensively with 10mM $NaPO_4$ pH 7.4 containing 0.2 % LDAO or DDM, respectively.

Table 3.1: Stains, Plasmids and Oligonucleotides

STRAIN	**CHARACTERISATION**	**REFERENCE**
Top10F`		Invitrogen
BL21DE3Omp8	porin deficient	Prilipov *et al.*, 1998
AG100		Jellen-Ritter and Kern, 2001
AG100ΔytfM	*‚ΔytfM::kan*	This study
PLASMID	**CHARACTERISATION**	**REFERENCE**
pARAJS2	*ara*BAD promoter, His^{10} tag, Amp^R	Stegmeier and Andersen, 2006, submitted
pARAJS2ytfM		This study
pARAJS2ytfM^C		This study
pARAytfM		This study
pNB2	*lacI*^{q}, tac promoter, Cm^R	Stegmeier *et al.*, 2006, submitted
pNBytfM		This study
pARAJS2yaeT		Stegmeier and Andersen, 2006, submitted
pARAJS2yaeT^C		Stegmeier and Andersen, 2006, submitted
pKD4		Datsenko and Wanner, 2000
pKD46		Datsenko and Wanner, 2000
OLIGONUCLEOTIDES		
YtfM_up	GCTTACTCTGCTTAAGCGGATCTGCCCATGGCGCGAACGTCC	
YtfM_down	CGCCGAGGCTGATTTTTGTACATAAACTCATAATTCTGG	
YtfM_NdeI_up	GGATATTCAGGAGAACATATGCGCTATATCCGACAG	
YtfMCterm_up	CGATGAGTACGAATCGAGAGCTCTGGCAGAACTG	
YtfM-KO-up	CGGCGTTCCAGGGGCAGGAAAAAAGGATATTCAGGAGAAAATATGGAGAAAAAAATCACTGG	
YtfM-KO-down	GTCGAAATCACCGGTTTCAATCCAGCCCAGCGTGCCGCGTGCGAACCCCAGAGTCCCGCTCA	
YtfM-FR_up	GCGGGGGAAACCAGATGC	
YtfM-FR_down	CAAGCCCGACAGCCAGATG	
pBADseq_up	CCTGACGCTTTTTATCGC	
pETseq_down	CCTCAAGACCCGTTTAGAG	

3.3.3. CD and FTIR spectroscopy

Protein samples used for structural analysis had a concentration of 3-20 mg/ml in 10mM $NaPO_4$ pH 7.4/ 0.2% LDAO for YaeT and 10mM $NaPO_4$ pH 7.4/ 0.2% DDM for YtfM. A

JASCO J 720 spectropolarimeter was used to record the CD spectra in range of 190-260 nm. The spectral band width used was 1 nm, response time of 1 sec with standard sensitivity. Before the measurement of the sample spectra, the spectra of air and buffer were also recorded. Each spectrum was recorded by accumulating 4 scans with a speed of 50 nm per minute. All data were analyzed using several spectrum analysis programs (Whitmore and Wallace, 2004). IR spectra were measured with a Bruker VECTOR 22 FTIR spectrometer equipped with a MCT detector (Mercury Cadmium Telluride). The detector was cooled using liquid nitrogen. The background spectra recorded was air (no protein on the diamond). The protein was then spread on the crystal and was allowed to dry for few a minutes before measuring the spectrum. 50 interferograms were averaged, apodized with a Blackman-Harris-4-term function, zero-filled and Fourier-transformed to yield a nominal spectral resolution of 2 cm^{-1} with an encoding interval of 1 cm^{-1}. The spectra were processed and visualized using windows OPUS version 3.1 software.

3.3.4. Sucrose-step gradient and NADH-oxidase activity test

For the sucrose-step gradient experiment cell membrane pellets were prepared of TOP10F´ cells containing the expression plasmids in the same manner as described previously. Membrane pellets of 200ml initial culture were carefully homogenized in 1ml 10mM Tris pH7.5 and loaded on a sucrose-step gradient containing 3ml 30%/ 5ml 50%/ 2ml 70% sucrose. The gradient was centrifuged at 114000xg at 4°C for 16h using a Beckmann SW40Ti rotor. Afterwards fractions of 1ml were collected and investigated by immunoblot. The fractions containing the inner respectively outer bacterial 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 by Mizuno and Kageyama (1978).

3.3.5. SDS-PAGE and western blotting

Prior to electrophoresis protein or whole cell samples were mixed with sample loading buffer and if necessary incubated for 10 min at 100°C. SDS-PAGE was performed according to the Laemmli gel system (Laemmli, 1970). For Western blots, a tank blot system (Amersham Biosciences) was used as described previously (Towbin *et al.*, 1979). The anti-His and the HRP-linked anti-mouse antibodies were purchased from Amersham Biosciences as well as the ECL Western Blotting Detection Reagents.

3.3.6. Electrophysiological experiments

YtfM protein samples were stored in 10mM $NaPO_4$/ 1% DDM pH 7.4 and stored at 4°C before used in electrophysiological experiments. The methods used for black lipid bilayer experiments have been described previously (Benz *et al.*, 1978). The experimental setup consisted of a Teflon cell with two water-filled compartments 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 1-10mM Tris pH 7.5 depending on the salt concentration. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) 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 20mV. Multi-channel experiments were performed in 100mM KCl with stirring to allow equilibration of added substances. The YtfM samples were always added to the trans (negative) site of the Teflon cell, 1M citrate to both compartments after the current was stationary. After citrate addition samples were taken for pH measurement.

Zero-current membrane potentials were measured by establishing a 5-fold salt gradient (100mM to 500mM KCl) across black lipid bilayer membranes containing at least 100 channels (Benz *et al.*, 1979).

3.3.7. *ytfM* knock-out

The chromosomal *ytfM* knock-out in AG100 pNBytfM was performed as described for *yaeT* (Datsenko and Wanner, 2000; Stegmeier and Andersen, 2006, submitted). An YtfM-KO_up primer, an YtfM-KO_down primer and pKD4 as template were used for PCR to produce an *ytfM* knock-out fragment. The knock-out fragment contained solely the two *ytfM* flanking regions and the kanamycin resistance gene (kan^R) leading to a substitution of the first 1339 bases of the chromosomal *ytfM*. AG100 pNBytfM pKD46 was grown at 30°C in LB medium supplemented with 0.2% arabinose and 1µM IPTG for 4-5 hours before transformation of the knock–out fragment. After transformation the cells were immediately added to LB medium and left at room temoerature (RT) overnight. Correct transformants were selected on LB agar

plates supplemented with 1µM IPTG at RT. The loss of *ytfM* was verified by colony-PCR using primers that bind up- and downstream of *ytfM*: YtfM-FR_up and YtfM-FR_down. The resulting strain was denoted AG100ΔytfM.

To allow the cells to forfeit the pNBytfM plasmid cultures and subcultures were grown for 40h at 37°C with kanamycin as selection antibiotic. After the cells were streaked out on LB agar plates containing kanamycin, single colonies were checked for the loss of chloramphenicol resistance by transferring on LB agar plates containing chloramphenicol. Chloramphenicol sensitive colonies were used for colony PCR to verify the result.

3.3.8. Growth experiments

Overnight cultures of AG100 pARAJS2, AG100ΔytfM and AG100ΔytfM pARAytfM grown under selective conditions and 30nM arabinose for AG100ΔytfM pARAytfM were diluted 1:10, grown for further 3h, and then used for the growth curve. The cultures were diluted to an initial number of $2x10^7$ cells/ml into 50ml fresh LB (for AG100ΔytfM pARAytfM supplemented with 30nM arabinose) and cultivated at 37°C in baffle and round-bottomed flasks. The OD_{600} was measured every hour. For electron microscopy and SDS-PAGE, bacteria samples were taken from cultures in exponential phase.

3.3.9. Electron microscopy

Pelleted bacteria were fixed with 2.5% buffered glutardialdehyde followed by a fixation with 2 % osmiumtetroxid according to standard procedures (Prüfert *et al.*, 2004). Ultrathin sections of Epon 812 embedded specimens were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Zeiss EM10 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

3.3.10. Mobility test

After adjusting all cell cultures to 1×10^9 cells/ml, 2µl of culture were dropped in the centre of a Petri disc containing 20 ml 0.3% LB agar and incubated at 37°C for 18h. The halos formed by mobile cells were documented with a digital camera. For verification of growth whole Petri disc contents were melted and OD_{600} was checked.

3.3.11. Bioinformatics

Using the text search tool of the Protein Information Resource (PIR, http://pir.georgetown.edu/pirwww/search/textsearch.shtml) searching in the iProClass database for the PFAM IDs PFAM07244 and PFAM01103, and for the text strings YaeT and YtfM revealed together 447 hits. By sequence alignment about 100 proteins were identified, which belong to the closest homologues to the *E. coli* YtfM. A search for PFAM ID PF04357 revealed 122 proteins, which belong to the family of YtfN proteins. A search for YtfP and PFAM ID PF03486 revealed 282 proteins. Sequence aligment was performed with Multalin program from Poole bioinformatique Lyonnaise PBIL (http://npsa-pbil.ibcp.fr) (Corpet., 1988).

3.4. Results

3.4.1. Sequence homology of YaeT and YtfM

YtfM is beside YaeT the second member of the Omp85 superfamily found in *Escherichia coli*. A databank search revealed about 100 YtfM homologous proteins, which can be separated in subfamilies according to their phylogenetic origin (see Appendix 3.6). Sequence alignment of the YtfM family with selected representatives of YaeT homologous proteins revealed that both protein families could be well distinguished by the length of the amino-terminal end. Proteins of the YaeT family have around 760 - 840 residues, YtfM homologous proteins are about 150 residues shorter. Repeating POTRA (polypeptide-transport-associated) subdomains were identified at the amino-terminus in both families (Sanchez-Pulido *et al.*, 2003). In the case of YaeT homologues there are five, whereas YtfM homologous proteins comprise just three POTRA repeats. The sequence alignment shows highly conserved regions within the POTRA subdomains, which makes a proper alignment of the amino-terminal domains possible. Following the POTRA domains there is a region of about 100 residues with no similarity between YtfM and YaeT families. Moreover, even between the individual YtfM subfamilies the variability is significant. In contrast, the carboxy-terminal end following this variable region is highly conserved in YaeT and YtfM families (Figure 3.1).

Figure 3.1: Schematic presentation of an alignment of representatives of the YaeT family with three YtfM subfamilies deriving form α-, β-, and γ-proteobacteria. Gaps in the light grey bar represent gaps in the alignment. Dark grey areas represent conserved regions. The black bars mark the five POTRA subdomains found in the YaeT family.

3.4.2. Localization and purification of YtfM

For YaeT we could show that amino-terminal half comprising the five POTRA subdomains is located in the periplasmic space whereas the carboxy-terminal half is integrated in the outer membrane (Stegmeier and Andersen, 2006, submitted). Membrane fractionation by sucrose-step gradient revealed that YtfM is also an outer membrane protein. The YtfM mutant YtfM^C lacking the amino-terminal POTRA repeats could also be located in the outer membrane (data not shown), which shows that the carboxy-terminal 325 amino acids anchors YtfM in the outer membrane independent on the amino-terminal POTRA repeat domain.

Amino-terminal His-tagged versions of YtfM and YtfM^C (HisYtfM and HisYtfM^C) were expressed in *E. coli* and extracted by detergent from cell envelopes obtained by French pressure of cells. Proteins were purified by Ni-NTA affinity chromatography and analysed by SDS-PAGE for heat-modifiable mobility, which is characteristic for outer membrane proteins (Beher *et al.*, 1980). Interestingly, for boiled and non-boiled samples of HisYtfM (Figure 3.2) as well as HisYtfM^C (data not shown) no distinct mobilities were observed.

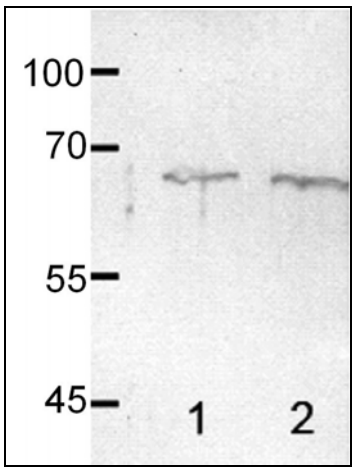


Figure 2: HisYtfM after purification using NiNTA sepharose detected via silver nitrate staining. Protein samples on a SDS polyacrylamid gel after incubation at 100°C for 10min (lane 1) or room temperature (lane 2). Positions of molecular mass markers (in kDa) are shown on the left.

3.4.3. Structure comparison of YaeT and YtfM

By two independent methods, CD and FTIR-spectroscopy, we have evaluated roughly the portion of secondary structure for YtfM, YaeT and of mutants of both proteins, $YtfM^C$ and $YaeT^C$, lacking the POTRA domains except the last 20-30 amino acids of the last domain (Table 3.2). The secondary structure composition of both full length proteins shows that the β-sheet content of YaeT is a little higher than that of YtfM (Table 3.2 and Figure 3.3). Interestingly, both methods reveal a clear difference between the carboxy-terminal domains of YaeT and YtfM. In the case of $YaeT^C$ the β-sheet content is about 45-55%, which can be seen by the typical minimum at 218 nm in the CD spectra and the dominant peak at 1630 cm^{-1} in the FTIR spectra (Figure 3.3;3.4; Jackson and Mantsch, 1995). This is in accordance with models of Omp85 suggesting that the membrane domain is composed of 12 or 16 β-sheets, respectively (Voulhoux *et al.*, 2003; Ertel *et al.*, 2005). For $YtfM^C$ the spectra reveal that the secondary structure composition is not changed compared to the full length protein. This means that the β-strand content of $YtfM^C$ is about half of that of $YaeT^C$ and suggests that the folding of the carboxy-terminal domain of YtfM is different to YaeT. For the α-helical contents one should notice that the given ranges may differ to some extent from the effective values. The region for α-helical structures in FTIR spectra (around 1652-1660) and CD (205-210 nm) are in close proximity to regions which indicate random structures and may cause some errors during evaluation.

Table 3.2: Composition of secondary structure of YtfM, YaeT, $YtfM^C$ and $YaeT^C$ analysed by CD and FTIR spectroscopy. Ranges are given, which cover the output of both methods.

Protein	Residues	% alpha	% beta	% random
HisYaeT	812	15-20	25-35	45-60
HisYtfM	578	15-25	20-25	50-65
$HisYaeT^C$	430	10-15	45-55	30-45
$HisYtfM^C$	378	15-25	20-25	50-65

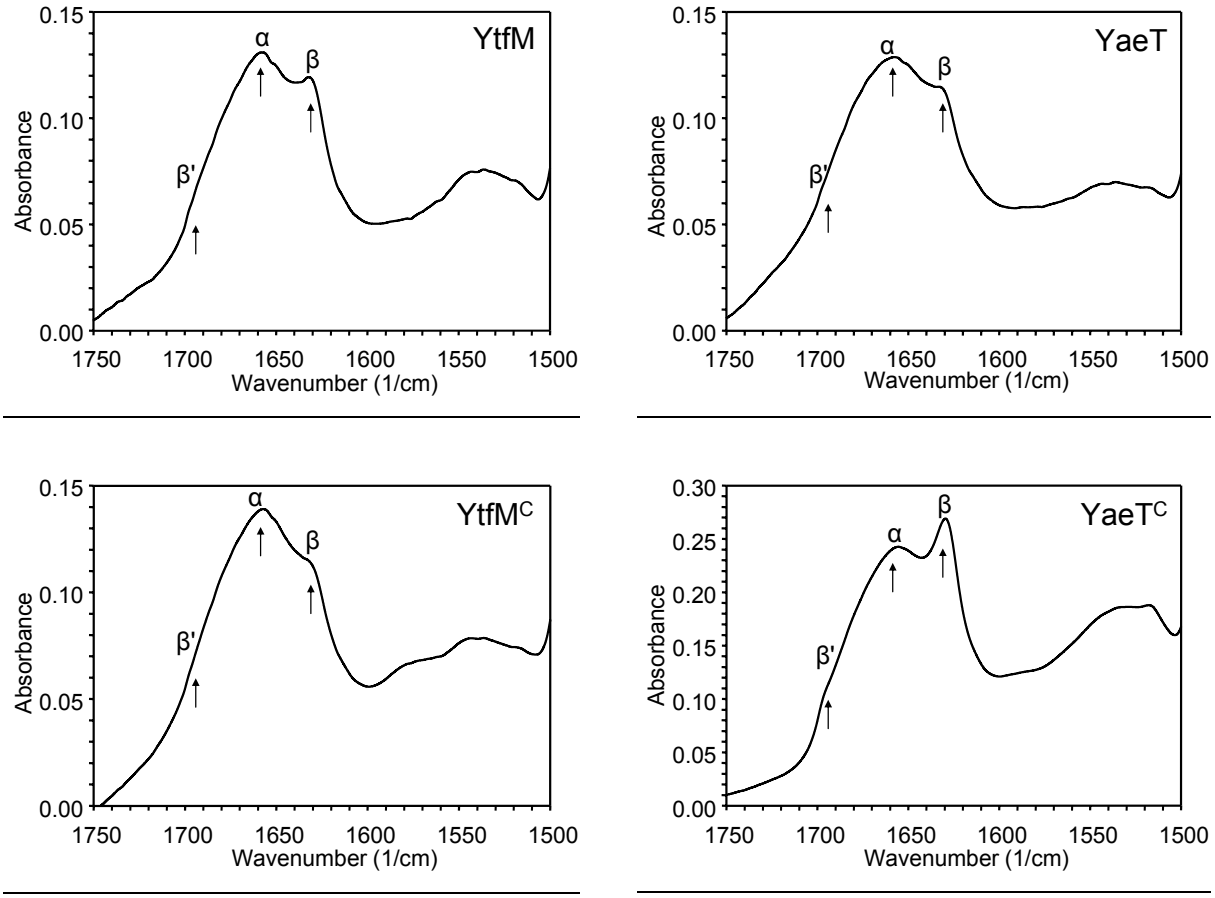


Figure 3.3: FTIR spectra showing the proteins YtfM, YtfM^C, YaeT and YaeT^C. Protein solutions of 3-20 mg/ml protein in $NaPO_4$ buffer containing 0.2% DDM or0. 2% LDAO, respectively, were used. Peak positions for β-strands (1630nm, 1695nm) and α-helices (1658nm) are indicated in the spectra.

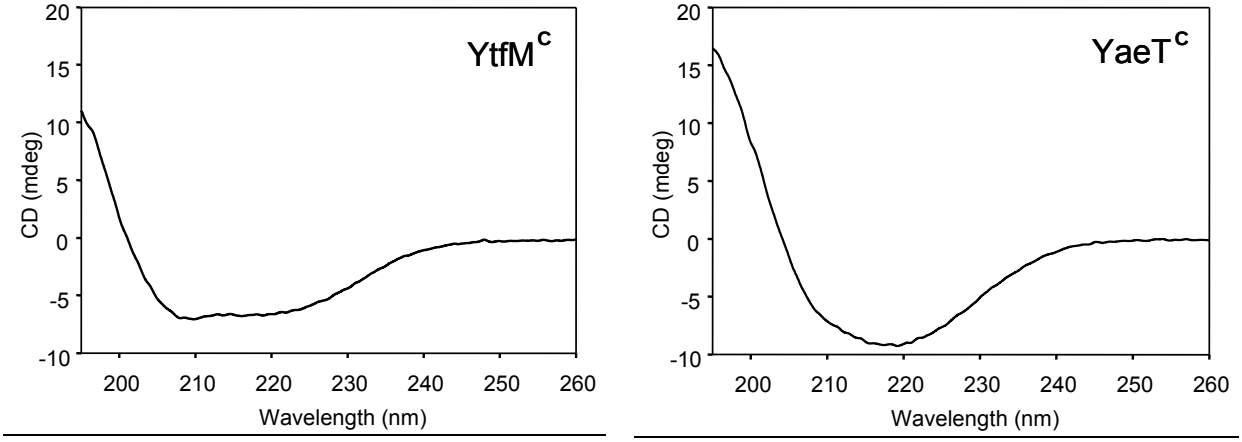


Figure 3.4: CD spectra of the secondary structure conformation of YtfM^C and YaeT^C. Protein solutions of 3-20 mg/ml protein in $NaPO_4$ buffer containing 0.2% DDM or 0.2% LDAO, respectively, were used. The spectrum for YaeT^C shows the distinct minimum at 218nm indicating a high β-strand content.

3.4.4. Bilayer experiments

Purified HisYtfM and HisYtfM^C were added to a black lipid bilayer apparatus to test pore-forming ability. Both proteins induced a stepwise increase of membrane conductance meaning that they form water filled pores in black lipid membranes (Figure 3.5).

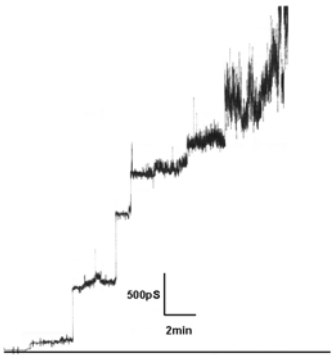


Figure 3.5: Single channel recordings of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of 10 ng/ml HisYtfM protein. The aqueous phase contained 1M KCl. The applied voltage was 20 mV.

However, insertion events were not homogenous and often accompanied by strong increase of conductance noise. Measured in 1M KCl, conductance steps between 200 pS and 500 pS were most frequently observed. Histograms showing the distribution of induced conductance steps document the high variability of single channel conductance for HisYtfM and HisYtfM^C (Figure 3.6). However, distribution of the single channel conductance is similar for both proteins, which means that the carboxy-terminal domain comprises the pore forming domain and that the amino-terminal POTRA repeats have no influence on the characteristic of the induced pores. YtfM pores were similar to that observed for YaeT (Stegmeier and Andersen, submitted). However, very large conductance steps of e.g. over 6 nS observed with YaeT under the same conditions were absent in YtfM measurements.

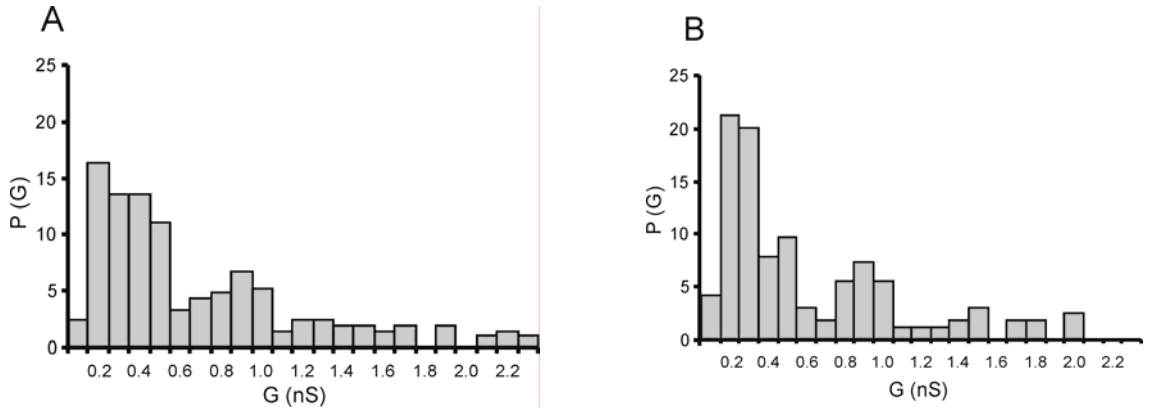


Figure 3.6: Histogram of conductance steps observed for 208 and 166 reconstituted HisYtfM (A) and HisYtfM^C (B) channels in membranes formed by diphytanoyl phosphatidylcholine/n-decane. The single channel conductance is highly variable in a range between 0.1 and 2.3 nS in both cases. P(G) is the probability that a given conductance increment G is observed in the single channel experiments. The aqueous phase contained 1 M KCl, the applied voltage was 20 mV.

By multi-channel experiments applying a KCl gradient across the membrane, we could determine slight anion selectivity. Under zero current conditions a 5-fold KCl gradient revealed a potential of 16.9 ± 3.1 mV, negative on the diluted side, which means that the pores are anion selective with a permeability ratio P_K/P_{Cl} of 0.35. This is a clear difference to YaeT, which is cation selective (P_K/P_{Cl} = 5.7; Stegmeier and Andersen, submitted). Furthermore, another difference between YtfM and YaeT is the pH dependence of the channels. Lowering pH from 8.0 to 4.6 by adding citrate to both compartments leads to a conductance decreased of about 20-25% in the case of YtfM (Figure 3.7) and 70-75% in the case of YaeT (data not shown).

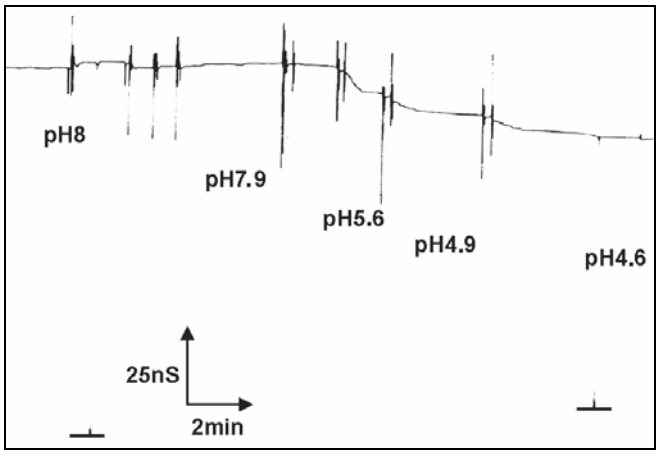


Figure 3.7: Influence of pH on membrane conductance induced by HisYtfM. The membrane was formed from diphytanoyl phosphatidylcholine/ n-decane. The aqueous phase on both sides of the membrane contained 1M KCl. 5µl protein sample (5 ng/ml) were added to the trans site. A stable conductance was reached with approximately 200 inserted HisYtfM channels. pH was lowered by adding citrate pH 3 to both sides of the membrane. The baseline is indicated before and after addition of citrate. The applied voltage was 20 mV.

3.4.5. Chromosomal deletion of *ytfM*

We have constructed the conditional *ytfM* knock-out strain AG100ΔytfM. To get *ytfM* knock-out mutants we complemented the strain by the plasmid pNBytfM encoding *ytfM* under the plac promoter. Furthermore, it was necessary to incubate the Petri dishes for selection of the *ytfM* knock-out mutants for 48 hours at room temperature since incubation at 30°C or 37°C never resulted in a single knock-out colony. Interestingly, the *ytfM* gene did not turn out to be essential since knock-out mutants that were allowed to forfeit pNBytfM, lost the plasmid and AG100ΔytfM cells were still viable. However, several attempts to knock-out *ytfM* without complementation by pNBytfM failed, which shows the importance of YtfM.

3.4.6. Growth experiments

For studying the phenotype of the *ytfM*-knock-out, wild-type, *ytfM* knock-out and complemented *ytfM* knock-out cells were grown in baffle flasks and round-bottomed flasks. It is not surprising that wild-type cells grow faster and reaches higher cell densities in baffle flasks compared to round bottomed flasks. Surprisingly, the effect of the *yftM* knock-out was dependent on the growth conditions. In baffle flasks a drastic effect was observed comparing AG100 and AG100ΔytfM cells (Figure 3.8). The growth rate of the knock-out strain was strongly reduced and cell density in the stationary phase was also smaller compared to wild-type. When grown in round bottomed flask the difference was not so pronounced. This result indicates that the knock-out strain is more affected when forced to grow fast. The growth defect of the knock-out strain could be complemented by the pARAytfM plasmid. After a longer lag-phase compared to the wild-type strain, AG100ΔytfM pARAytfM showed similar growth when expression of YtfM was induced by 30 nM arabinose. The growth rates were not as high as that of wild-type. However, cells reached maximal cell densities comparable to wild-type. The successful complementation of *ytfM* let us conclude that no polar effects due to the chromosomal deletion are responsible for the growth defect of the knock-out strain.

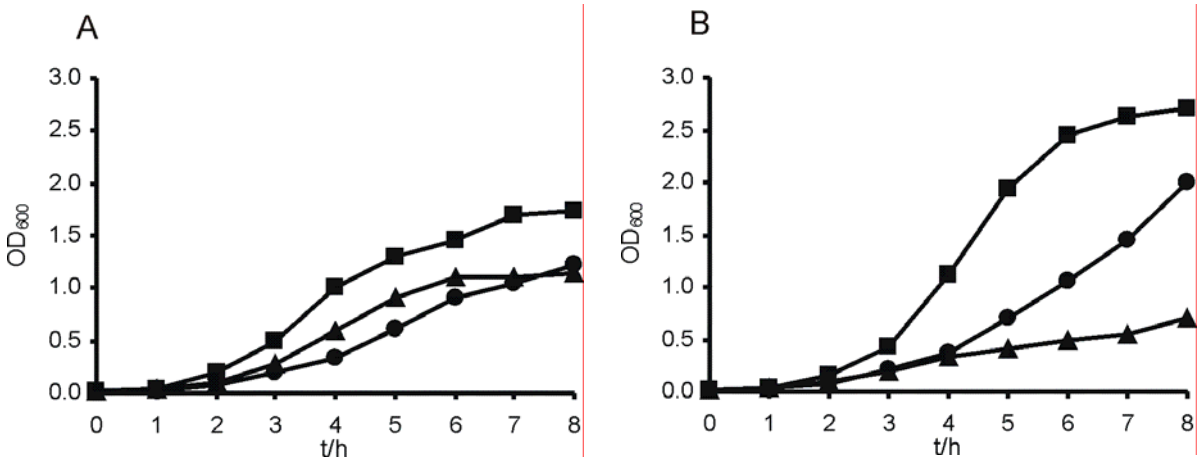


Figure 3.8: Growth curves of the wild-type (square), *ytfM* knock-out (triangle) and complemented *ytfM* knock-out (circles) grown in round-bottomed flasks (A) and baffle flasks (B) at 37°C.

3.4.7. Effects of *ytfM* knock-out on outer membrane composition

To check if the *ytfM* knock-out has any effect on the topology of the cells, AG100 and AG100ΔytfM cells were visualized by electron microscopy. Cells taken from exponentially grown cultures were fixed with glutardialdehyde and osmiumtetroxid and prepared for electron microscopy. The micrographs did not show any difference concerning the cell

envelop of AG100 and AG100ΔytfM (Figure 3.9). Additionally, western blots with antibodies against OmpF and TolC revealed no difference in the amount of the major *E. coli* porins. Also, on Coomassie stained gels no difference was observed for prominent OMP like OmpA between the wild-type and the knock-out strain (data not shown). Furthermore, we tested the sensitivity towards antibiotics and noxious compounds. MIC tests for vancomycin, novobiocin, erythromycin, rhodamine 6G, benzalkonium chloride and SDS revealed the same results for AG100 and AG100ΔytfM. All these experiments demonstrate that the *ytfM* knock-out has no influence on the topology and the OM composition and OM permeability of the cell.

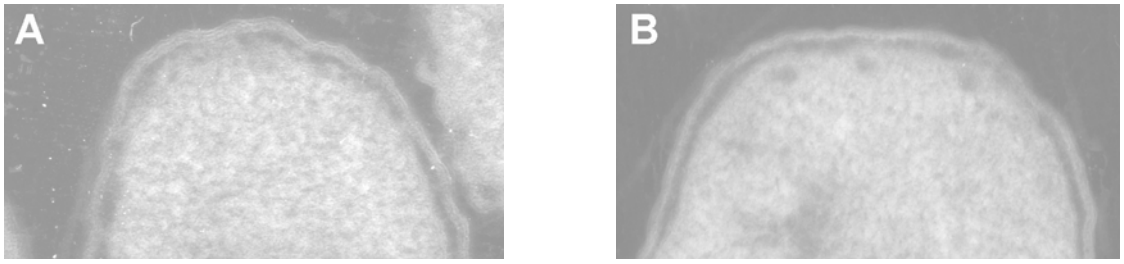


Figure 3.9: Electron micrographs of a section of *E. coli* cells AG100 (A) and AG100ΔytfM (B). Cells were grown in LB medium to exponential phase, then fixed with glutardialdehyde and osmiumtetroxid and prepared for electron microscopy. Original magnification: 63 000x.

3.4.8. Mobility test

To test whether YtfM depleted cells are still mobile, we performed a mobility test on agar plates. It can be seen that AG100ΔytfM forms a halo indicating that the cells are still mobile (Figure 3.10). In comparison to AG100 the halo was obviously smaller. This can be explained to some extent by the reduced growth of the knock-out mutant. Standardised measurements of the OD600 of the melted agar plates revealed more than 2-fold more AG100 cells compared to AG100ΔytfM. Nevertheless, we can not entirely exclude an effect of the *ytfM* knock-out on cell mobility.

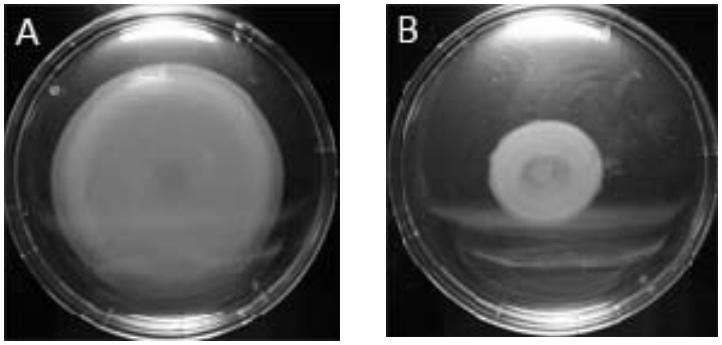


Figure 3.10: Mobility test of AG100 (left) and AG100ΔytfM (right) cells. 2µl cell culture were dropped into the centre of 0.3% agar plates and incubated 18 hours at 37°C. Afterwards the amount of cells was determined by measuring OD600 of the melted agar plates. AG100 (OD600 = 0.358) grew more than 2-fold better than AG100ΔytfM (OD600 = 0.148).

3.5. Discussion

The discovery that Omp85 of *N. meningitidis* plays a crucial role in the biogenesis of the outer membrane was an important step in understanding this vital process (Voulhoux *et al.*, 2003). Several studies were published recently dealing with the Omp85 homologous protein YaeT from *E. coli* (Werner and Misra, 2005; Doerrler and Raetz, 2005; Stegmeier and Andersen, submitted). It could be shown that YaeT is an essential outer membrane protein, which is part of a multi-protein complex required for membrane insertion of outer membrane proteins. Omp85 homologues are widespread in bacteria and are also found in eukaryotes. Beside transport *into* membranes Omp85 homologues mediate also transport *across* membranes as e.g. the Omp85 homologues involved in two partner secretion systems (Jacob-Dubuisson *et al.*, 1999; Könninger *et al.*, 1999). Among the six clusters of Omp85 homologous proteins is a cluster, for which no function is described yet (Yen *et al.*, 2000). One representative of this cluster is YtfM of *E. coli*.

The presence of two genes coding for an YtfM or a YaeT homologous protein in a single chromosome suggests that both proteins provide, although similar, but different functions. We could show that YtfM can not substitute for YaeT and vice versa, which confirms this suggestion. Like YaeT is YtfM also an outer membrane protein. Using the YtfM mutant $YtfM^{C}$ with the amino-terminal 243 residues deleted we prove that the lack of amino-terminal domain comprising the three POTRA repeats does not affect membrane insertion. This confirms topology predictions and own studies on YaeT that the POTRA repeats form an independent domain located in the periplasm, whereas the carboxy-terminal domain is located in the outer membrane (Voulhoux *et al.*, 2003; Stegmeier and Andersen, 2006 submitted).

We have reconstituted purified YtfM and $YtfM^{C}$ in black lipid membranes and observed that both proteins have channel forming activity. However, the induced conductance steps were inhomogeneous and insertion events are often accompanied by an increase of the conductance noise. Similar observations are made for YaeT and the corresponding carboxy-terminal domain of YaeT (Stegmeier and Andersen, submitted). Although the single channel conductance of YtfM and YaeT is in the same range, there is a major difference concerning the ion selectivity of the channels. YtfM is shown to be anion selective whereas YaeT channels are cation selective. Ion selectivity originates from fixed charges in the lumen of the pores. Therefore, our measurements imply that the net charge composition of membrane spanning β-strands of YtfM and YaeT is different. Another difference is the pH sensitivity of both channels. Whereas acidic pH leads to a strong reduction of YaeT-induced conductance,

an effect, which is also observed with other outer membrane pore forming proteins (Andersen *et al.*, 2002b; Muller and Engel, 1999; Andersen *et al.*, 2002c), the changed pH has less influence of YtfM induced conductance.

Beside these electrophysiological differences, YaeT and YtfM show also different heat modifiable mobility in SDS gels. YaeT band shifts to a smaller molecular weight, when loaded unboiled on the gel, which means that it adopts an SDS stable, compact structure. YtfM does not show this behaviour. A further difference is, that YaeT becomes degraded during purification (Stegmeier and Andersen, submitted), which is not observed for YtfM. Our structural analysis of the membrane domains of YtfM and YaeT confirms also differences between both proteins. CD and FTIR data reveal that the carboxy-terminal half of YaeT is mainly composed of β-sheets. In contrast, the β-sheet portion of the corresponding part of YtfM is about half as much. This speaks against a similar folding as suggested for Omp85 or YaeT assuming that 12 or 16 β-strands form the membrane domain (Voulhoux *et al.*, 2003; Ertel *et al.*, 2005). The sequence alignment shows that the carboxy-terminal half of the membrane domains of YtfM and YaeT are highly conserved in contrast to the remaining part. This suggests that the conserved parts adopt similar structures in both proteins. Based on structure predictions, sequence alignments and published Omp85 models we have built a topology model for this part of the protein showing eight β-strands. Interestingly, when counting the charges, which face to the channel lumen, it becomes obvious that there is a net positive charge in YtfM, which supports our electrophysiological measurements. Therefore, we conclude that these parts contribute to the membrane spanning domain.

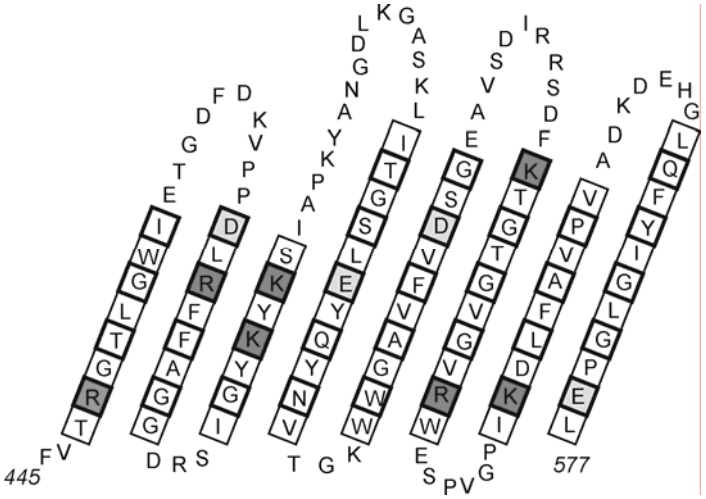


Figure 3.11: Topology model of the carboxy-terminal 133 residues of YtfM. The model was built based on structure predictions, sequence alignments and published Omp85 models. Residues facing to the channel lumen are surrounded by bold boxes. Boxes of charged residues are shaded light grey (negatively charged residues) and dark grey (positively charged residues), respectively.

The inhomogeneous and noisy single channel conductance of YaeT and YtfM suggests that the membrane inserted parts do not form a rigid structure. It could well be that the instability is caused by a partly unfolding of the protein possibly due to the lack of interacting proteins. For YaeT it is known that it is part of a multiprotein complex (Wu *et al.*, 2005). For YtfM interacting proteins are not known.

An analysis at the chromosomal environment of the *E. coli ytfM* gene suggests that it forms a potential operon with two other genes, *ytfN* and *ytfP*. A genome wide screening revealed that there exist 95 operons in 78 different species comprising genes coding for YtfM and YtfN homologous proteins, whereas operons comprising genes for YtfM, YtfN, and YtfP are restricted to *E. coli* and *Salmonella* and *Shigella* species. Therefore, we conclude that YtfM and YtfN form a functional unit independent on YtfP. YtfN homologous proteins are very large proteins varying in length between 710 and 2078 residues. For none of these proteins a function is described. Several proteins are annotated as exported, periplasmic, or outer membrane protein, which originates from the presence of a signal sequence at the amino-terminal end. Therefore, one just can speculate about the function of YtfM and YtfN. It is known from several transcriptomic and proteomic analysis that both genes are transcribed and expressed (Taoka *et al.*, 2004; Corbin *et al.*, 2003; Gevaert *et al.*, 2002 Champion, *et al.*, 2003). Because YtfN possess a signal sequence, which means it is secreted into the periplasmic space, it seems likely that YtfM and YtfN interact in the cell envelope. Considering the function of YtfM homologous proteins we propose two possible scenarios: firstly, YtfM acts as a transport protein, like e.g. the TPS proteins, and transports YtfN across the outer membrane into the surrounding medium. The second possibility is that YtfN is also an outer membrane protein, which is inserted specifically by YtfM. It is also thinkable that both protein remain in contact and form an outer membrane complex. However, to prove these speculations further experiment needs to be done.

The construction of an YtfM knock-out strain allowed us to investigate the function of YtfM. YtfM showed to be not essential as YaeT, although construction of the chromosomal *ytfM* knock-out was only possible in the presence of a plasmid encoded copy. We could show that lack of YtfM did not affect the amount of major proteins in the outer membrane or the appearance of the cell envelope, all phenotypes observed in Omp85 or YaeT, respectively knock-out strains (Voulhoux *et al.*, 2003; Werner and Misra, 2005; Doerrler and Raetz, 2005). We further tested susceptibility against noxious compounds as well as effects on the mobility of the cells may be due to disarrangements in the outer membrane, but no differences were found comparing knock-out and wild-type strain. The only phenotype we observed was

reduced growth. Interestingly, this phenotype was more pronounced, when cells were grown in optimal growth conditions. Reasons for reduced growth could have various origins, as e.g. limited uptake of essential nutrients or negative effects of YtfN lacking its *in vivo* partner.
However, the function of YtfM remains to be elucidated. We assume that it is most likely linked to YtfN. Further studies concerning the localization of YtfN and its interaction with YtfM will help to understand the function of the *ytfMN* operon. The wide distribution of *ytfMN* operons suggests that it plays an important role.

3.6. Appendix

3.6.1 Sequence alignment of YtfM homologous proteins

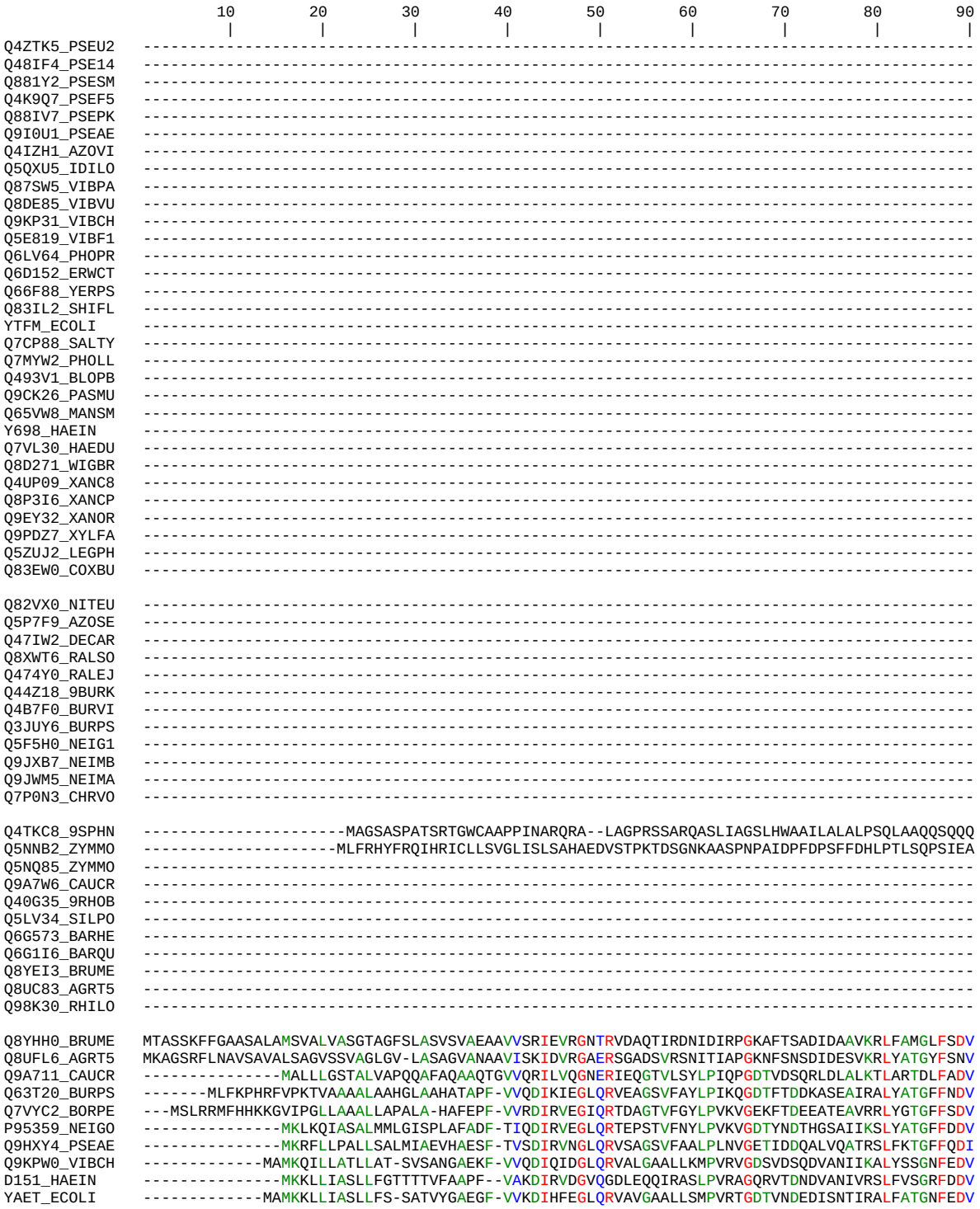
For clarity only one sequence of each genus or species was used (Table 3.3 shows the other sequences). Deltaproteobacteria, Bacteroidetes and Spirochaetes were omitted for clarity. For comparison YaeT from *E. coli*, Omp85 from *Neisseria gonorrhoe*, D15 of *Haemophilus influenzae* and seven other sequences of YaeT homologous proteins representing Alpha- Beta- and Gammaproteobacteria are included in the alignment.

Sequences were aligned using the MULTIALIN-platform of PBIL (Pole Bio-Informatique Lyonnaise) (Corpet, 1988). The matrix used is BLOSUM62 with variable parameters for gap length and gap penalty. At few positions, multialignments are manipulated subsequently by eye. The colour code was applied for separate alignment of each group (from top to bottom: Gamma-, Beta-, Alphaproteobacteria and YaeT-homologue).

On the top the hydrophatie index of each residue of the YtfM sequence is presented as histogram.

Table 3.3: Sequences that are not considered in the alignment

Sequence in alignment	Similar protein of the same genus/species
Q5NNB2_ZYMMO	Q9X3V8_ZYMMO
Q8UC83_AGRT5	Q7CWM5_AGRT5
Q8YEI3_BRUME	Q8G3A2_BRUSU
Q44Z18_9BURK	Q4M089_9BURK
Q3JUY6_BURPS	Q62LE4_BURMA; Q63W94_BURPS ; Q2T092_BURTH
Q7CP88_SALTY	Q5PJ80_SALPA; Q8XEL5_SALTI ; Q57GH2_SALCH
Q83IL2_SHIFL	Q3YUC7_SHISS; Q31TG0_SHIBS; Q328B7_SHIDS
YTFM_ECOLI	YTFM_ECO57; Q8CVG8_ECOL6
Q66F88_YERPS	Q8ZB95_YERPE
Y698_HAEIN	Q4QMM2_HAEI8
Q8DE85_VIBVU	Q7MPD6_VIBVY
Q9EY32_XANOR	Q5GUR4_XANOR; Q8PEY4_XANAC
Q9PDZ7_XYLFA	Q87E20_XYLFT
Q5ZUJ2_LEGPH	Q5X4B1_LEGPA; Q5WVP5_LEGPL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Q48IF4_PSE14
Q881Y2_PSESM
Q4K9Q7_PSEF5
Q88IV7_PSEPK
Q9I0U1_PSEAE
Q4IZH1_AZOVI
Q5QXU5_IDILO
Q87SW5_VIBPA
Q8DE85_VIBVU
Q9KP31_VIBCH
Q5E819_VIBF1
Q6LV64_PHOPR
Q6D152_ERWCT
Q66F88_YERPS
Q83IL2_SHIFL
YTFM_ECOLI
Q7CP88_SALTY
Q7MYW2_PHOLL
Q493V1_BLOPB
Q9CK26_PASMU
Q65VW8_MANSM
Y698_HAEIN
Q7VL30_HAEDU
Q8D271_WIGBR
Q4UP09_XANC8
Q8P3I6_XANCP
Q9EY32_XANOR
Q9PDZ7_XYLFA
Q5ZUJ2_LEGPH
Q83EW0_COXBU
Q82VX0_NITEU
Q5P7F9_AZOSE
Q47IW2_DECAR
Q8XWT6_RALSO
Q474Y0_RALEJ
Q44Z18_9BURK
Q4B7F0_BURVI
Q3JUY6_BURPS
Q5F5H0_NEIG1
Q9JXB7_NEIMB
Q9JWM5_NEIMA
Q7P0N3_CHRVO
Q4TKC8_9SPHN
Q5NNB2_ZYMMO
Q5NQ85_ZYMMO
Q9A7W6_CAUCR
Q40G35_9RHOB
Q5LV34_SILPO
Q6G573_BARHE
Q6G1I6_BARQU
Q8YEI3_BRUME
Q8UC83_AGRT5
Q98K30_RHILO
Q8YHH0_BRUME
Q8UFL6_AGRT5
Q9A711_CAUCR
Q63T20_BURPS
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P95359_NEIGO
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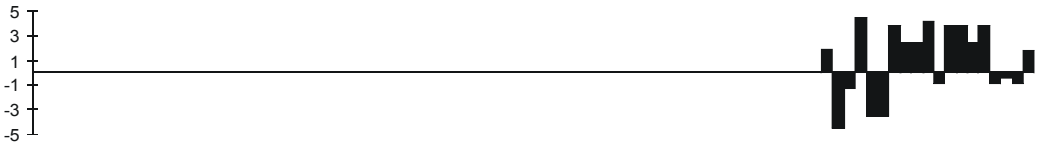
D151_HAEIN
YAET_ECOLI

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                       |         |         |         |         |         |         |         |         |
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Q48IF4_PSE14   ------------------------------------------------------------MKLFRLFSSLFLMTMSAAAL
Q881Y2_PSESM   ------------------------------------------------------------MKLFRLFSSLFLMTVSAAAL
Q4K9Q7_PSEF5   ---------------------------------------------------------MIASAPMKFPGRFTSGFLLLFTSCAAL
Q88IV7_PSEPK   ------------------------------------------------------------MRRMTYSGRLTWGLVFWVASFAAW
Q9I0U1_PSEAE   ------------------------------------------------------------MRLTQGLFRFPLA-GLLCLSTAAF
Q4IZH1_AZOVI   ------------------------------------------------------------MRASIHTGTFLGLLFWGSAVF
Q5QXU5_IDILO   ------------------------------------------------------------MRYFRFILFSFSILFNGLAL
Q87SW5_VIBPA   ------------------------------------------------------------MIKKALPIIVGLLVVSQSA
Q8DE85_VIBVU   ------------------------------------------------------------MIKKVLPLLVSSLFASNLV
Q9KP31_VIBCH   ---------------------------------------------------------MSPLYNESKSSMRRKSLPALISLWLFSAVS
Q5E819_VIBF1   ------------------------------------------------------------MIKKLPLFIIVCLLAISSQ
Q6LV64_PHOPR   ------------------------------------------------------------MNDVLKRFGLTVVAMVFATPVL
Q6D152_ERWCT   ----------------------------MTIFIAKRRFTSKNRFIGKKSVTGQKGVAEQKNVLAGCGAPRYRILGLMCALLMLAPDS
Q66F88_YERPS   ------------------------------------------------------------MLRYPILCFMC-LLLATPIA
Q83IL2_SHIFL   ------------------------------------------------------------MRYIRQLCCVSLLCLSGSA
YTFM_ECOLI     ------------------------------------------------------------MRYIRQLCCVSLLCLSGSA
Q7CP88_SALTY   ------------------------------------------------------------MPHIRQLCWVSLLCLSSSA
Q7MYW2_PHOLL   ------------------------------------------------------------MSRYPVLCFLCVSIVTPIA
Q493V1_BLOPB   ---------------------------------------------------------MFKDLKKYMIIRLHLIIYFFMPLI
Q9CK26_PASMU   ---------------------------------------------------MKLLSNTKASLIT----KTVFFIGSVLLSTVSL
Q65VW8_MANSM   ---------------------------------------------------MKIFSDKITHFVSVWLLKAVMFLALLISSPAIA
Y698_HAEIN     ------------------------------------------------------------MKKKSLKLTALFLALSCFPAFA
Q7VL30_HAEDU   ------------------------------------------------MNFNFRFFITGCFLTCSQIIFAEQLATTSQQEILAERPQLT
Q8D271_WIGBR   ------------------------------------------------------------MYKKFFYFSLSFYFLIFLS
Q4UP09_XANC8   ------------MPHRVRRPDRLPCEDLCMYVHDVHAYMTHGMALSTPRRPINRPSRSSRYPAPMRFILRVRPVFLLLCILAG--SAFA
Q8P3I6_XANCP   ------------MPHRVRRPDRLPCEDLCMYVHDVHAYMTHGMALSTPRRPINRPSRSSRYPAPMRFILRVRPVFLLLCILAG--SAFA
Q9EY32_XANOR   ------------------------------------------------------------MLKVAFALFLLCTLFVPLQAVA
Q9PDZ7_XYLFA   ---------------------------------------MKSLRCFWNVRFITL-SMLPRYTAQM--LRRFIPPMLSLWLLFAS-LAQA
Q5ZUJ2_LEGPH   ------------------------------------------------------------MNRFAYLIILIILYFP--LFAQDKKA
Q83EW0_COXBU   ---------------------------------------------------------MKEHLCTIFILFCWFFSGLAWAQQLPA

Q82VX0_NITEU   ------------------------------------------------------------MLLSCCMLYLLWCGTVLAAAADEKEK
Q5P7F9_AZOSE   ------------------------------------------------------------MTAFRIHALLRLWFVALCLGLSSGTVF
Q47IW2_DECAR   ------------------------------------------------------------MIARRLPLLLLLCLAQPVVA
Q8XWT6_RALSO   ---------------------------------------------------------MTGRQRPSR-FRLLATPAAALLAGCVLSVACTA
Q474Y0_RALEJ   ---------------------------------------MRTERTESARVAAAPDRTVRPEA-RSWTPARLRRVFLSALAVAWLLP
Q44Z18_9BURK   ---------------------------------------MAGQSNQQ-AHTAHHAGPRAARDRAG----AAARVARAALAGCLAACFALP
Q4B7F0_BURVI   ---------------------------------------MAGPANHHHADTAHDAAARSARDSRRRSRRGAAAAARAAVAGCVAAFFALP
Q3JUY6_BURPS   ----------------------------------------------------------------------MLLAGC-AAVLAAP
Q5F5H0_NEIG1   ---------------MPSEASRPVRTVLESKSATPMHDTRTMMIKPTALLLPALFFFPHAYAPAADLSENKAAGFALFKSKSPDTESVK
Q9JXB7_NEIMB   ---------------MPSEASRPVRTVLKSKAATPMHDTRTMMIKPTALLLPALFFFPHAYAPAADLSENKAAGFALFKNKSPDTESVK
Q9JWM5_NEIMA   ----------------------------------MHDTRTMMIKPTALLLPALFFFPHAYAPAADLSENKAAGFALFKNKSPDTESVK
Q7P0N3_CHRVO   ------------------------------------------------------------------------------MAAAQAQ

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Q5NQ85_ZYMMO   ----------------------------------------MAPLDNWSDLGVDWPD-LQKTDSNT-EGDLSPSVNSKADNQPKSSKSKIS
Q9A7W6_CAUCR   ----------------------------------------MRSARDGGGRHSERDTSPARDYSRGGGASRGERDLVLGRTTLALAAVT
Q40G35_9RHOB   ------------------------------------------------------------------------------MGGRQIIASAL
Q5LV34_SILPO   ------------------------------------------------------------------------MNLTGPLGARLRGLVTA
Q6G573_BARHE   ----------------------------------MVYQTGVFLKSGTTRSVLIGFCLVLAFPKSLAAFELFGIPLFGHKKINSSSNVDG
Q6G1I6_BARQU   ----------------------------------MVYQAGVLLKSGTIRSVLIGLCLIFTFPQPLAAFEFFGIPFFGQKKINSSSNGSG
Q8YEI3_BRUME   ------------------------------------------------------------MAFEIFGIHLWGKDKKQDPDIID-
Q8UC83_AGRT5   ---------------------------------------------------MTFALYPAFARDAFAFKLFGMRLWGSEEPE-VEVIN-
Q98K30_RHILO   ---------------------------------------------------MLCATALVAESRPAAAFELFGIKLWGSSNDEDADIVD-

Q8YHH0_BRUME   RIHQSGSTLIVQVKERSVVNNVLFQGNKKIKDPDLARAVQ---LKPRAPFDMATMEADKEAIKAAYSHIGRSDATVNARTVDLGQGRVNV
Q8UFL6_AGRT5   SMRVSGSTLVVTVNENQLVNQVVFNGNRKIKDDKLAGIVQ---TQPMGPFNQAIVTADIARIKEAYSAIGRSDVEITTQTVSVGQGRVNI
Q9A711_CAUCR   KIEMLGGDLVVKVVENPIINQVVFEGNSSLKEDKLKDEVQ---IRPRGIFTRAKVQADVQRIIELYRRSGRISATVTPKVVELPQKRVDL
Q63T20_BURPS   RIATQGGVVIVQVQERPAIASIDFTGIKEFDKDNLNKALKAVGLSQGRYYDKALVDKAEQELKRQYLTRGFYAAEVSTTVTPVDANRVSI
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P95359_NEIGO   RVETADGLLLLTVIVCPTIGSLNITGAKMLQNDAIKKNLESFGLAQSQYFNQATLNQAVAGLKEEYLGRGKLNIQITPKVTKLARNRVDI
Q9HXY4_PSEAE   QLGRDGNVLVVTVVERPSISSIEIEGNKAISKEDLLKGLKQSGLAEGEIFQRATLEGVRNELQRQYVAQGRYSAEINAEVIPQPRNRVAL
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D151_HAEIN     KAHQEGDVLVVSVVAKSIISDVKIKGNSVIPTEALKQNLDANGFKVGDVLIREKLNEFAKSVKEHYASVGRYNATVEPIVNTLPNNRAEI
YAET_ECOLI     RVLRDGDTLLVQVKERPTIASITFSGNKSVKDDMLKQNLEASGVRVGESLDRTTIADIEKGLEDFYYSVGKYSASVKAVVTPLPRNRVDL
```


```
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                         |         |         |         |         |         |         |         |         |
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Q881Y2_PSESM    AQARLDVRIKPANP----DLKSNVEGYVGDLGD-----RDAKA------LRNFSLGAEQQAEKAAQALGYYQAQIDSEIKDGED------
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Q88IV7_PSEPK    GQSELLVKVKPANK----ALKANVEGYIGTLGD-----RDEEA------LLRFSRGAEEQARKAAQALGYYQAQIDTEVKPASDADHP--
Q9I0U1_PSEAE    AESQLSVRVTPANA----ALKANIEAYVGSLGE-----RDEAA------LQRFRRNAEAQAEKAAQALGYFQAQIDSEVKDGKP------
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Q87SW5_VIBPA    YA-DVSLKLKGIDG----ALEDNVKAYLSSIPE-----KDYST------SLRFQARLDQSITEALNALGYYHAKISYSISEGND------
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Q82VX0_NITEU    KPDPVDVTITAP------ASLKKLLEKHMTL--PAKPFRDEID----QAAY-ARKIR-QESIELLATEGYFTPEIEFDEKK-GNGKA---
Q5P7F9_AZOSE    AQVPLQVELDAP------DSVRPLLERYLRILEPDGQVLPEVQAD---RVALARRTR-REATELLATEGYFTPEIRLQREPDG-------
Q47IW2_DECAR    GELDLQ----AP------DNITKLLAPYL--------------PD---EAGSPGKLQ-GLLSEILATEGYFSPVFEFAEKGDG-------
Q8XWT6_RALSO    ARAAYEVRIEAP------RPLRDLLERHLDLARYQD--RKDITDD---QLQYMVETIGEQVTKLTATEGYFVPKATAQLEGPPE------
Q474Y0_RALEJ    AQAAYKVEVEAP------KPLREILENHLDLSRYKD--RTDLSPN---QFAYMVETVGDQVRQFASTEGYFDPVTTARVEGEGD------
Q44Z18_9BURK    AHAKYDVDIDAP------RSIRKLLKTHLDIARFAK--RDDISDD---QFDFLVTATPQQVRDLTATAGYFSPVVRTDVRTRDG------
Q4B7F0_BURVI    AYAKYDVDIDAP------RAIRKLLGAHLDIARFAK--RDDISDD---QFDFLVTATPQQVRDLTATAGYFSPVVRTDVRSRDG------
Q3JUY6_BURPS    AFAKYAVEIDAP------RSVRSLLKQHLDIARFAK--REDLSED---QFEFLVTATPQQVRELAATAGYFSPVVRTDVRTTGG------
Q5F5H0_NEIG1    LKPKFPVRIDTQD-----SEIKDMVEEHLPLITQQQ--EEVLDKE---QTGFLAEEAPDNVKTMLRSKGYFSSKVSLTEKDG--------
Q9JXB7_NEIMB    LKPKFPVLIDTQD-----SEIKDMVEEHLPLITQQQ--EEVLDKE---QTGFLAEEAPDNVKTMLRSKGYFSSKVSLTEKDG--------
Q9JWM5_NEIMA    LKPKFPVRIDTQD-----SEIKDMVEEHLPLITQQQ--EEVLDKE---QTGFLAEEAPDNVKTMLRSKGYFSSKVSLTEKDG--------
Q7P0N3_CHRVO    AGIEYSVDIDAP------SDLAQLLRDNLELVEGQK--DPDVDPG---WLDALVKSTPDEAKKLLETEGYFGARVDASLEGGN-------

Q4TKC8_9SPHN    VEITDSLVLAFPAQDEGFTVRNEFVER-FDALSNIKAFDDGETNVAQL--AARAAADEELLNNLLRNYGFYDGLV--------VRTLGEI
Q5NNB2_ZYMMO    AKESDDLRYKVDVKGLSNIDTDQAFKN-YSVLLSNKNKAD---SLSVI--GARATNDQELINRILRSQGYYDGKA--------ALSITPI
Q5NQ85_ZYMMO    SVSENNLGYHYTVKITGLPPREHTWQSSFNGLSTLYMTRRKRANIAQI--RRRIRDDSENLTALLQSGGYYDASV--------ESRLTPI
Q9A7W6_CAUCR    WLSASAAWADEPMAQIQGVEDRALRDAIQRALSDSKQPPR---SRSEA--RRRARQAGEDAIAVLRAEGYYAYTV--------EPDVTEG
Q40G35_9RHOB    VACALAGAGQAQDIQLSAPGVDEDLTDRLRASSLLLQETEEVRTAQ-DL-IAAARADYGRLVAALYDAGHFSPVVRIDVDGREAARIPPF
Q5LV34_SILPO    GLLLVPVNASALEAVLTAPGASEELQDRLRQSSAALSASARGQDSPLEL-LSSALSDYRTLVQVLYDAGHFSPVVHIRLDGREAAAIDPF
Q6G573_BARHE    AEKFYNVDVVAS------PGASSEGVKIVKAVSSLVIDRDKALSSSSGL-LAKARSDYRAILSALYAEGYYGGVISIKINGLEAADLSPV
Q6G1I6_BARQU    TEKFYKVDVVAS------PGASAEGVKIVKAVSSLVADQDKALPSSSGL-LAKARSDYRVILSALYADGRYGGVISIRINGLEAADLSPV
Q8YEI3_BRUME    -PKTYSVDVTTTGDRKNADGKEADLKSVIEGASGLVSDADKPASGSAGL-LAKARGDYRRILSALYGEGRYGGTISIKVDGREAADIPPD
Q8UC83_AGRT5    -PVKYAVTLDA------ADADKSLKSSSLENSSLLLADKDKPASGDLGL-LIKARDDRDRLIAALYENARYGGIVNVTVAGKNVDDLPPN
Q98K30_RHILO    -PLRYAVTITA-------PDADKDLVKRLENASALKGDEDRPVSGSLGL-MAKARSDREQLVAALFADARYEGVVTITIDGKPLDELPPD

Q8YHH0_BRUME    VYEINEGSRTKIANIEFVGNQAFSGRRLRDVISTKRSNPLSWLTRNDVYDEGRLQADEETLRRFYYNRGYADFRVLSSNAVLDPSTNEY-
Q8UFL6_AGRT5    AFVINEGERTKIGRIDFIGNNSYSDGRLAAVINTKKSNMLSFLTRKDVYNEDKLRADEEALRQFYYNRGYADFRVVSSDAVLDESKNEY-
Q9A711_CAUCR    VFEINEGAKSGVLGINFLGNAEYSDNDLRDVIVTKESRWYKILTSNDNYDPDRIEYDREQLRKHYRNRGYFDFRVISSVAELAPDKNGF-
Q63T20_BURPS    LFAVAEGPSAKIRQINFIGNKAFKTSTLRDEMQLSTPNWFSWYTKNDLYSKEKLTGDLENVRSYYLNRGYLEFNIESTQVSISPDKKDM-
Q7VYC2_BORPE    SFDVFEGEVAKIREIRVVGSKAFSEGELLDQFDLTTPGWLTWYTNTDKYSREKLEGDIERLRSFYLDQGYLEFTVEPPQVTISPDRKDI-
P95359_NEIGO    DITIDEGKSAKITDIEFEGNQVYSDRKLMRQMSLTEGGIWTWLTRSDRFDRQKFAQDMEKVTDFYQNNGYFDFRILDTDIQTNEDKTRQ-
Q9HXY4_PSEAE    KININEGTVAAISHINVVGNTVFSEEDLTDLFELKTTNWLSFFKNDDKYAREKLSGDLERLRSYYLDRGYINMDIASTQVSITPDKKHV-
Q9KPW0_VIBCH    KFVFTEGVSAKIQQINFIGNQVFSDEELLSRFNLNVDVAWWNFLADDKYKKQVLAGDIEALRTYYLDRGYLKFQVDSTQVAISPDKKGV-
D151_HAEIN      LIQINEDDKAKLASLTFKGNESVSSSTLQEQMELQPD-SWWKLWGN-KFEGAQFEKDLQSIRDYYLNNGYAKAQITKTDVQLNDEKTKV-
YAET_ECOLI      KLVFQEGVSAEIQQINIVGNHAFTTDELISHFQLRDEVPWWNVVGDRKYQKQKLAGDLETLRSYYLDRGYARFNIDSTQVSLTPDKKGI-
```


```
YTFM_ECOLI      -----RQVLIAKVTPGVPVLIGGTDVVLRGGARTDKDYLKLL------DTRPAIGTVLNQGDYENFKKSLTSIALRKGYFDSEFTKAQLG

                      280       290       300       310       320       330       340       350       360
                       |         |         |         |         |         |         |         |         |
Q4ZTK5_PSEU2    ------PSLVINIQPGEPIHLRNVVIRVEGPAAAMKAFR-VPS-----SDALKTGAVLNHGNYEDAKRLIQNQASRYGFFSGHFTRQRLA
Q48IF4_PSE14    ------PRLVINIQPGEPIHLRKVVIRVEGPAASLKAFR-VPK-----SDALKTGAVLNHGNYEDAKRLIQNQASRYGFFSGHFTSQRLA
Q881Y2_PSESM    ------PRLIINIQPGEPIHLRNVVIRVDGPAAALQAFK-VPK-----SDALKTGAVLNHGNYEDAKRLIQNQASRYGFFSGRFTRQRLA
Q4K9Q7_PSEF5    ------PRLTLRIDPGEPVHLRNVTVRVEGPAASLKSFR-LPD-----GDQLKPGAVLNHGHYEDAKRLIQNQASRYGFFSGRFTQQKLS
Q88IV7_PSEPK    ------PKLIISINPGEPIRLRNVTVRIEGPASEMKAFR-VPD-----SKALRPGEQLNHGHYEDAKRLIQNQASRYGFFSGRFSSQRLA
Q9I0U1_PSEAE    ------PKLTLKVVPGEPVRLRQVNIQVLGEAASLESFR-LPS-----GKQLKPGAKLNQGVYEDAKRLIQNQASRYGFFQGRFSTQRLS
Q4IZH1_AZOVI    ------PTLVLRVVPGEPVRLRRVEIRVEGPAARLEAFR-VPQ-----SSRLRPGAQLNHGRYESVKSSIKSQASRLGFFSGHFTRQHLF
Q5QXU5_IDILO    ------WSAAYQIELNDPVKIAKNMWEIRGDAAEDEAFENIE-----NNSLKEKQRFIHSTYEQVKSRLLSLASQRGYHDARFIKHNVE
Q87SW5_VIBPA    -----E--LIVNIHKGLPVKIKVMDVVISGEAKEDEEFTNLIA-----KSPLKVGRILNQGEYDSLKSGIRNLALQRGYFNGDFKLNKLE
Q8DE85_VIBVU    -----E--LTAHITLGEPVRVETFDVRIEGEASNDKDFLQLLE-----KSPLKQGAILNHGDYDTLKSSIRNLALQKGYFKGDFIETRLE
Q9KP31_VIBCH    -----R--LRAAVTLGEVTRLSEVDIVIRGEAEGDRDFQRLIR-----RSGLRVDAPLNHSLYDNLKSGIRNLALQKGYFNGDFQASRLE
Q5E819_VIBF1    -----DRTLLVNIDAGQPVIIYVSDIVISGEAAADEDFINAVD-----NSGLNLGQVINHGRYESLKSTLQNLALRKGYFDGDFTTNRLE
Q6LV64_PHOPR    -----KSSIAVTVEKGPITHIAKSHIVLLGMAESDPDFIGLVK-----GSGLGLGESLNHGKYESLKSSLSSLALRKGYFDASLTKSVME
Q6D152_ERWCT    -----RPVLIATVTPGEPVKIAGVNITLRGGAHDDKDYQQLVK-----DDRPEIGSVLNHSDYDRFKSGLNGLSLRKGYFDARFQQSQLG
Q66F88_YERPS    -----RSVLIAKVVPGEPVLIAGVDIVLQGGAKTDPDYQALVR-----RDTPKIGSVLNHGDFDNFTSSLTGLALRRGYFDANMIKSQLG
Q83IL2_SHIFL    -----RQVLIAKVTPGVPVLIGGTDVVLRGGARTDKDYLKLL------DTRPAIGTVLNQGDYENFKKSLTSIALRKGYFDSEFTKAQLG
YTFM_ECOLI      -----RQVLIAKVTPGVPVLIGGTDVVLRGGARTDKDYLKLL------DTRPAIGTVLNQGDYENFKKSLTSIALRKGYFDSEFTKAQLG
Q7CP88_SALTY    -----RQVLIARVTPGQPVLIGGTEVILRGGARTDKDYLALL------KTRPAIGTVLNQGDYDNFKKSLTSVSLRKGYFDSEFIKSQLG
Q7MYW2_PHOLL    -----RSVLTAKVTVGEPVRIADVNVVLEGGAKTDGDYAKLVK-----SKSPKKGTILNHDEYENLKGSLTGLAIRKGYFDAVMKKSQLG
Q493V1_BLOPB    -----SDLLVIKVEPGDPVVVTEVNVVIRGDGVKDVDYQKIIK-----DSKSFIGKRLNHNDYEQFKNKLYNLALCKGYFDAKFQSSQLI
Q9CK26_PASMU    -----KDQLIAHVKIGKPVKIADVDFVLQGEATQDPEFIAVTK------NIPEKGSILNHETYDNYKSHIQKIALSRGYFDGDFLVSRLE
Q65VW8_MANSM    -----PDLLIARVDIGKPTLIEDTDIVITGDALHDEYFKRLEK------KVPAKGTVLDHETYEDYKTELQKLAVQRGYFDADFPVHQLQ
Y698_HAEIN      -----RDLLIAHVTPGEPTKIAGTDVQIEGEAAQDENFNALRK------NLPKDGVLVEHQTYDDYKTAISRLALNRGYFDGNFKISRLE
Q7VL30_HAEDU    -----PLLTLNVQLDNQKVTIDEVDIQLQGDANTDEDFIELKK------TAPNQGDSLAHNAYDDFKSHIEGLAFKKGYFDGQWLYHRLE
Q8D271_WIGBR    -----RNIITIYVVQGDFIKMLDSKIILNGDSKNDTDYNAWIK-----NNFPKIGSQLNHYKYEKFKQGLLDISIKKGYFDADFEVSELQ
Q4UP09_XANC8    -----RLTVVITVNRGEPVRVRQSHISITGWAEQDRYLGQDLK-----QFEPREGQVFSHPQYEASKVRITRRLAERGYFDADFTQRRVA
Q8P3I6_XANCP    -----RLTVVITVNRGEPVRVRQSHISITGWAEQDRYLGQDLK-----QFEPREGQVFSHPQYEASKVRITRRLAERGYFDADFTQRRVA
Q9EY32_XANOR    -----KVTVVITVDRGEPVRVRTSHISITGWAEQDRYLGQDLK-----QFEPREGQVFSHPQYEASKVRITRRLAERGYFDADFTQRRVA
Q9PDZ7_XYLFA    -----HITVVIYVDKGEPVRVRQAHVAMTGAAAQDHYLQRDLE-----DFKPKLGEIFNHPTYEASKVRITRRLAERGYFDADFTHRRVE
Q5ZUJ2_LEGPH    -----III---KVQPGSQIRIASIKAMLTGEGAQNPLLRKTLK-----ELPLHIGDPLFSEQYEKAKQNIINTAENMGYLHGVFKKAEIL
Q83EW0_COXBU    -----FWFGHFTVNPGPRMQFTQVKLQITGPGAYDRAFLHLYE-----NFPVKAGDFFDSEKYESAKNDLFNVAAGRGFFKARMIRNQIL

Q82VX0_NITEU    ----KAKAYELRITPGPRTRVAAVTIEFQGALAADEPAYRARAEQLRAEWPLTPGKVFRSARWEDAKSALLSSIANRDFATARIVESQAK
Q5P7F9_AZOSE    -------RWQLSVEPGPRASIAAVELLFDGDLAGDGETRAERRAALRQAWALAPGESFRQSAWDDAKARLLDGVAVRDYAAARIIDSRAE
Q47IW2_DECAR    -------L-KLTLDPGLRTTINSLDLLIDGPV------EAGTRKALIDDWALPVGQPFRQEGWNTAKQQILSRLLAVEHADARLLDSEAS
Q8XWT6_RALSO    -----RRTVRVKVEAGPRTTIDSVALDFTG-AIAQEP---KRIDALKKAWGLPQGAPFRQSGWDKAKEDSLAALQSKRYYAARQTASQAR
Q474Y0_RALEJ    -----KRVVHVTVDPGARTIIRNVDIKVDGPAATRSP---EQVAEVQKKWGLPVGDPFRQEDWDKAKEDALVTLQSRSYFGARLAQSQAR
Q44Z18_9BURK    -----KRDVHIAVDPGKQTVVSTVDLTFKGPVETEDP---KQETATRFAFSMKPGDPFTQSDWDDAKGAALRQLQSRRYLGAKIVSSEAR
Q4B7F0_BURVI    -----KRDVRVAVDPGPQTVVSAVDLTFKGPVDAEDP---KQEAATRFAFSLKPGDPFTQSDWDGAKSAALKQLQSRRYLGAKIVSSEAR
Q3JUY6_BURPS    -----ERRVTVSVDPGAQTLVSAVDLKFDGPVASEDP---KQETATRLAFSLKPGDPFTQAGWDDAKNAALKQLQSRRYLGAKITASEAR
Q5F5H0_NEIG1    -------AYTVHITPGPRTKIANVGVAILGDILSDGN-LAEYYRNALENWQQPVGSDFDQDSWENSKTSVLGAVTRKGYPLAKLGNTRAA
Q9JXB7_NEIMB    -------AYTVHITPGPRTKIANVGVAILGDILSDGN-LAEYYRNALENWQQPVGSDFDQDSWENSKTSVLGAVTRKAYPLAKLGNTQAA
Q9JWM5_NEIMA    -------AYTVHITPGPRTKIANVGVAILGDILSDGN-LAEYYRNALENWQQPVGSDFDQDSWENSKTSVLGAVTRKAYPLAKLGNTRAA
Q7P0N3_CHRVO    -------RVRVKVSPGEPVLIDDVTIRLDGDIRQQDD-YQQRLAQVLEAWTLPMGAPYRQADWDAGKRAVLRKVQADRFPKARIVSSEAR

Q4TKC8_9SPHN    QEADEIPAVRFDIVPGPRYRFGVIDLGSLVTAPDAN-------E-LRAAFEIESGDPLDNDRIIAEQLDLDIALGETGYPFAEIAAPSLL
Q5NNB2_ZYMMO    EKGQY--NVRYDVNAGSIYKLGQINLTGSEEEP-LR-------I-ARVALNLHRGDPIIATHIEQAENNILITLPHYGYPFAKIGDRTIL
Q5NQ85_ZYMMO    IEKHKV-VIEFKVNAGQLYRFTDVKVNGLDKEDTPQ-------LGLQKIYPIHKDDPVDQAAIISADQDLEKQLGHLGFPFAQITQPDIV
Q9A7W6_CAUCR    DPPRAI--VRITPGPAFLLADPHIDWSGSPPDEGVR-------QRAVAAMRLTEGEPGRSADVVGAEGRIVAQVAKLGYADVAAEPREVV
Q40G35_9RHOB    GGPRSVNQITIRVEPGPVFQLGTAEIAPLAP------------DTELPGEFRPGGAVTTGLLRDTASAAIGAWRDNGYATAEVSDQQIT
Q5LV34_SILPO    STPARIERIEIAVDPGPAFRFGTARVEPLSA------------ETRLSDAFAPGQPATTGAIRDAAQTGILGWREAGYAKARIGDQRIV
Q6G573_BARHE    TQLPKQSKIVITIDAGPQYVFSVAGIDKVAPFVKDK----SNKMPTVEELGYKVGFVAKSETILKTEQWAIEGWRRQGYAKAKVIKSEVV
Q6G1I6_BARQU    TQLPAQSHIVITIDAGPRYIFSFAGIDKVAPFLKDK----TNRMPTIEELGYKVGAVAKSETVLKAERWAIEGWRRQGYAKAKIVKSEVI
Q8YEI3_BRUME    TEIPNNAKVAITVDPGPQFLFSRTAISNIAPPPGNR----RDKVQTPEEAGFAPGQEAKSGTILKAERLAVEAWRQEGYAKARVTGEDVV
Q8UC83_AGRT5    PVFDHSTPVPMVITVTPGPKFTLGNVRLEGDVTG----------RNLDEYGLIAGGDAGSLAIIRAGNKLIDDLKAEGRPLAKLTKREAV
Q98K30_RHILO    AEFKGPQPIPVTINIAVGPKFTLGTIRLEGDAAG----------LMSADYGLISGGDAGSGAVLKAEALIVRTLKEQGRPLAKVTDRRIV

Q8YHH0_BRUME    -------TITITVDEGPRYTFGDVSVESTVDGVDTQ-------ALDRLVK-TRTGKPYSAKEIEDSVLSVTESVAGSGYAFAKVEPRG-D
Q8UFL6_AGRT5    -------TISITVDEGKKYDFGNVAVESTVPGVDGS-------ELQGLVE-TRQGASYSAKEVQQSMEAISKRVAGEGYPFARVTPRG-D
Q9A711_CAUCR    -------AVTYTLEEGPKYKFGKITVETELKKLDGN-------LLAQILP-VRTGQLYEDERIEQATDALTFAAGAAGFAFVDVRPRY-V
Q63T20_BURPS    -------YLTVALHEGEPYTVSSVKLAGNLLDR-QA-------ELEKLVK-IKPGDRFSAEKLQQTTKAIVDKLGQYGYAFATVNAQP-E
Q7VYC2_BORPE    -------YITITVHEGEPYKVREVKLAGNLMGL-DS-------EINNLVE-IKPGEVFSAAKANNSAKAITNYLGDLGYAFANVNPNP-Q
P95359_NEIGO    -------TIKITVHEGGRFRWGKVSIEGDTNEVPKA-------ELEKLLT-MKPGKWYERQQMTAVLGEIQNRMGSAGYAYSEISVQP-L
Q9HXY4_PSEAE    -------YITVNINEGEKYTIRDVKLTGDLKVPEE--------EVKRLLL-VQKGQVFSRKVMTTTSDLITRRLGNEGYTFANVNGVP-E
Q9KPW0_VIBCH    -------YITLNLNEGEPYTVSKVQFRGELMGKEA--------EFTSLIP-FEIGETYNGSAVTRLEESVKKVLGESGYAYPQVRTIP-E
D151_HAEIN      -------NVTIDVNEGLQYDLRSARIIGNLGGMSA--------ELEPLLSALHLNDTFRRSDIADVENAIKAKLGERGYGSATVNSVP-D
YAET_ECOLI      -------YVTVNITEGDQYKLSGVEVSGNLAGHSA--------EIEQLTK-IEPGELYNGTKVTKMEDDIKKLLGRYGYAYPRVQSMP-E
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YTFM_ECOLI      IALGLHKAFWDIDYNSGERYRFGHVTFEG-SQI---RDEYLQNLVPFKEGDEYESKDLAELNRRLSATGWFNSVVVAPQFDKARETK---
                        370       380       390       400       410       420       430       440       450
                        |         |         |         |         |         |         |         |         |
Q4ZTK5_PSEU2    IDPRAGVADIELVYESGPRYTLGKVMFSGDAPF---DDDLLKRMVPFKENTPYDSQLIAELTQAMQGSGYFEGVRVDAAPTAAE------
Q48IF4_PSE14    IDPRAGLADIELVYDSGPRYSLGKVMFSGNAPF---DDDLLKRMVPFKENTPYDSQLIAELTQAMQGSGYFEGVRVDAAPTAAD------
Q881Y2_PSESM    IDPRAGVADIELVYDSGLRYSLGKVMFSGNTPF---DEDLLKRMVPFKENTPYDSQMIAELTQAMQASGYFEGVRVDAAPTAAE------
Q4K9Q7_PSEF5    VDPQAGVADIDLVYDSGPRYALGKVSFSGDSLF---DEDLLRRMVPFKAGAPYDSELIAELNQALQSSGYFEGVRVDAAPTAAD------
Q88IV7_PSEPK    VDPQAGVADIELVYQSGPRYRLGAVTFGGDTPL---DEDLLQRMVSFKPGTPYDSELVAELNNDLQSSGYFEGVRVDAAPTAAV------
Q9I0U1_PSEAE    IDPRAGIADIDLVYDSGQRYTFGKVSFDGDSII---EEELLRRMVPFKAGQPYDSELIAELNQNLQSSGYFEGVRVDAAPTQAQ---ADG
Q4IZH1_AZOVI    IDPVANVADIELVYASGPRYRLGDVTFQGETPF---DEAFMRRVVPFQAGDPYDSGQIAKLQDNLQTLGYFEGVRVDADPAGVS------
Q5QXU5_IDILO    IDVAENTADIEVIFDSGKRYEYGQVTFEQD-IL---DDELLHRFVPFEHGEGYSTQELSQLQVALGDSGYFSQVQVSPDWGNAE------
Q87SW5_VIBPA    VIPELNEANVRLHYDSGIRYHFGPVEITG-SQI---WENRVESMRPFEIGEPYLVSDVGEYNQNLSNTDWFSSVFVEPDLSKLEDGR---
Q8DE85_VIBVU    VAADLNQAFVRLHYDSGMRYHFGQTTITG-SQI---EENRVRSLQPFKAGDPYLVAKVGEYNQNLSNTDWFSSVFVEPDLSSLDDTR---
Q9KP31_VIBCH    VIPELNQARVILHFDSGIRYLFGATTVEG-SQI---DENRVMSLRPFKQGEPYLVSQVGEFNQNLSNTDWFSSVFVEPDLSQLDEGR---
Q5E819_VIBF1    IAPGRHQAIVRIHYDSGKRYSFGETSIDG-SQI---EEKRVRSIIPFQAGDPYLASQVGELNQYLSNTEWFSSVYVEPDIDAVGKTY---
Q6LV64_PHOPR    VAPSRNEAFITIEFSSGRRYNFGDTTFTG-SQI---DEDRLQSLIPFEKDDPYLASKLGEFNQRLSTVGWFSSIFVGGDVQHLDDG----
Q6D152_ERWCT    VMREEREAFWDIDFDSGERYRFGAVNFQG-AQI---RDDYLQNLVPFHEGDVYTSEDLGELNRRLSASGWFNSVVVSPNFEQSKSTK---
Q66F88_YERPS    VAAQLHEAFWDIDFDSGQRYRFGKVIFQG-SQI---REDYLQNLVPFHEGEFYTSDELAELNRRLAATNWFNSVVVSPDFQDAKESK---
Q83IL2_SHIFL    IALGLHKAFWDIDYNSGERYRFGHVTFEG-SQI---RDEYLQNLVPFKEGDEYESKDLAELNRRLSATGWFNSVVVAPQFDKARETK---
YTFM_ECOLI      IALGLHKAFWDIDYNSGERYRFGHVTFEG-SQI---RDEYLQNLVPFKEGDEYESKDLAELNRRLSATGWFNSVVVAPQFDKARETK---
Q7CP88_SALTY    IALGRHQAFWDIDYDSGERYRFGPVTFEG-SQI---RDEYLQNLLPFKEGDEYESKDLAELNRRLSATGWFNSVVVAPEFEKSRKTK---
Q7MYW2_PHOLL    VAKERHEAFWDFVFNSGERYHFGQVRYRG-SQI---REDYLNHLVPFSEGDFYTSEQLAELNRRLAETGWFNSAVVSPDFNSARKNGDG-
Q493V1_BLOPB    VIPSRCQSIWNIDFYSGQRYVFERIKFHG-SQI---KEDYLKNISNIRSGEYYSATSVMELNRRFNYTNWFESVSISSDYMRFQQKK---
Q9CK26_PASMU    VRPSTQQAWWRLDYDSGERYRFGEVTFEN-AQI---REDYLRNMINFNKGQPYLINDLSTLTNNYTSSNWFSSVLMQPVLDEEHK-----
Q65VW8_MANSM    VMPSTRQAWWRMDFNSGSRYRYGEISFEH-SQI---REDYLRNMLEIKSGDEYLINDVSNMTNNFSSSGWFQSVLVRPELHEDSK-----
Y698_HAEIN      ISPETHQAWWRMLFDSGVRYHYGNITFSH-SQI---RDDYLNNILNIKSGDPYLMNNLSDLTSDFPSSNWFSSVLVQPNVNHKSK-----
Q7VL30_HAEDU    VYPQNHSVDWRLAYDSGKRYRYGEINFID-NQI---KEEYLTNILKIAPQQYYYASDLSLMTADYLSSNWFSSVLVEPKLNEKAK-----
Q8D271_WIGBR    VFPNYNVARWFIVFNSGYRYRFGKINFYG-SKI---HKSYLKNISNNYYNKLYNIELISDLNNKLISTNWFNSVTVSPDIYNNSKYKN--
Q4UP09_XANC8    VTRAEHAADIDLNWDSGRRYDMGTVRFDYDYF----RPGLFEPLVYWDEGSYYHEGKLDRLRESLTKLDYFSTIDIQPKPEEADDQG---
Q8P3I6_XANCP    VTRAEHAADIDLNWDSGRRYDMGTVRFDYDYF----RPGLFEPLVYWDEGSYYHEGKLDRLRESLTKLDYFSTIDIQPKPEEADDQG---
Q9EY32_XANOR    ITRAEHAADIDLNWDSGRRYDMGKVRFDYDYF----RDGLFDPLVYWDEGSYYHEGKLDRLRESLTKLDYFSTIDIQPKPEEADDQG---
Q9PDZ7_XYLFA    VTRAAHAADIDLIWESGRRYDMGPVRFHYDYF----HEGLFNPLVYWDEGSYFHEGKLDRLRESLTKLDYFSSIDIQPKPEEADPEG---
Q5ZUJ2_LEGPH    IDEHKKSAQITLIFDTGPQYYFGQVQFDPTYI----SPQLLHRFVPFDPGQPYATDQVLKLNDYLSNSGYFSSVLVKPQITDAQT-----
Q83EW0_COXBU    VDLKNYRSTVIIVFDTGPRFLFGTTDFSATPF----HESFLHRFLQYRKGHYFSQDKVRRTREGLANSDYFSTVVVTPEPQKAQGL----

Q82VX0_NITEU    VDPDTAQATLSITIDSGPAFRYGELQITGLERY--KPDVVQNSV-PFRPGDPYRREQLLAFQAALQNSQLFNAAAVTVKPDPMQ------
Q5P7F9_AZOSE    VDPDNATVQLSVTVDSGPRFFLGPLEVSGLQDL--PPDFVERFNM-LEPGEPFDQERLLALQTVLQNSPQFGSVVVDIERDPALA-----
Q47IW2_DECAR    IDTEAHRADLRAHYDAGPRYRFGPLHVEGLQNY--SPELIERYNRSVQPGQPYREDKLNALQTTLQSTPYFASVQTMLDRDAVEVHDDGT
Q8XWT6_RALSO    VDPDENKADLSVAYDSGPAYTLGPLDVQGLSRY---PRSIIDHVNPLIVGEDYSADRLQALAAAIQGQPYFANAIVDL-------GDDPN
Q474Y0_RALEJ    IEPDELRADLSARYDSGPAYLLGPLRVSGTRRY---PEQIIYNVNPLNEGEPYRVERLLELQRAIQNQPYFSNVQVDLVTPEGTAANPPD
Q44Z18_9BURK    IDPRTQRATLAVAFDSGPTFTIGKVDVDGVRRY---PEKIVTNVNPLSEGEIYDVQRIAELQRQLQNTPYYASVAIDV-------GDDTS
Q4B7F0_BURVI    VDPRTQRATLAVTFDSGPTFTIGKLDVDGVRRY---PEKIVTNVNPLSEGEIYDAQRIAELQRQLQNTPYYASVAIDV-------GDDTS
Q3JUY6_BURPS    IDPRTRAATLALMLDSGPTFTIGALDVSGVRRY---PEKIVHDVNPLAAGEIYDAKRITELQRQLQNTPYYASVAIDV-------ADDVA
Q5F5H0_NEIG1    VNPDTATADLNVVVDSGRPIAFGDFEITGTQRY---PEQTVSGLARFQPGTPYDLDLLLDFQQALEQNGHYSGASVQADFDRLQ------
Q9JXB7_NEIMB    VNPDTATADLNVVVDSGRPIAFGDFEITGTQRY---PEQIVSGLARFQPGMPYDLDLLLDFQQALEQNGHYSGASVQADFDRLQ------
Q9JWM5_NEIMA    VNPDTATADLNVVVDSGRPIAFGDFEITGTQRY---PEQIVSGLARFQPGTPYDLDLLLDFQQALEQNGHYSGASVQADFDRLQ------
Q7P0N3_CHRVO    IDPATRRATLEVKLDSGPMIGFGKITVNGVKRY---PEKVALGMADFNPGSPYSQDKVLAYQSALEQDPHFSSVIVSADFDHIV------

Q4TKC8_9SPHN    IDHDRSEGDLTLPVEPNGKYAFGQVI--SDRPEF-LSSRHLQRIARFEAGDVYQRSLQLDLRRAITATGLVSRVSIDVQEVQPPADGDV-
Q5NNB2_ZYMMO    LDDETHTGDYTLPVNAGNVGSYGSIIVSNNKHIV-LDAKHISHIARFKEGQRYDSQMVDDLRQALAATSLFSHVSVEPIATGRKREDGS-
Q5NQ85_ZYMMO    VDHSNFTGSLVMNVETGKPQKFGEIIL-NKNSQP-LDTHHLNEIARFHPGTPYDSVLLDDLRRAVIQTSLVSTIQLKPV----PDPDHP-
Q9A7W6_CAUCR    VDHADRTVRPTFRIMAGELVRLNGVDVVTKGRT---NPEWVGRLAPWVAGDVYDPEDVAELERRLRDTAVYDSISVSLAGTDKASAEGY-
Q40G35_9RHOB    ARNGDAVLDVRVRVEPGQIVQFGTLVPRGQER---MRVERILAIAGLPQGETFSPDTLDRVEGRLQDTGVFSAIALQEGD-VGPD-----
Q5LV34_SILPO    VRHAPAELDAEIRLLPGPKLRFGQMVVQGDSD---VRPEAIARIAGFPSGQVYSPDQVQRVGTRLRRTGAFALVSLTEHENPNPD-----
Q6G573_BARHE    ADHVARLIDAQITVDPGRQAYYGPLSVRNVSKKPAVDSAYIAWMTGLKLGQRYDSEAVTKANERLVRLDVFRAVNIHEAETINPN-----
Q6G1I6_BARQU    ADHAARLVDAQIAVDSGREAYYGPLSVRNVSKRPRVDSAYIAWMTGLKPGQKYDSDGLAKANERLARLNVFRAVNIREAETINPD-----
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Q8UC83_AGRT5    ANHATNTVDITMAAEGGPVAPLGAVTVTGEKT---VDGDFIRRYSRLNGGEPYSPEKLRKAADRLRQLGVFSSLTIKEAGTLARD-----
Q98K30_RHILO    ADHATSTLDVTLTVAAGPVAGYGATTVEGTEK---VDRDFTEYMTGLKRGKQYSPQEISDARDRLLALEVFNSVTFKEADKLDAD-----

Q8YHH0_BRUME    RNFENHTISVVYSVDQGPRAYIQRIEIRGNDK---TRDYVIRREFDLNEGDAFNQVMVQRAKRRLEVLDFFQTVNISTAPGSEP------
Q8UFL6_AGRT5    RDMSGNTIGVTYIVDQGERAYVERIEIRGNTR---TRDYVIRREFDISEGDAFNQTIITAAKRRLEALGYFSKVNISTAGGSAP------
Q9A711_CAUCR    PNRETKTVDVVFQVREGPRVYVDRIDIVGNTR---TLDYVLRRELEVAEGDAYNRVLVDRSKNNMRRLGFFKEVEIEDAPGSAP------
Q63T20_BURPS    IDQATHKVGLTLVVDPSRRVYVRRINIVGNTR---TRDEVVRREMRQLESSWFDSSRLALSKDRVNRLGYFTDVDVTTVPVEGTN-----
Q7VYC2_BORPE    LDRAKHEADVTFYVDPSRRVYVRRIQIGGNTR---TRDEVVRREMRQQEAAWYDAGDIKVSRDRVDRLGYFNEVNVKTDPVPGSP-----
P95359_NEIGO    PNAGTKTVDFVLHIEPGRKIYVNEIHITGNNK---TRDEVVRRELRQMESAPYDTSKLQRSKERVELLGYFDNVQFDAVPLAGTP-----
Q9HXY4_PSEAE    AHDDDKTVSVTFVVDPGKRAYVNRINFRGNTK---TEDEVLRREMRQMEGGWASTYLIDQSKARLERLGYFKEVNVETPAVPGTD-----
Q9KPW0_VIBCH    FDDEKQQVSLVVHVEAGKRVYVRDIRFVGNNS---TRDEVLRREMRQMEGSWLNSKDIETGKTRLNRLGFFETVEVQTVRVPGSE-----
D151_HAEIN      FDDANKTLAITLVVDAGRRLTVRQLRFEGNTV---SADSTLRQEMRQQEGTWYNSQLVELGKIRLDRTGFFETVENRIDPINGSN-----
YAET_ECOLI      INDADKTVKLRVNVDAGNRFYVRKIRFEGNDT---S KDAVLRREMRQMEGAWLGSDLVDQGKERLNRLGFFETVDTDTQRVPGSP-----
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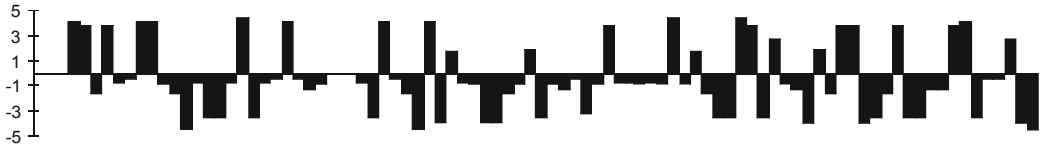


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YTFM_ECOLI      ---VLPLTGVVSPRTENTIETGVGYS---TDVGPRVKATWKKPWMNSYGHSLTTSTSISAPEQTLDFSYKMPLLKNPLEQYYLVQGGFKR

                        460       470       480       490       500       510       520       530       540
                        |         |         |         |         |         |         |         |         |
Q4ZTK5_PSEU2    -NQVIPVTVQLETRKPRTMGLGLGYS---TDVGPRGKANWTRHWANPQGHSYGFESEISAPRQNIGLWYDVPL-DPPLTDKLRYAGGYQY
Q48IF4_PSE14    -HQVIPVTVQLETRKPRTMGLGLGYS---TDVGPRGKANWTRHWANPQGHSYGFESEISAPRQNVGLWYDVPL-DPPLTDKLRYAGGYQY
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Q4K9Q7_PSEF5    -HEVIPVAVQLETRKPRTMGLGLGYS---TDVGPRVKANWTRHWVNPQGHSYGWEAEVSAPRQNVGLWYDIPL-DPPLTDKLRFAGGYQN
Q88IV7_PSEPK    -GEEVPVDVHLETRKPRTMGLGLGFS---TDVGPRGKANWTRHWVNPQGHSYGWETELSAPRQNVGLWYDIPL-DPPLTDKLRFAGGYQN
Q9I0U1_PSEAE    ARQAIPVAVRLEARKPRTMGVGLGFS---TDVGARARFNWTRHWVNAEGHSLGFESEISAPRQNVGAWYEIPL-DPPLTDKLRFTSGYQF
Q4IZH1_AZOVI    -GEMVPVLVHLQMRKPRSFGIGVGFS---TDVGPRGRANWTRHWANPQGHSYGVEMELSQPKQNVGLWYDIPL-DPPMTDKLRFVGGYQY
Q5QXU5_IDILO    -EHTVPISVSSEPNFRNQYQYGVGYG---TDTGARISASLNRRWVNTQGHQLRGMTQLSEVESRVGANYIIPG-QNPQSDYYQIRAEASD
Q87SW5_VIBPA    ---ELPIKVSLAPAAKNQIETGIGYS---TDTGVRGTLKWKKPWVSARGHSFNTALSLSKPEQTITAGYKIPL-DDVLREYYQLQFGLKH
Q8DE85_VIBVU    ---ELPMKVSLAPQAKNQIETGIGYA---TDVEFRGTLKWKKPWVNELGHSFDSSLSISKPEQTVTAGYQIPL-EDVLHDYYRIQYAMKH
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Q6LV64_PHOPR    ---TVPIAVNLAPQVRNQIETGIGYS---TDVGMRLKLNWKKPWLNSAGHSLSIKTELSKVQPKIEAAYKIPL-DDVLNDYYQVIGGIRY
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Q66F88_YERPS    ---ILPLDAVVTPRTENTVELGGGYA---TDVGPRLTASWRKPWMNSFGHSLTTTTALSAPEQTLDFSYRIPLLRNPLEQYYLIQGGFRR
Q83IL2_SHIFL    ---VLPLTGVVSPRTENTIETGVGYS---TDVGPRVKATWKKPWMNSYGHSLTTSTSISAPEQTLDFSYKMPLLKNPLEQYYLVQGGFKR
YTFM_ECOLI      ---VLPLTGVVSPRTENTIETGVGYS---TDVGPRVKATWKKPWMNSYGHSLTTSTSISAPEQTLDFSYKMPLLKNPLEQYYLVQGGFKR
Q7CP88_SALTY    ---ILPLKGVVSPRTENTIETGVGYS---TDVGPRVKASWKKPWMNSYGHSLTTSTSISAPEQVLDFSYKMPLLKNPLEQYYLVQGGFKR
Q7MYW2_PHOLL    ---MLPLEAVLTPRSKNYVELGGGYG---SDVGPRVKAKWTKPWLNSRGHSLSASINLSAPEQVIDATYKMPLKVNPLEQYYALQTGYKR
Q493V1_BLOPB    ---KIILDIFLYPCAKNNFATGSGYT---IDTGPRTKIIWKKPWINAYGHSLETNCSLSKPEQAFDLSYKVPLFSNPLEEYYLLQGGLIH
Q9CK26_PASMU    ---IVNVDVLLQPRKKNSMEVGIGWA---SDVGPRLQLGWTKPWINNRGHSFRTNLYVSAPKQTLEATYKMPLLKNPLNYYYEYSAGLEN
Q65VW8_MANSM    ---TIDLHLLMYPKKKNAMEVGLGYS---SDVGARAQIGWTRPWINNRGHSLHSDLYVSSPKQTFEITYKMPLLKNPMRYYYEFSTGIEN
Y698_HAEIN      ---TVDVEIILYPRKKNAMELGVGFS---TDGGVHGQIGWTKPWINSRGHSLRSNLYLSAPKQTLEATYRMPLLKNPLNYYYDFAVGWEG
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Q82VX0_NITEU    -HEAVPVIVTLTEAQSKHIGAGLGYS---SNNGARGEVNYRDYNFLGRAWNLSSLLRLEQKRQTFSTRVDTL----PDANHFQYSSGVRV
Q5P7F9_AZOSE    --AAVPIRVQVTEAQSRYLGFGVGYS---TNTGFRTEMSWRDVNFFDRGWELATGLRLEQRRNAAYADVFLP----PAPAGHRDSFGAAI
Q47IW2_DECAR    --ATAPILVRVRERSPHRVSFGAGAS---SNTGARVEFNYHTPNLFAQAWELDSGLRLEQKRQTAYADVFLP----PDERNRRHSVGVMA
Q8XWT6_RALSO    NPVAAPVRVRVREYPPNRITSGVGYS---TDTGAQVEGRYQYLNLFDKAWVLDTQARIEQRRQYLFGSVTLP----PDGKRYVNSLYGSL
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Q44Z18_9BURK    KPERTPVHVKVSEFPYNNIRGGVGFA---TDTGPHVQGSYTYLDTFGAAWPLTVSGRLDQIQQYGQVQLSMP----PGEKGWTNSVLASY
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Q5F5H0_NEIG1    -GDRVPVKVSVTEVKRHKLETGIRLD---SEYGLGGKIAYDYYNLFNKGYIGSVVWDMDKYETTLAAGISQP----RNYRGNYWTSNVSY
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Q5LV34_SILPO    --GTLDFNATFEDLPKRRITFGAELS---SRDGLDLSAIWIHRNLFGAAERLRLEATVRNIGG-------TEDIDGRIGFRLDRPDRLGP
Q6G573_BARHE    --GSLPLTLVVQERKPRRFGAGGSYS---TLDGAGFEAYWMHKNLFGHAERLKIETKINGVGGNKNQSYDFKSFNYLLGSTFIKPGILTP
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YAET_ECOLI      --DQVDVVYKVKERNTGSFNFGIGYG---TESGVSFQAGVQQDNWLGTGYAVGINGTKNDY-QTYAELSVTNPYFTVDGVSLGGRLFYND
```


```
YTFM_ECOLI      TD--------------LNDTESDSTTLVASRYWDLSSGWQRAINLRWSLDHFTQG-------------EITNTTMLFYPGVMISRTRS

                          550       560       570       580       590       600       610       620       630
                           |         |         |         |         |         |         |         |         |
Q4ZTK5_PSEU2    EEIA------------GTDSLSKLLTAGPEWHSKLPSGWERVISLKWQREEYRLG-------------DDAGLSTLLMPGISYSYLRS
Q48IF4_PSE14    EEIA------------GTDSLSKLLTAGPEWHSKLPSGWERVISLKWQREEYRLG-------------DDSGLSTMLMPGISYSYLRS
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Q9I0U1_PSEAE    EDLV------------DTESKLLTLGGEWHSKRPDGWQRVVSLNWMREEYKLG-------------DDSGLSSFLMPGIGYSLLET
Q4IZH1_AZOVI    EELA------------DTDTLSRLLKVGPEWHRRLDNGWQQIFSLKWQREEFRYG-------------DDNGLSNLLLPGVAYSYLES
Q5QXU5_IDILO    KDNE------------G--QTNRLYRFGASSVVAMGK-WQREYGLNWQRDDFEIG-------------ESSGTSQFLIPQASWNYVTA
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Q8DE85_VIBVU    VD--------------SRDTKSLESNLAVERHWLLDNRWHRTVFVRYLIENYEQGL-------------QDDVAQFVLPGVSFSRSRV
Q9KP31_VIBCH    LD--------------KRDTESLESNLSLERHWQLDGGWHRTVFIRYLLENYRQGL-------------QDDNSQFLLPGMTYTRTRT
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Q6D152_ERWCT    ED--------------LNDTKSDGTTVNVARYWDLSSGWQRAINLRWSLDHFTQA-------------SVTDTTMLIYPGVSINRTRQ
Q66F88_YERPS    TD--------------LNDTNSDTTTLNVARFWDLSSGWQRAINLRWSLDHFTQG-------------RVTDTTMLLYPGVSINRTRQ
Q83IL2_SHIFL    TD--------------LNDTESDSTTLVASRYWDLSSGWQRAINLRWSLDHFTQG-------------EITNTTMLFYPGVMISRTRS
YTFM_ECOLI      TD--------------LNDTESDSTTLVASRYWDLSSGWQRAINLRWSLDHFTQG-------------EITNTTMLFYPGVMISRTRS
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Q493V1_BLOPB    ED--------------THNVQSSVTTINIARYWNCSHKWQRSINVHWHFNHCSNN-------------YIIKNIMLIYPGINISRIRK
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Q8P3I6_XANCP    EQ--------------TEYIDLRNVKLTGSRSGQINERWSAIASINALRERWRFSSGSDFTD-------AVYETSTLIYPQLQANYVNV
Q9EY32_XANOR    EQ--------------TDYIDLRNVKLTGSRSGQINERWSAIASINALRERWRFSSGDDFEG-------AVYETSTLIYPQLQANYINV
Q9PDZ7_XYLFA    EQ--------------TKYIDLRNVKLSAARSGQINRHLTATASLNALRGRWRYRADDGTNT-------VAYQQSTLVYPQLEASYVDV
Q5ZUJ2_LEGPH    LN--------------YNAGYSNATLLSLSQLHNVNQFQRALSLNALYESFHYSL-------------QPNTDQFLLYPKANITFSKT
Q83EW0_COXBU    QD--------------QDTGKGRSGRLSVSYQTNVGNWQQIISLTALRERYNLRD-------------LPRTNAGVLYPSISWEHRHA

Q82VX0_NITEU    ERT-------------DIKNLQTFNQRVDFSRIRTTANSILQVGVNWQREQREPSGAP-------------KTTNETLALDLWYRYHDI
Q5P7F9_AZOSE    ERS-------------TVEGLTLMTQAVGVARTRTRGNIDTRLALRLQHEQRDPDGAP-------------STSRNSLTANWTWIRRRV
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Q8XWT6_RALSO    DRT-------------NLSGTDTRSYRSGFKQTRVRGIYETSFTIDFYYDDLRPEGAA-------------RELSKALVPGFAWTRRDV
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Q4B7F0_BURVI    TNT-------------NVSGTRIYSARVGAQRTRSGQFIDYAYSLMYYQDRLDQNSAG-------------PTTSRALVPQWSTRRNV
Q3JUY6_BURPS    TNT-------------NVSDTRIYSARVGAQRTRTGQFIDYSYSLMFYQDRLDQNGAG-------------PTTSRALVPQWATRRNV
Q5F5H0_NEIG1    NRS-------------TTQNLEKRAFSGGIWYVRDRAGIDARLGAEFLAEGRKIPGSDVD-----------LGNSHATMLTASWKRQLL
Q9JXB7_NEIMB    NRS-------------TTQNLEKRAFSGGVWYVRDRAGIDARLGAEFLAEGRKIPGSAVD-----------LGNSHATMLTASWKRQLL
Q9JWM5_NEIMA    NRS-------------TTQNLEKRAFSGGIWYVRDRAGIDARLGAEFLAEGRKIPGSDID-----------LGNSHATMLTASWKRQLL
Q7P0N3_CHRVO    KSE-------------TVQGLKTNTVDAGVWRIRSRGNIEARMGVEFLKESAEAGG--VL-----------TSDTHVTQLVYGWTQRAV

Q4TKC8_9SPHN    ETD-----------AVDARTIALRGTFERVSNLLFQKDFSWLVGAEVLATDERNVVRAAQTPNPLPR---ETFFIGSIFGEATI--DDT
Q5NNB2_ZYMMO    RYD-----------AYNGYSFSLGGSLSRQSTQLWQKRWTYSIGAELTQTNERSYDFSRS--ERLTR----SYLIAALPGQLGY--DRS
Q5NQ85_ZYMMO    KRR-----------AYDARTATLSASFERETNIIWQKKWVWSIGGQFIGSDERDNGYYTY---GAGH---HQYLIAALPGQLGY--DSS
Q9A7W6_CAUCR    DTD-----------AYNETGATVGVDLTRRRQTTAYR--TFGVSFDLSQTKEQVNRNGL-----IAG---RKLNLATLAGLAAYAWDFS
Q40G35_9RHOB    DTT----------AYLELGLNYFDDPAFTFTNVGAEVGVEHRFDRRLTGTLGLAVDYYDI---QTPFGNDQII-VLSTPLGMTW--DRR
Q5LV34_SILPO    DDS----------IFYIGEAERRNRTHYQITRAVLGIGARRTFSDTLYAEAGVGFNYGDA---DDAYGSGRKFRYFGLRSTVEW--DKR
Q6G573_BARHE    DTD---------FRAELK--VQQDVLENYTTKAIRGRLGITHIFNNNLSGQIAVEVSKGYS---RDIYFGNRDFTTIGLPFGLIY--DSR
Q6G1I6_BARQU    DTN---------FGAELK--IQQDVLDNYTTKAIRAKLGITHIFNSNLSGQTAVEGSNGYS---SDVYFGSRNFTTIGLPSGLIY--DSR
Q8YEI3_BRUME    DTD---------FVATLD--AKREVLDAYTETSINAKTGFTQIFSDELSGALYANASQGHF--VDDVFGK-RDFTTAGLEGNLLY--DSR
Q8UC83_AGRT5    RTT---------FKTSLI--AKTENPDTYRAKTLTGTAGFAYELNDTDTAAAGLEVQWADT---EDAFGK-NEYLTTSIPLEFVR--DTR
Q98K30_RHILO    ASK---------FFAGFK--TVLEHPDAYDHFSVKGSTGLSYELDKQQTVSAEVALDYSRI---TDAFGK-HTYLIASVPLQYVY--DNR

Q8YHH0_BRUME    YRVNDD------YDVEQTGGTIRFGLPITDNFSAGIAYSLVQEKYDLFRGDAENY--YAPA-----LLEAAENSPWLRSSVSYSLTYSSI
Q8UFL6_AGRT5    SKSEDY------YNYDEQGFALRVTAPITENLSTTFKYTYKQINYE-GKGDWQNNANLAEP-----YQALIRGEDWTQSILSNTLNYNTL
Q9A711_CAUCR    YDLSEF----AAYDTKSVGGDVRFGFPLTNDSSMSLRYTVRQDEVSVADSLCASG---SVS-----QILCLQRGAYITSLIGYGLRIDKR
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P95359_NEIGO    FDPRKASTSVKQYKTTTAGGGVRMGIPVTEYDRVNFGLAAEHLTVNTYNKAPKRYADFIKQ--YGKTDGADGSFKGLLYKGTVGWGRNKT
Q9HXY4_PSEAE    TDYDELDVDVASYSVNSLGAGMSIGYPISETSRLTYGLSVQRDQIDTGRYTVDEIYDF---------LDKEGDNFTN-FKASIGWSESTL
Q9KPW0_VIBCH    FEASEAGIV--DYTNESYGTSLTWGFPFDELNRFEFGIGYTHNKIGNLTPYLQVENFLAAQA---SNIDSGGNLLTDDFDINLSWTRNNL
D151_HAEIN      YDNSKSDTSS-NYKRTTYGSNVTLGFPVNENNSYYVGLGHTYNKISNFALEYNRNLYIQSM------KFKGNGIKTNDFDFSFGWNYNSL
YAET_ECOLI      FQADDADLS--DYTNKSYGTDVTLGFPINEYNSLRAGLGYVHNSLSNMQPQVAMWRYLYSMGEHPSTSDQDNSFKTDDFTFNYGWTYNKL
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YTFM_ECOLI      RGGL-MPTWGDSQRYSIDYSNTAWGSDVD-FSVFQAQNVWIRTLYD--RHRFVTRGTLGWIETGDFDK-----VPPDLRFFAGGDRSIRG

                                                                  xxxxxxxx                 xxxxxxxxx   xx
                        640       650       660       670       680       690       700       710       720
                        |         |         |         |         |         |         |         |         |
Q4ZTK5_PSEU2    DNRI-DPHQGYRLQFDTQVAKEGMLSDAN-LVHANVLLKGLTTVAQ--NHRFLGRVQFGGNLTDGYTS-----IPPSLRFFAGGDQSVRG
Q48IF4_PSE14    DNSI-DPHQGYRLQFDTQVAKEGMLSDAN-LVHANVLLKGLTTVAQ--NHRFLGRVQLGGNLTDGYTS-----IPPSLRFFAGGDQSVRG
Q881Y2_PSESM    DNRI-DPHQGYRLQFDTQVAKEGMLSDAN-LVHANVLLKGLTTVAQ--NHRFLGRVQFGGNLTDGYTS-----IPPSLRFFAGGDQSVRG
Q4K9Q7_PSEF5    DNRI-DPHNGYRIQFDTKLAKEGVGSDTN-LLYGTVLLKGLTTVWD--NHRFLGRVQFGGSATNGYKS-----IPPSLRFFAGGDQSVRG
Q88IV7_PSEPK    DNRI-DPHNGYRLQFDVQGAKEGLVSDTN-LLHGNVLLKGLTTLGH--NHRFLGRVQFGGSATNGFKNN----IPPSLRFFAGGDQSVRG
Q9I0U1_PSEAE    DNKV-DPSHGYRLQFNVKGAKEGLLADAD-VLHVDAMAKGLTSFAG--GHRLLGRLQVGGIATNDYKS-----IPPSLRFFAGGDQSVRG
Q4IZH1_AZOVI    DHPL-DPSRGYRLQVEMAAASEALLSDAN-LIYSSVMLKGLTTLAG--RHRLLGRLQFGGNFTDEYTT-----VPPSLRFFAGGDQSVRG
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Q87SW5_VIBPA    RGGS-MPMWGDKQSVTVEYGDPALLSETR-VLRLLGRSSWIRGIGE--NHRGLFRLEGGANITEEFEK-----LSPSLRFFAGGDNNIRG
Q8DE85_VIBVU    RGGS-MPSWGDKQSITFEYGDDNLLSETR-VLRVQGRTTWIRSLGE--NHRGLARLEAGANLTEEFEK-----LSPSLRFFAGGDNNLRG
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YTFM_ECOLI      RGGL-MPTWGDSQRYSIDYSNTAWGSDVD-FSVFQAQNVWIRTLYD--RHRFVTRGTLGWIETGDFDK-----VPPDLRFFAGGDRSIRG
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Y698_HAEIN      RGGS-FATWGDVQKITFDLSKRIWLSESS-FIKVQASSAWVRTYAE--NHRVVARAEIGYLHTKGIEK-----IPPTLRFFAGGDRSVRG
Q7VL30_HAEDU    DGNR-FPLWGDSQRITVNWGAKIFGSDVS-FYSWKASSTWVRTYLD--RHRLFLRGEVGQVHTKDFHQ-----IPPTLRYFAGGDMSIRG
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                xxxxx                                          xxxxxxxxxxx          xxxxxxxxxxx
                       730       740       750       760       770       780       790       800       810
                        |         |         |         |         |         |         |         |         |
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Q8UC83_AGRT5    YSYQEISPY----------------------------NAAGDATGGRSYVVGSVEARIK--VTDT-----IGLVPFFDAGVVSDGVTPDF
Q98K30_RHILO    YAYQGIGPK----------------------------DFTGQPIGGLSFFETSVEMRIA--VTDT-----IGIVPFVDAGTVSTKSVPNF

Q8YHH0_BRUME    FKFNGIGPYQDAK------------------------NGKRYWMGGTTYFSGTAEVQFPMPVLPE--SLGVRGAFFADAATL--------
Q8UFL6_AGRT5    FKNDGIGPRIGSD------------------------S-----IGGTTYFAASAEVTAPMPGVPE--DFGLRLAGFVDAGTM--------
Q9A711_CAUCR    FEIAGIGPRDISS------------------------SFNS--MGAKLYAISTFELTVPT-FLPE--QYGIKAALFSDVGTAGLLDDVDR
Q63T20_BURPS    YEPSSLGPR---D------------------------TKTNDPIGGSKMVVGNIELTFPLPGTGY--DRTLRVFTFLDGGNVWGNAPGGT
Q7VYC2_BORPE    YEGASLGPR---D------------------------RLTGDYIGGSRRMVANAQLYLPFPGASK--DRTLRWFVFTDAGQVAAGSGMSC
P95359_NEIGO    YESGTLGPKVYDE------------------------YGEKISYGGNKKANVSAELLFPMPGAKD--ARTVRLSLFADAGSVWDGRTYTA
Q9HXY4_PSEAE    FKDSTLGPRSTPSVARNPDGTPMKNQGPDSKGRYTDPDQDPEAFGGNILITGGAELLFPLPFVKD--QRQLRTVLFWDVGSTFDTDCPTK
Q9KPW0_VIBCH    FGSNSAGPKAVYRDYSGSNNGSD--------------TATDDSVGGNAIALASVELIVPTPFASEEARNQIRTSIFYDMASVWDTEFDY-
D151_HAEIN      FAYGSIGPNAIYAEYGNGSGTGTFK------------KISSDVIGGNAIATASAELIVPTPFVSDKSQNTVRTSLFVDAASVWNTKWKSD
YAET_ECOLI      FQSNTIGPKAVYFPHQASNYDPDYDYECATQDGAKDLCKSDDAVGGNAMAVASLEFITPTPFISDKYANSVRTSFFWDMGTVWDTNWD--
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YTFM_ECOLI      --------------------SDFKTGTGVGVRWESP-VGPIKLDFAVPVADKDE---HGLQFYIGLGPEL-
                                    xxxxxxxxxx       xxxxxxxxx        xxxxxxxxxxx
                         820       830       840       850       860       870       880
                          |         |         |         |         |         |         |
Q4ZTK5_PSEU2    --------------------PDLKTGVGFGVRWVSP-VGPLRLDLAHALDD-DG----GIRLHFSMGPEL-
Q48IF4_PSE14    --------------------PDLKTGVGFGVRWVSP-VGPLRLDLAHALDD-DG----GIRLHFSMGPEL-
Q881Y2_PSESM    --------------------PDLKTGVGFGVRWVSP-VGPLRLDLAHALDD-DG----GIRLHFSMGPEL-
Q4K9Q7_PSEF5    --------------------PNLKTGVGVGVRWVSP-VGPIRVDLAHAMND-DG----GIRLHFSMGPEL-
Q88IV7_PSEPK    --------------------PSLKTGVGFGVRWVSP-VGPLRLDLAKALDD-EG----GIRLHFSMGPEL-
Q9I0U1_PSEAE    --------------------PSIKTGVGFGVRWVSP-VGPLRLDLAHALDD-DG----GFRLHFSMGPEL-
Q4IZH1_AZOVI    --------------------PSLKTSAGFGVRWVSP-VGPLRVDLAHGI-D-DG----SFRLHFSMGPEL-
Q5QXU5_IDILO    --------------------EKLKQSVGFGIRWISP-IGSVRLDLAQAIDEPDK-----PWRLHFTLGPDL-
Q87SW5_VIBPA    --------------------PEWKSGAGVGIRWASP-VGPVSFDFAWGLDEKPN---NEFRIHFSLGPEL-
Q8DE85_VIBVU    --------------------PDWKTGTGLGIRWASP-VGPIRIDFAWGLDAKPG---DEFRLHFTLGPEL-
Q9KP31_VIBCH    --------------------PEWKKGVGTGIRWISP-VGPIRLDFAWGLDAAPG---DEFKIHFTLGPEL-
Q5E819_VIBF1    --------------------PDWKMGTGVGIRWASP-VGPVRLDFAWGLDAEPG---DEFKIHFTLGPEV-
Q6LV64_PHOPR    --------------------PEWYSGTGVGVRWASP-VGPISIDFAWGLEKET----DKFQLHFTLGPEL-
Q6D152_ERWCT    --------------------SNFKTGTGVGVRWASP-IGPVKLDVAMPIGDAEKK--NDVQFYIALGPEL-
Q66F88_YERPS    --------------------SDLKTGAGVGVRWASP-VGPIKLDIAAPIGDNET---HGVQFYIGLGPEL-
Q83IL2_SHIFL    --------------------SDFKTGTGVGVRWESP-VGPIKLDFAVPVADKDE---HGLQFYIGLGPEL-
YTFM_ECOLI      --------------------SDFKTGTGVGVRWESP-VGPIKLDFAVPVADKDE---HGLQFYIGLGPEL-
Q7CP88_SALTY    --------------------SDFKTGTGVGVRWASP-VGPVKLDFAVPVGDKDE---HGLQFYIGLGPEL-
Q7MYW2_PHOLL    --------------------SNFKTGVGIGVRWASP-VGPIKFDLARPIGDSES---RNIQFYIGLGSEL-
Q493V1_BLOPB    --------------------NSFKSGIGIGVRWQLP-IGPIKLDVATPLIHKGKINHHFLYLYVSLGPDL-
Q9CK26_PASMU    --------------------KELRYGAGMGVRWASP-VGAIKFDIATPVRDKDNS--KNIQFYIGLGAEL-
Q65VW8_MANSM    --------------------SELRYGAGMGVRWASP-VGAIKFDIATPIRDKDDS--KNIQFYIGLGTEL-
Y698_HAEIN      --------------------KELRYGTGVGVRWASP-VGAIKFDIATPIRDKDNS--KNIQFYIGLGTEI-
Q7VL30_HAEDU    --------------------RALHSGAGVGVRWISP-IGAIKFDIATPVKSPNNK--RGVQFYIGLGSEL-
Q8D271_WIGBR    --------------------KNLHTGIGLGIRWPAN-FGTIKLDIAKPISFFKE---NSLEFYIGLGSEL-
Q4UP09_XANC8    --------------------PDWRTGIGFGLRWRSP-VGPVRVDIAHGLNDPDS----QFQLYIDIGANL-
Q8P3I6_XANCP    --------------------PDWRTGIGFGLRWRSP-VGPVRVDIAHGLNDPDS----QFQLYIDIGANL-
Q9EY32_XANOR    --------------------PDWHTGIGFGLRWRSP-VGPVRVDIAHGLNDPDA----QFQLYIDIGANL-
Q9PDZ7_XYLFA    --------------------PDWHTGVGIGLRYRSP-VGPVRVDIARGLNSPDS----KIQLYIDIGANL-
Q5ZUJ2_LEGPH    --------------------KNIQYDIGGGLMWVSP-IGPIKVGLAQSVDNKMERIGHNPRLVISMGPDL-
Q83EW0_COXBU    --------------------EKLKIGVGPGIVLLTP-VGMFELTIANAISEPKKPWV----IQFSMGSVL-

Q82VX0_NITEU    --------------------FDLAIGYGIGARWRSP-AGPFALDLARRHDTGT------LRLHFSIAVAF-
Q5P7F9_AZOSE    --------------------FALKTGYGVGARWKSP-AGPLAVDLAYGHDEKR------LRLHFGIAIAF-
Q47IW2_DECAR    --------------------IRLAVGYGLGARWRSP-AGPIGVDLAYGERTRQ------VQLHFSLAIPF-
Q8XWT6_RALSO    --------------------RRWFNGVGVGVRWKSP-VGPIQLDLAYGIQQHQ------FRPSVALGIAF-
Q474Y0_RALEJ    --------------------VKIYNGVGIGARWRSP-VGPVQLDVGYGVQKQQ------FRPHISLGVAF-
Q44Z18_9BURK    --------------------RVFYPGVGVGARWRSP-VGPINVDVAYGLRNHS------VRPYLTLGIAF-
Q4B7F0_BURVI    --------------------RVFYPGVGIGARWRSP-VGPINVDVAYGLRNRS------VRPYLTLGIAF-
Q3JUY6_BURPS    --------------------KVFYPGAGVGVRWRSP-VGPVNFDLAYGLKNKS------VRPYLTLGIAF-
Q5F5H0_NEIG1    --------------------MKLKHGSGLGVRWFSP-LAPFSFDIAYGHSDKK------IRWHISLGTRF-
Q9JXB7_NEIMB    --------------------MKLKHGSGLGVRWFSP-LAPFSFDIAYGHSDKK------IRWHISLGTRF-
Q9JWM5_NEIMA    --------------------MKLKHGSGLGVRWFSP-LAPFSFDIAYGHSDKK------IRWHISLGTRF-
Q7P0N3_CHRVO    --------------------FQPEHANGVGVRWMSP-VAPLSFDIAKAERDGK------IRWNLSLGLAF-

Q4TKC8_9SPHN    E--------------------TVKFGAGLGLRYATG-FGPIRFDVGVPLNPGPN--DSSVAVYVSLGQAF-
Q5NNB2_ZYMMO    H--------------------NLRFGTGLGARYYTA-FGPFRIDLATPIARQPG--ESRISVYISIGQAF-
Q5NQ85_ZYMMO    S--------------------GLRLGTGLGLRYYTS-FGPMRVDLGTPLHRHAG--ENRIGVYVSLGQAF-
Q9A7W6_CAUCR    E--------------------DFRAGAGLGVRYDLG-FGPIRADIAAPLGRKG--DPKFQIYLSIGQSF-
Q40G35_9RHOB    G--------------------DWHAGAGLGVRYDTP-FGPIRVDLATPVRGEGV--GEDVYIYIGIGHAF-
Q5LV34_SILPO    G--------------------SDSHAGAGLGIRYDLGGLGPLRLDLAYPTSGNTD--G-GLQFYLGIGQAF-
Q6G573_BARHE    S--------------------KKMKWGTGIGGRYMTN-LGPLRIDLAFPLKREKG--DPRIGFYVGIGQAF-
Q6G1I6_BARQU    S--------------------QKMKWGAGLGGRYMTG-LGPLRIDLAFPLKREKG--DPRVGFYVGIGQAF-
Q8YEI3_BRUME    S--------------------EQMRVGVGGGLRYLTS-LGPIRLDVAVPLNRRSG--DPNYGFYVGIGQAF-
Q8UC83_AGRT5    S--------------------DIRAGAGIGLRYATP-FGPLRLDVAMPLEKYDG--GNNFGIYAGIGQSF-
Q98K30_RHILO    S--------------------DVKVGAGVGLRYVTP-FGPLRIDAAVPLNRDPN--DPHFGIYAGIGQAF-

Q8YHH0_BRUME    YGN--DTPDISGDD------KKLRASVGVSLMWASP-FGPLRFDYAFPVAKADT--DKVQNFNFGVSTKF-
Q8UFL6_AGRT5    YGNKVSTSQTVKDD------NSIRASAGIGVMWASP-FGPIRVDYAIPIAKEDY--DEEQRFRFGMSNTF-
Q9A711_CAUCR    QRSPGVFDPNIKDN------LGLRASAGISIDWKSP-MGPIRFDISRILSKEDY--DRTETFRFSTSTRFQ
Q63T20_BURPS    STGAN--------G--------LRYGYGIGLAWISP-IGPLKLSLGFPLQKHEG--DQYQKFQFQIGTAF-
Q7VYC2_BORPE    TAGKPDSEVEDPCG--------WRFSAGIGLSWQSP-LGPLQLSYARPLNSKSG--DDTQAFQFQIGTGF-
P95359_NEIGO    AENGNNKSVYSENAHKSTFTNELRYSAGGAVTWLSP-LGPMKFIYAYPLKKPE--DEIQRFQFQLGTTF-
Q9HXY4_PSEAE    TTTNCDGIKT----------DNLASSVGVGLTWITA-LGPLSFSLATPIKKPDN--AETQVFQFSLGQTF-
Q9KPW0_VIBCH    -RGKADYGNQYYYDYSDP--TNYRSSYGVALQWVSP-MGPLVFSLAKPIKKYEG--DDEEFFTFTIGRTF-
D151_HAEIN      KNGLESDVLKRLPDYGKS--SRIRASTGVGFQWQSP-IGPLVFSYAKPIKKYEN--DDVEQFQFSIGGSF-
YAET_ECOLI      ---SSQYSG--YPDYSDP--SNIRMSAGIALQWMSP-LGPLVFSYAQPFKKYDG--DKAEQFQFNIGKTW-
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CHAPTER 4

4. Chimeric adaptor (membrane fusion) protein reveals importance of hairpin domain for functionality in multidrug efflux pumps

4.1. Abstract

Drug efflux pumps are tripartite export machineries in the cell envelope of Gram-negative bacteria conferring multidrug resistance. Protein structures of all three components are solved, but the exact interaction sites are still unknown. We could confirm that the hybrid system composed of the *Pseudomonas aeruginosa* channel tunnel OprM and the *Escherichia coli* inner membrane complex, formed by the adaptor protein (membrane fusion protein) AcrA and the transporter AcrB of the resistance nodulation cell division (RND) family, is not functional. However, cross-linking experiments show that the hybrid exporter assembles. Exchange of the hairpin domain of AcrA by the corresponding hairpin from the adaptor protein MexA of *P. aeruginosa* restored functionality. This shows the importance of the MexA hairpin domain for functional interaction with the OprM channel tunnel. Based on these results we have modeled the interaction of the hairpin domain and the channel tunnel on a molecular level for AcrA and TolC as well as MexA and OprM, respectively. The role of this interaction for functional efflux pumps is discussed.

4.2. Introduction

MultiDrugResistance (MDR) becomes an increasing problem in medical treatment of pathogenic bacteria (Cosgrove and Carmeli, 2003). In Gram-negative bacteria multidrug

efflux pumps play an important role in resistance by expelling antimicrobial agents and thus reducing their concentration in the cells (Zgurskaya and Nikaido, 2000). Hence, they may raise antibiotic resistance by several orders of magnitude, rendering antibiotics clinically useless (Poole, 2001). An important group of multidrug efflux pumps is based on a tripartite assembly, which is composed of an outer membrane exit duct of the TolC family, an energized inner-membrane transporter and a periplasmic adaptor protein, also known as membrane fusion protein (Andersen *et al.*, 2001; Andersen, 2003). These subunits, each members of an extensive protein family, are assumed to assemble into a transporter unit during export of the substrates to span both the inner and outer membrane and create a bridge across the periplasm. Crystal structures of proteins belonging to any of the involved families are known. The trimeric outer membrane proteins TolC of *E. coli* and OprM of *P. aeruginosa* are representatives of channel tunnels, forming a canon shaped hollow conduit, which is anchored in the outer membrane by a β barrel, the channel domain and protrude into the periplasm with a 100 Å long α barrel, the tunnel domain (Koronakis *et al.*, 2000; Akama *et al.*, 2004b). AcrB of *E. coli* is the first crystallized member of the RND transporter family (Murakami *et al.*, 2002). It also forms trimers, which look like a jellyfish. The 50 Å thick membrane embedded part has a diameter of 80 Å. Periplasmic loops form the headpiece protruding 70 Å into the periplasm. The headpiece is divided into two stacked parts. The bottom part is called the pore domain. In its centre is a cavity, which is connected laterally with the periplasm by three openings, which might serve as conduits for substrate entry. The upper part of the cavity is linked by a central pore with a funnel like structure characterizing the top part of the headpiece. Recently, a direct interaction between the rim of the funnel and the periplasmic end of the channel tunnel could be detected by site directed disulfide cross-linking (Tamura *et al.*, 2005). The third component, the adaptor proteins, is also essential for a functional efflux pump. Whereas the inner and outer membrane proteins could be crystallized in their native oligomerisation state, the first member of the adaptor protein family MexA of *P. aeruginosa* crystallized as tri-decamer, which represents most likely not the native form of the protein (Akama *et al.*, 2004a; Higgins *et al.*, 2004). However, in parts the structure of the monomers verified the former structural model of adaptor proteins (Johnson and Church, 1999) showing a 47 Å long α-helical hairpin domain connected to a flattened β-sandwich domain folded like the already known lipoyl domain from biotinyl/lipoyl carrier proteins. Beside these already predicted domains, a third domain, the α/β domain could be solved showing a six-stranded β-barrel with a short α-helix. It is expected that there exists at least a fourth domain comprising the N- and C-terminus of the protein because the structure of the 28

N-terminal and 101 C-terminal residues could not be solved (Higgins *et al.*, 2004). Recently, the structure of AcrA comprising the residues 45 to 312 of the 397 amino acid protein was also solved by crystallography in a non-native oligomerisation state (Mikolosko *et al.*, 2006). The solved structural domains were highly similar to those of MexA with the exception that the α-helical hairpin has a length of 58 Å.

All models of multidrug efflux pumps propose that adaptor proteins mediate contact of the RND transporter with the outer membrane channel tunnel (Akama *et al.*, 2004a; Higgins *et al.*, 2004; Fernandez-Recio *et al.*; 2004). Biochemical experiments have supported this hypothesis showing that AcrA and TolC can be cross-linked, independent of the presence of any externally added substrate (Husain *et al.*, 2004). For the AcrAB-TolC and the MexAB-OprM efflux pumps it could be shown by pull-down assays that all three proteins form a tight association (Mokhonov *et al.*, 2004; Tikhonova and Zgurskaya, 2004). Concerning the assembly of drug efflux pumps, it is still an open question, which domains of the adaptor protein are necessary for functional interaction with the channel tunnel. Gerken and Misra have shown that point mutations in the α/β domain of AcrA could reverse the hypersensitive phenotype of a TolC mutant, which suggests that this region might have direct contact to the channel tunnel (Gerken and Misra, 2004).

In the present study, we have investigated the impact of the hairpin domain for the interaction of the periplasmic adaptor protein and the outer membrane channel tunnel. We have established a genetic background uncoupling the expression of multidrug efflux compounds from cellular regulation. By chemical cross linking we could show that a hybrid efflux pump assembles even when it is not functional. Functionality could be restored by the exchange of the hairpin domain of the adaptor protein. A model for the interaction of adaptor proteins with channel tunnels is presented.

4.3. Materials and Methods

4.3.1. Bacterial strains, plasmids and culture conditions

All bacterial strains were grown at 37°C in LB medium or on LB agar plates with appropriate antibiotics (Sigma). 100µg/ml ampicillin, 50µg/ml kanamycin and 40µg/ml chloramphenicol were used for selection of plasmids in the *E. coli* strains Top10F` (Invitrogen), AG100, DC14 (Jellen-Ritter and Kern, 2001). The *E. coli tolC*-knock-out strains AG100TC, DC14TC (this

work) were grown with reduced antibiotic concentrations (50µg/ml ampicillin, 25µg/ml kanamycin and 10µg/ml chloramphenicol).

The *tolC* knock-out in AG100 and DC14 was performed following the method of Datsenko and Wanner (2000) using a knock-out fragment produced by PCR with the primer pair TolC-KO_up and TolC-KO_down and pKD3 as template (for all primers see Table 4.1). The loss of *tolC* was verified by PCR. The resulting strains were denoted AG100TC and DC14TC, respectively.

Table 4.1: Table of Oligonucleotides

TolC-KO_up	CGCGCTAAATACTGCTTCACCACAAGGAATGCAAATGAAGAAGTGTAGGCTGGAG CTGCTTC
TolC-KO_down	CCGTTACTGGTGTAGTGCGTGCGGATGTTTGCTGAACGACTGCATATGAATATCCT CCTTA
pGEX_Nde_QC_up	CACACAGGAAACAGTACATATGTCCCCTATACTAGGTTATTGG
pGEX_Nde_QC_down	CCAATAACCTAGTATAGGGGACATATGTACTGTTTCCTGTGTG
NB_ACYC_up	GCATTAAAGCTTGTCGACGATAAGCTGTCAAACATGAG
NB_ACYC_down	CTATTTAACGACCCTGTCATGAACCGACGACCG
NB_Gex_up	CGTTATCCCCTGATTCCATGGATAACCGTATTACC
NB_Gex_down	CATTTATCAGGGTTATTGTCTCGAGAGCGGATACATATTTG
MCS_NB_up	CCGGTCGACTGCAGAGCTCGAGATCTTAAGCTTGTACAC
MCS_NB_down	CCGGGTGTACAAGCTTAAGATCTCGAGCTCTGCAGTCGA
TolC_up	CCACAAGGAATGCTCATGAAGAAATTGC
TolC_down	GTCGTCATCAGTTACGGAATTCGTTATGACCG
TolC_NB_up	GCTAACAGGAGGAATTACATATGAAGAAATTGCTCCCC
TolC_NB_down	CAGCCAAGCTCGAGACCGTTTAAACTC
OprM_up	GGGGCAATCATATGAAACGGTCC
OprM_down	GCGATCAAGCTTGGGGATCTTC
AcrAB_up	CTCGAGGTTTACTCATGAACAAAAACAGAGG
AcrAB_down	GGCATGTCTTAACGGCTCGAGTTTAAGTTAAGACTTGG
AcrA_down	CGGCTCCTGTTTAAGTTAAAGCTTGGACTGTTCAGGCTG
dHPup	GGTGTCTCTCTCTATCAGATTGATCCGGGGCTTGCGTACACCAAAGTCACCTCTCC GATTAGCGG
dHPdown	CCGCTAATCGGAGAGGTGACTTTGGTGTACGCAAGCCCCGGATCAATCTGATAGA GAGAGACACC
AcrAMexAHP_up	GTGTCTCTCTCTATCAGATTGATCCCGCCACCTACGAGGCCGAC
AcrAMexAHP_down	GACTTTGGTGTAAGCCAGGTTGATCCGCGCCTGCTCCAC

Plasmids were constructed using standard recombinant DNA techniques (Sambrook *et al.*, 1988). PCRs were performed using Pfu Turbo DNA polymerase according to the manufacturers' manual and DNA was purified using the Nucleospin Extract Kit (Macherey & Nagel). Plasmids were isolated using the Nucleospin plasmid Kit (Macherey & Nagel). All

DNA modifying enzymes were supplied by MBI Fermentas or New England Biolabs. All constructed plasmids were verified by DNA sequencing (SEQLAB).

Initially, a NdeI cutting site was introduced into pGEX-3X (Amershan Biosciences) in front of the GST gene by Quick Change PCR using the primer pair pGEX_Nde_QC_up/down. The vector pNB-pre is the product of the ligation of the SalI and PagI digested PCR product using the primer pairs NB_ACYC_up and NB_ACYC_down and pACYC184 as template and the NcoI and XhoI digested PCR product using the primer pairs NB_Gex_up and NB_Gex_down and pGEX-3XNdeI as template. A multi cloning site (MCS) cassette with 5' CCGG overhang formed by MCS_NB_up and MCS_NB_down was inserted in XmaI/CIAP (calf intestine alkaline phosphatase) digested pNB-pre vector. The orientation of the MCS cassette was verified by DNA sequencing and the resulting plasmids were denoted pNB1 and pNB2, respectively. These expression vectors comprise the origin of replication (p15rep) and the Cm resistance gene (*cat*) of pACYC184 (Rose, 1988), the *lacI*^{q} gene, the tac promoter and the glutathione S-transferase (GST) gene of pGEX-3X (Amersham Biosciences) as well as a MCS following the GST gene with cutting sites for SalI, PstI, SacI, XhoI, BglII, AflII, HindIII and XmaI (order corresponds to the orientation in pNB1).

The pNB vectors are used to express the channel tunnels. The *tolC* gene was first amplified using the primers TolC_up and TolC_down and cloned into pBADMyc/His$_C$ using NcoI/EcoRI. The resulting vector pBADtolC was used as template for amplifying *tolC* linked to the Myc/His sequence using the primers TolC_NB_up and TolC_NB_down. *tolC*-(Myc/His) was cloned into pNB1 and pNB2 using the restriction endonucleases NdeI and XhoI. pNB2oprM was constructed after amplification of *oprM* with primers OprM_up and OprM_down and ligated into a NdeI/HindIII digested pNB2tolC vector leading to *oprM* connected to the Myc/His sequence.

The primers AcrAB_up/down were used to amplify *acrAB* and the PagI/XhoI digested PCR product was inserted into the NcoI/XhoI digested pBADHis$_C$ vector resulting in pBADacrAB. For constructing AcrA mutants, *acrA* was cloned into a AflIII/HindIII digested pUC18 vector using the primers AcrAB_up and AcrA_down for NcoI/HindIII restriction sites.

pUC18acrA serves as template for the one step hairpin sequence deletion of *acrA* via Quick Change PCR using the primers dHP_up and dHP_down (Geiser *et al.*, 2001). For exchange of the AcrA hairpin against the MexA hairpin the hairpin sequence of *mexA* was amplified using AcrAMexAHP_up and AcrAMexAHP_down as primers. The PCR product itself was used as a primer pair for a Quick Change PCR of pUC18acrA. The *acrAΔHP* and *AcrAMexAHP*

sequences were excised by Bpu1102I digestion and inserted into the Bpu1102I digested pBADacrAB vector resulting in pBADacrABΔHP and pBADacrABMexAHP, respectively.

4.3.2. Determination of minimal inhibitory concentration

The bacterial strains were grown overnight in LB media supplemented with 50µg/ml ampicillin and 10µg/ml chloramphenicol. Afterwards, the cultures were diluted into 5ml fresh LB media supplemented with 50µg/ml ampicillin, 10µg/ml chloramphenicol, 0.1µM IPTG and 0.01% arabinose and grown for 3 hours at 37°C. The cultures were diluted and added to a 96-well-plate (Cellstar) using an initial concentration of 100 cells/ well. In every well 25µg/ml ampicillin, 5µg/ml chloramphenicol, 0.1µM IPTG, 0.01% arabinose and antimicrobial agents in following concentrations were given: novobiocin 0, 2.5, 5.0, 7.5, 10, 15, 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 270 µg/ml – erythromycin 0, 4.7, 9.4, 18.8, 25, 37.5, 50, 75, 100, 150, 200, 300 µg/ml – rhodamine 6G 0, 7.8, 15.6, 31.3, 62.5, 93.8, 125, 187.5, 250, 375, 500, 750 µg/ml – benzalkonium chloride 0, 0.6, 1.3, 2.5, 4.1, 5, 8.1, 10, 16.3, 20, 32.5, 40 µg/ml – sodium dodecyl sulfate (SDS) 0, 1.25, 2.5, 5, 10, 12.5, 20, 25, 40, 50, 80, 100 mg/ml. The 96-well-plates were incubated for 24h at 37°C without shaking and growth was verified using an ELISA Reader (Molecular Devices). The MIC was determined using at least seven MIC tests per strain.

4.3.3. Cross-linking experiments and protein purification

The cross-linking experiments were performed as previously described (Zgurskaya and Nikaido, 2000; Balakrishnan *et al.*, 2001) with minor changes. The bacterial strains were grown to OD_{600} 0.5 in LB with appropriate antibiotic selection (50 µg/ml ampicillin, 10 µg/ml chloramphenicol) and cultivated for further 3 hours after induction of the expression plasmids. Cells were washed with 150 mM NaCl, 20 mM phosphate buffer pH 7.4, concentrated 10-fold and treated with 1 mM dithiobis succinimidyl propionate, DSP (Fluka) cross-linker at 37°C for 30 minutes. The reaction was stopped with 40 mM Tris-HCl pH 7.4. The cells were spun down and freeze thawed repeatedly in 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 1 mM PMSF. Lysed cells were centrifuged 10 minutes at 16,000 *g* after adding 10 mM $MgSO_4$. Pellets were solubilised in 8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, 1 % Triton-100, 0.2 % Sarkosyl, pH 8.0 and incubated for 1 hour at RT. After an ultracentrifugation step at 149,000 *g* for 30 minutes the supernatants were mixed with 50 µl Ni-NTA Sepharose resin

(Amersham)/10 ml culture and incubated overnight at 4°C. Ni-NTA Sepharose resin was washed extensively with 8 M urea, 10 mM Tris, 100 mM imidazole, 100 mM NaH_2PO_4, 1 % Triton-100, pH 6.3 and 500 mM NaCl, 20 mM Tris, 100 mM imidazole, 0.1 % SDS, pH 8.0. Proteins were eluted with 8 M urea, 50 mM Tris, 2%SDS and 700 mM imidazole pH 4.5. The cross-linker was reduced with 80 mM dithiothreitol (DTT) for 30 minutes at 37°C. As a control, the same protocol was performed in parallel without adding DSP.

4.3.4. SDS-PAGE and western blotting

Prior to electrophoresis equal numbers of cells or aliquots of purified proteins were incubated in sample loading buffer for 10min at 100°C. SDS-PAGE was performed according to the Laemmli gel system (Laemmli, 1970). For Western blots, a tank blot system (Amersham Biosciences) was used as described previously (Towbin *et al.*, 1979). The anti-His and the HRP-linked anti-mouse, respectively HRP-linked anti-rabbit antibodies were purchased from Amersham Biosciences, the AcrA specific antibody from rabbit was produced by Pineda Antibody Services, Berlin. ECL Western Blotting Detection Reagents (Amersham Biosciences) were used for detection.

4.3.5. Growth curve

The bacterial strains were grown overnight in LB medium supplemented with 50µg/ml ampicillin and 10µg/ml chloramphenicol. Afterwards, the cultures were diluted into 50 ml fresh LB media supplemented with 50µg/ml ampicillin, 10µg/ml chloramphenicol, 0.1µM IPTG and 0.01% arabinose and the initial number of cells was adjusted to $5x10^7$/ml by measuring the OD_{600}. The OD_{600} was measured frequently and novobiocin (final concentration 20µg/ml) was added when OD_{600} reached 0.3-0.4.

4.3.6. Protein modeling

AcrA and MexA structures were shown using the Homology module of InsightII-software package (Accelrys) based on their structures (Mikolosko *et al.*, 2006; Akama *et al.*, 2004a). The binding sites of the channel tunnels with adaptor proteins were first identified by bioinformatical approach (see text) and then supported by molecular modeling using the CharmM module of the InsightII software package (Accelrys).

Table 4.2: Strains and Plasmids

Strain	**Characterisation**	**Reference**
AG100		Jellen-Ritter and Kern, 2001
AG100TC	Δ*tolC*	this work
DC14	*ΔacrAB::kan*	Jellen-Ritter and Kern, 2001
DC14TC	Δ*tolC*, *ΔacrAB::kan*	this work
Plasmid	**Characterisation**	**Reference**
pKD3		Datsenko and Wanner, 2000
pACYC184		Rose, 1988
pGEX-3X		Amersham
pGEX-3XNdeI	NdeI cutting site in front of GST gene	this work
pNB-pre	p15 origin of replication, *cat* from pACYC184 (Cm^R), $lacI^q$, tac promoter, GST gene from pGEX-3X	this work
pNB1	pNB-pre + MCS (SalI-PstI-SacI-XhoI-BglII-AflII-HindIII-XmaI)	this work
pNB2	pNB-pre + MCS (XmaI-HindIII-AflII-BglII-XhoI-SacI-PstI-SalI)	this work
pBADMyc/His_C	pBR322 origin, *ara*BAD promoter, *araC*, Myc/His sequence	Invitrogen
pBADtolC	pBADMyc/His_C + *tolC*	this work
pNB1tolC	pNB1 + *tolC*, Myc/His sequence, GST gene excised	this work
pNB2tolC	pNB2 + *tolC*, Myc/His sequence, GST gene excised	this work
pNB2oprM	pNB2 + *oprM*, Myc/His sequence, GST gene excised	this work
pBADHis$_C$	pBR322 origin, araBAD promoter, *araC*, Amp^R	Invitrogen
pBADacrAB	pBADHisC + *acrAB*	this work
pUC18		Yanisch-Perron *et al.*, 1985
pUC18acrA	pUC18 + *acrA*	this work
pUC18acrAΔHP	pUC18acrA without *acrA* hairpin sequence	this work
pUC18acrAMexAHP	pUC18acrA, *acrA* hairpin substituted by *mexA* hairpin sequence	this work
pBADacrABΔHP	pBADacrAB without *acrA* hairpin sequence	this work
pBADacrABMexAHP	pBADacrAB, *acrA* hairpin substituted by *mexA* hairpin sequence	this work

4.4. Results and Discussion

4.4.1. Construction of a bacterial strain with an inducible, cellular regulation independent AcrAB/TolC multidrug efflux pump

The AcrAB/TolC multidrug efflux pump is responsible for high resistance levels of *E. coli* strains against various noxious components (Sulavik *et al.*, 2001). However, it is known that expression of the three compounds is regulated by diverse factors like growth rate, cell density, or the presence of noxious compounds (Miller and Sulavik, 1996; Rahmati *et al.*, 2004; Rand *et al.*, 2002; Rosenberg *et al.*, 2003). Therefore, investigation of the interaction between components of bacterial multidrug efflux pumps by determination of the minimal inhibitory concentration (MIC) requires a genetic background, which uncouples the transcription of the three genes *tolC*, *acrA*, and *acrB* from cellular regulation mechanisms. We have constructed two plasmids, pBADacrAB with the genes *acrA* and *acrB* under the control of an arabinose inducible araBAD promoter, as well as pNB1tolC with *tolC* under the control of the IPTG inducible tac promoter. Both plasmids were transformed into DC14TC, an *E. coli* K12 strain with chromosomal deletions of *acrAB* and *tolC*. In MIC tests we determined susceptibility of strains against the antibiotics novobiocin and erythromycin, the detergents sodium dodecyl sulphate and benzalkonium chloride as well as the dye rhodamine 6G, all substances shown to be substrates of the AcrAB/TolC efflux pump (Sulavik *et al.*, 2001).

First, the amount of IPTG and arabinose were varied to find conditions in which MIC test revealed similar resistance levels for DC14TC pBADacrAB pNBtolC as obtained with wild-type strain AG100 pBAD pNB. The concentration of arabinose and IPTG for induction were set to 660 µM (=0.01%) and 0.1 µM, respectively, revealing MIC values for the reconstituted wild-type system identical to the wild-type strain AG100 pBAD pNB. Increase of the arabinose concentration to e.g. 1320 µM resulted in higher resistance levels (>300 µg/ml for novobiocin), which is in accordance with results observed before when over expressing efflux pump components (Augustus *et al.*, 2004). For novobiocin we show that the absence of either TolC or AcrA and AcrB leads to high susceptibility as observed for the strain with all three proteins missing (Table 4.3). Interestingly, cells expressing TolC but not AcrAB were slightly more resistant to novobiocin compared to cells lacking TolC. The explanation for this is that TolC could also interact with other inner membrane complexes to form efflux pumps, which are able to expel novobiocin to a certain extent. The data from Sulavik and coworkers (Sulavik *et al.*, 2001) support this interpretation, because they found that deletion of *acrAB*

together with genes of four other inner membrane complexes, resulted in a further reduction of resistance against novobiocin compared to single *acrAB* knock-out strains. It should be mentioned that in contrast to the araBAD promoter, which is tightly regulated, the tac promoter is leaky, which means that the presence of the plasmid pNB1tolC was sufficient to mediate resistance to a certain level even without IPTG induction. However, we induced the channel tunnel expression with 0.1 µM IPTG to have a defined expression of the protein. Thus, the strain DC14TC pBADacrAB pNBtolC with genes coding for the efflux pump AcrAB/TolC under control of inducible promoters provide an appropriate genetic background for studying the functionality of the protein components by MIC tests.

Table 4.3: Minimal inhibitory concentrations (MIC) of five antimicrobial agents for *E. coli* DC14TC expressing efflux pump components in various combinations (CT, channel tunnel; AP, adaptor protein; Nov, novobiocin, R6G, rhodamine 6G; Ery, erythromycin, Benz, benzalkonium chloride; SDS, sodium dodecyl sulphate; n.m., not measured)

		MIC (µg/ml)				
CT	**AP**	**Nov**	**R6G**	**Ery**	**Benz**	**SDS**
-	-	2.5	4.7	7.8	1.3	1250
TolC	-	7.5	n.m	n.m	n.m	n.m
-	AcrA	2.5	n.m	n.m	n.m	n.m
OprM	-	2.5	n.m	n.m	n.m	n.m
-	AcrAMexAHP	2.5	n.m	n.m	n.m	n.m
TolC	AcrAΔHP	7.5	4.7	7.8	1.3	1250
TolC	AcrA	210	200	500	16	100000
TolC	AcrAMexAHP	120	150	500	16	100000
OprM	AcrA	2.5	4.7	7.8	1.3	1250
OprM	AcrAMexAHP	80	100	375	10	50000

4.4.2. Components of the hybrid efflux pump AcrAB/OprM interact but are not functional

To study the interaction between the inner membrane complex and the outer membrane channel we expressed a hybrid multidrug efflux pump. Previous results show that the

MexAB/OprM efflux pump of *P. aeruginosa* can be functionally expressed in *E. coli* (Srikumar *et al.*, 1998). Thus, we used OprM to replace the channel tunnel TolC in *E. coli*. Immunoblots show that the amount of OprM is slightly reduced compared to TolC independent on the presence or absence of AcrAB (Figure 4.1). This could be explained by the fact that OprM is not a native *E. coli* protein. Cells expressing the hybrid multidrug efflux pump AcrAB/OprM were susceptible against the tested drugs with MIC values comparable to the TolC deficient strain. Even higher induction of OprM did not increase resistance of the cells (data not shown). This shows clearly that OprM is not functional with AcrAB, which supports previous reports (Tikhonova *et al.*, 2002).

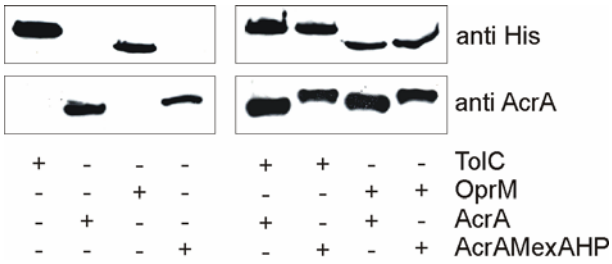

Figure 4.1: Expression of components of multidrug efflux pumps. Equal amounts of cells 3 hours after induction at 0.5 OD_{600} with 0.01% arabinose and 0.1 µM IPTG of *E. coli* DC14TC expressing different combinations of drug efflux components were subjected to SDS-PAGE and blotted. Proteins were detected by anti-His or anti-AcrA antiserum.

In the next step we tried to find out, if the hybrid efflux pump is not functional because the inner membrane complex AcrAB of *E. coli* and the channel tunnel of *P. aeruginosa* did not assemble. Co-purification of AcrA with OprM after chemical cross-linking shows that the hybrid efflux pump components are in contact (Figure 4.2). This means that the inner and outer components of the hybrid efflux pump can interact, but that this interaction is not sufficient to form a functional efflux pump.

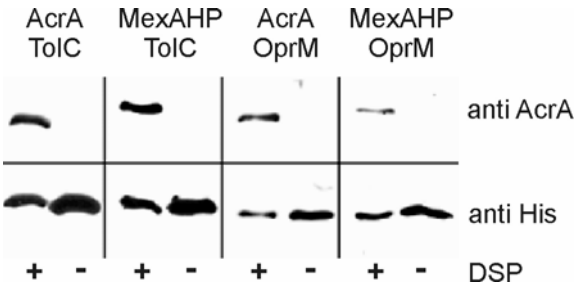

Figure 4.2: Interaction of channel tunnels and adaptor proteins proven by cross-linking. Purification of His-tagged channel proteins in presence and absence of DSP. Co-purified adaptor proteins were released by addition of DTT before SDS-PAGE and Western Blot. Proteins were detected by anti-His or anti-AcrA antiserum. AcrAMexAHP is abbreviated MexAHP.

4.4.3. Exchange of the AcrA by MexA hairpin confers functionality of the AcrAB/OprM hybrid efflux pump

Almost all models of efflux pumps assume that the hairpin subdomain of the adaptor protein provides contact with the outer membrane component (Akama *et al.*, 2004a; Fernandez-Recio *et al.*, 2004; Eswaran *et al.*, 2004). The importance of the hairpin was shown by an AcrA mutant. 74 residues (97-170), forming the hairpin, were replaced by the tri-peptide DPG resulting in the AcrAΔHP mutant. When expressed together with AcrB and TolC, the amount of AcrAΔHP detected by immunoblot was over 100-fold lower compared to AcrA wild-type (data not shown) and the construct failed to mediate resistance (Table 4.3). This suggests that deletion of the hairpin domain destabilizes the protein explaining why the efflux pump is not functional. However, we can exclude that contact with the outer membrane component is necessary for correct folding of the adaptor protein. The protein level of AcrA wild-type is not reduced in a TolC deficient background (Figure 4.1).

Based on the observation that interaction of AcrAB with OprM is not sufficient to form a functional drug efflux pump, we constructed an AcrA adaptor protein with the hairpin replaced with the MexA hairpin. We used a 218 bp long PCR fragment comprising the MexA hairpin and the adjacent regions of the lipoyl domain of AcrA as primers in a Quick Change PCR. It allowed a precise exchange of the hairpin without introducing new residues. This showed to be important because initial attempts of hairpin exchange, by ligation of *mexA* hairpin sequence into self created restriction sites in *acrA*, lead to point mutations resulting in a less stable protein and reduced MIC levels (data not shown). Western blot analysis shows that the amount of mutant protein (denoted AcrAMexAHP) is slightly reduced compared to AcrA wild-type protein, independent on the presence or absence of TolC or OprM (Figure 4.1). Unexpectedly, the hybrid protein had an apparently higher molecular weight. However, we confirmed that it is the mature form of AcrAMexAHP by over-expressing the proteins, which revealed an additional band with higher molecular weight corresponding to the unprocessed form (data not shown). Interaction of the chimeric protein with TolC as well as OprM was proven by co-purification after chemical cross-linking (Figure 4.2).

Although it is known that the hybrid efflux pump MexAB/TolC is not functional in *E. coli* (Srikumar *et al.*, 1998; Tikhonova *et al.*, 2002), co-expression of the AcrAMexAHP mutant with AcrB and TolC establishes a resistance phenotype. This means that TolC tolerates the exchange of the AcrA hairpin with the MexA hairpin. When the chimeric adaptor protein AcrAMexAHP was expressed with AcrB and OprM, the MIC values for all tested compounds were significantly increased compared to cells expressing the hybrid efflux pump

AcrAB/OprM (Table 4.3). The resistance level did not reach that of the AcrAB/TolC efflux pump. This might be explained to a certain extent by the slightly smaller amounts of OprM and AcrAMexAHP compared to TolC and AcrA, respectively. However, the experiment clearly shows that the exchange of the AcrA hairpin with the MexA hairpin makes OprM useful for a functional efflux pump.

This result was also further confirmed by susceptibility test in liquid culture. When novobiocin was added to an exponential culture expressing AcrAB/OprM (final concentration 20 µg/ml), the growth stopped immediately as observed for cells lacking at least one efflux pump component (Figure 4.3). In contrast, the addition of novobiocin to cells expressing OprM and AcrB with AcrAMexAHP led to a short lag time followed by continuous growth. Taken together, our results show that the exchange of the AcrA hairpin with that of MexA is sufficient to revert the hybrid efflux pump AcrAB/OprM from a non functional into functional export machinery.

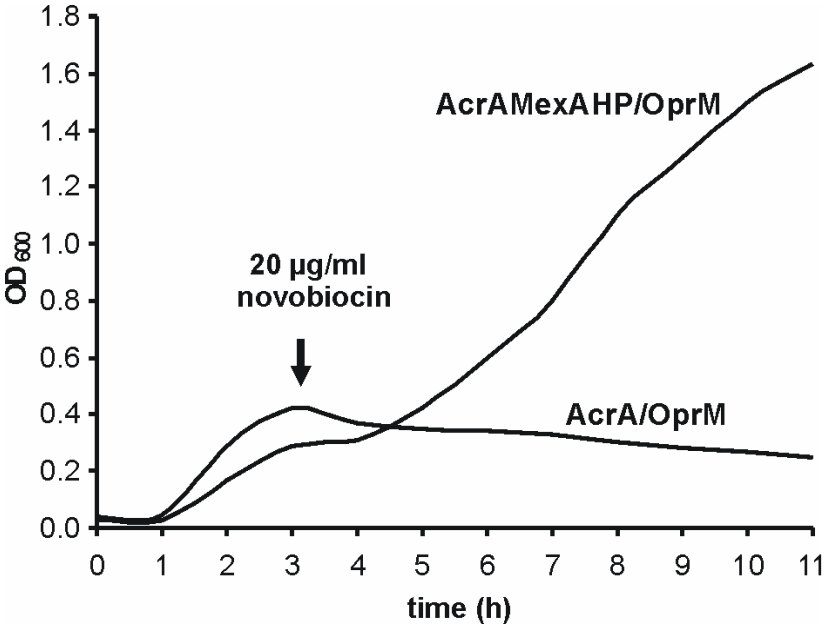

Figure 4.3: Effect of novobiocin on growth of cells expressing OprM in combination with AcrA wild-type or AcrAMexAHP. Protein expression was induced by 0.1µM IPTG and 0.01% arabinose from the beginning. Novobiocin was added to final concentration of 20µg/ml when OD_{600} reached 0.3-0.4.

4.4.4. Model for MexAHP/OprM interaction

The crystal structure of OprM shows that the periplasmic entrance of OprM is almost closed (Akama *et al.*, 2004b) and it is obvious and already shown for TolC that it is necessary that it is open to allow export (Eswaran *et al.*, 2003). It is believed that opening is induced by specific interaction with the inner membrane complex (Koronakis *et al.*, 2000; Andersen *et al.*, 2002a). Our results show that the exchange of the MexA hairpin is sufficient to convert

the non functional hybrid efflux pump AcrAB/OprM into functional export machinery. Therefore, we assume that the MexA hairpin inheres the quality to open OprM tunnel entrance, whereas interaction of AcrA hairpin with OprM is not able to induce opening. Since structural information of OprM and MexA is available several models of efflux pumps are proposed. Exceptional is the model of Gerken and Misra suggesting that the position of the adaptor protein is shifted towards the outer membrane in a way that the α/β domain of the adaptor protein interacts directly with the tunnel (Gerken and Misra, 2004). This assumption supports their interpretation of a direct interaction of these two domains. However, experiments performed by isothermal titration calorimetry speak against a direct interaction of the α/β domain with the tunnel (Touze *et al.*, 2004). Another argument against the model of Gerken and Misra is that the hairpins include the equatorial domain, therefore making a direct interaction of the hairpins with the tunnel forming helices impossible. All other models agree that the hairpin interacts with the tunnel helices. However, the models differ in the oligomeric state of the adaptor protein proposing trimers, hexamers, or nonamers (Akama *et al.*, 2004a; Fernandez-Recio *et al.*, 2004; Eswaran *et al.*, 2004). Based on a detailed analysis of the structures, we describe the first model for interaction between the channel and the adaptor protein on a molecular level. We assume that six adaptor proteins interact with the tunnel domain. This assumption is supported by biochemical evidence for a 2:1:1 stoichiometry for the MexAB/OprM efflux pump components (Narita *et al.*, 2003). Channel tunnels are evolved most likely by gene duplication events, deduced from sequence homology between the amino- and carboxy-terminal halves (Gross, 1995; Andersen *et al.*, 2000). In order to characterise residues, which are involved in interaction with MexA, we have determined the amino acids at the tunnel domain of OprM, which are conserved at both halves of the protein indicating that they are part of the MexA binding site (coloured light yellow in Figure 4.4). It is not surprising that leucine and valine residues at the interface between two adjacent helices are conserved, since they play a role in maintaining the tunnel structure. However, there are also conserved hydrophobic residues facing outwards. A region close to the periplasmic end comprises conserved aromatic residues located at helix H3 and H7. Conserved valine and leucine residues further contribute to a hydrophobic surface of H7 and H8 and H3 and H4, respectively. Charged residues, which are conserved, are rather rare. There are just three residues, all located at the more outward-facing helices H3 and H7. When looking at the residues on the MexA hairpin, it is striking that the surface of the downwards helix (Hdown) on the opposite side to the lipoyl and β-barrel domain is covered by small residues like glycine, alanine, or serine (coloured black). These residues are often found in helices with

close contact to other proteins. The only charged residues at this side of the MexA hairpin are two aspartate residues. Bringing the MexA hairpin and the OprM structure together in a way that Hdown interacts with the hydrophobic patch between the helix pairs H7/H8 and H3/H4, respectively, the structures fit perfectly in both cases. Salt bridges can be formed between the conserved positively charged residues at helix H3 and H7 and the negative charges at the hairpin surface. An arginine residue at the side of Hup comes in close vicinity to the negatively charged conserved residue at helix H3 and H7, respectively and could also form a salt bridge (Figure 4.4, circled residues). Other charged residues on the surface of the OprM tunnel come in close vicinity to asparagine and glutamine residues, allowing interaction via hydrogen bonds. It should be noted that no equally charged residues would face each other in this model, which means that there are no repelling forces. We assume that this perfect assembly of the hairpins with the tunnel helices is prerequisite to induce the conformational change which opens the tunnel entrance. Primarily, the proper interaction of MexA hairpin with the binding site formed by H7, H8, and H3 might be important to induce the outwards movement of the inner coiled coils formed by H7 and H8.

4.4.5. AcrA structure explains incompatibility with OprM

In contrast to MexA hairpins, AcrA hairpins are not able to functionally interact with OprM. To get a structural insight as to why the interaction is not functional, we first checked the compatibility of AcrA with the natural partner, TolC (Figure 4.4). Generally, the surface of AcrA hairpin is characterised by more charged residues compared to MexA. When looking at the charge distribution of the AcrA hairpin, it fits perfectly to residues at the TolC tunnel surface of helices H7, H8, and H3. Six salt bridges can be formed, when AcrA hairpin assembles with this part of the TolC tunnel (circled residues). Like Hdown of MexA, the surface of Hdown of AcrA is covered by small residues, which agrees well with the hydrophobic area between H7 and H8 of TolC. The mode of interaction of AcrA hairpin with the second binding site formed by H3, H4, and H7$^{\#}$ is slightly different. In contrast to H3, there are no charged residues at H7$^{\#}$, which could interact with AcrA. However, corresponding charged groups are found at H4, which could form salt bridges with the residues in the middle of Hup. Small residues at the surface of H7$^{\#}$ and at Hup allow a close contact of the two proteins. As in the assembly of AcrA with TolC H7/H8/H3, there are no electrostatic conflicts in the assembly with H3/H4/H7$^{\#}$, which means that the AcrA model seems reasonable.

There are two major differences between the hairpin of MexA and AcrA, which could be responsible for the fact that AcrA hairpin is not able to interact functionally with OprM. Firstly, the surface of AcrA, which interacts most likely with the tunnel helices, is covered by three more charged residues compared to MexA. The additional charges do not conflict when AcrA is assembled with the H3/H4/H7$^{\#}$ hairpin binding site of OprM. This might explain why even AcrA and OprM interact as shown by cross linking experiments. When trying to model the binding of AcrA to the H7/H8/H3 hairpin binding site of OprM, which might be crucial for opening the tunnel entrance, there is a conflict due to repelling charges (marked by a green asterisk). The second difference between the AcrA and MexA hairpins is the size of the hairpin. The AcrA hairpin is 11 Å longer due to seven additional residues per helix. Assuming that at least the H3/H4/H7$^{\#}$ hairpin binding site of OprM binds AcrA, the longer extension of the AcrA hairpin leads to a gap between the end of the tunnel and the top of the transporter.
Recently, it was shown for TolC and AcrB by disulfide cross-linking that there is close contact between the two proteins (Tamura *et al.*, 2005). Because it might be possible that the interaction between the channel and the RND transporter is also necessary for a functional efflux pump, the missing contact between OprM and AcrB could also contribute to the failure of the AcrAB/OprM hybrid efflux pump.

4.4.6. TolC is compatible with AcrAMexAHP

The interaction between the RND transporter and the channel tunnel could also explain why the hybrid efflux pump MexAB/TolC is not functional (Tikhonova *et al.*, 2002). However, in our hybrid efflux pump AcrAMexAHP/AcrB/TolC there is functional interaction between the MexA hairpin and TolC. This could be explained by the fact that the MexA hairpin is covered by very few charged residues, which do not conflict with charges on the potential hairpin binding sites of TolC, even though the hairpin is two helical turns shorter than that of AcrA. One must also consider that TolC serves as outer membrane component of diverse export machineries, requiring an interaction site which is compatible with very distinct hairpin domains. Additionally, comparing the tunnel domains of TolC and OprM it is obvious that OprM is tighter closed compared with TolC. Therefore, a less specific interaction with the hairpin could be sufficient for a functional efflux pump. Furthermore, in this hybrid efflux pump the channel tunnel TolC assembles with its native RND transporter AcrB, which might facilitate tunnel opening mediated by MexA hairpin.

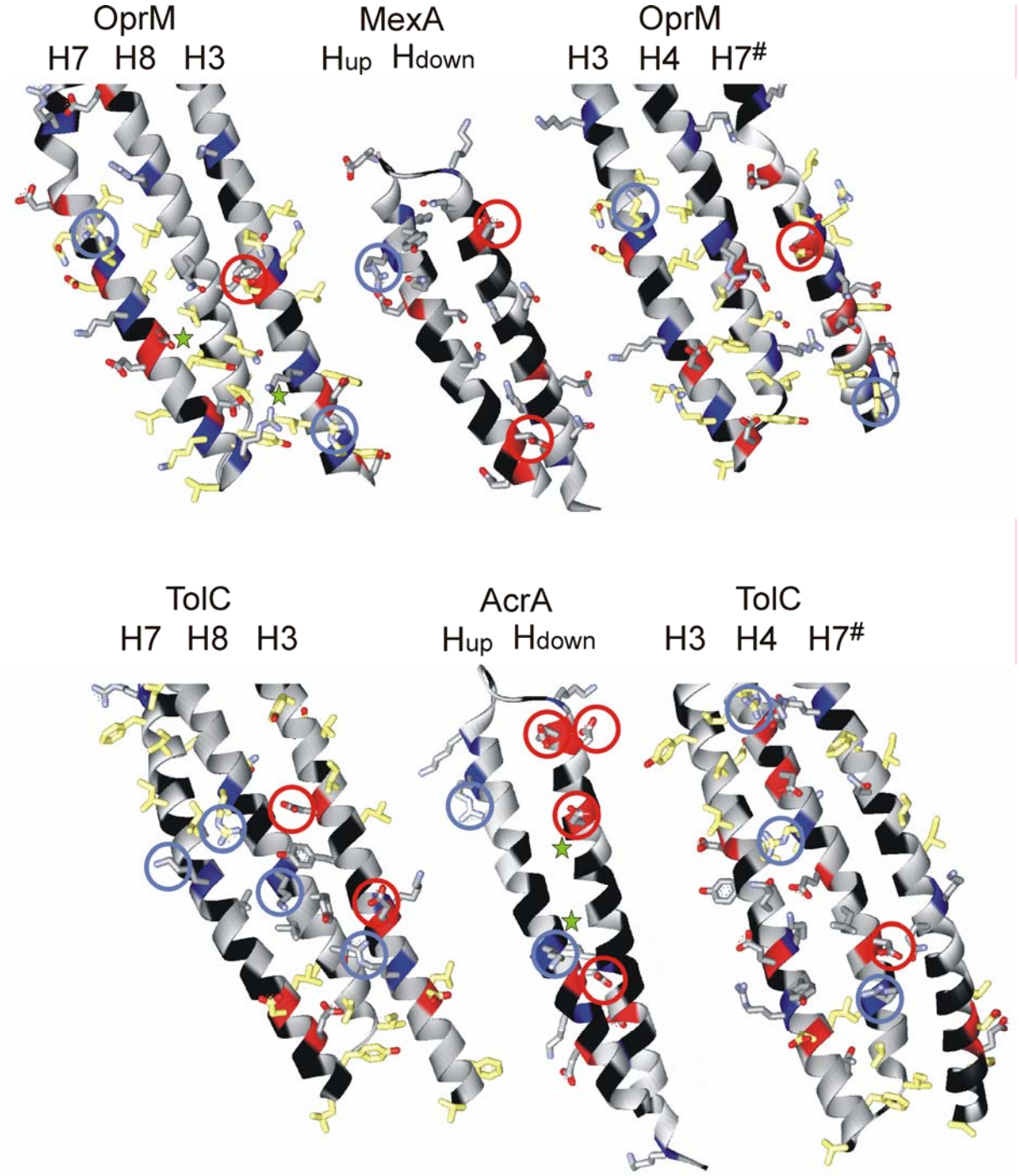

Figure 4.4: Structures of channel tunnel helices forming the tunnel domain below the equatorial domain and adaptor protein hairpins. The channel tunnel structures are split for clarity. The helix pairs H7/H8 and H3/H4 are shown with the adjacent helix of the same protomer (H3) and of the adjacent protomer ($H7^{\#}$). The adaptor protein hairpins are shown in an orientation with lipoyl and β-barrel domain facing backwards. Side chains of residues, which are conserved in both halves of channel tunnels, are coloured in light yellow. The helices are coloured black at positions with small side chains (glycine, alanine, and serine), red at positions with negatively, and blue with positively charged residues. The circles point out residues, which might form salt bridges for channel tunnel/adaptor protein interaction. Residues, which conflict in the assembling of AcrA with OprM, are marked by green asterisks. For further explanation see text. PDB files used: 1WP1 (OprM), 1EK9 (TolC), 1VF7 (MexA), 2F1M (AcrA).

4.5. Conclusion

We have elucidated the role of the hairpin domain for the assembling and function of efflux pumps. However, the mechanism of drug efflux remains yet to be elucidated. Prerequisite is the understanding of the efflux pump assembly. Integrating our experimental data and structural analysis we have presented the first detailed model of the interaction between the adaptor protein and channel tunnel of two different efflux pumps. This will help to design mutants, which will covalently link outer and inner membrane components making co-purification and crystallization of the complete drug efflux machinery possible. Knowledge of the binding sites will also help to design efflux drug inhibitors needed to combat multi-resistant bacterial strains.

CHAPTER 5

5. Summary

5.1. Summary

In recent times the family of Omp85 proteins came into the focus of research, since Omp85 of *Neisseria meningitidis* and its ortholog YaeT of *Escherichia coli* were shown to be essential for outer membrane biogenesis. Both proteins belong to one of six clusters which comprise the Omp85 family and mediate insertion of outer membrane proteins into the outer membrane. Interestingly, there exists a homologue to YaeT in *E. coli* and almost all proteobacteria, denoted YtfM. These proteins of the Omp85 family belong to a separate cluster and their function is unknown until today.

In this study YaeT and YtfM were investigated by using biochemical and electrophysiological methods as well as bioinformatical and structural analysis. In addition, knock-out strains were constructed to further study the relevance of these proteins *in vivo*. The prediction that Omp85 proteins are composed of two domains, a periplasmic amino-terminal POTRA (polypeptide translocation associated) domain and a carboxy-terminal domain anchoring these proteins in the outer membrane, was confirmed by the construction of mutants. It could be shown that the carboxy-terminal part of the proteins is able to insert into the outer bacterial membrane, even if the POTRA domain is removed. Furthermore, pore-forming activity in the black-lipid bilayer was observed for both full-length proteins as well as their carboxy-terminal membrane located parts. The channels formed by both proteins in the black lipid bilayer showed variable single channel conductance states rather than a defined value for conductance. In 1M KCl, e.g. YaeT forms pores with a channel conductance of 100 to 600 pS containing a most abundant value at 400 pS. This variability is at least reasonable for YaeT due to a prerequisite

flexibility of its channel for OMP insertion. YaeT was identified to form a cation selective, YtfM an anion selective channel, which is less pH dependent than YaeT. Another feature of the YaeT channel is that its selectivity and conductance is influenced by charged detergent molecules indicating an accumulation of these molecules in hydrophobic pockets inside the compact channel. YaeT revealed heat-modifiable mobility in SDS-PAGE which is characteristic for β-barrel OMPs, whereas YtfM did not show this behaviour. This result could be explained by sequence alignment and structural comparison of YaeT and YtfM via CD and FTIR spectra displaying much higher β-strand content for the carboxy-terminal part of YaeT compared to YtfM.

Since the carboxy-terminal parts were shown to have pore forming ability and are inserted in the OM *in vivo*, the substitution of the essential protein YaeT by its carboxy-terminal mutant was attempted in a *yaeT* knock-out strain. The carboxy-terminal half of YaeT was not sufficient to compensate depletion of the full-length protein indicating an important role of the amino-terminus for cell viability. In contrary, YtfM is shown to be a non-essential protein and lack of YtfM had no effects on the composition and integrity of the OM. However, chromosomal deletion of *ytfM* remarkably reduced the growth rate of cells.

This study provides the first detailed investigation of the structure of YaeT and describes its electrophysiological behaviour, which could be a basis for further studies of YaeT and its substrate proteins. Furthermore, YtfM was characterised and its *in vivo* function was investigated revealing YtfM as the second Omp85 family protein of importance in *E. coli*.

In a second part of this study assembly and function of multidrug efflux pumps were investigated. Drug efflux pumps are tripartite export machineries in the cell envelope of Gram-negative bacteria conferring multidrug resistance and therefore causing severe problems for medical treatment of diseases. Protein structures of all three efflux pump components are solved, but the exact interaction sites are still unknown. Assembly of a hybrid exporter system composed of the *Pseudomonas aeruginosa* channel tunnel OprM, the *E. coli* adaptor protein AcrA and its associated transporter AcrB could be shown by chemical cross-linking, even though this efflux pump is not functional. Exchange of the hairpin domain of AcrA by the corresponding hairpin from the adaptor protein MexA of *P. aeruginosa* restored functionality tested by antibiotic sensitivity assays. This shows the importance of the MexA hairpin domain for functional interaction with the OprM channel tunnel. Interestingly, the hybrid protein was also able to assemble with TolC as outer membrane component to form a functional efflux pump indicating a higher flexibility of TolC compared to OprM concerning

interaction partners. Based on these results, an interaction model of the hairpin domain and the channel tunnel on molecular level for AcrA and TolC as well as MexA and OprM, respectively, is presented. This model provides a basis for directed mutagenesis to reveal the exact contact sites of the hairpin of the adapter protein and the outer membrane component.

5.2. Zusammenfassung

Seitdem vor kurzem gezeigt wurde, dass die Proteine Omp85 aus *Neisseria meningitdis* und YaeT aus *Escherichia coli* essentiell für die Biogenese der Außenmembran Gram-negativer Bakterien sind, rückte die Familie der Omp85 Proteine ins Zentrum des wissenschaftlichen Interesses. Beide Proteine gehören zu einer von sechs Untergruppen der Omp85 Proteinfamilie und vermitteln den Einbau von integralen Proteinen in die äußere Membran. In fast allen Proteobakterien existiert ein weiteres Protein der Omp85 Familie. Dieses Protein, in *E. coli* YtfM genannt, gehört zu einer anderen Untergruppe und seine Funktion ist bis heute gänzlich unbekannt.

In dieser Arbeit wurden YaeT und YtfM biochemisch und elektrophysiologisch charakterisiert, sowie bioinformatisch und strukturell analysiert. Des Weiteren wurden Bakterienstämme mit chromosomalen Deletionen der beiden zugehörigen Gene hergestellt, um die Relevanz der beiden Proteine *in vivo* zu untersuchen. Für Omp85 aus *N. meningitdis* wurde vorhergesagt, dass das Protein aus zwei strukturellen Untereinheiten besteht, einer periplasmatischen amino-terminalen Domäne, der POTRA – („polypeptide-transport-associated“ -) Domäne und einer carboxy-terminalen Domäne, die das Protein in der Außenmembran verankert. Diese Vorhersagen wurden für die Omp85 Homologen YaeT und YtfM mit Hilfe von geeigneten Mutanten bestätigt. Es konnte gezeigt werden, dass der carboxy-terminale Teil beider Proteine, YaeT und YtfM, auch bei Fehlen der amino-terminalen Domäne noch in der äußeren Membran zu finden ist. Bestätigt wurde dieses Ergebnis durch „Black Lipid Bilayer“ Experimente. Für beide nativen Proteine, sowie deren carboxy-terminale Mutanten, konnte der Einbau in eine künstliche Membran und Porenbildung gezeigt werden. Anders als bei herkömmlichen Porinen, die normalerweise eine sehr gut definierte Leitfähigkeit haben, musste man den beiden untersuchten Proteinen eher eine Leitfähigkeitsspanne zuordnen. Bei Messungen in 1M KCl wurden für YaeT beispielsweise Poren mit einer Leitfähigkeit von 100 bis 600 pS aufgezeichnet, wobei 400 pS der am häufigsten vorkommende Wert ist. Diese Variabilität ist bei YaeT zumindest dadurch begründbar, dass für den Einbau anderer Proteine in die äußere Membran ein flexibler Kanal mit einer möglichen lateralen Öffnung notwendig ist. Die YaeT Poren sind kationenselektiv, für YtfM wurde ein anionenselektiver Kanal nachgewiesen, welcher zusätzlich im Vergleich zu YaeT weniger pH anfällig ist. Weiterhin haben die elektrophysiologischen Experimente gezeigt, dass die Ionenselektivität und Leitfähigkeit von YaeT durch Detergenzmoleküle

beeinflussbar ist. Dies lässt hydrophobe Bereiche im Kanalinnern vermuten, an denen sich die Moleküle mit ihrem unpolaren Teil anlagern und dann durch ihre polaren Köpfe die Nettoladungen im Kanalinnern verändern. Mittels Gelelektrophorese konnte für YaeT das typische Laufverhalten von Außenmembranproteinen gezeigt werden. Wenn YaeT ungekocht auf ein SDS-Gel aufgetragen wurde, wanderte es schneller, als wenn man es vorher auf 100°C erhitzte, was für eine kompakte Struktur spricht. Für YtfM wurde dieses Verhalten nicht festgestellt. Die Erklärung für diesen Unterschied lieferten ein Sequenzvergleich beider Proteine, sowie die strukturelle Untersuchung mittels CD- und FTIR-Spektroskopie, welche im Vergleich zu YtfM einen deutlich höheren β-Faltblatt-Anteil für den carboxy-terminalen Teil von YaeT ergaben.

Da der carboxy-terminale Teil der Proteine in der Außenmembran zu finden ist und porenformende Aktivität besitzt, wurde versucht, YaeT durch seine carboxy-terminale Mutante in einem Knock-out Stamm zu ersetzen. Dies schlug jedoch fehl, was auf eine bestimmte Funktion der amino-terminalen Hälfte *in vivo* hindeutet. Im Vergleich zu YaeT ist YtfM kein essentielles Protein und sein Fehlen beeinflusst die Zusammensetzung und die Funktion der äußeren Membran nicht. Die chromosomale Deletion von *ytfM* führte jedoch zu einer deutlichen Reduktion der Wachstumsrate.

Diese Arbeit zeigt die erste genaue Untersuchung der Struktur von YaeT, sowie dessen elektrophysiologische Eigenschaften, und kann als Grundlage für weitere Studien der Wechselwirkung von YaeT mit anderen Proteinen dienen. Weiterhin wurde YtfM charakterisiert und auf seine Funktion *in vivo* untersucht, was die Wichtigkeit eines zweiten Omp85 Proteins in *E. coli* veranschaulicht.

Im zweiten Teil der Arbeit wurde der Zusammenbau und die Funktionalität von Multidrug Efflux Pumpen untersucht. Efflux Pumpen sind dreiteilige Exportapparate in der Zellhülle von Gram-negativen Bakterien, die den Organismen Resistenz gegen diverse Stoffe verleihen und somit große Probleme bei der Behandlung von Infektionskrankheiten schaffen. Mittlerweile sind die Strukturen aller drei Pumpenkomponenten gelöst, ihre Interaktionsstellen sind allerdings noch nicht bekannt. Mit einem chemischen „Cross-linker“ konnte der Zusammenbau eines hybriden Exportsystems bestehend aus der Außenmembrankomponente OprM aus *Pseudomonas aeruginosa*, dem Adapterprotein AcrA aus *E. coli*, sowie dessen zugehörigem Innenmembrantransporter AcrB, nachgewiesen werden. Der Zusammenbau ist insofern erstaunlich, da diese hybride Effluxpumpe nicht funktionell ist. Die Funktionalität dieser Effluxpumpe, nachgewiesen durch

Antibiotikasensitivitätstests, konnte jedoch durch den Austausch der „Hairpin“-Domäne von AcrA durch den „Hairpin“ von MexA wieder hergestellt werden. Dieses Ergebnis zeigt deutlich die Wichtigkeit dieser Haarnadelstruktur für die funktionelle Interaktion mit der Außenmembrankomponente OprM. Interessanterweise konnte das hybride Adapterprotein eine funktionelle Effluxpumpe mit TolC als Außenmembrankomponente bilden, was für eine höhere Flexibilität von TolC im Gegensatz zu OprM bezüglich der Interaktionspartner spricht. Aufgrund der oben genannten Ergebnisse wurde ein Interaktionsmodell der „Hairpin“-Domäne von AcrA bzw. MexA mit den Außenmembranproteinen TolC und OprM auf molekularer Ebene erstellt. Dieses Modell kann nun als Vorlage für zielgerichtete Mutationen dienen, um die Interaktionsstellen des Adapterproteins mit der zugehörigen Außenmembrankomponente genau zu beschreiben.

CHAPTER 6

6. Appendix

6.1. References

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