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Assembly of outer-membrane proteins in bacteria and mitochondria

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The cell envelope of Gram-negative bacteria consists of two membranes separated by the periplasm. In contrast with most integral membrane proteins, which span the membrane in the form of hydrophobic α-helices, integral outer-membrane proteins (OMPs) form β-barrels. Similar β-barrel proteins are found in the outer membranes of mitochondria and chloroplasts, probably reflecting the endosymbiont origin of these eukaryotic cell organelles. How these β-barrel proteins are assembled into the outer membrane has remained enigmatic for a long time. In recent years, much progress has been reached in this field by the identification of the components of the OMP assembly machinery. The central component of this machinery, called Omp85 or BamA, is an essential and highly conserved bacterial protein that recognizes a signature sequence at the C terminus of its substrate OMPs. A homologue of this protein is also found in mitochondria, where it is required for the assembly of β-barrel proteins into the outer membrane as well. Although accessory components of the machineries are different between bacteria and mitochondria, a mitochondrial β-barrel OMP can be assembled into the bacterial outer membrane and, vice versa, bacterial OMPs expressed in yeast are assembled into the mitochondrial outer membrane. These observations indicate that the basic mechanism of OMP assembly is evolutionarily highly conserved.

Introduction

The cell envelope of Gram-negative bacteria is composed of two membranes, the inner membrane and the outer membrane, which are separated by the periplasm containing the peptidoglycan layer. While the inner membrane is a phospholipid bilayer constituted of glycerophospholipids, the outer membrane is highly asymmetrical, containing glycerophospholipids in the inner leaflet and lipopolysaccharides (LPSs) exposed to the cell surface (Fig. 1). The outer membrane functions as a permeability barrier protecting the bacteria against harmful compounds, such as antibiotics and bile salts, from the environment. Most nutrients pass this barrier via a family of integral outer-membrane proteins (OMPs), collectively called porins (Fig. 1). These trimeric proteins form open, water-filled channels in the outer membrane, which allow for the passage of small hydrophilic solutes, such as amino acids and monosaccharides, via passive diffusion (Nikaido, 2003). Other OMPs have more specialized transport functions, such as the secretion of proteins and the extrusion of drugs, or function as enzymes or structural components of the outer membrane (Koebnik *et al.*, 2000). Besides integral OMPs, the membrane also contains lipoproteins, which are attached to the membrane via an N-terminal lipid moiety.

All constituents of the outer membrane are synthesized in the cytoplasm or at the inner leaflet of the inner membrane. An area of intense research is how these components are transported and assembled into the outer membrane. An obvious model organism to study such fundamental questions is *Escherichia coli*, but *Neisseria meningitidis* has also proven to be a very suitable organism to address these questions. *N. meningitidis* normally resides as a commensal in the nasopharynx but occasionally causes sepsis and meningitis. Besides generally useful features, such as a relatively small genome size (~2200 genes) and natural competence and recombination proficiency, which facilitate the construction of mutants, the organism has several properties particularly useful for the study of outer membrane biogenesis. Firstly, in contrast with *E. coli*, *N. meningitidis* is viable without LPS (Steeghs *et al.*, 1998). Such mutants defective in LPS biosynthesis still produce an outer membrane into which OMPs are assembled (Steeghs *et al.*, 2001). Since *N. meningitidis* is viable without LPS, the genes encoding the components of the LPS transport route can be knocked out and the properties of such mutants can be studied (Bos *et al.*, 2004; Tefsen *et al.*, 2005). Secondly, studies on OMP assembly in *E. coli* are thwarted by a stress response that is activated when unfolded OMPs accumulate

Abbreviations: LPS, lipopolysaccharide; OMP, outer-membrane protein; POTRA, polypeptide transport-associated [domain]; PPIase, peptidyl-prolyl *cis/trans* isomerase.

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in the periplasm. Activation of this stress response, which is dependent on the alternative σ factor σ^E, results in the increased production of periplasmic chaperones that aid in OMP assembly and of the protease DegP that degrades these unfolded OMPs (Ruiz & Silhavy, 2005). In addition, small regulatory RNAs are produced that inhibit the translation of the mRNAs for OMPs by stimulating their decay (Johansen *et al.*, 2006; Papenfort *et al.*, 2006). Thus, OMP synthesis is inhibited under these conditions until unfolded OMPs are cleared from the periplasm. Consequently, mutations resulting in OMP assembly defects do not normally result in the extensive accumulation of unfolded OMPs in the periplasm, but in decreased OMP levels (Chen & Henning, 1996; Sklar *et al.*, 2007b). Since other signals such as altered LPS structure (Tam & Missiakas, 2005), and even cytoplasmic signals (Costanzo & Ades, 2006) can also trigger the σ^E-dependent stress response, decreased OMP levels do not necessarily reflect an OMP assembly defect. Since this σ^E-dependent stress response is absent in *N. meningitidis* (Bos *et al.*, 2007a), unfolded OMPs normally accumulate in the periplasm of assembly-defective *N. meningitidis* mutants, which facilitates these studies. This paper focuses on the current knowledge of OMP biogenesis in bacteria and on the evolutionary conservation of the OMP assembly machinery.

Structure of bacterial OMPs

Whereas most integral membrane proteins, including bacterial inner-membrane proteins, span the membrane in the form of α-helices entirely composed of hydrophobic amino acids, bacterial OMPs present an entirely different structure (Fig. 1). These proteins form β-barrels composed of antiparallel amphipathic β-strands (Koebnik *et al.*, 2000). The hydrophobic residues in these β-strands are exposed to the lipid environment of the membrane, whereas the hydrophilic residues point towards the interior of the protein, which is the aqueous channel in the case of porins. These β-barrel structures are very stable, usually withstanding incubation in 2 % SDS (i.e. as present in standard sample buffer for SDS-PAGE) at ambient temperature. This property explains the heat-modifiable behaviour of many OMPs in SDS-PAGE analysis: the native form of these proteins migrates differently in the gel compared with the heat-denatured form (Dekker *et al.*, 1995; Nakamura & Mizushima, 1976). Also, natively folded OMPs are usually highly resistant to proteases. Heat modifiability and protease resistance are facile parameters to probe the folding of OMPs into their native configuration.

Transport of OMPs across the bacterial inner membrane

The unusual structure of bacterial OMPs is probably imposed by their biogenesis pathway. OMPs are synthesized in the cytoplasm as precursors with an N-terminal signal sequence, which marks them for transport across the inner membrane via the Sec system (Fig. 2). The protein-conducting channel of the Sec system, which is composed of the integral membrane proteins SecY, SecE and SecG (Driessen & Nouwen, 2008), releases OMPs and periplasmic proteins at the periplasmic side of the membrane. The SecYEG translocon is also implicated in the assembly of integral inner-membrane proteins. When large hydrophobic protein segments are inserted into the translocon, the channel opens laterally to allow for the insertion of these proteins into the inner membrane (Fig. 2; Driessen &

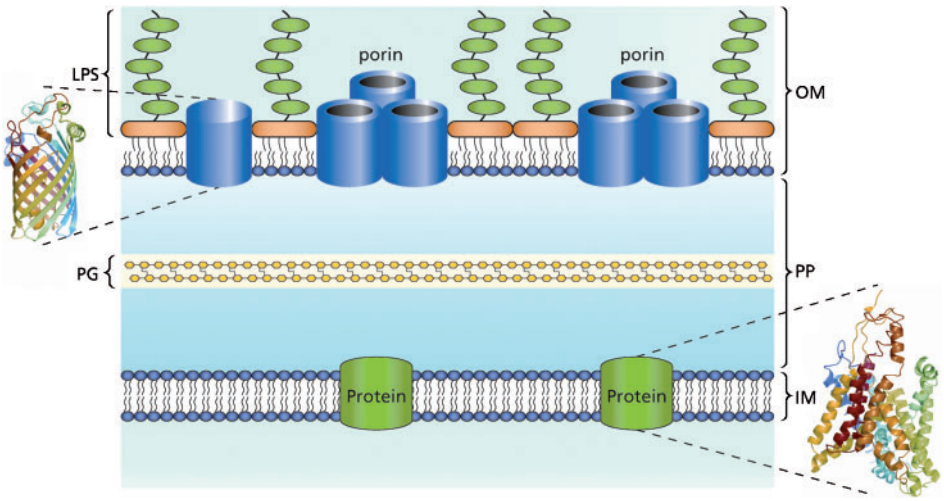

Fig. 1. Structure of the Gram-negative bacterial cell envelope. OM, Outer membrane containing LPS in the outer leaflet of the bilayer and porins as the major protein components; PP, periplasm containing the peptidoglycan layer (PG); IM, inner membrane. Examples of a typical β-barrel structure of an OMP, i.e. the LPS deacylase LpxR from *Salmonella typhimurium* (PDB file 3FID) (Rutten *et al.*, 2009), and of a typical α-helical inner-membrane protein, i.e. the SecYE translocon of *Thermus thermophilus* (PDB file 2ZQP) (Tsukazaki *et al.*, 2008), are shown on the left and the right, respectively.

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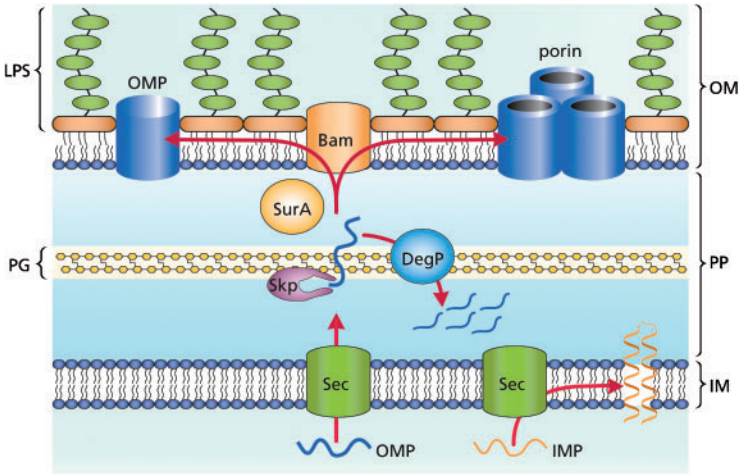

Fig. 2. Model for the biogenesis of bacterial OMPs. Porins and other OMPs are synthesized in the cytoplasm as precursors with a signal sequence, which is cleaved off during or immediately after their transport to the periplasm via the Sec translocon. While still engaged with the Sec translocon, the nascent OMPs are bound by the chaperone Skp, which prevents their aggregation in the periplasm. Folding is initiated when they arrive at the Bam complex in the outer membrane and is, at least for some OMPs, aided by the chaperone SurA. The Bam complex mediates their assembly into the outer membrane. How exactly the nascent OMPs pass the peptidoglycan layer is unknown, but the Bam complex components extend into the periplasm (Fig. 3a, b) and some of them might modulate the peptidoglycan to facilitate the passage of the OMPs. The main function of DegP is probably the degradation of misfolded OMPs. The Sec complex also processes nascent inner-membrane proteins (IMPs) and opens laterally to insert them into the inner membrane. OM, PP, PG and IM are defined in the legend to Fig. 1.

Nouwen, 2008). Thus, the presence of similar hydrophobic segments in OMPs would prevent them from reaching their final destination, while the amphipathic β-strands that constitute the transmembrane segments of OMPs are compatible with transport via the SecYEG translocon to the periplasm. Indeed, the insertion of hydrophobic segments into the outer membrane porin PhoE of *E. coli* was shown to affect the biogenesis of the protein (Agterberg *et al.*, 1990).

Transport of OMPs through the periplasm

In *E. coli*, three chaperones have been reported to guide nascent OMPs during their intermediate periplasmic stage (Fig. 2): Skp, SurA and the protease DegP, which also has chaperone qualities (Spiess *et al.*, 1999). Recent structural analysis showed that DegP in its activated state can form large oligomeric cage-like structures of 12 or 24 subunits that could harbour a folded OMP in its central cavity without degrading it (Krojer *et al.*, 2008). None of these chaperones is essential in *E. coli*, but double mutants show synthetic, often lethal, phenotypes, suggesting redundancy in chaperone activities. Detailed analyses of single and double mutants suggested the existence of two parallel pathways of chaperone activity in the periplasm, a major SurA-dependent route and an alternative Skp- and DegP-dependent route that deals with substrates that fall off the SurA pathway (Rizzitello *et al.*, 2001; Sklar *et al.*, 2007b). However, *skp* and *degP* mutations have also been reported to show a synthetic phenotype (Schäfer *et al.*, 1999), which is inconsistent with the idea that these chaperones operate within the same pathway. Furthermore, a recent proteomic analysis indicated that SurA has only a few substrates, including the OMP LptD, which is involved in LPS biogenesis, and that the reduced levels of many other OMPs in *surA* mutants may be solely a consequence of activation of the σ^E-dependent stress response (Vertommen *et al.*, 2009). The study of Vertommen and colleagues argues against the hypothesis that the SurA pathway is the major periplasmic chaperone pathway for OMPs in the periplasm.

An alternative explanation for the synthetic phenotypes of double chaperone mutants is that these proteins have different, but complementary functions (Bos *et al.*, 2007a; Walther *et al.*, 2009b). Skp selectively binds unfolded OMPs (Chen & Henning, 1996; de Cock *et al.*, 1999), presumably while they are still engaged with the Sec translocon (Harms *et al.*, 2001). The crystal structure of this trimeric protein has been solved (Korndörfer *et al.*, 2004; Walton & Sousa, 2004); it resembles a jellyfish that can hold nascent OMPs between its tentacles, thereby preventing their aggregation in the aqueous environment of the periplasm (Walton *et al.*, 2009). SurA appears to play a role in the folding of OMPs into their native configuration (Lazar & Kolter, 1996; Rouvière & Gross, 1996). SurA is a peptidyl-prolyl *cis/trans* isomerase (PPIase) with two PPIase domains, which, however, appear to be dispensable for the chaperone qualities of the protein (Behrens *et al.*, 2001). In this model, Skp is a 'holding chaperone' that prevents folding and aggregation of OMPs in the periplasm, whereas SurA acts as a 'folding chaperone' that assists in the folding of OMPs once they arrive at the assembly machinery in the outer membrane.

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The synthetic lethality of a *skp surA* double mutant is explained by an increased requirement for a holding chaperone when the folding of the OMPs is compromised by the absence of SurA, and, vice versa, efficient folding is increasingly important when the holding chaperone Skp is absent. The main role of DegP in this model is to prevent toxic accumulation of misfolded OMPs in the periplasm, either by degrading them (Fig. 2) or by sequestering them within the multimeric cage, thereby preventing them from engaging with the assembly machinery in the outer membrane (Bos *et al.*, 2007a; Walther *et al.*, 2009b). Obviously, this role of DegP becomes more important when the activity of Skp or SurA is compromised.

The role of the periplasmic chaperones has also been studied in *N. meningitidis*, where the σ^E-dependent stress response is absent (E. Volokhina, M.P. Bos & J. Tommassen, unpublished results). An important role for Skp in OMP biogenesis in this organism has been confirmed. However, inactivation of the *surA* gene had no notable effect on OMP assembly; this is consistent with the aforementioned proteomics study in *E. coli* (Vertommen *et al.*, 2009), which suggested that SurA has only a very restricted number of substrates. Furthermore, inactivation of *surA* in an *skp* mutant of *N. meningitidis* did not aggravate the OMP assembly defect of the *skp* single mutant. A homologue of DegP is non-existent in *N. meningitidis*, but there is a homologue of the closely related protease DegQ (Bos *et al.*, 2007a). Inactivation of this *degQ* gene caused no OMP assembly defect and again no synthetic phenotype was observed when the mutation was combined with an *skp* or *surA* mutation (E. Volokhina, M.P. Bos & J. Tommassen, unpublished results). Thus, at least in *N. meningitidis*, Skp appears to be the major periplasmic chaperone involved in OMP biogenesis.

The bacterial OMP assembly machinery

After travelling through the periplasm and reaching the outer membrane, OMPs have to fold and insert into this membrane. The first component of the OMP assembly machinery identified was a protein known as Omp85 in *N. meningitidis*. Homologues of Omp85 were identified in all available Gram-negative bacterial genome sequences (Voulhoux *et al.*, 2003; Voulhoux & Tommassen, 2004), and previous attempts to inactivate the gene in *Haemophilus ducreyi* and *Synechocystis* sp. were reported to be unsuccessful (Reumann *et al.*, 1999; Thomas *et al.*, 2001), suggesting an important function for the protein. Furthermore, the *omp85* gene was found to be located in many genome sequences immediately upstream of the *skp* gene encoding the periplasmic OMP chaperone, suggesting that Omp85 might be involved in OMP biogenesis as well. To assess the function of Omp85, the gene was cloned under an IPTG-inducible promoter (Voulhoux *et al.*, 2003). In the absence of IPTG, the resulting mutants stopped growing and all OMPs examined were found to accumulate as unfolded proteins as shown (amongst other characteristics) by their protease sensitivity and their lack of heat modifiability. These results demonstrated an essential role of Omp85 in OMP assembly.

Non-denaturing SDS-PAGE (Voulhoux *et al.*, 2003) and cross-linking experiments (Manning *et al.*, 1998) indicated that Omp85 is part of a multi-subunit complex in *N. meningitidis*. These results were confirmed in *E. coli*, where the Omp85 homologue is now called BamA (Bam stands for β-barrel assembly machinery). BamA forms a complex with four lipoproteins, BamB–E (Fig. 3a) (Wu *et al.*, 2005; Sklar *et al.*, 2007a). Whereas Omp85/BamA homologues are present in all Gram-negative bacteria, the accessory lipoproteins are less well conserved. For example, in the *N. meningitidis* Bam complex, the BamB component is lacking and this complex contains an additional component, RmpM, an OMP with a peptidoglycan-binding motif (Fig. 3b) (Volokhina *et al.*, 2009). In the case of *Caulobacter crescentus*, the BamC component is absent and a different protein with a peptidoglycan-binding motif, the lipoprotein Pal, is present as an additional component (Anwari *et al.*, 2010). In some alphaproteobacteria, both BamB and BamC appear to be absent (Gatsos *et al.*, 2008). Also, the function of the accessory lipoproteins is less vital. In *E. coli*, BamD is the only essential lipoprotein component of the complex, whereas mutational loss of the other lipoproteins causes only mild OMP assembly defects (Malinverni *et al.*, 2006; Sklar *et al.*, 2007a). However, even in the closely related bacterium *Salmonella enterica*, BamD appears to be dispensable (Fardini *et al.*, 2009). Also, in *Neisseria gonorrhoeae*, a viable knockout mutant in the *bamD* homologue, designated *comL*, has been described (Fussenegger *et al.*, 1996) but the gene appears essential for viability and OMP assembly in *N. meningitidis* (Volokhina *et al.*, 2009). Thus, the Bam complex in bacteria consists of one essential central component, Omp85/BamA, and a variable number of accessory components, the importance of which is variable and depends on the specific component and the bacterium being studied.

Interaction of substrate OMPs with BamA/Omp85

Electrophysiological experiments demonstrated that purified BamA reconstituted into planar lipid bilayers forms narrow ion-conductive channels (Robert *et al.*, 2006; Stegmeier & Andersen, 2006). The physiological significance of these channels is still unclear, but this property could be used to study the interaction of the protein with its substrate OMPs. Addition of denatured OMPs to BamA-containing planar lipid bilayers increased the conductivity of the pores, demonstrating a direct interaction between BamA and its substrates (Robert *et al.*, 2006). Since addition of periplasmic proteins to the bilayers had no such effect, this interaction between BamA and its substrates was specific.

The specificity of the interaction between BamA and its substrates indicated the presence of a recognition signal within these substrates. Previously, a signature sequence was recognized at the C terminus of the vast majority of bacterial OMPs (Struyvé *et al.*, 1991). This signature consists of a phenylalanine (or occasionally tryptophan) at the

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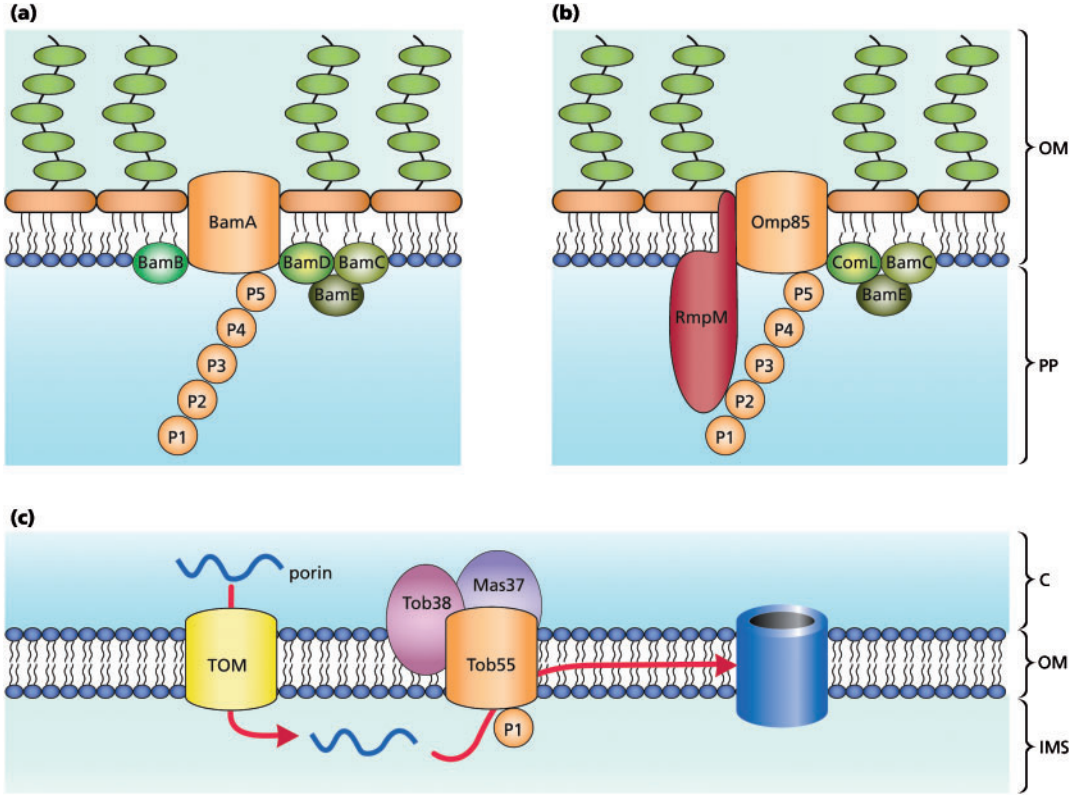

Fig. 3. Comparison of the composition of the β-barrel OMP assembly complexes in (a) *E. coli*, (b) *N. meningitidis* and (c) mitochondria. Homologous components in the various systems are coloured identically. The N-terminal periplasmic part of the bacterial BamA/Omp85 consists of five POTRA domains (P1–P5), whereas the mitochondrial homologue Tob55 contains only one such domain (P1). In mitochondria, the precursors of β-barrel OMPs, such as mitochondrial porin, are first imported from the cytoplasm (C) of the eukaryotic cell via the TOM complex into the intermembrane space (IMS) before they are assembled via the TOB complex into the outer membrane (OM). OM and PP are defined in the legend to Fig. 1.

C-terminal position, a tyrosine or a hydrophobic residue at position –3 relative to the C terminus, and also hydrophobic residues at positions –5, −7 and –9 from the C terminus. Furthermore, the importance of the C-terminal Phe *in vivo* was demonstrated by its deletion or substitution in porin PhoE (Struyvé *et al.*, 1991). Such mutations severely affected the assembly of the protein into the outer membrane. Of note, however, is that Phe was not absolutely essential: while a mutant protein deleted for the C-terminal Phe accumulated in periplasmic inclusion bodies when it was highly expressed (Struyvé *et al.*, 1991), it was still assembled into the outer membrane when expression levels were reduced (de Cock *et al.*, 1997). This observation could be explained if the mutation decreases but does not abrogate the recognition of the mutant protein by the assembly machinery resulting in its periplasmic aggregation. So, reduced expression will decrease the aggregation kinetics, thereby increasing the time span needed for the assembly machinery to deal with the suboptimal mutant protein.

The hypothesis that the C-terminal Phe is part of the recognition signal for BamA was confirmed in planar lipid bilayer experiments with reconstituted BamA (Robert *et al.*, 2006). In contrast with wild-type PhoE, the mutant protein lacking the C-terminal Phe did not stimulate the conductivity of the BamA channels. However, at higher concentrations, it blocked the BamA channels, indicating that it can still interact with BamA but differently from the wild-type protein. The latter result indicates that either the recognition signal is not completely disrupted by the deletion or the PhoE protein contains additional signals. This is consistent with the observation that a mutant protein lacking the C-terminal Phe can still be assembled *in vivo* if the expression level is low (de Cock *et al.*, 1997). The existence of a C-terminal recognition signal in PhoE was further confirmed by using synthetic peptides (Robert *et al.*, 2006). Like the full-length PhoE, a synthetic peptide comprising its last 12 aa stimulated the conductivity of the BamA channels, while control peptides did not.

Omp85/BamA was predicted to consist of two domains, an N-terminal periplasmic domain and a C-terminal domain embedded as a β-barrel into the outer membrane (Fig. 3a and b) (Voulhoux *et al.*, 2003). The periplasmic part was predicted to consist of five repeated domains, named polypeptide transport-associated (POTRA) domains

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(Sánchez-Pulido *et al.*, 2003). Considering their periplasmic location, it seems likely that these POTRA domains interact with the substrate OMPs. The structures of BamA fragments containing several POTRA domains have been solved by X-ray crystallography (Kim *et al.*, 2007; Gatzeva-Topalova *et al.*, 2008) and NMR spectroscopy (Knowles *et al.*, 2008). Although these domains show only very limited sequence identity, they have a common structure consisting of a three-stranded β-sheet overlaid with two α-helices. It was suggested that these POTRA domains interact with the substrate OMPs and/or with the accessory lipoproteins of the Bam complex by β-augmentation (Kim *et al.*, 2007). NMR experiments indeed revealed that several peptides derived from porin PhoE could weakly bind to either side of the β-sheets in the POTRA domains (Knowles *et al.*, 2008). Unfortunately, a C-terminal fragment of PhoE could not be tested in those experiments because of solubility problems.

OMP biogenesis in mitochondria

Other than in the outer membranes of Gram-negative bacteria, integral β-barrel membrane proteins are also found in the outer membranes of mitochondria and chloroplasts, probably reflecting the endosymbiont origin of these eukaryotic cell organelles. It should be noted that these organelles also contain α-helical OMPs (Walther & Rapaport, 2009), which will not be discussed further here. Soon after the discovery that Omp85/BamA is an essential component of the bacterial OMP assembly machinery (Voulhoux *et al.*, 2003), several research groups identified a homologue in mitochondria and showed that it is involved in the assembly of β-barrel proteins into the mitochondrial outer membrane (Gentle *et al.*, 2004; Kozjak *et al.*, 2003; Paschen *et al.*, 2003). This protein was named either Omp85, Sam50 or Tob55, and will be referred to from here on as Tob55. Tob55 was shown to be part of a complex (called the TOB or SAM complex) with at least two other proteins, which are known under various names, i.e. Tob38/Sam35 and Mas37/Tom37/Sam37 (Fig. 3c) (Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004). These accessory components are exposed to the cytosolic side of the outer membrane and show no homology to the lipoprotein components of the bacterial Bam complex.

Much of the genome of the endosymbiont that evolved into mitochondria has been transferred to the nucleus. Consequently, most mitochondrial proteins are synthesized in the cytoplasm of the eukaryotic cell from where they are transported into the mitochondria via the TOM complex in the outer membrane and the TIM complexes in the inner membrane (Chacinska *et al.*, 2009). Also the precursors of β-barrel OMPs are synthesized in the cytoplasm from where they have direct access to the mitochondrial outer membrane. Nevertheless, these proteins are first imported via the TOM complex into the intermembrane space of the mitochondria (i.e. the equivalent of the bacterial periplasm) (Rapaport & Neupert, 1999; Krimmer *et al.*, 2001; Model *et al.*, 2001) to approach the assembly machinery from the same side as occurs in bacteria (Fig. 3c). This extension of the biogenesis route is consistent with an evolutionarily conserved assembly mechanism.

Mitochondrial β-barrel OMPs must carry a signal that is recognized by the assembly machinery in the outer membrane. This signal, termed the β-signal, was recently identified by Kutik *et al.* (2008). Like the C-terminal signature sequence in bacterial OMPs described above, this β-signal is located near the C terminus of the OMPs. However, it is never located at the very end and is always followed by another 1–28 residues. As shown in Table 1, the bacterial and mitochondrial signals, although not identical, appear to be rather similar and are probably evolutionarily related. Curiously, whereas the bacterial OMP signature sequence is recognized by the conserved central component BamA/Omp85 of the assembly machinery (Robert *et al.*, 2006), the β-signal in the mitochondrial OMPs appears to be recognized by the accessory component Tob38 (Kutik *et al.*, 2008). It should be noted, however, that the N-terminal POTRA domain of Tob55 has also been reported to interact with substrate proteins (Habib *et al.*, 2007).

Comparison of β-barrel OMP assembly in bacteria and mitochondria

Comparison of β-barrel OMP assembly in bacteria and mitochondria reveals several similarities but also considerable differences. Firstly, the substrates in both cases are β-barrel proteins. However, while all bacterial OMPs

Table 1. Comparison of the β-signal of mitochondrial OMPs and the C-terminal signature sequence of bacterial OMPs, which are recognized by their respective OMP assembly machineries

The β-signal of the mitochondrial porin VDAC from *Neurospora crassa* and the signature sequence of the bacterial porin PhoE from *E. coli* are included in the comparison as examples. The one-letter code for amino acids is used. X, Any amino acid; ϕ, hydrophobic residue; π, polar residue; n, 1–28 residues. The mitochondrial β-signal is given in bold type.

	Sequence											
Mitochondrial β-signal	X	X	$\boldsymbol{\pi}$	**X**	**G**	**X**	**X**	$\boldsymbol{\phi}$	**X**	$\boldsymbol{\phi}$	(X)n	
Bacterial signature	X	ϕ	X	ϕ	X	ϕ	X	Y/ϕ	π	F	–	–
VDAC	T	H	K	V	G	T	S	F	T	F	E	S
PhoE	I	V	A	V	G	M	T	Y	Q	F	–	–

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appear to contain an even number of β-strands (Koebnik *et al.*, 2000), the only mitochondrial β-barrel OMP of which the structure has been solved, i.e. the voltage-dependent anion channel VDAC or mitochondrial porin, is a 19-stranded β-barrel (Bayrhuber *et al.*, 2008; Hiller *et al.*, 2008; Ujwal *et al.*, 2008). It is interesting to note that a mutant form of porin PhoE lacking the first N-terminal β-strand has been reported to be functionally assembled, albeit inefficiently, into the *E. coli* outer membrane (Bosch *et al.*, 1988), demonstrating that the bacterial Bam complex can deal with β-barrels with an odd number of strands. Secondly, the OMP assembly machineries contain a conserved central component, Omp85/BamA in bacteria and Tob55 in mitochondria. However, Tob55 is considerably smaller than its bacterial homologues. It contains only a single POTRA domain at its N terminus (Fig. 3c), while the bacterial proteins contain five of these domains (Fig. 3a and b). A deletion analysis in *N. meningitidis*, however, revealed that a mutant expressing an Omp85 variant with only a single POTRA domain was viable and assembled OMPs into the outer membrane with only slightly decreased efficiency in the case of larger OMPs (Bos *et al.* 2007b). Thirdly, the bacterial and mitochondrial machineries contain several accessory components, which, however, show no mutual homology. Fourthly, signals for recognition by the assembly machineries have been identified near the C termini of both bacterial and mitochondrial β-barrel OMPs. These signals are similar but not completely identical. Moreover, they are recognized by different components of the assembly machineries, i.e. by Omp85/BamA in the bacterial system and by Tob38 in the mitochondrial system.

A mitochondrial β-barrel OMP can be assembled into the bacterial outer membrane

The similarities between the bacterial and mitochondrial β-barrel OMPs and their assembly machineries suggest a common evolutionary origin. However, as described above, there are also considerable differences between the systems. Therefore, it was of interest to determine whether a mitochondrial OMP can be assembled into the bacterial outer membrane. To test this possibility, VDAC of *Neurospora crassa* was genetically fused to a signal sequence to mediate transport across the bacterial inner membrane via the Sec system, and the construct was expressed in *E. coli* (Walther *et al.*, 2010). Cell fractionations, protease-sensitivity assays and immunofluorescence microscopy showed that VDAC was assembled into the bacterial outer membrane where it formed functional pores. Furthermore, assembly into the outer membrane was dependent on the C-terminal β-signal in VDAC and on the expression of a functional *E. coli* BamA protein (Walther *et al.*, 2010). These results demonstrated that the bacterial OMP assembly machinery can still deal with the β-barrel OMPs that evolved in mitochondria.

Bacterial OMPs can be assembled into the mitochondrial outer membrane

It was also of interest to determine whether the β-barrel OMP assembly machinery that evolved in mitochondria is still able to handle bacterial OMPs. This question was more complicated to address, since β-barrel OMPs in mitochondria first have to be taken up via the TOM complex before reaching the TOB complex from the right side of the membrane (Fig. 3c). The mitochondrial β-barrel OMPs do not contain a cleavable signal for their targeting to mitochondria but rather an uncleavable internal signal. The nature of this signal has not been characterized and may be dispersed over the entire polypeptide rather than being confined to a discrete segment (Walther & Rapaport, 2009). Such a signal would be difficult to fuse genetically to a bacterial OMP. However, it was also proposed that β-barrel-specific structural elements are recognized by the mitochondrial import machinery (Walther & Rapaport, 2009), in which case, bacterial OMPs might also be recognized. To test this possibility, porin PhoE of *E. coli* was expressed in *Saccharomyces cerevisiae* without its signal sequence, which would presumably lead the protein to the endoplasmic reticulum (Walther *et al.*, 2009a). The protein was found to accumulate in the mitochondria of the yeast in a TOM-dependent manner. Similar results were obtained for a diverse set of other bacterial OMPs. Thus, apparently, the bacterial OMPs contain the appropriate signals to be taken up into mitochondria via the TOM complex. These results indicate that no eukaryote-specific import signals were required to evolve in mitochondrial β-barrel OMPs to ensure their import into mitochondria when, during endosymbiont evolution, their structural genes were transferred to the nucleus.

The accumulation of PhoE in the mitochondria was also dependent on a functional TOB complex. The protein was inserted into the mitochondrial outer membrane in its native trimeric state and it was detectable at the surface of intact mitochondria with PhoE-specific monoclonal antibodies that recognize conformational epitopes (Walther *et al.*, 2009a). The efficiency of the assembly into the mitochondrial outer membrane was dependent on the expression level; at low expression levels, all PhoE detected was correctly assembled into the trimeric configuration, whereas at high expression levels considerable amounts of the protein also accumulated as aggregates, presumably in the mitochondrial intermembrane space (Walther *et al.*, 2009a). Thus, apparently, the capacity of the TOB complex to deal with the heterologous substrate protein is limited. Assembly of PhoE into the mitochondrial outer membrane was also dependent on its C-terminal signature sequence; when the mutant PhoE protein lacking the C-terminal Phe was expressed in *S. cerevisiae*, it was taken up into the mitochondria but it was not assembled into the outer membrane in its native trimeric state (Walther *et al.*, 2009a). Thus, collectively, bacterial OMPs can be assembled into the mitochondrial outer membrane and this assembly depends on their C-terminal signature

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sequence and on the mitochondrial TOM and TOB complexes.

Conclusions

In recent years, much progress has been made in studies on the biogenesis of bacterial OMPs. This progress is mostly related to the identification of the components of the machinery that assemble these proteins into the outer membrane and also on the resolution of the structures of the periplasmic chaperones involved, some in complex with their substrate OMPs. Progress was also stimulated by the discovery of a similar machinery for the insertion of β-barrel OMPs into the mitochondrial outer membrane. The basic mechanism of OMP assembly is conserved to such an extent that a mitochondrial OMP can be assembled *in vivo* into the bacterial outer membrane, and vice versa, bacterial OMPs can be assembled into the mitochondrial outer membrane. It is likely that a similar mechanism operates in chloroplasts (Hsu & Inoue, 2009). Thus, results in these fields will be mutually profitable. Mechanistic insight into the assembly process and the function of the individual components of either of these systems is still very limited. Much progress is to be expected in the near future from the resolution of the structures of the components or, perhaps, of the entire machineries and from the development of reconstituted systems with purified components to study the assembly process *in vitro*.

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