The Mitochondrial Porin, VDAC, Has Retained the Ability to Be Assembled in the Bacterial Outer Membrane

Dirk M. Walther,†,1 Martine P. Bos,2 Doron Rapaport,*1 and Jan Tommassen*2

1Interfaculty Institute for Biochemistry, University of Tübingen, Tübingen, Germany
2Department of Molecular Microbiology and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands
†Present address: Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, 82152 Martinsried, Germany
*Corresponding author: E-mail: j.p.m.tommassen@uu.nl; doron.rapaport@uni-tuebingen.de.
Associate editor: Martin Embley

Abstract

β-Barrel proteins are present in the outer membranes (OMs) of Gram-negative bacteria, mitochondria, and chloroplasts. Their assembly requires a machinery of which the central component, called Omp85 (BamA) in bacteria and Tob55 (Sam50) in mitochondria, is evolutionarily conserved. An open question is whether the signals in β-barrel OM proteins required for assembly via this multicomponent machinery are also conserved. To address this question, we have expressed (Voulhoux et al. 2003). Its homologue in Neurospora crassa fused to a bacterial signal sequence for transport across the bacterial inner membrane. The protein was assembled in the bacterial OM where it formed pores. Assembly of VDAC was dependent on its β-signal, which is required for assembly in the mitochondrial OM, and on the bacterial Omp85 assembly machinery. These results demonstrate that the basic mechanism of β-barrel assembly in the OMs of bacteria and mitochondria is conserved.

Key words: Escherichia coli, membrane protein, mitochondria, Omp85, outer membrane, porin.

Introduction

The cell envelope of Gram-negative bacteria consists of an inner membrane and an outer membrane (OM), which are separated by the peptidoglycan-containing periplasm. The integral OM proteins (OMPs) generally have a β-barrel structure (Koebnik et al. 2000). Similar β-barrel proteins are found in the OMs of mitochondria and chloroplasts, probably reflecting the endosymbiont origin of these eukaryotic cell organelles.

Bacterial OMPs are synthesized in the cytoplasm with an N-terminal signal sequence for transport across the inner membrane into the periplasm via the Sec system. Their subsequent assembly into the OM is mediated by a machinery, the central component of which is a protein designated Omp85 (Bos et al. 2007a). Omp85 is an essential OMP, the function of which was first identified in Neisseria meningitidis (Voulhoux et al. 2003). Its homologue in Escherichia coli, designated BamA (formerly YaeT), was found to be part of a multisubunit complex, the Bam complex, further consisting of four lipoproteins, BamB–E (formerly YfgL, NlpB, YfoO, and SmpA, respectively; Wu et al. 2005; Sklar et al. 2007).

In mitochondria, the precursors of β-barrel OMPs are imported from the cytosol into the intermembrane space via the translocase of the outer mitochondrial membrane complex (Pfanner et al. 2004; Paschen et al. 2005). Their subsequent assembly into the OM requires the topogenesis of mitochondrial outer membrane β-barrel proteins (TOB) complex, also named the sorting and assembly machinery (SAM) complex, which includes a homologue of Omp85, known as Tob55/Sam50 (Kozjak et al. 2003; Paschen et al. 2003; Gentle et al. 2004). Two additional components of this complex, Mas37/Sam37 and Tob38/Sam35/Tom38, are largely exposed to the cytosol and share no obvious sequence similarity with the lipoproteins of the bacterial Bam complex (Wiedemann et al. 2003; Ishikawa et al. 2004; Milenkovic et al. 2004; Waizenegger et al. 2004).

How do these assembly machineries recognize their substrates? The vast majority of bacterial OMPs contain a C-terminal signature sequence, the most prominent feature of which is a Phe (or Trp) residue at the ultimate C-terminal position (Struyvè et al. 1991). This sequence was found to be essential for efficient OMP assembly in vivo (Struyvè et al. 1991) and to directly interact in vitro with the Omp85/BamA component of the assembly machinery (Robert et al. 2006). Recently, a signal was identified in mitochondrial β-barrel OMPs required for assembly via the TOB/SAM complex (Kutik et al. 2008). Like the bacterial signature sequence, this so-called β-signal is constituted by the last predicted β-strand of the OMP, which, however, never includes the ultimate C-terminal residue(s). Although different, the eukaryotic β-signal likely evolved from the bacterial signature sequence. Strikingly, the β-signal is in mitochondria not recognized by the conserved component Tob55 (the Omp85 homologue) but rather by the peripheral subunit Tob38 of the assembly machinery (Kutik et al. 2008).

Thus, even though there are clear similarities in OMP assembly between bacteria and mitochondria with respect to both the machinery and the signals involved, there
are also noticeable differences. Therefore, it is of interest to study whether, nevertheless, the basic mechanism of β-barrel OMP assembly is evolutionarily conserved. In a previous study, we showed that bacterial OMPs, when expressed in yeast, are assembled in the mitochondrial OM (Walther et al. 2009), demonstrating that the primordial bacterial OMP signature sequence is still properly recognized and processed by the mitochondrial machinery. Of note, this result does not necessarily imply that the evolved mitochondrial OMPs will also be recognized and properly processed by the bacterial machinery. For example, as noted above, the β-signal in mitochondrial OMPs is recognized by the Tob38 subunit of the assembly machinery, a component for which there is no equivalent in bacteria. Furthermore, although all bacterial OMPs of which the structure has been solved contain an even number of β-strands (Koebnik et al. 2000), the only available structure of a mitochondrial OMP, that is, that of the voltage-dependent anion channel (VDAC), comprises 19 β-strands (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008). Also, by the presence of lipopolysaccharides (LPSs) and the absence of sterols, the lipid composition of the bacterial OM is very different from the mitochondrial one; such distinct membrane composition could affect OMP assembly. Thus, to further investigate the possibility that OMP assembly is evolutionarily conserved, we have studied here the fate of the mitochondrial porin VDAC from Neurospora crassa upon its synthesis in E. coli.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli K-12 strain MC4100 has been described by Casadaban (1976). Strain CE1224 contains a deletion of the phoE gene and a mutation in the regulatory gene ompR preventing expression of the porins OmpF and OmpC (Tommassen et al. 1983). Strain CE1265 also contains phoE and ompR mutations and additionally contains a mutation in the phoR gene leading to the constitutive expression of the pho regulon (Tommassen et al. 1983). Strain BW25113 and its degP mutant derivative JW0157 were obtained from the National BioResource Project (NIG, Japan): E. coli. Unless specified otherwise, the strains were grown at 37 °C in L-broth (Tommassen et al. 1983) or in a peptone-based inorganic-phosphate (P_i)-poor medium (Levinthal et al. 1962), both supplemented, if necessary for plasmid maintenance, with 25 μg/ml chloramphenicol. Strain WD401, bearing a deletion of the chromosomal bamA gene and either plasmid pWTD30-9 or pWTD30, containing temperature-sensitive or wild-type alleles of bamA, respectively (Doerrler and Raetz 2005), was grown in the same media as above supplemented with 100 μg/ml ampicillin.

Construction of Plasmids

DNA fragments encoding full-length N. crassa VDAC or its C-terminally truncated variants were amplified by polymerase chain reaction (PCR) using the oligonucleotides shown in the supplementary table S1 (Supplementary Material online). The PCR products were digested with PstI and BamHI and ligated into PstI- and BglII-digested plasmid pJP29, which contains the E. coli phoE gene under its authentic promoter (Bosch et al. 1986). In the resulting constructs, the VDAC gene is fused in frame to the signal sequence—encoding part of phoE. To produce a VDAC variant without signal sequence in E. coli, a PCR fragment was generated and cloned in PacI- and BglII-digested pJP29. Plasmid pJP29_U, encoding solely the signal sequence of PhoE (Oomen et al. 2004), was used as an empty vector control.

Bacterial Cell Fractionation, Electrophoresis, and Western Blotting

Escherichia coli cell envelopes were isolated (Lugtenberg et al. 1975) and extracted with urea (Voulhoux et al. 2003) according to the published procedures. Inner membrane and OMs from midexponential-phase cultures were separated by sucrose density gradient centrifugation essentially as described by Burghout et al. (2004) except that twice the amount of lysozyme was used and that centrifugation in an SW41 rotor was carried out for 65 h at 36,000 rpm (220,000 × g). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE; Lugtenberg et al. 1975) and western blotting, and nicotinamide adenine dinucleotide (NADH)- oxidase activity was determined as a marker of the inner membrane. The antisera used to detect E. coli porins was raised against PhoE and cross-reacted with the related porins OmpC and OmpF. Immunodecorated proteins were detected on western blots by enhanced chemiluminescence and, if appropriate, the bands were quantified by densitometry.

Protease Digestion

For proteinase K treatment, mitochondria or bacterial envelopes were resuspended in 10 mM 3-(N-Morpholino)propanesulfonic acid–KOH, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 or 20 mM Tris–HCl, 2 mM EDTA, pH 7.4, respectively. Proteolysis was carried out for 15 min on ice and terminated by addition of 2 mM phenylmethylsulfonyl fluoride before resiolation of the membranes.

Immunofluorescence Microscopy

Escherichia coli cells or isolated N. crassa mitochondria were fixed with 2% (v/v) formaldehyde and immobilized on polylysine-coated cover slips by centrifugation. After blocking with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), immunostaining was performed with a polyclonal antiseraum raised against isolated, native N. crassa VDAC and Alexa-488–conjugated goat antirabbit-IgG antibodies in PBS, 0.5% BSA. Bright field and fluorescence images were taken with a Zeiss Axioskop microscope.

In vivo Pore Measurements

Susceptibility of strains to antibiotics was determined on plates by measuring the growth inhibition zones around
<table>
<thead>
<tr>
<th>Mitochondrial signal</th>
<th>X</th>
<th>X</th>
<th>π</th>
<th>X</th>
<th>G</th>
<th>X</th>
<th>X</th>
<th>φ</th>
<th>X</th>
<th>(X)n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial signal</td>
<td>X</td>
<td>φ</td>
<td>X</td>
<td>φ</td>
<td>X</td>
<td>φ</td>
<td>X</td>
<td>Y</td>
<td>φ</td>
<td>F</td>
</tr>
<tr>
<td>full length VDAC</td>
<td>T</td>
<td>H</td>
<td>K</td>
<td>V</td>
<td>G</td>
<td>T</td>
<td>S</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>VDAC Δ2 C terminus</td>
<td>T</td>
<td>H</td>
<td>K</td>
<td>V</td>
<td>G</td>
<td>I</td>
<td>S</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>VDAC Δ5 C terminus</td>
<td>T</td>
<td>H</td>
<td>K</td>
<td>V</td>
<td>G</td>
<td>I</td>
<td>S</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>VDAC Δ6 C terminus</td>
<td>T</td>
<td>H</td>
<td>K</td>
<td>V</td>
<td>G</td>
<td>I</td>
<td>S</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
</tbody>
</table>

**Fig. 1.** Comparison of the bacterial and mitochondrial β-barrel-OMP assembly signals and the C termini of the VDAC variants used in this study. 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 bordered by thick lines.

filter discs containing the antibiotics (Oomen et al. 2004). To determine the rate of permeation of the chromogenic β-lactam antibiotic nitrocefin (Calbiochem), cells were transformed with plasmid pBR322 encoding β-lactamase. Nitrocefin degradation by the intact cells was determined as described in Oomen et al. (2004) except that the measurements were carried out during 90 s in Tris-buffered saline containing 5 mM MgCl₂.

**Results**

**Expression of VDAC in E. coli**

To investigate whether the *E. coli* Bam complex can deal with a mitochondrial β-barrel protein, we genetically fused VDAC from *N. crassa* to the signal sequence of the bacterial porin PhoE to allow for transport across the inner membrane. In addition to the full-length protein, two VDAC variants were initially constructed to investigate whether the β-signal, which is required for the assembly of β-barrel proteins into the mitochondrial OM, is also needed for their assembly into the bacterial OM. Deletion of two amino acid residues from the C terminus does not affect the β-signal identified by Kutik et al. (2008) and positions a Phe residue at the C terminus like in the bacterial OMP signature sequence (fig. 1). Deletion of five residues does affect the β-signal and eliminates the Phe residue (fig. 1). The constructs generated are under control of the phoE promoter, which allows for high-level expression when the cells are grown under Pᵢ limitation. Because they are located on a medium-copy plasmid, low-level expression was anticipated under Pᵢ-replete conditions.

Cells of the wild-type *E. coli* strain MC4100 containing the relevant plasmids were grown in Pᵢ-replete L-broth, and whole-cell lysates were analyzed by SDS-PAGE and western blotting. Immunodetection of the blots revealed that full-length VDAC was expressed under these conditions (fig. 2A). The specificity of the VDAC antisera was confirmed by the absence of a signal in the strain carrying the empty vector (fig. 2A). After ultrasonic disruption of the *E. coli* cells, VDAC fractionated with the membranes like the endogenous porins OmpF and OmpC, whereas the cytoplasmic chaperone SecB was recovered in the supernatant (fig. 2A). Deletion of two residues from the C terminus did not influence protein levels, and like the full-length protein, this mutant form was also recovered in the membrane fraction (fig. 2A). However, the mutant protein lacking five C-terminal residues was not detected under these conditions, possibly because this variant was not assembled into the OM and thus was proteolytically degraded. Similarly, VDAC was not detected when expressed without a signal sequence (fig. 2A). To verify the proteolytic degradation of the mutant protein lacking five C-terminal residues, the plasmid encoding this construct was introduced into strain JW0157, which lacks the major periplasmic protease DegP, and its isogenic parental strain BW25113. Because the mutant protein was detected only in the degP mutant (fig. 2B), it appears to be a substrate for the periplasmic protease in the wild-type strain.

**Assembly of VDAC in the Bacterial OM**

We wished to determine in which membrane VDAC was located. To separate inner membranes and OM, we applied sucrose density gradient centrifugations. VDAC fractionated with the marker OM proteins OmpF and OmpC and not with the inner-membrane marker NADH-oxidase (fig. 2C). Next, we monitored the integration of VDAC into the OM in protease-digestion experiments. In its native environment, the mitochondrial OM, the protein is resistant to proteinase K, whereas Tom40, which has a large loop exposed to the surface of the mitochondria, is degraded (fig. 2D). Also in the bacterial OM, VDAC was largely resistant to proteinase K, like the endogenous porins OmpC and OmpF and unlike OmpA, which has a large periplasm-exposed domain and was degraded under these conditions (fig. 2D). Furthermore, akin the bacterial porins, VDAC could not be extracted from the bacterial membranes with urea (fig. 2D), confirming that it was integrally inserted into the OM.

If VDAC is indeed assembled into the bacterial OM, it might be detectable at the bacterial cell surface with specific antibodies. The low-level background expression in uninduced MC4100 cells was insufficient for detection of VDAC by immunofluorescence microscopy. To increase the expression levels, the plasmid encoding VDAC was introduced into *E. coli* strain CE1224, which does not produce endogenous porins. Growing the transformed cells under Pᵢ limitation resulted in drastic overproduction of VDAC, even to such an extent that a small fraction of unprocessed precursor proteins was detected (fig. 3A). Using immunofluorescence microscopy, the protein was detected at the bacterial cell surface, like it was at the surface of isolated mitochondria (fig. 3B). The specificity of the signal observed was confirmed by its absence in bacteria carrying the empty vector. Also, the mutant protein lacking two C-terminal amino acid residues was detected at the bacterial cell surface, but not the one lacking five amino acids (fig. 3B), although it was abundantly produced under these conditions (fig. 3C). Collectively, these results indicate that VDAC assembles into the bacterial OM and that assembly is dependent on the β-signal.

**Pore Formation by VDAC in the E. coli OM**

VDAC is the main pathway by which metabolites cross the outer mitochondrial membrane. Thus, if VDAC is correctly assembled into the bacterial OM, it should form large
pores. To test this possibility, the plasmid encoding VDAC was introduced into *E. coli* strain CE1265, which does not produce endogenous porins. Due to a mutation in the regulatory gene *phoR*, this strain expresses the *pho* regulon constitutively but not to the same extent as does a fully induced strain like CE1224 (fig. 3A). The formation of pores in the OM was determined in an antibiotic sensitivity test in which the zone of growth inhibition around a disc containing the antibiotic tested is a measure for the diffusion of the antibiotic through the pores. When the bacteria produced VDAC, they were more sensitive to all antibiotics tested (table 1), indicating that VDAC forms pores in the bacterial OM. To substantiate this conclusion, the cleavage of nitrocefin by periplasmic β-lactamase was measured in intact cells, a process in which the permeation of this chromogenic antibiotic through the OM is rate limiting. The synthesis of VDAC resulted in a drastically increased rate of nitrocefin cleavage in intact cells (fig. 4A) consistent with the formation of aqueous pores in the OM. In both assays, expression of *phoE* from the same vector resulted in a more moderate increase in antibiotic uptake (table 1 and fig. 4A) consistent with the observation that VDAC has a considerably larger

**Fig. 2.** Mitochondrial VDAC from *Neurospora crassa* is localized to the OM in *Escherichia coli*. (A) Whole-cell lysates, cell envelopes, and soluble fractions (supernatant) were isolated from MC4100 cells containing plasmids encoding VDAC (full length), C-terminal truncated variants lacking five (Δ5 C-term.) or two (Δ2 C-term.) amino acids, VDAC without signal sequence (Δsignal) or the empty vector. The fractions were analyzed by SDS-PAGE and western blotting using antisera directed against VDAC, *E. coli* porins (OmpC/F), or the cytoplasmic protein SecB. (B) Whole cells of *E. coli* strain BW25113 and its *degP* mutant derivative JW0157, both containing plasmids encoding either full-length VDAC or mutant VDAC lacking five C-terminal residues, were analyzed by SDS-PAGE followed by western blotting using antisera directed against VDAC or OmpA. (C) Inner membranes and OM from MC4100 cells expressing full-length VDAC were separated by sucrose density gradient centrifugation. Fractions were analyzed by SDS-PAGE and western blotting using antisera directed against VDAC or *E. coli* porins. NADH-oxidase activity was assessed as an inner-membrane marker. Signals or activities for each protein in each fraction were normalized to the amount detected in the peak fraction. (D) Mitochondria isolated from *N. crassa* (left) and suspensions of cell envelopes from *E. coli* strain MC4100 expressing *N. crassa* VDAC (right) were either left intact (input), digested with the indicated concentrations of proteinase K (PK), or extracted with 6 M urea and separated into an extractable (supernatant) and a membrane-embedded (pellet) fraction. Samples were subjected to SDS-PAGE, followed by either staining with Coomassie Brilliant Blue to detect the OmpC, OmpF, and OmpA proteins or western blotting using antibodies against VDAC and Tom40. Tom40, mitochondrial OMP with a protease-accessible loop; OmpA, *E. coli* OMP with protease-accessible periplasmic extension; OmpC and OmpF, protease-protected OMPs.
pore size than the *E. coli* porins (Freitag et al. 1982). Collectively, similar to its role in mitochondria, the integration of VDAC into the bacterial OM promotes the flux of solutes across this membrane.

### VDAC Assembly in the *E. coli* OM is Dependent on the β-Signal

In the nitrocefin-uptake assay, expression of the mutant VDAC lacking two residues from the C terminus promoted cleavage of the antibiotic at efficiencies similar to those obtained upon expression of the full-length protein (fig. 4A), suggesting that this mutation does not affect assembly and pore formation. In contrast, nitrocefin cleavage was much less promoted by expression of the variant lacking five residues (fig. 4B). The latter variant could be detected in the constitutive strain CE1265 although clearly less than the full-length protein (fig. 4B). The protein was largely degraded when the cell envelope fraction of these cells was treated with proteinase K and it could be extracted from these membranes with urea, whereas the full-length protein produced in this strain was protease resistant and not extractable from the membranes with urea (fig. 5A). Thus, it appears that a large fraction of the detected mutant protein was not inserted into the OM; these polypeptides only fractionate with the membranes, presumably because they form dense aggregates that are pelleted with the membranes during the centrifugation procedure (Tommassen 1986). Nevertheless, a small portion of the polypeptides was resistant to proteinase K and was not extracted with urea (fig. 5A); these polypeptides are most likely correctly assembled into the bacterial OM as they facilitated nitrocefin degradation in intact cells to some extent.
Proteins and western blotting using antibodies against the indicated proteins. Used in nitrocefin-uptake experiments were analyzed by SDS-PAGE to compensate for the disruption of the nus, is assembled as efficiently as is the wild-type VDAC. To investigate whether the presence of a C-terminal Phe could compensate for the disruption of the C-terminal residues where it presents a Phe (fig. 1). In contrast to the mutant protein lacking two residues, which still retains an intact β-signal and presents a Phe residue at the C terminus, the mutant protein lacking four C-terminal residues is only very inefficiently assembled into the bacterial OM. The results presented so far indicated that the mutant protein lacking five C-terminal residues is inefficiently assembled into the bacterial OM. Thus, BamA is required for the efficient assembly of VDAC into the bacterial OM.

**Discussion**

Integral membrane β-barrel proteins are found in the OM of Gram-negative bacteria and mitochondria. Their assembly requires an evolutionarily conserved protein of the Omp85 family, but significant adaptations of the assembly machineries have occurred during evolution. The accessory proteins, that is, four lipoproteins in the case of bacteria and two cytosol-exposed membrane-associated proteins in mitochondria, do not show any sequence similarity. Also, the Omp85-like component adapted during evolution; besides a membrane-embedded β-barrel domain, the bacterial proteins contain five periplasm-exposed POTRA domains, whereas the mitochondrial variant contains only one such domain. Of note, a single POTRA domain was recently demonstrated to be sufficient for Omp85 function in N. meningitidis (Boe et al. 2007b), but in E. coli BamA, three of these domains were essential although also the other two were important for optimal functioning (Kim et al. 2007).

Not only the assembly machineries but also the signals they recognize underwent adaptations during evolution. In addition, these signals are recognized by different components of the machinery, that is, by Omp85 in the bacterial system (Robert et al. 2006) and by Tob38 in the mitochondrial system (Kutik et al. 2008). We recently demonstrated that, despite these variations, the primordial bacterial OMP signature sequence is still properly recognized and processed by the mitochondrial machinery (Walther et al. 2009), suggesting that the basic mechanism of β-barrel assembly is dependent on BamA. To determine whether assembly of VDAC into the OM of E. coli depends on the Bam complex, we made use of a temperature-sensitive (ts) bamA mutant strain (Doerrler and Raetz 2005). This strain fails to grow at 44 °C, but even at a permissive temperature, it shows an assembly defect of OMPs as evidenced by decreased levels of these proteins. These reduced levels probably result from degradation of misassembled OMPs by periplasmic proteases, such as DegP. The plasmid encoding VDAC was introduced into this mutant strain and into an isogenic strain carrying a wild-type bamA gene, and the bacteria were grown at 32 °C under Pi-limiting conditions to induce VDAC synthesis. The bamA(ts) mutation did not affect growth under these conditions (fig. 6A). Like the amounts of correctly assembled endogenous porins and OmpA, the amount of membrane-inserted VDAC was drastically reduced in the bamA(ts) mutant strain (fig. 6B). In contrast, levels of the periplasmic enzyme alkaline phosphatase (PhoA), the inner-membrane-located enzyme leader peptidase, and the cytoplasmic chaperone SecB in the cell lysates were unaffected (fig. 6B). Therefore, in spite of the presence of a C-terminal Phe, the mutant protein lacking four C-terminal residues presents a Phe residue at the C terminus, whereas the mitochondrial variant contains only one POTRA domain. Of note, a single POTRA domain was recently demonstrated to be sufficient for Omp85 function in N. meningitidis (Boe et al. 2007b), but in E. coli BamA, three of these domains were essential although also the other two were important for optimal functioning (Kim et al. 2007).

Not only the assembly machineries but also the signals they recognize underwent adaptations during evolution. In addition, these signals are recognized by different components of the machinery, that is, by Omp85 in the bacterial system (Robert et al. 2006) and by Tob38 in the mitochondrial system (Kutik et al. 2008). We recently demonstrated that, despite these variations, the primordial bacterial OMP signature sequence is still properly recognized and processed by the mitochondrial machinery (Walther et al. 2009), suggesting that the basic mechanism of β-barrel assembly is dependent on BamA.
protein assembly might be evolutionarily conserved. To test this hypothesis, we investigated here whether a mitochondrial OMP, VDAC, can be assembled into the OM of bacteria, which was indeed the case, and it was shown to be dependent on a functional Bam complex. Of note, the structure of VDAC, that is, a 19-stranded \( \beta \)-barrel (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008), deviates from those of bacterial OMPs, which all contain an even number of \( \beta \)-strands (Koebnik et al. 2000). However, this difference apparently does not prevent the incorporation of VDAC into the bacterial OM. Consistent with this observation, a mutant form of porin PhoE lacking the N-terminal transmembrane \( \beta \)-strand was previously reported to be functionally incorporated into the \( E. coli \) OM (Bosch et al. 1988), demonstrating that, indeed, the bacterial Bam machinery can deal with \( \beta \)-barrels with an odd number of strands.

Efficient assembly of VDAC into the bacterial OM was dependent on its \( \beta \)-signal, which is also required for its assembly in the mitochondrial OM (Kutik et al. 2008). Thus, the bacterial system is able to decode the evolved signals in the mitochondrial OMPs even though they are recognized in the authentic mitochondrial system by a component that has no equivalent in the bacterial system. At very high-expression levels, a mutant form of VDAC that lacks five C-terminal amino acid residues and is thereby affected in its \( \beta \)-signal was undetectable at the bacterial cell surface in immunofluorescence microscopy experiments (fig. 3B). However, at lower expression levels, at least some of the total quantity of the mutant protein produced appeared

**FIG. 5.** VDAC assembly into the \( E. coli \) OM depends on an intact \( \beta \)-signal. (A) The majority of VDAC molecules lacking the five C-terminal residues is not properly integrated into the OM. Cell envelopes from \( E. coli \) strain CE1265 expressing either the full-length VDAC (upper part) or the mutant VDAC (lower part) were treated with proteinase K (PK) or extracted with urea as described in the legend to figure 2D and analyzed by SDS-PAGE and either staining with Coomassie Brilliant Blue to visualize OmpA or western blotting to detect the VDAC variant. (B) Cell envelopes of \( E. coli \) strain MC4100 expressing either full-length VDAC or the mutant protein lacking four C-terminal residues were extracted with urea as described in the legend to figure 2D and analyzed by SDS-PAGE and western blotting using antiserum against VDAC.

**FIG. 6.** BamA is required for membrane insertion of VDAC in \( E. coli \). (A) \( E. coli \) cells containing either a ts allele or wild-type \( bamA \) were grown overnight in L-broth at 30 °C. Cells were resuspended at time 0 in \( P_i \)-poor medium (LPi) or the same medium supplemented with 660 \( \mu \)M \( K_2HPO_4 \) (HPi) and growth at 32 °C was determined by measuring the optical density at 660 nm. (B) \( P_i \)-starved cells containing either the ts \( bamA \) allele or the wild-type allele grown in LPi or HPi were harvested after 6 h (arrow in panel A). Cell envelopes were isolated and extracted with 6 M urea to determine the amount of membrane-inserted VDAC and \( E. coli \) OMPs. Whole-cell lysates were analyzed to determine the effect of the \( bamA \) ts mutation on other proteins. Samples were analyzed by SDS-PAGE, followed either by staining with Coomassie Brilliant Blue to visualize PhoE, OmpC, OmpF, and OmpA or by western blotting using antisera against the following controls: PhoA, periplasmic alkaline phosphatase, which is expressed upon \( P_i \), limitation; leader peptidase, an inner-membrane protein; SecB, a cytoplasmic protein.
to be incorporated into the OM (figs. 4A and 5A), demonstrating that an intact β-signal is not absolutely essential for OM assembly of VDAC in *E. coli*. Similarly, an intact C-terminal signature sequence is not absolutely essential for the correct assembly of a bacterial OMP into the OM. At high-expression conditions, a mutant form of porin PhoE lacking the C-terminal Phe residue was not incorporated at all in the *E. coli* OM, but formed dense periplasmic aggregates that pellet with the membrane fraction during centrifugation and could be visualized in the cell by electron microscopy (Struyve et al. 1991; de Cock et al. 1997). However, at lower expression conditions, this mutant protein was assembled into the OM (de Cock et al. 1997). Presumably, the lower aggregation kinetics at low-expression conditions increases the time span for the Bam machinery to deal with substrates with an imperfect recognition signal. Similarly, when the bacterial OMP PhoE was highly expressed in yeast, it accumulated as unfolded aggregates in the mitochondria, whereas it was efficiently assembled into the mitochondrial OM at lower expression conditions (Walther et al. 2009).

Also, the lipid composition could potentially affect the assembly of integral membrane proteins. Indeed, LPS, an abundant lipid component of the bacterial OM, has been implicated in the biogenesis of OMPs in *E. coli* (Bos et al. 2007a), although viable LPS-deficient mutants of *N. meningitidis* have been described in which OM assembly appears unaffected (Steeghs et al. 2001). The lipid composition of the bacterial OM is, by the presence of LPS and the absence of sterols, entirely different from that of the mitochondrial OM, but this difference is apparently not an insurmountable impediment to the assembly of VDAC into the bacterial OM.

In conclusion, it appears that signal recognition is flexible enough to allow for assembly of a mitochondrial OMP into the bacterial OM. Together with the previous observation that bacterial OMPs expressed in yeast are assembled into the mitochondrial OM (Walther et al. 2009), these data allow the conclusion that the basic mechanism of β-barrel protein assembly is evolutionarily conserved.

**Supplementary Material**

Supplementary table S1 is available at *Molecular Biology and Evolution* online (http://mbe.oxfordjournals.org/).

**Acknowledgments**

We would like to thank Ria van Boxtel and Frank Beckers for technical assistance, and W.T. Doerrler and the National BiobResource Project (NIG, Japan): *E. coli* for providing the ts *bamA* and the *degP* mutant strains, respectively. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 446-TP A30 and the Netherlands Research Council for Earth and Life Sciences (ALW) from the Netherlands Organization for Scientific Research (NWO).

**References**


