Species-Specificity of the BamA Component of the Bacterial Outer Membrane Protein-Assembly Machinery

Elena B. Volokhina,a Jan Grijpstra, Frank Beckers, Erika Lindh,b, Viviane Robertc, Jan Tommassen, Martine P. Bos*

Department of Molecular Microbiology and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

Abstract

The BamA protein is the key component of the Bam complex, the assembly machinery for outer membrane proteins (OMP) in gram-negative bacteria. We previously demonstrated that BamA recognizes its OMP substrates in a species-specific manner in vivo. In this work, we further studied species specificity in vivo by testing the functioning of BamA homologs of the proteobacteria *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bordetella pertussis*, *Burkholderia mallei*, and *Escherichia coli* in *E. coli* and in *N. meningitidis*. We found that no BamA functioned in another species than the authentic one, except for *N. gonorrhoeae* BamA, which fully complemented a *N. meningitidis* bamA mutant. *E. coli* BamA was not assembled into the *N. meningitidis* outer membrane. In contrast, the *N. meningitidis* BamA protein was assembled into the outer membrane of *E. coli* to a significant extent and also associated with BamD, an essential accessory lipoprotein of the Bam complex. Various chimeras comprising swapped N-terminal periplasmic and C-terminal membrane-embedded domains of *N. meningitidis* and *E. coli* BamA proteins were also not functional in either host, although some of them were inserted in the OM suggesting that the two domains of BamA need to be compatible in order to function. Furthermore, conformational analysis of chimeric proteins provided evidence for a 16-stranded β-barrel conformation of the membrane-embedded domain of BamA.

Introduction

Gram-negative bacteria are characterized by a cell envelope consisting of an inner and an outer membrane (OM), which are separated by the peptidoglycan-containing periplasm. While integral inner membrane proteins are α-helical, all but two known integral OM proteins (OMPs) are β-barrels [1–3]. Only recently, the mechanisms of the assembly of OMPs into the OM have started to come to light. Previously, we showed that the Omp85 protein is an essential part of the OMP assembly machinery in *Neisseria meningitidis*; depletion of this essential protein in a conditional mutant strain resulted in the accumulation of unassembled forms of all OMPs analyzed [4]. Later, a similar function was shown for the Omp85 homologs in *Escherichia coli* [5-7], *Pseudomonas aeruginosa* [8] and *Borrelia burgdorferi* [9]. Omp85 homologs have been renamed BamA, for β-barrel assembly machine component A. Interestingly, a BamA homolog is also present and required for the assembly of β-barrel OMPs in mitochondria [10-12]. In *E. coli*, four lipoproteins associated with BamA (EcBamA) have been identified: BamB, BamC, BamD, and BamE [6,13]. We established that *N. meningitidis* BamA (NmBamA) is also associated with BamC, BamD (ComL) and BamE, and, additionally, with the RmpM protein [14]. BamB is not present in neisserial strains.

BamA was predicted to consist of an N-terminal part localized in the periplasm and a membrane-embedded β-barrel in its C-terminal part [4]. In the predicted periplasmic part, five polypeptide-transport-associated (POTRA) domains are located [15]. Crystallographic and NMR structural studies have shown a similar fold for all POTRA domains: a β-sheet of three β-strands overlaid with a pair of antiparallel α-helices [16-19]. For the β-barrel domain, originally a 12-stranded β-barrel topology was predicted [4], which leaves a 61-amino-acid
Lack of Cross-Species Functionality of BamA

region between the POTRA and β-barrel domains, which we
designated the hinge region [20]. However, the crystal structure
of the two-partner secretion-system component FhaC of
 Bordetella pertussis, which shares limited sequence similarity
with BamA, showed that this protein consists of two POTRA
domains and a 16-stranded β-barrel, suggesting that the BamA
β-barrel might also contain 16 β-strands, with four additional β-
strands located in the postulated hinge region [21,22].

We have shown that EcBamA interacts with OMPs via the
so-called signature sequence present at the C terminus of the
substrates [23]. This sequence is characterized by a
phenylalanine or a tryptophan at the C-terminal position and
hydrophobic amino-acid residues at positions -3, -5, -7, and -9
from the C terminus [24]. EcBamA interacted in vitro with E.
coli-derived C-terminal signature sequences, but not with those
found in neisserial OMPs, the defining difference being the
nature of the penultimate C-terminal amino acid [23]. These
results are consistent with the observation that neisserial
OMPs are not efficiently assembled in E. coli. This apparently
high species-specificity of the Bam machinery seems to be at
odds with the observation that the Bam machinery of E. coli
can properly assemble a mitochondrial β-barrel OMP [25] and
with the finding that bacterial OMPs were properly assembled
into the mitochondrial OM of Saccharomyces cerevisiae [26].
To gain further insight into the species-specificity of the
functioning of the Bam machinery, we investigated here
whether BamA homologs from various Gram-negative bacteria
are able to substitute each other in vivo.

Materials and Methods

Bacterial Strains and Growth Conditions

E. coli strains TOP10F (Invitrogen), DH5α (laboratory
collection), and the EcBamA-depletion strain UTP::bamA
[27] were grown either on Luria-Bertani (LB) agar plates or in
liquid LB medium on a shaker at the indicated temperatures.
When necessary, the media were supplemented with an
appropriate antibiotic (25 µg/ml chloramphenicol or 50 µg/ml
kanamycin), 0.4% glucose, 0.02% L-arabinose or 0.5 mM
isopropyl-β-D-thiogalactopyranoside (IPTG). N. meningitidis
strain HB-1[28], an unencapsulated derivative of the serogroup
Neisseria gonorrhoeae stain FA1090 from our laboratory collection were grown at 37°C in candle
jars on GC agar plates (Oxoid), supplemented with Vitox
(Oxoid), and, when necessary, with an antibiotic (10 µg/ml
chloramphenicol or 80 µg/ml kanamycin). Liquid cultures were
obtained by growing N. meningitidis for 6 h in tryptic soy broth
(Becton Dickinson) in the absence or presence of 1 mM IPTG.

Plasmid Constructions

The plasmids and primers used are listed in Tables S1 and
S2, respectively. DNA fragments containing bamA genes were
obtained by PCR using genomic DNA from N. meningitidis
HB-1 plus primer pair NmF/NmR, N. gonorrhoeae FA1090
DNA plus primer pair NmF/NmR, Burkholderia mallei Bogor
DNA (provided by the Central Veterinary Institute, Lelystad,
The Netherlands) plus primer pair BmF/BmR, Bordetella
pertussis Tohama I DNA (provided by the Netherlands Vaccine
Institute, Bilthoven, The Netherlands) plus primer pair BpF/BpR
and E. coli DH5α DNA plus primer pair EcF/EcR, and cloned into
cPCR-TOPO. We used the sequences of the locus tags
NM0182, NGO1801, BMA1547, BP1427 and b0177,
respectively (www.ncbi.nlm.nih.gov/gene), to design primers.
The primers carried restriction sites allowing for subcloning of
the genes behind the IPTG-inducible promoter on plasmid
pFP10-c-ɪbpa, which is able to replicate in N. meningitidis
as well as in E. coli. As a result of the cloning strategy, the original
signal sequences were substituted by that of the lactoferrin-
binding protein A (LbpA) of N. meningitidis (MNKKHSFPPLTLTALAIATAFPSYA). The Ndel-AatII fragments, carrying
N. meningitidis, N. gonorrhoeae, and E. coli bamA
genences were ligated into Ndel-AatII-digested pFP10-c-ɪbpa,
yielding pFP10-NmbbamA, pFP10-NgbbamA, and pFP10-
EcbamA, respectively. The fragments containing B. pertussis
and B. mallei bamA were excised using the Ndel site introduced
by the forward primers and the KpnI site of pCRII-
TOPO and introduced into Ndel-KpnI-digested pFP10-
EcbamA, yielding pFP10-BbpbamA and pFP10-BmbbamA,
respectively.

A construct encoding the C-terminal 317 amino acids of
NmBamA fused to the signal sequence of LbpA was created by
PCR using primer pair Omp85-F481-NmR and pFP10-
NmbbamA as template. The resulting PCR product was cloned
into pCRII-TOPO and ligated into Ndel-KpnI-digested pFP10-
EcbamA, yielding pFP10-BbpbamA and pFP10-BmbbamA,
respectively.

The N- and C-terminal domains of NmBamA and EcBamA
were swapped resulting in chimeras. Two chimeric bamA
genences were designed encoding proteins in which the N-
terminal and C-terminal domains of NmBamAand EcBamA
were exchanged within a small region of high homology around
the start of the predicted 12-stranded β-barrel [4]. The
construct designated EcNmbNm encodes a protein carrying the
N-terminal part of EcBamA and the C-terminal part of
NmBamA, while construct NmEcEc encodes the reverse
chimera. The numbers indicate the most C-terminal amino acid
of the BamA domain present in the N-terminal part of the
chimera (counting the C-terminal signal sequence). For the
EcNmbNm construct, two overlapping PCR products were made:
one using the EcF and HingeR primers and pFP10-EcbamA
as template and the other using the HingeF and NmR primers and
pFP10-NmbbamA as template. The purified PCR products were
mixed, melted and annealed and combined with EcF and NmR
as external primers in a second PCR to obtain the complete
chimeric genes. For the NmEcEc chimera, also two PCR
products were generated: one using NmF and HingeR
primers and pFP10-NmbbamA as template, and the other with
HingeF and EcR as primers and pFP10-EcbamA as template.
The second PCR was performed using purified PCR products
and NmF and EcR as external primers. The obtained chimeras
were introduced into pCRII-TOPO. The Ndel-AatII fragments
were subsequently ligated into pFP10-c-ɪbpa digested with
Ndel and AatII, yielding constructs pFP10-EcNmbNm and pFP10-

Another set of chimeras, designated EcEcEcNm and NmEcEc,
was constructed to produce hybrid proteins where domain
exchange occurred at the C terminus of the predicted POTRA5
domain [18] with the N-terminal domain of the chimeras comprising 423 amino-acid residues. To make the EcNm construct, a megaparamper was created using primers EcNmF and Omp85R2, and pFP10-NmbamA as the template. The purified megaparamper was combined with the EcR primer and pFP10-EcbamA as the template for a second PCR and the product was cloned into pCRRI-TOPO. The chimeric fragment was then introduced into pFP10-NmbamA, using the Ndel site and the KpnI site present in the N. meningitidis bamA gene, yielding pFP10-EcNm-Nm. For the NmEc chimera, megaparamper1 was generated using primer pair NmEcR-Omp85F3 and pFP10-NmbamA as the template. For the second PCR, megaparamper1 was combined with the EcR primer and pFP10-EcbamA as the template. The chimeric fragment obtained was cloned into pCRRI-TOPO, yielding pCRRI-NmEc-a. Megaparamper2 was produced using primers Omp85F4 and Omp85R7 and pFP10-NmbamA as template. Megaparamper2 was then combined with EcR and pCRRI-NmEc-a as template in a PCR. The resulting PCR product was cloned into pCRRI-TOPO, yielding pCRRI-NmEc-b. The missing 5' part of NmbamA was added through insertion of a Sall-Nott fragment of pCRRI-NmbamA, a precursor of pFP10-NmbamA, into Sall-Nott-restricted pCRRI-NmEc-b, yielding pCRRI-NmEc-c. Finally, an Ndel-KpnI fragment of pCRRI-NmEc-c was ligated into Ndel-KpnI-digested pFP10-EcbamA, yielding pFP10-NmEc. Plasmid pRV-His-NmbamA was created by insertion of a Nott-AatII fragment, encoding NmbamA with an additional HHHHHHQQDF amino-acid sequence between the signal sequence and the N terminus of the mature BamA protein, from pEN11-His-Omp85 into Nott-AatII restricted pRV2000. All constructs were verified by sequencing.

RT-PCR

Reverse transcription-PCR (RT-PCR) was performed as described [29] except that conventional PCR instead of real-time PCR was performed for amplification of cDNA. Primer couples Q-for-1/Q-rev-Ngo, Q-for-2/Q-rev-Eco, Q-for-3/Q-rev-Bper and Q-for-1/Q-rev-Bmal were used to amplify the bamA cDNAs of N. gonorrhoeae, E. coli, B. pertussis and B. mallei, respectively.

Complementation Assays

To test whether various BamA variants could complement BamA deficiency in E. coli, pFP10-based plasmids containing bamA variants under the control of an IPTG-inducible promoter were introduced into EcBamA-depletion strain UTP::bamA, which produces EcBamA from an arabinose-inducible promoter. Growth of the resultant strains in LB with indicated supplements was assessed at 37°C or on LB plates at 22°C. To evaluate BamA complementation in N. meningitidis, it was tested whether the chromosomal copy of bamA could be disrupted, when bamA variants were expressed from the pFP10-derived plasmids. To this end, pRV1300 was used as the template to generate a PCR product containing a fragment upstream of N. meningitidis bamA, a kanamycin-resistance cassette and a 3' fragment of bamA, as described [4]. The PCR fragment was used to transform HB-1 cells carrying bamA variants on plasmid. Transformations were done in the presence of IPTG and transformants were selected on GC agar plates supplemented with 1 mM IPTG and kanamycin and analyzed by PCR using primer pair G and Omp85R2 that hybridize upstream and in the 3' part of NmbamA.

Isolation of Cell Envelopes

To isolate cell envelopes, bacteria from liquid cultures were collected by centrifugation, resuspended in 50 mM Tris-HCl, 5 mM EDTA (pH 8.0) containing protease inhibitor cocktail “Complete” (Roche) and stored overnight at -80°C. After ultrasonic disintegration (3 x 45 s at level 8, output 40%, Branson sonifier 450; Branson Ultrasonics Corporation), unbroken cells were removed by centrifugation (12,000 g, 15 min, 4°C). Cell envelopes were collected by ultracentrifugation (170,000 x g, 5 min, 4°C), dissolved in 2 mM Tris-HCl (pH 7.6) and stored at -20°C.

Urea Extraction

Thirty-ul samples of cell envelope preparations were incubated in 1 ml of 20 mM Tris-HCl, 100 mM glycine (pH 7.6), 6 M urea for 1 h at room temperature while rotating. The insoluble material was separated from the soluble fraction by ultracentrifugation (200,000 g, 1 h, 4°C). The pellet was dissolved in 30 μl of 2 mM Tris-HCl (pH 7.6), while the solubilized proteins were precipitated from the supernatant with 10% trichloroacetic acid (TCA) and dissolved in 30 μl H2O.

Trypsin Treatment

Exponentially growing cells were pelleted and resuspended in phosphate-buffered saline plus 1 mM MgCl2 and 0.5 mM CaCl2 to an optical density at 550 nm (OD550) of 2. Trypsin was added to 200-μl portions of this suspension. After incubation for 15 min at room temperature, 1 mM phenylmethanesulfonyl fluoride (PMSF) was added, and the bacteria were collected by centrifugation and boiled in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell envelopes were treated with 50 μg/ml trypsin at room temperature for the indicated time periods and subsequently boiled in SDS-PAGE sample buffer.

SDS-PAGE and Immunoblotting

Proteins were analyzed by standard denaturing SDS-PAGE as described [14], followed by staining with Coomassie Brilliant Blue or silver [30], or immunoblotting as described [14]. EcBamA was detected with a rabbit antiserum raised against the denatured full-length protein [23]. Rabbit antisera against the N-terminal (residues 22-464) and C-terminal (residues 455-797) regions of N. meningitidis BamA (α-N-NmbamA and α-C-NmbamA, respectively) were generously provided by Ralph Judd (University of Montana, USA). The antisera against E. coli BamD and BamB were generous gifts of Naoko Yokota and Hajime Tokuda (University of Tokyo, Japan). A monoclonal antibody (Mab) directed against the POTRA1 domain of NmBamA (α-POTRA1NmbA) came from GlaxoSmithKline Biologicals (Rixensart, Belgium).
Affinity Purification

Extraction and purification of His-tagged BamA from cell envelopes using Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA)-agarose beads (Qiagen) was performed as described [14].

Results

E. coli BamA does not function in N. meningitidis

To determine whether E. coli BamA could substitute for N. meningitidis BamA in vivo, a plasmid containing the EcbamA gene under control of a lac promoter was introduced into N. meningitidis strain HB-1. This resulted in IPTG-dependent expression of EcbamA in HB-1 (Figure 1A). As a control, HB-1 was also transformed with a similar plasmid containing N. meningitidis BamA. Next, we attempted to disrupt the chromosomal NmbamA copy in the transformants. Correct mutants were easily obtained when N. meningitidis bamA was expressed from plasmid. In contrast, PCR analysis showed that the few kanamycin-resistant transformants that were obtained from the cells expressing EcBamA still contained the wild-type copy of NmbamA on the chromosome. Apparently, EcBamA cannot replace N. meningitidis BamA.

The inability of EcBamA to substitute for NmbamA could be due to its failure to become correctly assembled in the meningococcal OM. To test this hypothesis, we analyzed the extractability of EcBamA with urea from these membranes. Correctly inserted OMPs are not usually extracted with urea, as demonstrated for the neisserial porins in Figure 1B (left panel). However, most of EcBamA present in neisserial cell envelopes was extractable with urea (Figure 1B, left panel), whereas, as expected, this was not the case for EcBamA in E. coli cell envelopes (Figure 1B, right panel). Correctly assembled β-barrel domains of OMPs are also usually resistant to proteases. Accordingly, the intact C-terminal β-barrel domain of EcBamA can be obtained by treating E. coli cell envelopes with trypsin [23] (Figure 1C). However, when neisserial cell envelopes containing EcBamA were treated with trypsin, no protected fragment was found (Figure 1C), indicating that no part of EcBamA is properly assembled into the OM. Together, these data demonstrate that EcBamA, for the most part, was not inserted in the neisserial OM and, therefore, cannot be expected to functionally replace NmbamA.

N. meningitidis BamA does not function in E. coli

To assess whether N. meningitidis BamA can substitute for E. coli BamA, we made use of the EcBamA-depletion strain UTP$\text{ec}$bamA in which chromosomal EcbamA expression is under control of an arabinose-inducible promoter resulting in a growth-arrest after prolonged growth in the absence of arabinose (Figure 2A). Introduction of pFP10-NmbamA into this strain did not rescue growth in the absence of arabinose even when NmbamA synthesis was induced with IPTG (Figure 2B), while a control strain containing EcBamA under IPTG control on pFP10-EcbamA continued to grow under these conditions (Figure 2C). The expression of N. meningitidis bamA in response to IPTG induction was confirmed on immunoblots (Figure 3A). Even under conditions of slow growth, i.e. on LB plates at 22°C, NmbamA did not support growth of E. coli (Figure S1). Thus, BamA of N. meningitidis does not function in E. coli. Interestingly, N. meningitidis BamA appeared to be inserted into the E. coli OM since it was not extracted from the membrane fraction with urea just as in neisserial cell envelopes (Figure 3B).

EcBamA is associated with several lipoproteins, i.e., BamB, BamC, BamD and BamE [6,13]. A failure to associate with...
these lipoproteins might explain the lack of function of neisserial BamA in \textit{E. coli}. To test this possibility, pRV-His-NmBamA encoding NmBamA with a His-tag at the N terminus of the mature protein was introduced into \textit{E. coli} DH5a and expression of the recombinant protein was induced with IPTG. Cell envelopes were extracted with Elugent and the His-tagged NmBamA was affinity purified with Ni$^{2+}$-NTA beads under native conditions (Figure 3C). A control purification was done on cells not producing the His-tagged NmBamA. Remarkably, substantial amounts of \textit{E. coli} BamD co-purified with the neisserial BamA (Figure 3C, lane 2), while no BamD was detected in the eluate of the control experiment with cells not expressing the His-tagged NmBamA (Figure 3C, lane 1). In contrast to this essential lipoprotein, the non-essential lipoprotein BamB did not specifically co-purify with NmBamA (Figure 3C). These results enforce the notion that at least a portion of NmBamA produced in \textit{E. coli} is correctly assembled.

**Complementation by Other BamA Homologs**

The observation that EcBamA and NmBamA could not functionally replace each other in vivo could be related to the evolutionary distance between \textit{E. coli}, belonging to the class of \textgamma-proteobacteria, and \textit{N. meningitidis}, a \textbeta-proteobacterium. Therefore, we next investigated whether BamA from other \textbeta-proteobacteria, i.e. those of \textit{B. pertussis}, \textit{B. mallei} and \textit{N. gonorrhoeae}, could functionally substitute BamA in \textit{N. meningitidis}. Strain HB-1 was transformed with plasmids containing these \textit{bamA} genes under \textit{lac} promoter control. \textit{N. gonorrhoeae} and \textit{B. pertussis} BamA protein production could be verified by immunoblotting utilizing the apparent cross-reactivity of the α-N-NmBamA and α-EcBamA antisera, respectively (Figure 4A). Expression of the \textit{B. mallei} bamA gene was demonstrated by RT-PCR (Figure 4B). Next, we tested whether the chromosomal \textit{bamA} gene could be inactivated while the cells were kept in the presence of IPTG. We only succeeded to obtain correct mutants when the gonococcal \textit{bamA} was expressed from plasmid. Thus, apparently, the meningococcal BamA protein can be functionally replaced by BamA of the closely related species \textit{N.}

Figure 2. Functionality of BamA variants in \textit{E. coli}. Growth of \textit{E. coli} strain UTP$_{bad}::$\textit{bamA} carrying no plasmid (panel A) or plasmids encoding the proteins indicated in the upper right hand side of the graphs was assessed by measuring the OD$_{600}$. Strains were grown in the presence of glucose (open circles), arabinose (closed diamonds) or IPTG (closed triangles) in LB at 37°C. Cultures were diluted into fresh medium at the time points indicated by arrows.

doi: 10.1371/journal.pone.0085799.g002
Lack of Cross-Species Functionality of BamA

Functionality of Chimeras of *N. meningitidis* and *E. coli* BamA

To determine which part of the BamA protein could be dictating the species specificity of functionality, two sets of chimeras were constructed in which the N- and C-terminal domains of *Nm*BamA and *Ec*BamA are swapped. For the first couple of constructs, designated Ec423Nm and Nm423Ec, the site of the domain exchange was chosen based on a model predicting a 12-stranded β-barrel in the C-terminal part of BamA [4] (Figure 5A,B). The second set of chimeras, designated Ec432Nm and Nm432Ec, was designed based on a FhC-like 16-stranded β-barrel [21]; in this case the domains were swapped directly after the C terminus of POTRA5 (Figure 5A,B). The chimeric genes were cloned behind an IPTG-inducible promoter and introduced into *N. meningitidis*. Synthesis of all hybrid proteins was detected when immunoblots with cell envelopes were probed with appropriate antisera (Figure 6A). However, the chromosomal copy of *Nm*bamA could not be inactivated during expression of any of the chimeras indicating that all four chimeras are non-functional in *N. meningitidis*.

To test whether the chimeric proteins were assembled in the OM, cell envelopes were treated overnight with trypsin. Such treatment of wild-type membranes yields three bands reactive with an antisemur directed against the C-terminal domain of *Nm*BamA indicated with I, II and III in Figure 6B [14]. Treatment of the membranes containing the Ec423Nm or Ec479Nm chimeras with trypsin also yielded bands I, II and III; this was expected since these membranes also contain native *Nm*BamA encoded by the chromosome. Remarkably, additional distinct fragments of 37 and 30 kDa were detected with the antiserum (indicated with asterisks in Figure 6B) indicating that the C-terminal β-barrel domain of these chimeras was assembled into the OM.

Next, we assessed functionality of the chimeric BamA proteins in *E. coli* by introducing the plasmids containing the chimeric constructs into the *Ec*BamA-depletion strain. All chimeric proteins were produced after growth of the cells in the presence of arabinose plus IPTG (Figure 7). Extraction of the membrane fraction with urea indicated that the chimeric proteins Ec423Nm and Ec479Nm were fully inserted into the *E. coli* OM, whereas the Nm423Ec and Nm432Ec proteins were each for ~50% extractable and, therefore, only partially inserted (Figure 7). The plasmid-encoded full-length *Ec*BamA was also partially extracted (Figure 7 bottom panel), in contrast to

gonorrhoeae, which shows 95% amino-acid sequence identity with the meningococcal one (Figure S2A), but not by those of *B. pertussis* or *B. mallei*, which are less similar (Figure S2B). We evaluated also whether the *B. pertussis* and *B. mallei* BamA proteins could substitute for *E. coli* BamA. Neither one allowed for growth of the *E.coli* BamA-depletion strain in the absence of arabinose and the presence of IPTG at 37°C (Figure 2D,E) or at 22°C (Figure S1) although they were expressed (Figure 4C), demonstrating that they cannot functionally substitute *Ec*BamA.

Figure 3. Expression and assembly of *N. meningitidis* BamA in *E. coli*. A: Expression of NmbamA in *E. coli*. *E. coli* strain UTP::bamA containing pFP10-NmbamA was grown in LB containing 0.02% arabinose in the absence or presence of IPTG. Cell envelopes were isolated and analyzed by immunoblotting with Mab α-POTRA1 from Nm. B: Assembly of NmBamA in the *E. coli* OM. Cell envelopes from *E. coli* strain UTP::bamA containing pFP10-NmbamAgrown in LB containing 0.02% arabinose and IPTG were extracted with urea (Ec). A control, cell envelopes of strain HB-1 were extracted with urea (Nm). Input (i), urea-insoluble (p) and -soluble (s) fractions were analyzed by SDS-PAGE and immunoblot analysis with Mab α-POTRA1 from Nm. C: Co-purification of Bam-complex components with His-tagged NmBamA in *E. coli*. Cell envelopes of *E. coli* DH5α cells containing pRV-His-NmbamA either induced (lanes 2) or not (lanes 1) with IPTG were extracted with Elugent and subjected to Ni²⁺-NTA purification. Shown are elution fractions analyzed by denaturing SDS-PAGE and silver staining (left panel) or immunoblotting (right panels) using antisera against the indicated proteins. As a positive control for BamB detection, cell envelopes derived from strain DH5α were also analyzed on blot (lane c). The arrow indicates the position of BamB.

doi: 10.1371/journal.pone.0085799.g003

PLOS ONE | www.plosone.org 6

December 2013 | Volume 8 | Issue 12 | e85799
endogenously expressed chromosomal EcBamA (Figure 1B). Apparently, the cells cannot assemble overexpression levels of EcBamA. Despite significant insertion, none of the chimeras appeared to complement the absence of endogenous EcBamA, since the strains stopped growing in the absence of arabinose and presence of IPTG at 37°C (Figure 2F-I) or at 22°C (Figure S1).

Figure 4. Expression of heterologous bamA variants in N. meningitidis and E. coli. A, B: N. meningitidis strain HB-1 and its derivatives were grown with IPTG to induce expression of B. pertussis (Bper), B. mallei (Bmal), N. gonorrhoeae (Ngo) and as control, E. coli (Eco) bamA genes from plasmids. A: Cell lysates were analyzed by SDS-PAGE and immunoblotting with the antisera indicated on the right. Note that also chromosome-encoded NmBamA is detected with the α-N-NmBamA antisera in the upper panel. B: RNA was isolated and treated or not with reverse transcriptase (RT). Specific cDNA was detected by conventional PCR followed by agarose gel electrophoresis. The -RT samples serve as controls for the absence of plasmid DNA in the RNA preparations. C: RNA was isolated from UTP\_BAD::bamA containing pFP10-BmbamA (Bmal) or pFP10-BpbamA (Bper) grown in the presence of arabinose and IPTG and processed as explained for panel B.

doi: 10.1371/journal.pone.0085799.g004

Figure 5. Schematic representation of the BamA chimeras used in this study. A: Two sets of chimeras were constructed based on two different topology models for BamA. In one model, the POTRA domains (P1-P5) are connected via a hinge region (H) to a 12-stranded β-barrel (left). In the other model, the POTRA domains are directly connected to a larger, 16-stranded β-barrel (right). E. coli-derived polypeptides are indicated in grey and N. meningitidis derived polypeptides in white. B: ClustalW alignment of partial sequences (residues 417-485, counting the signal sequence) of N. meningitidis and E. coli BamA. The C terminus of POTRA5 (T423) [18] and the N terminus of the predicted 12-stranded β-barrel (V483) [4] are indicated by arrows. The β-strands 1 through 4 plus the start of the fifth, forming the BamA β-barrel as determined in the recently published crystal structure of NgBamA [37], are indicated with grey arrows.

doi: 10.1371/journal.pone.0085799.g005
Analysis of the NmBamA β-barrel Domain

The simplest model explaining the correct assembly of the C-terminal parts of both the Ec423Nm and the Ec479Nm chimera into the neisserial OM would be that their membrane-embedded domains are fully comprised within the NmBamA-derived part. These should then have identical conformations. To test this notion, we treated intact cells with limited amounts of trypsin in order to target only the surface-exposed part of the BamA variants. Trypsin treatment of cells of the parent strain HB-1 or HB-1 cells expressing the Ec423Nm or Ec479Nm chimera did not affect chromosomally encoded wild-type NmBamA as deduced from similar reactivity with Mab α-POTRA1Nm, which specifically recognizes POTRA1 of N. meningitidis BamA and, therefore, only reacts with endogenous NmBamA (Figure 8A, left panels). Apparently, no readily accessible trypsin-cleavage sites are exposed at the cell surface in native NmBamA. Next, we probed the samples with an antiserum directed against EcBamA to detect specifically the chimeric proteins. Remarkably, an approximately 7-kDa fragment was cleaved off from the majority of the Ec479Nm protein whereas little or no cleavage was observed for the Ec423Nm chimera (Figure 8A, right panels).
proteins, designated starting with amino acid 481 of amino acid 420 of that extraction experiments indicated that implying that this domain contains 16 β-strands.

To test whether individual 16- or 12-stranded NmBamA β-barrels could be stably inserted in the OM, we produced such proteins, designated NmBamA (mature protein starting with amino acid 420 of NmBamA) and NmBamA (mature protein starting with amino acid 481 of NmBamA), in HB-1. Urea extraction experiments indicated that NmBamA was completely and NmBamA was only partially inserted in the OM (Figure 8B). Trypsin treatment of cell envelopes showed that NmBamA was completely protected from the protease, whereas NmBamA was for the most part degraded (Figure 8C). The NmBamA protein migrated at exactly the same position as the trypsin-protected fragment of EcBamA, suggesting that they possess a similar membrane domain, i.e. a stably integrated 16-stranded β-barrel.

Discussion

In this work we studied the species-specific functioning of BamA, the central component of the OMP insertion machinery, which is essential and conserved in Gram-negative bacteria. Previously, it was reported that the BamA protein of the cyanobacterium Anabaena sp. PCC 7120 did not function in E. coli, despite its insertion in the E. coli OM [31]. This lack of function may be due to the large phylogenetic distance between these bacterial species which is reflected in the distinct pore diameters, differing number of POTRA domains (3 versus 5) and differences in associated complex components for BamA proteins from cyanobacterial and proteobacterial origin [32-35]. We now show that the BamA species specificity extends to much more closely related species, since even BamA homologs of proteobacteria or even within one class of proteobacteria were generally not exchangeable.

Several factors might potentially contribute to this lack of cross-complementation: insufficient assembly of the heterologous BamA into the OM of the host, an inability to associate with the accessory lipoproteins into a fully functional OMP-insertion machinery, or failure of the heterologous BamA to efficiently recognize various OMP substrates. E. coli BamA was not assembled into the OM of N. meningitidis and therefore could obviously not compensate for the absence of neisserial BamA. In contrast, N. meningitidis BamA appeared to be inserted into the E. coli OM, at least to a substantial extent. Previously, we showed that neisserial OMPs are only poorly inserted into the E. coli OM. This defect was related to the nature of the penultimate amino acid of the OMP: in Neisseria this residue is almost invariably a positively charged one, whereas in E. coli this is only very rarely the case [23]. Replacing the lysine present in this position in the neisserial porin PorA by glutamine greatly enhanced PorA assembly in E. coli [23]. NmBamA is one of the few neisserial OMPs possessing a threonine instead of a positively charged residue at the penultimate position; therefore, it might be sufficiently recognized by the Bam complex to be inserted into the E. coli OM. Neisserial BamA was even able to associate in the OM of the host, an inability to associate with the accessory lipoproteins into a fully functional OMP-insertion machinery, or failure of the heterologous BamA to efficiently recognize various OMP substrates. E. coli BamA was not assembled into the OM of N. meningitidis and therefore could obviously not compensate for the absence of neisserial BamA. In contrast, N. meningitidis BamA appeared to be inserted into the E. coli OM, at least to a substantial extent. Previously, we showed that neisserial OMPs are only poorly inserted into the E. coli OM. This defect was related to the nature of the penultimate amino acid of the OMP: in Neisseria this residue is almost invariably a positively charged one, whereas in E. coli this is only very rarely the case [23]. Replacing the lysine present in this position in the neisserial porin PorA by glutamine greatly enhanced PorA assembly in E. coli [23]. NmBamA is one of the few neisserial OMPs possessing a threonine instead of a positively charged residue at the penultimate position; therefore, it might be sufficiently recognized by the Bam complex to be inserted into the E. coli OM. Neisserial BamA was even able to associate in the E. coli OM with the essential Bam-complex component BamD but not with BamB. The latter is consistent with the absence of a BamB homolog in N. meningitidis[14]. We did not test association of BamC and BamE, but, since these proteins have been shown to interact indirectly with BamA via BamD [13,36], they are likely to form part of the hybrid complex. Thus, despite its proper assembly into the OM and its association with at least the other essential Bam complex component, NmBamA could not functionally replace EcBamA. Therefore, the most likely explanation for its lack of function in E. coli is poor recognition of the host’s OMP substrates.

Paradoxically, whereas the lack of substrate recognition is apparently an issue when BamA proteins are exchanged...
between different proteobacteria, we recently showed that *E. coli* OMPs could be assembled into the OM of yeast mitochondria [25], and vice versa, that a mitochondrial OMP could be inserted into the *E. coli* OM [26]. However, these assemblies were efficient only when the OMPs were expressed at a low level with extensive amounts of unassembled OMPs accumulating at higher expression levels. It seems likely that also *NmBamA* can handle *E. coli* OMP substrates to some extent. However, due to the fact that for cellular survival it needs to deal with all OMPs under conditions of suboptimal recognition, it is not unexpected that *NmBamA* fails to sufficiently handle this load of substrates. Cells then likely die from lethal accumulation of periplasmic OMP aggregates.

Chimeric proteins comprising BamA domains derived from *N. meningitidis* and *E. coli* were not functional in either host. All evidence indicated that the two chimeras containing the C-terminal part of *N. meningitidis* BamA were inserted into the neisserial OM. However, the conformation of the β-barrel domain of the Ec<sub>479</sub>Nm protein was different as indicated by its accessibility to trypsin in intact cells. The apparent cleavage in one of its extracellular loops also explains the considerably smaller trypsin-resistant fragment detected after trypsinization of the Ec<sub>479</sub>Nm protein in cell envelopes (Figure 6B). In contrast, similar to wild-type *NmBamA*, the Ec<sub>423</sub>Nm chimera was resistant to trypsin digestion in intact cells. The most straightforward explanation for these results is that the protein segment between residues 423 and 479 does not constitute a hinge region between the POTRA domains and the β-barrel according to the original 12-stranded β-barrel model, but is actually part of the β-barrel. Consequently, in the Ec<sub>479</sub>Nm protein, four β-strands of the *NmBamA* β-barrel are substituted by the corresponding ones of *EcBamA* and this substitution results in a conformational change rendering a surface-exposed loop near the C-terminal end sensitive to trypsin. Overall, these data support a 16-stranded rather than the originally proposed 12-stranded membrane β-barrel.

Interestingly, during submission of this manuscript the structure of *N. gonorrhoeae* BamA, refolded from solubilized inclusion bodies, was elucidated [37] and found to consist of a large periplasmic domain attached to a 16-stranded β-barrel domain. Thus, our observations from in vivo assembled proteins are consistent with the reported BamA structure.

Despite apparently correct membrane insertion of the Ec<sub>423</sub>Nm and Ec<sub>479</sub>Nm chimeras, the proteins could not functionally replace *NmBamA*. Trypsin digestion of the chimeras in cell envelopes did not yield the intermediate fragments seen for native BamA. These fragments are indicative for association with other Bam complex members [14]. Therefore, the EcBamA-derived POTRA domains in both chimeras apparently fail to associate with the accessory Bam complex components in *N. meningitidis*, which would explain the lack of function of both chimeras in *N. meningitidis*. In

---

**Figure 8. Analysis of the β-barrel domain of *NmBamA*.** A: Intact HB-1 cells and HB-1 cells producing Ec<sub>423</sub>Nm or Ec<sub>479</sub>Nm were treated with the indicated concentrations of trypsin for 15 min and processed for immunoblotting with α-POTRA<sub>1Nm</sub> (left panels) or α-EcBamA (right panels) antibodies. B. Cell envelopes of HB-1 producing the proteins indicated at the right were treated with urea and processed for immunoblotting with α-C-NmBamA antiserum. C: Cell envelopes of HB-1 producing the proteins indicated on top were treated overnight with or without trypsin and analyzed on immunoblots with α-C-NmBamA antiserum. The # signs indicate the positions of the truncated 420<sub>Nm</sub> BamA and 481<sub>NmBamA</sub> variants, whereas the * signs indicate tryptic fragments derived from the chimeric Ec<sub>423</sub>Nm or Ec<sub>479</sub>Nm proteins (similar to those shown in Fig. 6B).

doi: 10.1371/journal.pone.0085799.g008
addition, it is entirely possible that the EcBamA-moiety of the chimeras does not efficiently recognize the neisserial OMP substrates. In E. coli, we expected at least the Ec423Nm chimeras to function. This hybrid consists of the periplasmic POTRA domains from EcBamA and the entire β-barrel from NmBamA. Like the wild-type EcBamA, it was inserted into the OM of E. coli as shown in urea-extraction experiments. With its periplasmic domain derived from EcBamA, this chimeric protein should have no problem recognizing E. coli OMP substrates or recruiting accessory Bam components. However, these features were apparently not sufficient to allow for complete functioning. The lack of function of this hybrid suggests that appropriate interactions between the POTRA domains and the β-barrel are essential for function. Indeed, the POTRAs domain was found to interact with several periplasmic loops in the crystal structure of MgbamA [37]. Such interaction may be essential and not be possible in the case of the Ec423Nm hybrid, which could also explain its lack of function.

In conclusion, our results demonstrate high species specificity in the functioning of the BamA component of the OMP assembly machinery. This specificity is most likely related to the requirement for an efficient recognition of the substrate OMPs and subtle species-specific differences in the recognition signals in these substrates. Furthermore, our results obtained with in vivo assembled BamA are consistent with the recently reported crystal structure of BamA [37]. Finally, our results indicate that specific interactions between the β-barrel and the POTRA domains might be essential for appropriate functioning.

References


