The β -Barrel Outer Membrane Protein Assembly Complex of *Neisseria meningitidis*[∇]

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The evolutionarily conserved protein Omp85 is required for outer membrane protein (OMP) assembly in gram-negative bacteria and in mitochondria. Its *Escherichia coli* homolog, designated BamA, functions with four accessory lipoproteins, BamB, BamC, BamD, and BamE, together forming the β -barrel assembly machinery (Bam). Here, we addressed the composition of this machinery and the function of its components in *Neisseria meningitidis*, a model organism for outer membrane biogenesis studies. Analysis of genome sequences revealed homologs of BamC, BamD (previously described as ComL), and BamE and a second BamE homolog, Mlp. No homolog of BamB was found. As in *E. coli*, ComL/BamD appeared essential for viability and for OMP assembly, and it could not be replaced by its *E. coli* homolog. BamE was not essential but was found to contribute to the efficiency of OMP assembly and to the maintenance of OM integrity. A *bamC* mutant showed only marginal OMP assembly defects, but the impossibility of creating a *bamC bamE* double mutant further indicated the function of BamC in OMP assembly. An *mlp* mutant was unaffected in OMP assembly. The results of copurification assays demonstrated the association of BamC, ComL, and BamE with Omp85. Semi-native gel electrophoresis identified the RmpM protein as an additional component of the Omp85 complex, which was confirmed in copurification assays. RmpM was not required for OMP folding but stabilized OMP complexes. Thus, the Bam complex in *N. meningitidis* consists of Omp85/BamA plus RmpM, BamC, ComL/BamD, and BamE, of which ComL/BamD and BamE appear to be the most important accessory components for OMP assembly.

Membrane-embedded β -barrel proteins are found in the outer membranes (OMs) of gram-negative bacteria, mitochondria, and chloroplasts. Only in recent years have cellular components required for the assembly and insertion of these OM proteins (OMPs) into the OM been identified. Omp85, which was first characterized in *Neisseria meningitidis*, is the key protein of the OMP assembly machinery (41). The function of Omp85 has been preserved during evolution, not only in gram-negative bacteria (8, 37, 44, 46) but also in mitochondria, where an Omp85 homolog, also known as Tob55 or Sam50, was shown to mediate the assembly of β -barrel proteins into the OM (15, 23, 27). Accordingly, bacterial OMPs are still recognized by the eukaryotic assembly machinery: when expressed in yeast, bacterial OMPs were found to be assembled into the mitochondrial OM in a Tob55-dependent manner (43). Omp85 in *Escherichia coli*, which was recently renamed BamA, for β -barrel assembly machinery (Bam) component A, is associated with at least four lipoproteins: BamB (formerly known as YfgL), BamC (NlpB), BamD (YfiO), and BamE (SmpA) (32, 46). In *E. coli*, BamB, BamC, and BamE are not essential, but the phenotypes of deletion mutants suggest that these proteins contribute to the efficiency of OMP assembly. Like BamA, BamD is an essential protein in *E. coli* (24, 26),

involved in OMP assembly (24). These lipoproteins are evolutionarily less well conserved; the mitochondrial Tob55 protein is associated with two accessory proteins, but they do not show any sequence similarity with the lipoproteins of the *E. coli* Bam complex (14).

Besides *E. coli*, *N. meningitidis* is one of the major bacterial model organisms for studies of OM assembly. As mentioned above, it was the first organism in which the function of Omp85 was identified (41), and also, the role of an integral OMP, designated LptD (formerly Imp or OstA), in the transport of lipopolysaccharide (LPS) to the cell surface was first established in *N. meningitidis* (3). With regard to OM biogenesis, *N. meningitidis* has several features that distinguish it from *E. coli*. For example, in contrast to *E. coli* (13), *N. meningitidis* mutants defective in LPS synthesis or transport are viable (3, 34), and OMPs are assembled perfectly well in such mutants (33). Furthermore, in OMP assembly mutants of *E. coli*, the periplasmic accumulation of unassembled OMPs is limited due to the induction of the σ^E extracytoplasmic stress response, which results in the degradation of unfolded OMPs (30) and the inhibition of their synthesis by small regulatory RNAs (20). In contrast, in *N. meningitidis*, most of the components involved in this response are absent (4), and unassembled OMPs continue to accumulate as periplasmic aggregates when OMP assembly is halted (41). However, the composition of the Bam complex and the role of accessory components in OMP assembly have not so far been studied in this organism. Therefore, to further understand the OMP assembly process in *N. meningitidis*, we have now analyzed the composition of the Bam complex and addressed the roles of the different components.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
TOP10F'	Cloning strain	Invitrogen
DH5 α	Cloning strain	Laboratory collection
<i>N. meningitidis</i> strains		
H44/76	Wild-type serogroup B strain	18
HB-1	H44/76 with the capsule locus replaced by an erythromycin resistance gene cassette	2
HB-1 Δ <i>bamE</i>	HB-1 with <i>bamE</i> replaced by a <i>kan</i> cassette	This study
HB-1 Δ <i>mlp</i>	HB-1 with <i>mlp</i> replaced by a <i>cat</i> cassette	This study
HB-1 Δ <i>bamC</i>	HB-1 with <i>bamC</i> replaced by a <i>kan</i> cassette	This study
HB-1 Δ <i>bamC</i> -2	HB-1 with <i>bamC</i> replaced by a <i>cat</i> cassette	This study
HB-1 Δ <i>comL</i> (pComL)	HB-1 containing pEN11-ComL with the chromosomal <i>comL</i> copy replaced by a <i>kan</i> cassette	This study
HB-1(pBamD)	HB-1 containing pEN11-BamD	This study
HB-1 Δ <i>bamE</i> (pBamE)	HB-1 Δ <i>bamE</i> transformed with pEN11-BamE	This study
HB-1 Δ <i>mlp</i> Δ <i>bamE</i>	HB-1 Δ <i>mlp</i> with <i>bamE</i> replaced by a <i>kan</i> cassette	This study
HB-1 Δ <i>omp85</i> (pHisOmp85)	HB-1 containing pEN11-HisOmp85 with the chromosomal copy of <i>omp85</i> replaced by a <i>kan</i> cassette	This study
H44/76- Δ <i>cld4</i>	H44/76 containing <i>rmpM::kan</i>	39
HB-1- Δ <i>cld4</i>	HB-1 containing <i>rmpM::kan</i>	This study
HB-1 Δ <i>rmpM</i>	HB-1 with <i>rmpM</i> replaced by a <i>kan</i> cassette	This study
HB-1 Δ <i>rmpM</i> -2	HB-1 with <i>rmpM</i> replaced by a <i>cat</i> cassette	This study
HB-1 Δ <i>rmpM</i> Δ <i>bamC</i>	HB-1 Δ <i>bamC</i> -2 with <i>rmpM</i> replaced by a <i>kan</i> cassette	This study
HB-1 Δ <i>rmpM</i> Δ <i>bamE</i>	HB-1 Δ <i>rmpM</i> -2 with <i>bamE</i> replaced by a <i>kan</i> cassette	This study
Plasmids		
pCRII-TOPO	TA cloning vector for PCR products	Invitrogen
pCRII- Δ <i>comL</i>	<i>comL</i> deletion plasmid	This study
pCRII- Δ <i>bamE</i>	<i>bamE</i> deletion plasmid	This study
pCRII- Δ <i>bamC</i> -1	<i>bamC</i> deletion plasmid containing a <i>kan</i> cassette	This study
pCRII- Δ <i>bamC</i> -2	<i>bamC</i> deletion plasmid containing a <i>cat</i> cassette	This study
pCRII- Δ <i>rmpM</i> -1	<i>rmpM</i> deletion plasmid containing a <i>kan</i> cassette	This study
pCRII- Δ <i>rmpM</i> -2	<i>rmpM</i> deletion plasmid containing a <i>cat</i> cassette	This study
pCRII- Δ <i>mlp</i>	<i>mlp</i> deletion plasmid	This study
pMB25	<i>imp</i> deletion plasmid	3
pEN11- <i>Imp</i>	<i>Neisseria</i> replicative plasmid containing H44/76-derived <i>imp</i> under <i>lac</i> promoter control	3
pEN11- <i>ComL</i>	pEN11- <i>Imp</i> with <i>imp</i> replaced by HB-1-derived <i>comL</i>	This study
pEN11- <i>BamE</i>	pEN11- <i>Imp</i> with <i>imp</i> replaced by HB-1-derived <i>bamE</i>	This study
pEN11- <i>BamD</i>	pEN11- <i>Imp</i> with <i>imp</i> replaced by DH5 α -derived <i>bamD</i>	This study
pCRII-POTRA1	pCRII-TOPO vector carrying H44/76-derived <i>omp85</i> lacking the region encoding POTRA1	5
pEN11-HisOmp85	pEN11- <i>Imp</i> with <i>imp</i> replaced by a gene encoding His ₆ -Omp85	This study
pRV1300	<i>omp85</i> deletion plasmid	41

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown on LB agar plates at 37°C. When necessary, an appropriate antibiotic (25 μ g/ml chloramphenicol or 50 μ g/ml kanamycin) was added for plasmid maintenance. *N. meningitidis* strains were grown at 37°C in candle jars on GC agar plates (Oxoid) supplemented with Vitox (Oxoid) and, when necessary, with an antibiotic (10 μ g/ml chloramphenicol or 80 μ g/ml kanamycin). Liquid cultures were grown in tryptic soy broth (TSB) (Becton Dickinson). To achieve depletion of proteins encoded by genes cloned behind an isopropyl- β -D-1-thiogalactopyranoside (IPTG)-inducible promoter, *N. meningitidis* cells grown overnight on plates containing 1 or 10 μ M IPTG as indicated below were resuspended in TSB without IPTG to an optical density at 550 nm of 0.1 and grown for 6 h. To induce the expression of IPTG-regulated genes, 0.5 mM IPTG was added at the start of the liquid culture.

Antibiotic sensitivity. Meningococci grown overnight on GC agar plates were resuspended in 100 μ l of TSB to an optical density at 550 nm of 0.025 and plated on GC agar plates. Paper discs containing 30 μ g of vancomycin (BD Biosciences) were placed on top of the agar. The plates were incubated at 37°C for 24 h, after which growth inhibition zones around the discs were measured in millimeters from the rim of the disk. All tests were repeated at least three times.

Plasmid and mutant constructions. Plasmids and primers used in this study are summarized in Tables 1 and 2, respectively. Primers were designed based on

the genome sequence of *N. meningitidis* serogroup B strain MC58 (www.tigr.org), which belongs to the same clonal complex as the strain used in this study, H44/76. Deletion constructs of *bamC*, *comL*, *bamE*, *rmpM*, and *mlp* were obtained by amplifying DNA fragments upstream and downstream of these genes by PCR using genomic DNA of strain HB-1 as template and primers indicated with Up-For and Up-Rev and Down-For and Down-Rev in Table 2. The fragments were cloned into pCRII-TOPO. Next, the upstream and downstream fragments of each gene were joined together in one plasmid by using the AccI sites that were introduced via the primers and the XbaI site in the vector. A kanamycin resistance gene (*kan*) cassette including the neisserial DNA uptake sequence, obtained from pMB25, was inserted into each plasmid after AccI restriction, yielding pCRII- Δ *bamC*-1, pCRII- Δ *comL*, pCRII- Δ *bamE*, and pCRII- Δ *rmpM*-1. A chloramphenicol resistance gene (*cat*) cassette was amplified by PCR from pACYC184 using primers P1 and P2 and cloned into pCRII-TOPO, yielding pCRII-*cat*. This cassette was used to create pCRII- Δ *mlp*, pCRII- Δ *bamC*-2, and pCRII- Δ *rmpM*-2 by AccI restriction and ligation. For allelic replacements, constructs containing the antibiotic-resistance cassette in the same transcriptional direction as the gene to be replaced were used. *N. meningitidis* was transformed as described previously (3), using PCR fragments obtained from the gene replacement constructs by using primer pair M13Rev and M13For. When appropriate, 50 μ M IPTG was added to the selection plates. The transformants were checked for the presence of the mutant alleles by PCR using the corresponding

TABLE 2. Primers used in this study

Primer	Sequence (5'-3')	Underlined restriction site
BamC-Up-For	ATGATGTGTTCCGCATCGTC	
BamC-Up-Rev	ATGTCGACGCAGCTTCCAAACAGA	AccI
BamC-Down-For	ATGTCGACTCGGACGGCATGGCTATATT	AccI
BamC-Down-Rev	ATGACGTCGGACGGCATCGTTGCCGTCG	
ComL-Up-For	ATGACGTCGAGCGACATTCGATATAGC	
ComL-Up-Rev	ATGTCGACGAGTGGCACAGGCACTCA	AccI
ComL-Down-For	AAGTCGACATGCCTTGCCAGCACG	AccI
ComL-Down-Rev	ATGTGGCTTGCCGATGTGTC	
BamE-Up-For	ATTTACCTCGCCGCACTTCACG	
BamE-Up-Rev	ATGTCGACGGTAGTGTAACACTGCTTGAATA	AccI
BamE-Down-For	ATGTCGACAGGAACACACATGACACCGC	AccI
BamE-Down-Rev	ATGCGCATTGTTTGAGGTCTCT	
Mlp-Up-For	CTTGGTAAACAGCCATTGTTCCCG	
Mlp-Up-Rev	ATGTCGACGTTCTGGAAGAGCCGCATC	AccI
Mlp-Down-For	ATGTCGACATGTTACTACCTGCCAATAC	AccI
Mlp-Down-Rev	AGTTTCGCGGCAGAGGATGCGG	
RmpM-Up-For	GAGACCGAAACCACGCGCTTG	
RmpM-Up-Rev	ATGTCGACGAAGCGAGCAATGCAACG	AccI
RmpM-Down-For	ATGTCGACAATCCGCAGCATCGTAAC	AccI
RmpM-Down-Rev	TTAGGCTCAATCGCTGCAACTGACGG	
ComL-For	ATCATATGAAAAAAATTCTTTTAAC	NdeI
ComL-Rev	ATGACGTCTTATCAGTGCCAGTAACGCCAC	AatII
BamE-For	ATCATATGGTGAACAAAACCCCTCATCCT	AccI
BamE-Rev	ATCGATCGTTATGGTTTGTCTGTGTT	PvuI
BamC-int	GTCGACGGCAAGTCTCCTGC	
ComL-int	ACGCGGCGCATATATCGCCGC	
BamE-int	TACTGCGCGACGCATTCCAT	
Mlp-int	CGCCCGCATTACGACGAAAG	
BamD-For	ATCATATGATGACGCGCATGAAA	NdeI
BamD-Rev	ATGACGTCTTATGTATTGCTGCT	AatII
His-up	GACTTCCACCACCACCACCACCAAGACTTCACCATCCAAGACATCCG	
His-down	GTCTTGGTGGTGGTGGTGGTGGTGGAAAGTCGGCAAGTGCCAAAGGCGA	
Omp85NotIF	AGCGGCCGCAAAACCGCATTCGCAACCAAGGAA	NotI
Omp85NotIR	TTTTAAGCTTTTAGAACGTCGTGCCGAGTTGGAAT	
P1	GTCGACGGATCCGTGTAGGCTGGAGCTGCTTC	AccI
P2	GTCGACGGATCCATGCCGTCTGAACATATGAATATCCTCCTTA	AccI
M13-For	GTAAAAACGACGGCCAGT	
M13-Rev	CAGGAAACAGCTATGAC	

Up-For and Down-Rev primers and for the absence of the wild-type alleles by PCR using primers annealing within the removed coding sequence (indicated with “-int” in Table 2) and the corresponding Down-Rev primer and/or by immunoblot analysis. An insertional *rmpM* mutation was created in HB-1 by transferring the *rmpM::kan* allele from H44/76- Δ cI4 into HB-1. To that end, HB-1 was transformed with a PCR product produced from H44/76- Δ cI4 chromosomal DNA with primers RmpM-Up-For and RmpM-Down-Rev.

N. meningitidis *comL* and *bamE* genes were amplified by PCR using genomic DNA of HB-1 as template and primer pairs *comL*-For/-Rev and *BamE*-For/-Rev, respectively. The resulting PCR products were cloned into pCRII-TOPO. The *comL* gene was subcloned into the neisserial replicative plasmid pEN11-Imp via *Nde*I/*Aat*II restriction and ligation, resulting in pEN11-*ComL*. The *bamE* gene was subcloned into this vector by using *Nde*I/*Pvu*I sites, yielding pEN11-*BamE*. In this vector, the expression of the inserted gene is driven by tandem *lac-tac* promoter/operator sequences. The *E. coli* *bamD* gene was PCR amplified from DNA of DH5 α using primer pair *BamD*-For/Rev. After the PCR product was cloned into pCRII-TOPO, *bamD* was subcloned into pEN11-Imp via *Nde*I/*Aat*II restriction and ligation, yielding pEN11-*BamD*.

To engineer a His tag at the N terminus of mature *N. meningitidis* Omp85, two overlapping DNA fragments were generated by PCR using primer pairs His-Up/Omp85NotIR and His-Down/Omp85NotIF and genomic DNA of HB-1 as template. The PCR products were purified and mixed for a second PCR with the external primers Omp85NotIF and Omp85NotIR, creating a DNA fragment encoding an Omp85 variant with an additional HHHHHHQDF amino acid sequence between the signal sequence and the N terminus of the mature protein. The resulting PCR product was cloned into pCRII-TOPO. The 5' fragment of the *omp85* allele obtained was excised by using the NotI site upstream of *omp85*.

and a SalI site within *omp85* and substituted for the corresponding fragment in pCRII-POTRA1, a plasmid encoding a mutant *N. meningitidis* Omp85 protein lacking its POTRA1 (polypeptide transport-associated 1) domain and containing an AatII site at the 3' end of *omp85*. The complete gene was subsequently introduced into pEN11-Imp by using NotI/AatII restriction, yielding pEN11-HisOmp85. The chromosomal copy of *omp85* in HB-1, containing pEN11-HisOmp85, was for the most part replaced by a *kan* cassette as described before (41).

Cell envelope isolation. To isolate cell envelopes, bacteria grown in TSB for 6 h 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 (three times for 45 s at level 8, output 40%, Branson sonifier 450; Branson Ultrasonics Corporation), unbroken cells were removed by centrifugation ($12,000 \times g$ for 15 min at 4°C). Cell envelopes were collected by ultracentrifugation ($170,000 \times g$ for 5 min at 4°C), dissolved in 2 mM Tris-HCl (pH 7.6), and stored at -20°C . When necessary, cell envelopes were treated with 200 $\mu\text{g}/\text{ml}$ lysozyme (Calbiochem) for 2 h at 37°C .

Trypsin digestion. The protease susceptibility of Omp85 in cell envelopes was tested by incubating samples with 50 µg/ml of trypsin (Sigma) overnight at room temperature. The samples were denatured by boiling in sample buffer and analyzed by regular sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Affinity purification. Cell envelopes were incubated in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), 2% Elugent (Calbiochem), 5 mM EDTA for 2 h at room temperature and centrifuged ($2,800 \times g$ for 30 min at room temperature). The buffer of the supernatant was exchanged for TBS, 0.1% Elugent, 20 mM imid-

azole using PD-10 columns (GE Healthcare). The extract was mixed with Ni^{2+} -nitrilotriacetic acid (Ni^{2+} -NTA)-agarose beads (Qiagen) for 1 to 2 h at 4°C while rotating. The beads were washed in TBS, 0.05% *n*-dodecyl- β -D-maltoside, 20 mM imidazole for 1 h at 4°C while rotating. After settling of the beads by gravity, proteins were eluted with TBS, 200 mM imidazole, 0.05% *n*-dodecyl- β -D-maltoside. The samples were analyzed by SDS-PAGE and immunoblotting. For immunoblotting, the fractions were concentrated 10-fold by precipitation with trichloroacetic acid.

SDS-PAGE and Western blot analysis. Protein samples were analyzed by regular SDS-PAGE (41). Alternatively, for semi-native SDS-PAGE, sample buffer lacking β -mercaptoethanol and containing only 1% SDS and running buffer containing 0.025% SDS (5) were used for analysis of Omp85 complexes, while sample buffer containing 0.1% SDS and no β -mercaptoethanol combined with running buffer containing 1% SDS was used for porin analysis. Furthermore, the gels contained no SDS and electrophoresis was carried out on ice at 12 mA. Proteins were visualized in the gels with Coomassie brilliant blue or silver (1). To enhance epitope recognition on immunoblots, native proteins were denatured within the gels by leaving the gels in steam for 20 min prior to blotting. Blotting was performed in a Bio-Rad wet blotting system in 25 mM Tris, 192 mM glycine, 0.02% SDS in 20% methanol, pH 8.3. The membranes were blocked for 1 h in phosphate-buffered saline (pH 7.6) supplemented with 0.5% nonfat dried milk (Protifar; Nutricia) and 0.1% Tween 20. The blots were incubated for 1 h with primary antibodies, washed, and then probed for 1 h with goat anti-rabbit or goat anti-mouse immunoglobulin G secondary antibodies conjugated with horseradish peroxidase or alkaline phosphatase (Southern Biotechnology Associates, Inc.) diluted in the blocking buffer. The signal was visualized with enhanced chemiluminescence (Amersham). When alkaline phosphatase-conjugated antibodies were used, the blots were incubated in 0.1 M Tris-HCl (pH 9.5), 0.1 mg/ml Nitro Blue Tetrazolium, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma-Aldrich) until color developed.

Antisera. Rabbit antisera against the N-terminal (residues 22 to 464) or C-terminal (residues 455 to 797) parts of *N. meningitidis* Omp85 and against *E. coli* BamD were generously provided by Ralph Judd (University of Montana) and by Naoko Yokota and Hajime Tokuda (University of Tokyo, Japan), respectively. A monoclonal antibody (MAb) directed against *N. meningitidis* Omp85 and anti-PilQ antiserum were produced by GlaxoSmithKline Biologicals (Rixensart, Belgium). Rabbit antisera were raised against synthetic peptides designed from the sequences of neisserial BamC (CDASALLGKLHSELRL), ComL (CVLETN FPKSPFLKQ), and BamE (CAEALDKDRQNTDKP) at Genosphere Biotechnologies (Paris, France). MAbs directed against PorA, PorB, and RmpM (reduction-modifiable protein M) were provided by The Netherlands Vaccine Institute (Bilthoven, The Netherlands). The anti-Imp antiserum was previously described (3).

RESULTS

Presence of genes encoding Bam components in neisserial genomes. In *E. coli*, the Omp85 homolog BamA is associated with the accessory lipoprotein components BamB, BamC, BamD, and BamE (32, 46). We performed BLAST searches in the genome sequence of *N. meningitidis* strain MC58 using *E. coli* protein sequences to identify the genes encoding similar lipoproteins. For BamC, we found a homolog (locus tag NMB0928) with relatively low similarity (24% identity and 37% similarity) but in the same chromosomal location as *bamC* in *E. coli* (i.e., downstream from the *dapA* gene). The homolog of BamD was identified previously in *Neisseria gonorrhoeae* and designated ComL, since a transposon insertion in the corresponding gene resulted in decreased competence (12). ComL/BamD of strain MC58 (NMB0703) shares 38% identity and 59% similarity with its *E. coli* counterpart. Searches for BamE homologs yielded two candidates: NMB0204 (37% identity and 65% similarity) and NMB1898 (25% identity and 47% similarity). Comparison of flanking genes did not provide any further clue as to which open reading frame would encode the functional homolog of BamE; the genes flanking *bamE* in *E. coli*, *recN* and *b2618*, are not found near NMB0204 or NMB1898. Given its greater similarity to BamE, we will refer

to the protein encoded by NMB0204 as the neisserial BamE homolog. The protein encoded by NMB1898 is annotated as Mlp, for meningococcal lipoprotein (47). We did not find a BamB homolog either in strain MC58 or in any other neisserial genome sequence present in databases at the NCBI. In *E. coli*, the *bamB* (*yfgL*) gene is present in a locus comprising *yfgK*, *yfgL*, *yfgM*, and *hisS*. In the neisserial genomes, a similar locus is found but without *yfgL*. A conserved region in the *E. coli* BamB was identified that is involved in interaction with BamA (42). Further BLAST searches with this conserved region in the available neisserial genomes yielded no hits. BamB has also been shown to contain seven binding motifs for pyrroloquinoline-quinone (21). Their relevance is not clear, because *E. coli* does not possess any pyrroloquinoline-quinone synthase (25). No proteins containing these domains are present in *N. meningitidis* (<http://smart.embl.de>). Thus, no BamB-like protein appears to be present in the neisseriae. The sequences and genetic organization of the *bamC*, *comL* (*bamD*), and *bamE* loci are highly conserved among the pathogenic neisseriae, as revealed by searches in the 19 currently available genome sequences of *N. gonorrhoeae* and *N. meningitidis* at the NCBI (data not shown) and as reported for *bamC* (7).

Construction of mutants defective in the synthesis of the accessory lipoproteins. To investigate whether the accessory lipoproteins identified would indeed function in OMP assembly in *N. meningitidis*, we attempted to create deletion mutants in HB-1 by replacing the corresponding genes with antibiotic resistance cassettes. Strains deficient for BamC and BamE were easily obtained, indicating that these proteins are not essential in *N. meningitidis* (Fig. 1A and B). The growth of both mutants in liquid medium was not significantly different from that of HB-1 (data not shown). The introduction into HB-1 Δ *bamE* of plasmid pEN11-BamE, which carries the *bamE* gene under the control of an IPTG-inducible promoter, resulted in a strain demonstrating regulatable *bamE* expression (Fig. 1B). To investigate the function of the second BamE homolog, Mlp, we also constructed a mutant lacking this gene and, additionally, a strain lacking both *bamE* and *mlp*. These mutants were easily obtained, demonstrating that Mlp is not essential either in a wild-type or in a *bamE* background.

Contrary to the results described above, we were not able to inactivate the *comL/bamD* gene. We were only able to inactivate this gene on the chromosome in a strain that expressed a complementing copy of *comL* from a plasmid, demonstrating that this gene is essential in *N. meningitidis*. Accordingly, the growth of the resulting strain, designated HB-1 Δ *comL*(pComL), was dependent on IPTG (Fig. 1C). This strain demonstrated an increased lag time, likely because the start culture contained fewer viable bacteria since the preculture had been substantially depleted for ComL during the overnight growth. However, in the logarithmic phase, the growth rate was similar to that of the parent strain, HB-1 (Fig. 1C). Interestingly, we could not inactivate the chromosomal *comL* gene when the *E. coli* homolog *bamD* was present on the complementing plasmid, even though we could demonstrate production of the BamD protein in the presence of IPTG with Western blotting (Fig. 1D). This lack of complementation was not due to toxicity of the BamD protein since the addition of a range of IPTG concentrations varying from 0 to 500 μ M to strain HB-1 con-

FIG. 1. BamC, BamE, and ComL mutants. (A) Cell envelopes derived from the strains indicated above the panel were separated by SDS-PAGE and immunoblotted with anti-BamC antiserum. The predicted molecular weight of BamC is 39,000; the bands below the 37,000-molecular-weight marker probably represent nonspecific reactivity of the antiserum. (B) Cell lysates of the strains indicated above the panel were separated by SDS-PAGE and immunoblotted with anti-BamE antiserum. HB-1ΔbamE(pBamE) was analyzed after growth in the presence (+) or absence (−) of 0.5 mM IPTG. (C) Growth of HB-1 and HB-1ΔcomL(pComL) in the absence (−) or presence (+) of 0.5 mM IPTG. HB-1ΔcomL(pComL) was pregrown overnight on plates containing 1 μM IPTG. OD₅₅₀, optical density at 550 nm. (D) Cell lysates of HB-1(pBamD) grown in the absence (−) or presence (+) of 0.5 mM IPTG were subjected to SDS-PAGE, blotted, and probed with anti-BamD antiserum. (E) Immunoblot, probed with anti-ComL antiserum, of cell envelopes of HB-1 and HB-1ΔcomL(pComL) grown in the absence (−) or presence (+) of 0.5 mM IPTG. In this case, HB-1ΔcomL(pComL) was pregrown overnight on plates containing 10 μM IPTG. Numbers to the left of gels are molecular weights (in thousands).

taining pEN11-BamD did not affect growth (data not shown). These results indicate a species-specific functioning of ComL/BamD. To obtain sufficient amounts of ComL-depleted cells for membrane preparations, HB-1ΔcomL(pComL) was pregrown overnight on plates containing 10 μM IPTG and, subsequently, in TSB for 6.5 h with or without IPTG. This procedure resulted in considerable growth in the absence of IPTG, yet cells became depleted for ComL as shown by immunoblotting (Fig. 1E). Of note, the amounts of ComL detected were not affected when the cell envelopes were treated with lysozyme prior to SDS-PAGE analysis (results not shown), demonstrating that ComL in *N. meningitidis* is not covalently associated with peptidoglycan, unlike what was previously reported to be the case in *N. gonorrhoeae* (12). Collectively, these data show that BamC, BamE, and Mlp are not essential in *N. meningitidis*, whereas ComL/BamD is essential even though a viable *comL* transposon insertion mutation in *N. gonorrhoeae* has been described previously (12).

Role of the accessory lipoproteins in OMP assembly. Next, we tested the impacts of the mutations on OMP assembly. Cell envelopes of the various mutants showed similar protein profiles when analyzed in denaturing SDS-PAGE (Fig. 2A). The most-abundant proteins in the meningococcal OM are porins PorA and PorB, which normally assemble into trimers that are detectable in semi-native SDS-PAGE. When OMP assembly in *N. meningitidis* is compromised due to Omp85 depletion, porins accumulate in their unassembled states and migrate at the positions of the denatured monomeric forms in semi-native SDS-PAGE (41). Using this assay, we found accumulations of unassembled porins, most prominently in the ComL/BamD-depleted strain and, to a lesser extent, also in HB-1ΔbamE (Fig. 2B). No unassembled porins were detected in the HB-1ΔbamC (Fig. 2B) or the HB-1Δmlp (Fig. 2C) strain. These observations were confirmed in immunoblots using anti-PorA

and anti-PorB antibodies (Fig. 2D). The assembly defects seen in the absence of BamE or upon depletion of ComL were complemented by the expression of *bamE* or *comL* in *trans*. In the immunoblot analysis, HB-1ΔbamC only occasionally showed a very slight defect in PorA assembly (Fig. 2D). We also tested the assembly of a nonprototypical OMP, PilQ. PilQ is a secretin that forms very stable multimers, possibly homododecamers, which are highly resistant to denaturation by SDS at high temperatures. PilQ assembly defects can therefore be detected in regular SDS-PAGE analysis by assessing the levels of monomeric PilQ (6, 9). PilQ assembly was unaffected in HB-1ΔbamC, since similar amounts of PilQ monomers were detected in this strain and in the parent strain (Fig. 2E). However, in HB-1ΔbamE and in the ComL/BamD-depleted strain, PilQ assembly was clearly diminished (Fig. 2E). Again, in both cases, the defect was restored upon the expression of *bamE* or *comL*, respectively, in *trans*. In *E. coli*, even mild OMP assembly defects can have a profound effect on the integrity of the OM, resulting in increased sensitivity to antibiotics (26, 32, 46). To evaluate whether the ΔbamC and Δmlp mutants could have such mild assembly defects, which are poorly detectable in the biochemical analyses described above, we tested the sensitivity of the strains to vancomycin in a disc diffusion assay. Like the wild-type strain, the ΔbamC and Δmlp mutants appeared completely resistant to this antibiotic, whereas the deletion of *bamE* clearly increased sensitivity, resulting in a clearing zone of 6 mm around the vancomycin-containing disk. This sensitivity was not further enhanced by the simultaneous absence of *mlp* (data not shown).

To further address a potential role for BamC in OMP assembly, we set out to construct a *bamC bamE* double mutant with the rationale that OMP assembly defects are possibly more pronounced in a double mutant than in either single mutant if both proteins are involved in this process. However,

FIG. 2. Role of putative Bam complex components in OMP assembly. (A to D) Cell envelopes were subjected to denaturing (d) or semi-native (n) SDS-PAGE and stained with Coomassie brilliant blue (A, B, C) or blotted and probed with anti-PorA or anti-PorB antibodies (D). Arrows point at unfolded porins. (E) Cell lysates were subjected to denaturing SDS-PAGE and immunoblotting with anti-PilQ antiserum. Only the relevant parts of the blot are shown. The strains are indicated below the panels as follows: 1, HB-1; 2, HB-1ΔbamC; 3, HB-1ΔbamE; 4, HB-1ΔcomL(pComL); 5, HB-1ΔbamE(pBamE); and 6, HB-1Δmlp. The prominent bands seen in lane 4 of panel A at the 28,000-molecular-weight marker likely represent a combination of phase-variable Opa proteins and ComL, which have similar predicted molecular weights. Numbers to the left of gels are molecular weights (in thousands). α, anti; +, present; -, absent.

we did not succeed in obtaining such a double mutant, either when we tried to introduce a $\Delta bamE$ *kan* mutation into a $\Delta bamC$ *cat* mutant strain or by the reverse approach. This was not due to lack of competence of the $\Delta bamC$ *cat* strain since this strain was easily transformable with an unrelated *kan* cassette-containing construct. These observations suggest that BamC and BamE function in the same process. Thus, together, our results demonstrate that ComL/BamD and BamE are required for efficient OMP assembly, whereas BamC and Mlp have a minor or no role in this process, respectively.

Characterization of an HMW Omp85 complex. Next, we wished to determine whether BamC, ComL/BamD, and BamE form a complex with Omp85/BamA, as in *E. coli*. Previously, we found that Omp85 is mostly present in a high-molecular-weight (HMW) complex when cell envelopes of *N. meningitidis* are analyzed by semi-native SDS-PAGE (41). To test whether this complex represents Omp85 with associated lipoproteins, we probed blots containing these complexes with antisera directed against Omp85 and the three lipoproteins. As expected, Omp85 was found in a HMW complex (Fig. 3A). However, none of the three lipoproteins was detected at this position (Fig. 3A). To verify the absence of the lipoproteins in the HMW Omp85 complex, we analyzed the electrophoretic mobility of this complex in cell envelopes of mutants deficient in the Omp85-associated lipoproteins. Indeed, the electrophoretic mobility of the complex from the mutants was not altered (Fig. 3B), confirming that BamC, BamE and ComL/BamD are not part of this complex. The absence of BamE and

the depletion of ComL resulted in higher levels of unfolded monomeric Omp85, consistent with their roles in OMP assembly. This defect was for the most part restored upon the expression of *bamE* or *comL* in *trans* (Fig. 3B).

Identification of RmpM in the HMW Omp85/BamA complex. Since we could not detect the accessory lipoproteins in the HMW Omp85 complex, this complex represents either a homo-oligomer of Omp85 or a complex of Omp85 with (an) other component(s). Lysozyme treatment of cell envelopes did not alter the amount of the complex or its electrophoretic mobility on a gel, demonstrating that peptidoglycan fragments are not present in the complex (data not shown). We previously found the RmpM protein to be associated with several neisserial OMP complexes, such as the porins and the lactoferrin receptor (19, 28). Therefore, we reasoned that perhaps this protein could also be present in the HMW Omp85 complex. RmpM is a two-domain protein with a so-called OmpA domain, which is thought to associate noncovalently with the peptidoglycan layer, in its C-terminal end (17). The 40-amino-acid N-terminal domain of RmpM, which is linked to the C-terminal domain via a proline-rich hinge region, is too small to form a membrane-embedded β -barrel. Also, RmpM does not contain an N-terminal cysteine residue which could be lipidated, thereby forming a membrane anchor. Instead, RmpM is thought to be associated with the OM through binding via its N-terminal domain to integral OMPs (17).

To assess the presence of RmpM in the Omp85 complex, we transferred the *rmpM* allele from a previously constructed

FIG. 3. Composition of the HMW SDS-resistant Omp85 complex. (A) Cell envelopes from strain HB-1 were analyzed by denaturing (d) or semi-native (n) SDS-PAGE, blotted, and probed with antisera indicated above the panels. (B) Cell envelopes derived from the strains indicated above the panels were analyzed by denaturing (d) or semi-native (n) SDS-PAGE, blotted, and probed with an anti-Omp85 MAb. HB-1 Δ bamE-(pBamE) was grown in the presence (+) or absence (–) of 0.5 mM IPTG as indicated. HB-1 Δ comL(pComL) was pregrown overnight on plates containing 10 μ M IPTG and subsequently grown in TSB in the absence or presence of 0.5 mM IPTG. (C to E) Cell envelopes of 1, HB-1; 2, HB-1 Δ cl4; 3, HB-1 Δ rmpM; and 4, H44/76- Δ cl4 were analyzed by semi-native SDS-PAGE and immunoblotting using an anti-Omp85 MAb. (E) Cell envelopes of HB-1 were incubated with (+) or without (–) 0.4 mM β -mercaptoethanol for 30 min prior to loading on the gel. The positions of various conformations of Omp85 are indicated as follows: UM, unfolded monomer; FM, folded monomer. Numbers to the left of gels are molecular weights (in thousands). α , anti.

rmpM mutant (39) into strain HB-1, yielding HB-1 Δ cl4. Western blot analysis with an anti-RmpM MAb confirmed the absence of the wild-type RmpM in the mutant (data not shown). The Omp85 complex of this strain migrated substantially faster in the gel (Fig. 3C, compare lanes 1 and 2), demonstrating that RmpM is part of this complex. However, Omp85 still migrated as a distinct complex at a position much higher than that of the monomer. We noticed that the *rmpM::kan* allele used to create this mutant was constructed by insertion of a *kan* cassette in the 3' end of the gene, thereby disrupting the OmpA domain but leaving the 5' end of *rmpM* intact. The resulting mutant allele could possibly encode a protein comprising the N-terminal 157 amino acids of RmpM, including the signal sequence. To test whether this protein would be sufficient for forming a complex with Omp85, we constructed an alternative *rmpM* mutant, designated HB-1 Δ rmpM, which had only 49 nucleotides left at the 5' end of the gene. Western blots of semi-native SDS-PAGE gels containing cell envelopes prepared from this mutant usually showed several separate HMW bands containing Omp85 plus significant quantities of denatured monomeric Omp85 (Fig. 3D, lane 3). However, occasionally, all of the Omp85 detected was found to migrate at its unfolded monomeric position (Fig. 3C, lane 3). This variability is likely

reflective of a very unstable complex. In contrast, mutants containing the *rmpM::kan* allele, either in an H44/76 or in an HB-1 background, reproducibly showed a distinct HMW Omp85 complex, migrating faster than that of the parental strain (middle lanes in Fig. 3C and D). Apparently, the N terminus of RmpM is required for the formation of an Omp85 complex that is stable during semi-native SDS-PAGE analysis. However, RmpM is not absolutely required for complex formation, since HMW forms of Omp85 were still detected in its absence, suggesting that other, unknown, components are associated with Omp85 or that Omp85 migrates as homo-oligomers.

Since RmpM is present in many different HMW OMP complexes (28), we could not unequivocally demonstrate the presence of RmpM in the HMW Omp85 complex by probing blots similar to those shown in Fig. 3A with anti-RmpM antibodies. However, an additional indication for the presence of RmpM in the Omp85 complex came from the observation that the stability of the complex decreased in the presence of a reducing agent (Fig. 3E). Whereas Omp85 does not contain any cysteine residues, RmpM contains four: one pair in the N-terminal and another pair in the C-terminal domain. Thus, the sensitivity of

FIG. 4. OMP assembly in RmpM mutants. (A to D) Cell envelopes were subjected to denaturing (d-) or semi-native (n) SDS-PAGE and stained with Coomassie blue (A, B), or blotted and probed with anti-PorA (C) or anti-PorB (D) antibodies. Cell envelopes were prepared from 1, HB-1; 2, HB-1-Δcl4; 3, HB-1ΔrmpM; and 4, HB-1ΔcomL(pComL) grown in the absence of IPTG. Folded porin monomers, dimers, and trimers are indicated by single, double, and triple asterisks, respectively. #, Unfolded monomeric porin. The ComL-depleted cell envelope sample is included as a control to show defective PorA assembly. (E) Cell lysates were subjected to denaturing SDS-PAGE and immunoblotting with anti-PilQ antiserum. Lane numbers refer to strains as indicated for panels A to D. Numbers to the left of gels are molecular weights (in thousands). α, anti.

the complex to reducing agents is consistent with the presence of RmpM in the complex.

Role of RmpM in OMP assembly. Since RmpM is part of the Omp85 complex, we wished to determine whether it has any role in OMP assembly. From the analysis of H44/76-Δcl4, which contains the *rmpM::kan* allele, we postulated previously that RmpM stabilizes OMP complexes (19, 28). To readdress this issue and investigate any effects on OMP assembly in a strain completely lacking RmpM, we analyzed porin assembly in HB-1ΔrmpM and, for comparison, in HB-1-Δcl4. As shown in Fig. 4A and B, the overall protein profile of cell envelopes in denaturing conditions was unaffected by the partial or complete absence of RmpM, except for the presence of the full-length RmpM protein in strain HB-1, which is best visualized when samples are heat denatured without reducing agent (Fig. 4A). In the semi-native SDS-PAGE analysis, we did not detect unassembled porins in either *rmpM* mutant (Fig. 4B), a finding confirmed for PorA in an immunoblot analysis (Fig. 4C). On this blot, cell envelopes of a ComL/BamD-depleted strain were included to show the PorA pattern of an OMP assembly mutant. These gels and blots showed that the porin trimers from the *rmpM* mutants migrated faster than those of the parent strain, consistent with the notion that these trimers in the wild-type strain actually represent hetero-oligomers consisting of porin trimers and RmpM (19). Furthermore, the blots revealed the presence of dimers and folded monomeric forms of the porins; these forms were not present or were present in much smaller amounts in the sample prepared from the parent strain (Fig. 4C and D). These dimeric and folded monomeric forms were not detected in the assembly-defective ComL-depleted strain (Fig. 4C, lane 4), indicating that their presence does not result from an assembly defect but from decreased stability of assembled trimeric porins in the absence of RmpM. Overall, these data show that RmpM is not required for porin folding and assembly but, rather, it stabilizes their trimeric forms. In the case of PorB, this stabilizing role appeared to be more important than in the case of PorA, since we did not detect any PorB trimers in the HB-1ΔrmpM mutant (Fig. 4D).

Interestingly, the assembly of PilQ was totally unaffected by the absence of RmpM, since the levels of PilQ multimers and monomers detected in the membranes of HB-1ΔrmpM were

similar to the levels in the parent strain (Fig. 4E). Also, the PilQ complex did not migrate faster in the gel in the absence of RmpM (Fig. 4B and E), demonstrating that RmpM is not associated with this complex. Thus, apparently, RmpM is not associated with all of the protein complexes in the OM.

Next, we constructed ΔrmpM ΔbamC and ΔrmpM ΔbamE double mutants to test for potential synthetic defects. The sensitivity of the ΔrmpM ΔbamE mutant to vancomycin was similar to that of the ΔbamE mutant, and the ΔrmpM ΔbamC mutant was, just like the ΔrmpM and ΔbamC single mutants, not sensitive to this compound at all. Thus, these data also indicate that RmpM has no direct role in OMP assembly.

Association of lipoproteins with the Bam complex. The absence of the lipoproteins from the HMW Omp85 complex detected in semi-native SDS-PAGE does not necessarily mean that they are not associated with Omp85/BamA in vivo. Possibly, they dissociate from the complex due to the prevailing conditions of semi-native SDS-PAGE. To further investigate the association of the lipoproteins with Omp85, we analyzed the protease accessibility of Omp85 in cell envelopes of the mutants, emanating from the idea that its protease sensitivity could increase when associated components are lost. Trypsin treatment of cell envelopes followed by immunoblotting with an antiserum directed against the C-terminal β-barrel domain of Omp85 resulted in the detection of three large, distinct fragments with apparent molecular weights of approximately 58,000, 48,000, and 40,000 (designated I, II, and III, respectively, in Fig. 5). In the case of the ΔrmpM, ΔbamC, and Δmlp strains, the digestion products obtained were similar to those in the parent strain (Fig. 5 and data not shown). Interestingly, in the ΔbamE strain, only fragment III was detected, demonstrating that Omp85 is more accessible to trypsin in the absence of BamE. In the case of the ComL/BamD-depleted strain also, only fragment III was detected (Fig. 5), indicating that newly inserted Omp85 proteins, which were inserted by preexisting functional Bam complexes, cannot find a ComL partner molecule and therefore become more accessible to trypsin. This altered digestion profile was in both cases completely reversible when the plasmid-borne copies of *bamE* and *comL*, respectively, were expressed (Fig. 5). The observation that the absence of RmpM or BamC did not affect the tryptic pattern of

FIG. 5. Protease accessibility of Omp85 in Bam complex mutants. Cell envelopes from the strains indicated above the lanes were treated with (+) or without (–) trypsin (tryp.) and subjected to denaturing SDS-PAGE followed by immunoblotting with antiserum against the C-terminal part of *N. meningitidis* Omp85. Tryptic fragments with apparent molecular weights of approximately 58,000, 48,000, and 40,000 are indicated with I, II, and III, respectively. Where relevant, the absence (–) or presence (+) of IPTG during growth of the strains is indicated. Numbers to the left of gels are molecular weights (in thousands).

Omp85 suggests that the association of ComL and BamE with Omp85 is not significantly changed in these mutants, indicating that RmpM and BamC are not required for the association of ComL and BamE with Omp85.

To obtain additional evidence for the association of RmpM, ComL, BamE, and possibly, BamC with Omp85, we purified the Bam complex from the neisserial OM by means of pull-down assays, using an N-terminally His-tagged Omp85 protein expressed in strain HB-1. The chromosomal copy of *omp85* was disrupted in this strain to ensure maximal copurification of Omp85 complex components. In the presence of IPTG, this strain grew indistinguishably from HB-1 (data not shown), demonstrating that the His-tagged version of Omp85 is functional. Detergent extracts of cell envelopes prepared from this strain were subjected to Ni^{2+} -NTA purification. As a control, we subjected extracts of cell envelopes from the parent strain HB-1 to similar procedures. Analysis of the elution fractions by SDS-PAGE and silver staining yielded only one specific band in the sample of the cells producing His-tagged Omp85 (Fig. 6A), which was confirmed to be Omp85 by immunoblotting (Fig. 6A). Next, the elution fractions were probed with antisera directed against RmpM, ComL, BamC, and BamE. RmpM and all three lipoproteins were detected specifically in the elution fraction derived from the His-Omp85-expressing strain and not found or in much smaller amounts in that of the control strain (Fig. 6A). The Imp/LptD protein, an OMP functioning in LPS transport to the cell surface (3), did not copurify with Omp85 (Fig. 6A), further demonstrating the specificity of the assay. These results demonstrate that RmpM, BamC, BamE, and ComL/BamD are associated with Omp85/BamA in *N. meningitidis* but, apparently, only the association with RmpM withstands the conditions of semi-native SDS-PAGE. Moreover, since the ratio of Omp85 to BamE in cell envelopes and elution fractions was very similar (Fig. 6B), the majority of BamE in the cell appears to be associated with Omp85, and

FIG. 6. Omp85-associated proteins in *N. meningitidis*. (A) Cell envelope extracts of strains expressing wild-type (1) or His-tagged (2) Omp85 were subjected to Ni^{2+} -NTA purification. Shown are elution fractions analyzed by denaturing SDS-PAGE and silver staining or immunoblotting, using antibodies against the indicated proteins. The lane indicated with c contains nonextracted cell envelopes of HB-1 as a control to show reactivity of the anti-Imp antiserum. Numbers to the left of gels are molecular weights (in thousands). (B) Cell envelopes containing His-tagged Omp85 (a) and a Ni^{2+} -NTA elution fraction derived from them (b) were blotted. The blot was cut horizontally, and the upper part probed with an anti-Omp85 MAb and the lower part with anti-BamE antiserum. Before exposure to film, the blots were pieced together again.

this complex remains stably associated during extraction from the cell envelopes.

DISCUSSION

In this work, we addressed the composition of the Bam complex in *N. meningitidis* and identified homologs of the Bam complex components BamC, BamD, and BamE, while a BamB homolog was not found. The absence of BamB in *Neisseria* was confirmed by searches using hidden Markov models developed for each of the Bam complex components of *E. coli* (14). Applied to alphaproteobacteria, these searches demonstrated that *Brucella* species also lack BamB and, in addition, BamC. BamC was also not detected in *Caulobacter* and *Rickettsia* species (14). Homology searches indicated that even the essential lipoprotein ComL/BamD appears to be absent from species such as *Borrelia burgdorferi*. Thus, the Bam complex composition in gram-negative bacteria is not very well conserved, except for the presence of Omp85/BamA, reinforcing the key function of this protein in the OMP assembly process.

We found the BamD homolog ComL to be essential in *N. meningitidis*, as it is in *E. coli*. In ComL-depleted cells, severe assembly defects of the porins and secretin PilQ were observed, indicative of a general OMP assembly defect. Remarkably, a viable *comL* mutant has been described for *N. gonorrhoeae* (12). This mutant contained a transposon insertion in *comL*, potentially resulting in the expression of a truncated ComL protein containing only 96 out of the 251 amino acid residues of the mature part of the protein. Possibly, the essential part of ComL is contained within these N-terminal 96 residues. Consistently, efforts to introduce transposons in the gonococcal *comL* gene upstream of this insertion site were unsuccessful (12), suggesting that *comL* is essential in *N. gonorrhoeae* as well. On the other hand, we cannot exclude the

possibility that species-specific differences exist in the BamD/ComL dependency of bacteria. In this respect, it is interesting to note that a viable *bamD* knockout mutant was also recently described in a close relative of *E. coli*, i.e., *Salmonella enterica* (11). The gonococcal *comL* mutant demonstrated a severe defect in transformation but not in DNA uptake or in piliation (10). These observations, together with data suggesting that the gonococcal ComL might be covalently attached to the peptidoglycan layer, since it was not released from this layer by boiling in 4% SDS, led to the suggestion that ComL functions in periplasmic DNA transport and perhaps acts as a “space maker” (10, 12). Although we could not confirm the peptidoglycan association in the case of the meningococcal ComL, such a function as space making could also be important for the periplasmic transport of OMPs.

The *bamC* mutant showed only very slight defects in OMP assembly. Similarly, *E. coli bamC* mutants demonstrated only very mild phenotypes: they were shown to be selectively sensitive only to rifampin (rifampicin) (26), and defects in growth and OMP assembly were only observed when *bamC* mutations were combined with mutations in other OMP assembly components (26, 32, 46). The *bamE* mutant, in contrast, demonstrated a clear defect in OMP assembly and a severely compromised OM integrity. Consistent with these results, an increased sensitivity of a neisserial *bamE* mutant to the cationic peptide polymyxin was reported before (38). Also in *E. coli*, *bamE* mutations compromised OM integrity (32, 36), but such mutants displayed only very mild OMP assembly defects (32). Thus, *bamC* and *bamE* single mutants demonstrate comparable phenotypes both in *E. coli* and in *N. meningitidis*. However, the phenotypes of double mutants are different in the two species. In *E. coli*, all three combinations of *bamB*, *bamC*, and *bamE* double mutations result in synthetic phenotypes which differ in severity, ranging from lethality in a *bamB bamE* mutant (32) to only modest defects in a *bamB bamC* mutant (46). The synthetic lethality of the *bamB* and *bamE* mutations suggests that the corresponding proteins might have overlapping functions and that the inactivation of the individual genes is compensated for by the presence of the other gene. Since *bamB* is naturally absent in *N. meningitidis*, we expected a severe OMP assembly defect or even lethality in a *bamE* mutant, which, however, was not the case. Interestingly, the simultaneous absence of BamC and BamE is not tolerated in *N. meningitidis*, whereas an *E. coli bamC bamE* mutant is viable even though it is severely defective in OMP assembly (32). The simplest but highly speculative explanation for these data would be that the neisserial BamC protein compensates for the absence of BamB in this organism. It seems that the second *bamE* homolog we identified, designated *mlp*, is not functionally related to BamE since a *bamE mlp* double mutant did not display any synthetic defects. We did not observe any OMP assembly or OM integrity phenotype of an *mlp* mutant at all. Hence, the function of Mlp remains to be resolved. In a microarray analysis, the gene was found to be induced under iron limitation conditions (16), which, however, we could not confirm in a recent proteomic analysis (40). Remarkably, all *N. gonorrhoeae* strains analyzed so far contain a frameshift mutation 72 bp into the coding sequence of *mlp*, resulting in premature termination of the reading frame (47 and data not shown), hence its name, *mlp*, for meningococcal lipoprotein.

Both BamE and Mlp consist of a so-called SmpA_OmlA domain. Interestingly, the closest homologs of Mlp in many bacteria, such as a 31-kDa protein from *Haemophilus somnus* (45), are extended with an OmpA domain, a peptidoglycan-binding domain thought to function in cell envelope stabilization.

The results of protease-accessibility experiments and pull-down assays clearly demonstrated that Omp85, RmpM, BamC, ComL, and BamE form a complex in *N. meningitidis*. The observation that *E. coli* BamD could not replace its meningococcal homolog might be due to failure to incorporate the heterologous protein into the neisserial Bam complex. Importantly, we did not copurify the Imp protein in the pulldown assays, demonstrating that the LPS transport machinery is not, or at least not strongly, associated with the OMP assembly machinery in *N. meningitidis*. This finding argues against a suggestion that one of the POTRA domains of *E. coli* BamA may bind Imp/LptD (22). Possibly, Imp-Omp85 association may be different in *E. coli*, but then, a His-tagged BamB protein also did not pull down Imp/LptD in this species (46).

A novel component of the Bam complex of *N. meningitidis*, RmpM, was identified by the analysis of an Omp85-containing HMW complex detected in semi-native SDS-PAGE and confirmed in pulldown assays. BamC, ComL/BamD, and BamE were not detected in this complex on the gel, presumably because they dissociated from this complex in the sample buffer, which contained 1% SDS. RmpM was previously shown to be present in oligomeric complexes of porins and TonB-dependent receptors in the neisserial OM (19, 28). Possibly, the association of RmpM with these OMPs is established while they are engaged with the Bam complex. Lack of RmpM did not result in general defects in OMP assembly, as inferred from porin and PilQ assembly. Rather, RmpM appears to stabilize oligomeric OMP complexes (19, 28), as was confirmed for the porins in the present study. Similarly, we observed that the HMW Omp85 complex was dramatically less stable in the $\Delta rmpM$ mutant, since large amounts of monomeric Omp85 were detected (Fig. 3C and D). The decreased stability of the Omp85 complex apparently did not impede its function and is possibly only detectable in the gel experiments. However, it is entirely possible that the stabilization of the complex is important when the bacteria grow in the hostile environment of the host. Interestingly, our results indicated that RmpM is not associated with the PilQ complex, perhaps reflecting structural differences between PilQ and classical β -barrel OMPs. Consistently, the stability of the oligomeric PilQ complex appeared unaffected in the *rmpM* mutant.

Even in the absence of RmpM, Omp85 was detected in HMW forms. Possibly other, still-unknown components are associated with Omp85 or Omp85 is present in this complex as homo-oligomers. This latter possibility would be consistent with our previous findings that in vitro-refolded *E. coli* Omp85/BamA appeared to form tetramers (29). Moreover, several members of the Omp85 superfamily, such as mitochondrial Tob55 (27), chloroplast Toc75 (31), and the two-partner secretion component HMW1B from *Haemophilus influenzae* (35), behave as multimers after extraction from the membrane.

In conclusion, the Bam complex of *N. meningitidis* consists at least of Omp85/BamA, BamC, ComL/BamD, BamE, and RmpM and differs from that of *E. coli* by the absence of a BamB homolog and the presence of RmpM. As in *E. coli*,

Omp85/BamA and ComL/BamD are essential components, while the relative importance in OMP assembly of the nonessential components BamC, BamE, and RmpM differs.

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