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Role of the Periplasmic Chaperones Skp, SurA, and DegQ in Outer Membrane Protein Biogenesis in *Neisseria meningitidis*▽†

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The periplasmic chaperones Skp, SurA, and DegP are implicated in the biogenesis of outer membrane proteins (OMPs) in *Escherichia coli*. Here, we investigated whether these chaperones exert similar functions in *Neisseria meningitidis*. Although *N. meningitidis* does not contain a homolog of the protease/chaperone DegP, it does possess a homolog of another *E. coli* protein, DegQ, which can functionally replace DegP when overproduced. Hence, we examined whether in *N. meningitidis*, DegQ acts as a functional homolog of DegP. Single *skp*, *surA*, and *degQ* mutants were easily obtained, showing that none of these chaperones is essential in *N. meningitidis*. Furthermore, all combinations of double mutants were generated and no synthetic lethality was observed. The absence of SurA or DegQ did not affect OMP biogenesis. In contrast, the absence of Skp resulted in severely lower levels of the porins PorA and PorB but not of other OMPs. These decreased levels were not due to proteolytic activity of DegQ, since porin levels remained low in a *skp degQ* double mutant, indicating that neisserial DegQ is not a functional homolog of *E. coli* DegP. The absence of Skp resulted in lower expression of the *porB* gene, as shown by using a P_{porB}-*lacZ* fusion. We found no cross-species complementation when Skp of *E. coli* or *N. meningitidis* was heterologously expressed in *skp* mutants, indicating that Skp functions in a species-specific manner. Our results demonstrate an important role for Skp but not for SurA or DegQ in OMP biogenesis in *N. meningitidis*.

After crossing the inner membrane (IM) and the periplasm, bacterial outer membrane proteins (OMPs) are assembled into the outer membrane (OM) by a machinery that, in *Neisseria meningitidis*, consists of the central component Omp85 (48), the essential lipoprotein ComL, two nonessential lipoproteins BamC and BamE, and the RmpM protein (47). In *Escherichia coli*, this machinery was recently renamed Bam (β-barrel assembly machinery) and is similarly composed of BamA (the homolog of Omp85), BamD (the homolog of ComL), BamC, BamE, and additionally, BamB, a lipoprotein that has no homolog in the genus *Neisseria* (39, 53). *E. coli* does not contain a homolog of RmpM.

In *E. coli*, several chaperones have been identified that play a role in the transit of OMPs through the periplasm (25). Among them, Skp, SurA, and DegP appear to be the most prominent ones. Skp was shown to bind unfolded β-barrel OMPs (6, 10), presumably while they emerge from the Sec machinery (13), and to stimulate their release from the IM, resulting in the formation of soluble periplasmic intermediates of these proteins (36). The crystal structure of Skp revealed a homotrimer forming a basket-like shape, which transiently shields substrates from the environment to prevent their aggregation (19, 51, 52). The SurA protein contains two peptidyl-prolyl isomerase (PPIase) domains flanked by N- and C-terminal sequences. The PPIase domains are dispensable for SurA's chaperone activity (1). Its crystal structure shows a core module formed by the N- and C-terminal segments and one PPIase domain, with the other PPIase domain extending away from this core (2). Consistent with its proposed OMP-specific chaperone activity (22, 33), SurA preferentially binds *in vitro* to peptides containing two consecutive aromatic residues or two aromatic residues separated by one other residue, a motif that is regularly present in OMPs (14).

DegP is thought to function both as a chaperone and as a protease. The protein belongs to the HtrA family of proteases, which is characterized by the presence of one or more PDZ domains and a trypsin-like protease domain (8). *E. coli* contains three members of this family, DegP, DegS, and DegQ. DegP and DegQ contain two PDZ domains and DegS only one. A variable region, called the Q-linker, is located between the first and second β-strand of the protease domain and is approximately 40 amino acids long in DegP, 20 amino acids in DegQ, and basically absent in DegS. DegQ is a functional substitute for DegP when overexpressed (50). Many other bacteria possess only one HtrA family member, which usually is a DegQ homolog, based on the number of PDZ domains present and the length of the Q-linker (18). Whether this single HtrA family member then fulfils all functions ascribed to DegP, DegS, and DegQ in *E. coli* is at present unclear.

The roles of these chaperones have been studied almost exclusively in the *Enterobacteriaceae*. Recently, it was reported that *surA* in *Bordetella pertussis* could not be inactivated, suggesting that it is an essential gene in that species (15). Thus, the OMP assembly process in *E. coli* is not the paradigm for all

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bacteria. In this respect, *N. meningitidis* has already revealed some insightful differences with *E. coli*; for example, in contrast to *E. coli*, this Gram-negative bacterium can survive and assemble OMPs when the synthesis or transport of lipopolysaccharide (LPS) is disturbed (4, 40). Also, the composition of the Bam complex in *N. meningitidis* is not identical to that of *E. coli* (47) and the phenotype of mutants depleted of Bam components is different in each species. In *E. coli*, the periplasmic accumulation of unfolded OMPs activates the σ^E response; consequently, unfolded OMPs are degraded by the periplasmic protease DegP and OMP synthesis is downregulated by small RNAs (17, 31). This regulatory pathway is missing in *N. meningitidis* (5), and unassembled OMPs accumulate in the periplasm when OMP assembly is inhibited (47, 48). The lack of such feedback mechanisms may allow for a clearer interpretation of mutant phenotypes.

Since *N. meningitidis* has proven to be very informative as a model organism in studies to understand OM biogenesis, we here undertook a systematic study into the role of the periplasmic chaperones Skp, SurA, and DegQ in OMP biogenesis in *N. meningitidis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used are described in Table 1. *E. coli* strains were grown at 37°C either on LB agar plates or in liquid LB medium, supplemented with 25 μg/ml of chloramphenicol or 50 μg/ml kanamycin when appropriate. To analyze the effect of Skp on OmpA levels, BW25113-derived strains were grown at 28°C in M9 medium supplemented with 2 μg/ml thiamine and 0.4% maltose (6) in the absence or presence of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). *N. meningitidis* strains were grown at 37°C in candle jars on GC agar plates (Oxoid) supplemented with Vitox (Oxoid) and, when necessary, with 10 μg/ml of chloramphenicol or 80 μg/ml of kanamycin. For liquid cultures, *N. meningitidis* was grown overnight on plates, from which it was swabbed into tryptic soy broth (TSB) (Becton Dickinson) to an optical density at 550 nm (OD_{550}) of 0.1 and grown for 6 h with shaking.

Plasmid and mutant constructions. The plasmids and primers used in this study are summarized in Tables 1 and 2, respectively. All *N. meningitidis* DNA fragments were obtained by PCR using genomic DNA from strain HB-1 as the template. Deletion constructs of *skp*, *surA*, *degQ*, NMB1332 (locus tag), and NMB1433 were obtained by amplifying DNA fragments upstream and downstream of these genes by PCR using the 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 using the AccI sites that were introduced via the primers and the XbaI site in the vector. A kanamycin resistance gene (*kan*) cassette that includes the neisserial DNA uptake sequence was obtained from pMB25 and inserted in some plasmids after AccI restriction, yielding pCRII-Δ*skp*1, pCRII-Δ*surA*, and pCRII-Δ*degQ*. Alternatively, a chloramphenicol-resistance gene (*cat*) cassette was obtained from pCRII-*cat* and inserted after AccI restriction, resulting in pCRII-Δ*surA*-1, pCRII-Δ*degQ*-1, pCRII-ΔNMB1332, and pCRII-ΔNMB1433. For allelic replacements, constructs containing the antibiotic-resistance cassette in the same transcriptional direction as the gene to be replaced were used.

To create an insertional *skp* mutant, a fragment consisting of the 3′ region of *omp85* and the 5′ region of *skp* was amplified using primers A and B, and another fragment consisting of the 3′ region of *skp* and the 5′ region of *lpxD* was amplified using primers C and D. The fragments were cloned into pCRII-TOPO, yielding pCRII-*skp*-AB and pCRII-*skp*-CD, respectively. The AccI-SpeI fragment of pCRII-*skp*-CD was then ligated into AccI/SpeI-restricted pCRII-*skp*-AB together with the AccI-restricted *kan* cassette released from pMB25, yielding pCRII-*skp*2. The final construct contained a mutant *skp* allele with a *kan* cassette inserted after nucleotide 68 in the same transcriptional direction as *skp* and a 2-nucleotide insertion directly downstream of the cassette. The *kan* cassette was replaced by the *cat* cassette from pCRII-*cat*, using AccI restriction and ligation, resulting in pCRII-*skp*2-*cat*.

For complementation experiments, *N. meningitidis skp* was obtained by PCR using primers Skp-For and Skp-Rev and cloned into pCRII-TOPO. From there, the gene was excised with NdeI and AatII and subcloned into NdeI/AatII-digested pEN11-Imp, producing pEN11-Skp. A similar strategy was used to construct a *degQ* complementation plasmid, using primers DegQ-for and DegQ-rev, resulting in pEN11-DegQ. *E. coli skp* was amplified by PCR from genomic DNA of strain DH5α using primers Skp-Eco-For and Skp-Eco-Rev and cloned into pCRII-TOPO. From there, the gene was excised with NdeI and AatII and subcloned into NdeI/AatII-digested pFP10-c-*lbpA*, producing pFP10-Skp_{Ec}. As a result of the cloning strategy, the signal sequence of the *E. coli* Skp was replaced with that of the *N. meningitidis* lactoferrin-binding protein A (LbpA) (MNKKH SFPLTLTALAIATAFPSYA). For comparison, pFP10-Skp_{Nm} was constructed in a similar fashion, using primers Skp-For2 and Skp-Rev, resulting in a construct encoding the neisserial Skp with the signal sequence of LbpA. Plasmid pEN11-NhhA was constructed by subcloning *nhhA* (locus tag NMB0992) from pPU100 into pEN11-Imp using NdeI/AatII restriction and ligation.

Plasmid pSMS1 contains a promoterless *lacZ* gene inserted into the *hrtA* (*h*igh *r*ate of *t*ransformation) locus of *N. meningitidis* (7). A 1,235-bp fragment of the *porB* promoter region, including its ribosome-binding site and the first 18 bp of the open reading frame (ORF), was obtained by PCR amplification using primers SMS-1 and SMS-7 and chromosomal DNA of strain HB-1 as the template. The PCR product was digested with BamHI, and the released insert was cloned into the BglII site of pSMS1 to generate a translational fusion of the 5′ end of *porB* to the *lacZ* gene. Correct orientation of the promoter relative to the *lacZ* gene was confirmed by PCR. The resulting plasmid, pSMS-1235porB, was verified by sequencing and used to transform strain HB-1.

Meningococci were transformed on plates by adding 1 μg of PCR product or plasmid in 10 mM $MgCl_2$ to a few freshly restreaked colonies for 6 h. Then, bacteria were plated on GC agar plates containing appropriate antibiotics for mutant selection. The transformants were restreaked on similar selection plates to ensure their antibiotic-resistant phenotype and to prevent contamination of any remaining DNA used for the transformation in subsequent PCRs. To verify the mutants, a few colonies of each transformant were resuspended in H_2O, boiled, and centrifuged for 5 min at 13,000 rpm in a microcentrifuge. The resultant supernatant was used as template in a series of PCRs. The knockout mutants were tested for the presence of the mutant allele by using the relevant Up-For and Down-Rev primers and for the absence of the wild-type allele by using internal primers annealing within the removed coding sequence combined with appropriate Up-For or Down-Rev primers. These internal primers were for the following constructs: for the Δ*degQ* mutant, DegQ-for and DegQ-for1; for the Δ*surA* mutant, SurA-for and SurA-rev; for the Δskp_1 mutant, Skp-for; for the ΔNMB1332 mutant, 1332-int; and for the ΔNMB1433 mutant, 1433-int. The presence of pEN11-Skp, pFP10-Skp_{Nm}, or pFP10-Skp_{Ec} was tested by PCR using a primer annealing within the *lac*/*tac* promoter region (primer lac-cass) combined with Skp-Rev or Skp-Eco-Rev. For all PCRs, wild-type bacteria were used as controls.

Antibiotic sensitivity. Meningococci grown overnight on GC agar plates were resuspended in 100 μl of TSB to an OD_{550} of 0.2 and plated on GC agar plates. Paper discs containing 30 μg 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 mm from the rim of the disk. All tests were repeated at least three times.

SDS-PAGE and immunoblot analysis. Denaturing and seminative SDS-polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting procedures were used as described previously (47). Protein bands in gels were stained with Coomassie brilliant blue. To enhance epitope recognition on immunoblots, native proteins were denatured within the seminative SDS-PAGE gels by leaving the gels in steam for 20 min prior to blotting.

Cellular fractions. Cell envelopes were prepared as described previously (47). Extracellular media were collected from cultures grown for 6 h in TSB. Bacteria were removed by centrifugation (6,000 × *g* for 10 min), and proteins were precipitated from the supernatant with 10% trichloroacetic acid (TCA).

Urea solubilization. Cell envelopes were incubated in 20 mM Tris-HCl, 100 mM glycine, 6 M urea (pH 7.6) for 1 h at room temperature. Nonsoluble material was separated from soluble material by ultracentrifugation (170,000 × *g* for 1 h). The resulting pellet was dissolved in 2 mM Tris-HCl (pH 7.6). Proteins in the supernatant were precipitated by 10% TCA.

Protease treatment. Intact cells were suspended in phosphate-buffered saline supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (pH 7.6) to an OD_{550} of 1 and treated with 50 μg/ml proteinase K (Merck) for 15 min at room temperature. After the addition of 1 mM phenylmethanesulfonyl fluoride (Sigma), cells were boiled in SDS-PAGE sample buffer. Cell envelopes in 2 mM Tris-HCl (pH 7.6) were treated with 50 μg/ml trypsin (Sigma) for 16 h at room temperature and subsequently boiled in SDS-PAGE sample buffer.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
TOP10F′	Cloning strain	Invitrogen
DH5α	Cloning strain	Laboratory collection
BW25113	Parent strain of the Keio collection	NBRP[a]
JW0173	BW25113 with *skp* replaced by a *kan* cassette	NBRP
JW0173(pSkp$_{Ec}$)	JW0173 containing pFP10-Skp$_{Ec}$	This study
JW0173(pSkp$_{Nm}$)	JW0173 containing pFP10-Skp$_{Nm}$	This study
N. meningitidis strains		
HB-1	Serogroup B strain H44/76 with the capsule locus replaced by an erythromycin resistance gene cassette	3
HB-1Δ*skp*1	HB-1 with *skp* replaced by a *kan* cassette	This study
*HB-1skp*2	HB-1 with a *kan* cassette inserted in *skp*	This study
HB-1Δ*surA*	HB-1 with *surA* replaced by a *kan* cassette	This study
HB-1Δ*surA*-1	HB-1 with *surA* replaced by a *cat* cassette	This study
HB-1Δ*degQ*	HB-1 with *degQ* replaced by a *kan* cassette	This study
HB-1Δ*degQ*(pDegQ)	HB-1Δ*degQ* containing pEN11-DegQ	This study
HB-1Δ*skp*1(pSkp)	HB-1Δ*skp*$_1$containing pEN11-Skp	This study
HB-1*skp*2(pSkp)	HB-1*skp*$_2$ containing pEN11-Skp	This study
HB-1*skp*2(pSkp$_{Ec}$)	HB-1*skp*$_2$ containing pFP10-Skp$_{Ec}$	This study
HB-1*skp*2Δ*surA*	HB-1*skp*$_2$ with *surA* replaced by a *cat* cassette	This study
HB-1*skp*2Δ*degQ*	HB-1*skp*$_2$ with *degQ* replaced by a *cat* cassette	This study
HB-1Δ*surA*Δ*degQ*	HB-1Δ*surA*$_{-1}$ with *degQ* replaced by a *kan* cassette	This study
HB-1(pNhhA)	HB-1 containing pEN11-NhhA	This study
HB-1*skp*2(pNhhA)	HB-1(pNhhA) with a *kan* cassette inserted in *skp*	This study
HB-1Δ*surA*(pNhhA)	HB-1(pNhhA) with *surA* replaced by a *kan* cassette	This study
HB-1Δ*degQ*(pNhhA)	HB-1(pNhhA) with *degQ* replaced by a *kan* cassette	This study
HB-1(pFrpB)	HB-1 containing pFP10-c-*frpB*	This study
HB-1*skp*2(pFrpB)	HB-1*skp*$_2$ containing pFP10-c-*frpB*	This study
HB-1Δ*surA*(pFrpB)	HB-1Δ*surA* containing pFP10-c-*frpB*	This study
HB-1Δ*degQ*(pFrpB)	HB-1Δ*degQ* containing pFP10-c-*frpB*	This study
HB-1*skp*2ΔNMB1332	HB-1*skp*$_2$ with NMB1332 replaced by a *cat* cassette	This study
HB-1*skp*2ΔNMB1433	HB-1*skp*$_2$ with NMB1433 replaced by a *cat* cassette	This study
HB-1*lacZ*	HB-1 with promoterless *lacZ* gene inserted in the *hrtA* locus	This study
HB-1*porB-lacZ*	HB-1 with P$_{porB}$-lacZ fusion inserted in the *hrtA* locus	This study
HB-1*porB-lacZ skp*2	HB-1 with P$_{porB}$-lacZ fusion inserted in the *hrtA* locus and a *cat* cassette inserted in *skp*	This study
Plasmids		
pCRII-TOPO	TA-cloning vector for PCR products	Invitrogen
pMB25	pCRII-TOPO with *imp* inactivated by a *kan* cassette	4
pCRII-*cat*	pCRII-TOPO with a *cat* cassette flanked by AccI sites	47
pCRII-Δ*skp*1	*skp* deletion plasmid containing a *kan* cassette	This study
pCRII-*skp*2	Plasmid containing *kan* cassette insertion in *skp*	This study
pCRII-*skp*2-cat	Plasmid containing *cat* cassette insertion in *skp*	This study
pCRII-Δ*surA*	*surA* deletion plasmid containing a *kan* cassette	This study
pCRII-Δ*surA*-1	*surA* deletion plasmid containing a *cat* cassette	This study
pCRII-Δ*degQ*	*degQ* deletion plasmid containing a *kan* cassette	This study
pCRII-Δ*degQ*-1	*degQ* deletion plasmid containing a *cat* cassette	This study
pCRII-ΔNMB1332	NMB1332 deletion plasmid containing a *cat* cassette	This study
pCRII-ΔNMB1433	NMB1433 deletion plasmid containing a *cat* cassette	This study
pSMS1	Plasmid containing promoterless *lacZ* gene inserted into the neisserial *hrtA* locus	6
pSMS-1235porB	pSMS1 with *porB* promoter region, including the first 18 bp of the ORF, cloned in front of *lacZ*	This study
pEN11-Imp	*Neisseria* replicative plasmid containing H44/76-derived *imp* under *lac* promoter control	4
pEN11-Skp	pEN11-*imp* with *imp* replaced by *N. meningitidis skp*	This study
pEN11-NhhA	pEN11-*imp* with *imp* replaced by *N. meningitidis nhhA*	This study
pFP10-c-*lbpA*	*Neisseria* replicative plasmid containing the *lbpA* gene of H44/76 under *lac* promoter control	27
pFP10-c-*frpB*	*Neisseria* replicative plasmid containing the *frpB* gene of H44/76 under *lac* promoter control	20
pFP10-Skp$_{Ec}$	pFP10-c-*lbpA* with the mature LbpA-encoding part replaced by that of *E. coli* Skp	This study
pFP10-Skp$_{Nm}$	pFP10-c-*lbpA* with the mature LbpA-encoding part replaced by that of *N. meningitidis* Skp	This study
pPU100	pET11a containing *nhhA*	44

[a] NBRP, National Bioresource Project (NIG, Japan): E. coli.

TABLE 2. Primers used in this study

Primer name	Sequence (5′–3′)	Underlined restriction site
Skp-Up-For	GTGGCAGAACACCTGACC	
Skp-Up-Rev	ATGTCGACCTGAAGGGCTTCAGACGGCATT	AccI
Skp-Down-For	ATGTCGACTTCAGACGGCATACCGAA	AccI
Skp-Down-Rev	ATGACGGTGTGCGAACCGATT	
Skp-For	ATCATATGACCCGTTTGACCC	NdeI
Skp-For2	ATCATATGCCGACACCTTCCAAAAAATCG	NdeI
Skp-Rev	ATGACGTCTCATCAGCGGGCGT	AatII
SurA-Up-For	TACGGCAACGACAGGATTA	
SurA-Up-Rev	ATGTCGACACGGTGCTCCTGCCAGGTT	AccI
SurA-Down-For	ATGTCGACGAGCAGGCGGGAATCCGGTT	AccI
SurA-Down-Rev	ATGACGTCGGCAACTTCTGAATCGTC	
SurA-For	ATCATATGATGAAAATCAAAGCCCTG	NdeI
SurA-Rev	ATGACGTCTTAGCGGATGTCGACATACGCGC	AatII
DegQ-Up-For	ATCCGACCACCGAGCTGAATTTC	
DegQ-Up-Rev	ATGTCGACATTCTACAACGTCCGTCC	AccI
DegQ-Down-For	ATGTCGACTCCGACGCGGCAGAACGCG	AccI
DegQ-dOwn-Rev	GCCTAAAGACAGCAGTACGC	
DegQ-for	ATCATATGTTCAAAAAATACCAATACC	NdeI
DegQ-for1	AATCCGATGTCGCCCTTCTG	
DegQ-rev	ATGACGTCTTATTGCAGGTTTAATGC	AatII
1332-up-for	ATCGGAATTCGGTTATGGGTATCGGCAG	
1332-up-rev	AGTCGTCGACGGCCACGCCGCTGATTG	AccI
1332-down-for	ATCGGTCGACTCGCAGGTGCATTGCAGG	AccI
1332-down-rev	CTTCTGCCACTGCTCGGGCG	
1332-int	CAAGCCGATAGTCGTCAACCTG	
1433-up-for	ATGCGAATTCTGACCAACTCGCTGCAG	
1433-up-rev	ATCGGTCGACTCGTGCCGCATGAGGCG	AccI
1433-down-for	ATCGGTCGACCTCTCCACACCGTTTTAC	AccI
1433-down-rev	CTGAAAGCTTGCACGTCGAAGGCGATGC	
1433-int	GCAACCGGCTTCGATTGCAG	
A	ATGCCGTCTGAACGCCGAAATCGAA	
B	TTTGGACTAGGTGTCGACGTGCGCGC	AccI
C	ACGTCGACACCTAGTCCAAAAAATCG	AccI
D	ATGCCGTCTGAAAACAGGCGGGCGACTTTGG	
Skp-Eco-For	ATCATATGCTGACAAAATTGCA	NdeI
Skp-Eco-Rev	ATGACGTCTTATTTAACCTGTTTC	AatII
lac-cass	TCTGGATAATGTTTTTTGCGCCGAC	
rmpMF	CAGGCTCCGCAATATGTTGA	
rmpMR	GTTGTCTTGAGCTTCGGCG	
lpxDF	GGACATTTCCGTTACCGCC	
lpxDR	CTGTCGTGGACTTCGGCTTT	
fabZF	ATCCAAAAACTCATCCCCCAC	
fabZR	GGTGACGTTTTTAATCGCGGT	
SMS-1	GCGCGCGGATCCAGGGCAATCAGGGATTTTTTC	BamHI
SMS-7	GCGCGCGGATCCAATGACGGGATTTTAGGTTTC	BamHI

Immunofluorescence microscopy. Labeling and immunofluorescence microscopy analysis of formaldehyde-fixed bacteria were performed as described previously (48).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (42). Primer couples lpxDF/lpxDR, fabZF/fabZR, and rmpMF/rmpMR (Table 2) were used to amplify the cDNAs of *lpxD*, *fabZ*, and *rmpM*, respectively. The *rmpM* transcript was used to normalize all data.

Antisera. Monoclonal antibodies (MAbs) against PorA and PorB were provided by the Netherlands Vaccine Institute (Bilthoven). The anti-PilE MAb was a generous gift from John Heckels (University of Southampton Medical School, United Kingdom). Mouse antisera directed against FrpB, PilQ, and fHbp and MAbs against Omp85, NspA, and NhhA were provided by GlaxoSmithKline Biologicals (Rixensart, Belgium). Mouse antiserum against *N. meningitidis* Skp (26) was generously provided by Gerardo Guillen Nieto (Center for Genetic Engineering and Biotechnology, Havana, Cuba), and rabbit antiserum against *E. coli* Skp was a kind gift from Mathias Müller (Universität Freiburg, Germany). Rabbit antisera against *N. meningitidis* Imp/LptD and *E. coli* SecB and a MAb against *E. coli* OmpA came from our laboratory stocks.

β-Galactosidase assays. Standard β-galactosidase assays were conducted as described previously (24). Data are presented as the mean results ± standard errors of the means from one representative experiment performed in triplicate.

RESULTS

Construction of *skp*, *surA*, and *degQ* mutants in *N. meningitidis*. Homology searches using *E. coli* Skp, SurA, and DegP sequences yielded locus tags NMB0181, NMB0281, and NMB0532, respectively, in the sequenced genome of *N. meningitidis* strain MC58. The Skp and SurA homologs were found in genetic locations similar to those in *E. coli*, i.e., downstream of the genes encoding Omp85/BamA and Imp/LptD, respectively (see Fig. S1 in the supplemental material). Imp is an OMP that is required for transport of lipopolysaccharide (LPS) to the cell surface (4) and was recently renamed LptD in

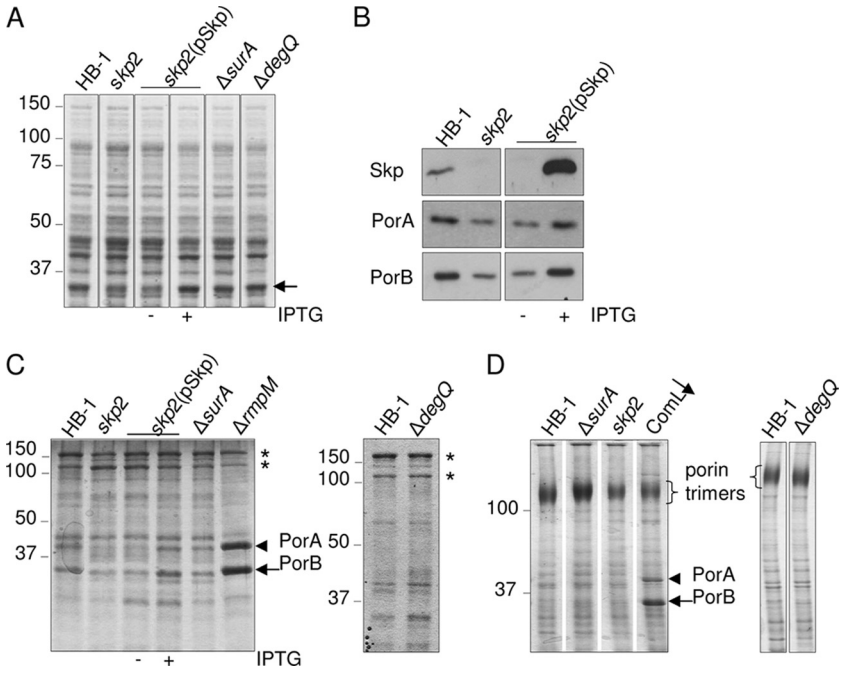

FIG. 1. Phenotypes of chaperone mutants. (A) Protein profiles of cell lysates observed by SDS-PAGE followed by Coomassie brilliant blue staining. An arrow indicates the band with the most variable intensity. (B) Identical amounts of cell lysates, based on the OD_{550} of the cultures, were immunoblotted with antibodies against the proteins indicated on the left. (C) Protein profiles of extracellular media. The bands indicated with asterisks represent two different secreted forms of IgA protease (45). (D) Assembly of porin trimers observed by seminative SDS-PAGE of cell envelopes followed by Coomassie brilliant blue staining. The lane indicated by ComL ↓ contains cell envelopes of a ComL-/BamD-depleted strain and was included to show the profile of a strain with a porin assembly defect (47). In panels C and D, monomeric forms of PorA and PorB are indicated by arrowheads and full arrows, respectively. When relevant, the presence or absence of 1 mM IPTG during growth is indicated. The positions of molecular mass standard proteins are shown in kDa at the left side of the gels.

E. coli (5). A reciprocal search using the amino acid sequence of NMB0532 on *E. coli* genome sequences showed that NMB0532 is more similar to DegQ than to DegP (the percentages of identity and similarity at the amino acid level to DegQ are 39% and 59% and to DegP are 34% and 56%). Also, the Gln-rich region called the Q-linker, characteristic of DegP, is absent in NMB0532. Therefore, we will refer to NMB0532 as DegQ. No other significant hits came up in searching the MC58 genome using DegP, DegQ, DegS, or COG0265, the conserved domain of the HtrA protein family, as queries. Thus, *N. meningitidis* appears to contain only one member of the HtrA protein family. To investigate the functions of the Skp, SurA, and DegQ proteins in *N. meningitidis*, mutants were constructed by replacing the main part of their coding sequences in strain HB-1 with antibiotic resistance cassettes (see Fig. S1 in the supplemental material). Correct mutants were easily obtained, demonstrating that none of these genes is essential. We found no growth defects for the Δ*surA* mutant in liquid medium, whereas the Δ*degQ* mutant demonstrated an enhanced lag time which was restored when a complementing copy of *degQ* was expressed from plasmid (see Fig. S2A and B in the supplemental material). In contrast, the Δ*skp* mutant, designated HB-1Δ*skp*1, grew significantly more slowly than any of the other strains (see Fig. S2A in the supplemental material). This defect in growth was not restored when *skp* was expressed from a complementing plasmid (data not shown), indicating that the Δskp_1 mutation could be polar. Indeed, qRT-PCR analysis revealed that the transcript levels of *lpxD* and *fabZ*, which are located downstream of *skp* in the same operon (11), were ~30 and ~9 times lower in HB-1Δskp_1 than in the parent strain. Therefore, we constructed an alternative *skp* mutant by the insertion of a *kan* cassette into the 5′ part of the *skp* gene without a concomitant deletion of *skp* sequences (see Fig. S1 in the supplemental material). This mutant, designated HB-1*skp*2, did not demonstrate any growth defect (see Fig. S2A in the supplemental material). The *lpxD* transcript level in the skp_2 mutant was ~5 times lower than that of the parent strain, indicating that, indeed, the polar effect of the skp_2 mutation is much smaller than that of the Δskp_1 mutation.

OMP assembly in the chaperone deletion mutants. The protein profiles of the cells and culture supernatants were analyzed by SDS-PAGE. The comparison of cell lysates yielded one obvious difference: the skp_2 mutant contained lower levels of a protein migrating at 35 kDa. This defect was indeed due to the absence of Skp, since it was restored upon the expression of *skp* from plasmid (Fig. 1A, compare lanes without and with [− and +] IPTG). The absence and presence of the Skp protein was confirmed by immunoblotting (Fig. 1B, upper panel). As the 35-kDa band represented one of the most abundant cellular proteins, we reasoned that it might be PorB, i.e., one of the two major porins present in this strain. This notion was confirmed by immunoblotting (Fig. 1B). Similar blots using an antibody against the other porin, PorA, showed that the levels of this protein also were affected by the absence of Skp. Extracellular protein profiles showed the expected presence of two processed forms of IgA protease (45) in the parent strain

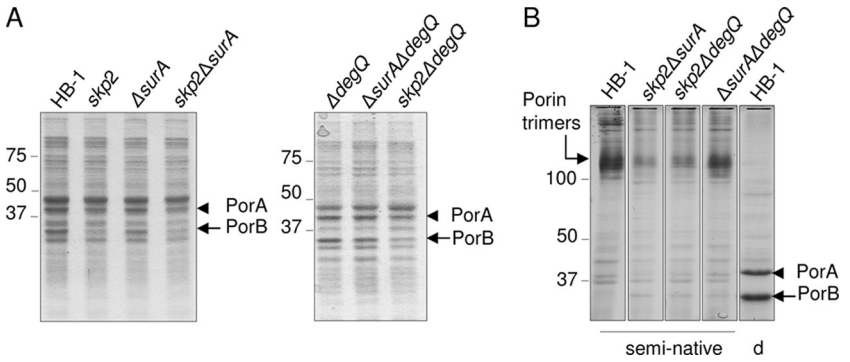

FIG. 2. Protein profiles of chaperone double mutants. (A) Cell lysates were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. (B) Cell envelopes prepared from the indicated strains were subjected to seminative or denaturing (d) SDS-PAGE followed by Coomassie brilliant blue staining. Molecular weight, in thousands, is shown beside the gels.

(Fig. 1C), which were similar in the mutants, demonstrating that none of the three chaperones is essential for the correct processing and secretion of this autotransporter. Neisserial strains are known to shed OM blebs spontaneously, a process that is considerably enhanced in mutants lacking the RmpM protein, which anchors the OM to the peptidoglycan (41). As a consequence, high levels of porins are present in spent media of such mutants (Fig. 1C). The porin levels in the extracellular media of the chaperone mutants showed no evidence for enhanced blebbing for any of these mutants (Fig. 1C). In fact, the levels of porins in the spent media of the Skp variants reflected the differential cellular porin levels. Thus, the lower porin levels in the skp_2 mutant are not due to increased blebbing.

Porins are present as trimers in the OM, which can be visualized by seminative SDS-PAGE, where they migrate as high molecular weight (HMW) complexes. Porin assembly defects are manifested by the appearance of porins migrating at their denatured monomeric position, as demonstrated in Fig. 1D for a strain depleted of ComL/BamD (47). No unfolded porins were detected in seminative SDS-PAGE analysis of cell envelopes of the various chaperone mutants (Fig. 1D). Consistent with its overall lower cellular porin levels, the amount of trimers was decreased in the skp_2 mutant, without concomitant accumulation of monomeric forms (Fig. 1D).

Lack of synthetic lethality of *skp*, *surA*, and *degQ* mutations in *N. meningitidis*. In *E. coli*, the simultaneous absence of any two of the chaperones Skp, SurA, and DegP leads to synthetic defects, often resulting in lethality (32, 36, 38). To analyze the potential existence of similar synthetic phenotypes in *N. meningitidis*, we constructed skp_2 Δ*surA*, skp_2 Δ*degQ*, and Δ*surA* Δ*degQ* double mutants. These mutants were all easily obtained, showing that none of the combinations is synthetically lethal. Protein profiles of cell lysates (Fig. 2A) and extracellular media (data not shown) of the skp_2 Δ*surA* and skp_2 Δ*degQ* double mutants revealed no other or stronger defects than those already observed in the skp_2 single mutant, i.e., lower levels of porins, most significantly of PorB. The porins were completely assembled into trimers, since we did not observe any unassembled monomeric porins in seminative SDS-PAGE (Fig. 2B). Of note, these results also demonstrate that the decrease in porin levels in the absence of Skp is not due to proteolytic degradation of unassembled forms by DegQ, as the levels of the porins were very similar in the skp_2 single and skp_2 Δ*degQ* double mutants (Fig. 2A) and no unassembled forms were detected in the double mutant (Fig. 2B).

In *E. coli*, the absence of periplasmic chaperones results in an increased sensitivity to antimicrobial agents, presumably because of a compromised OM integrity. We tested the sensitivity of the neisserial mutants to vancomycin in a disk diffusion assay. The parent strain, the skp_2, Δ*surA*, and Δ*degQ* single mutants and the Δ*surA* Δ*deg*Q double mutant were completely resistant to this antibiotic. Only the skp_2 Δ*degQ* and skp_2 Δ*surA* double mutants were slightly sensitive, as seen from the appearance of a clearing zone of 1.5 mm around the vancomycin-containing disk. For comparison, strains lacking BamE yielded clearing zones of 6 mm (47).

Assembly of OMPs other than porins. To test whether any of the chaperones is involved in the assembly of only a limited set of proteins, we investigated the levels and assembly of several OMPs and surface-associated proteins in the various mutants. We were particularly interested to see whether the absence of SurA would affect LptD assembly, as the LptD protein was recently reported to be one of the few "true" SurA substrates in *E. coli* (46). The cellular levels of LptD were not changed in the Δ*surA* mutant (see Fig. S3A in the supplemental material). The LptD protein migrates as two HMW bands in seminative SDS-PAGE (Fig. 3A). These two HMW forms of LptD appeared not to be affected in the Δ*surA* mutant (Fig. 3A) or in any of the other mutants (data not shown), suggesting that none of the chaperones is essential for LptD biogenesis. Next, we assessed the levels of FrpB (FetA), a TonB-dependent receptor that can be as highly expressed as the porins (20). To that end, a plasmid containing *frpB* under IPTG control was introduced into the various mutants. High levels of FrpB were detected after growth in the presence of IPTG in all strains (Fig. 3B). All FrpB produced was correctly assembled in the OM, as deduced from the lack of urea extractability of the protein (data not shown) and from the similar tryptic profiles of FrpB in the various mutants (Fig. 3C). Also, the levels and assembly (tested as described in reference 47) of the secretin PilQ, the OMP assembly factor Omp85/BamA, and the small 8-stranded β-barrel OMP NspA (43) were unaffected, as were the levels of the pilin subunit PilE (data not shown and Fig. S3A and B in the supplemental material, respectively).

NhhA is a trimeric autotransporter (44) whose membrane-

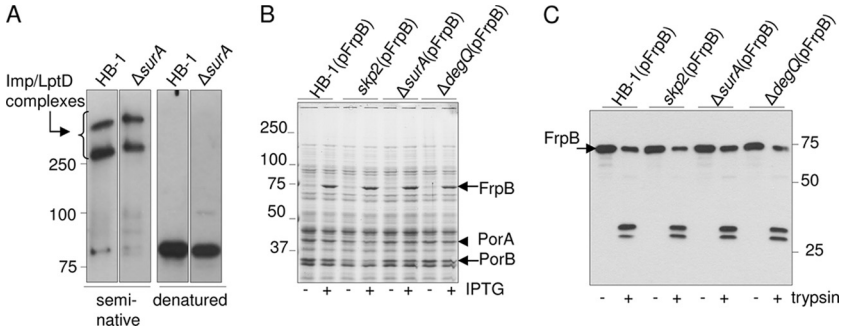

FIG. 3. Levels and assembly of OMPs in chaperone mutants. (A) Cell envelopes of the indicated strains were subjected to seminative or denaturing SDS-PAGE followed by immunoblotting with anti-LptD antiserum. (B) Cell lysates of the indicated strains grown with or without 1 mM IPTG were subjected to SDS-PAGE and Coomassie brilliant blue staining. (C) Cell envelopes of the indicated strains grown in the presence of IPTG were treated with urea and subsequently treated or not treated with trypsin, as indicated, and analyzed by immunoblotting with an anti-FrpB antiserum.

embedded translocator domain is formed by three monomers, each donating four β-strands to the 12-stranded β-barrel, resulting in a heat- and SDS-resistant molecule migrating at a much higher apparent molecular weight than expected from the predicted size of a monomer with a molecular weight of 60,000 (9, 44). We anticipated that chaperones might be required for the biogenesis of such a complex molecule. As natural levels of NhhA could not be detected in HB-1, we introduced a plasmid containing *nhhA* under IPTG control in HB-1 and subsequently constructed *skp*$_2$, Δ*surA*, and Δ*degQ* mutations in this background. Growth of the strains in the presence of IPTG resulted in comparable, huge levels of NhhA migrating in a HMW position in SDS-PAGE, indicating that the translocator domain is assembled correctly in all strains (see Fig. S3C and D in the supplemental material). Also, immunofluorescence experiments using an antibody against the passenger domain of NhhA showed similar positive staining of parent and mutant strains (data not shown), indicating that the chaperone mutants correctly assemble this protein. Factor H-binding protein (fHbp) is a surface-exposed lipoprotein (23) whose presence at the cell surface can be determined by using its high sensitivity to extracellular proteases. Both the levels and protease accessibility of fHbp were unaffected in the mutants (see Fig. S3E in the supplemental material). Thus, none of the chaperones Skp, SurA, and DegQ are required for the biogenesis of the cell surface-exposed lipoprotein fHbp, the OMPs PilQ, Omp85/BamA, Imp/LptD, NspA, and FrpB, or the trimeric autotransporter NhhA, even when this protein is highly overexpressed.

The absence of Skp affects *porB* expression. The lower levels of PorB in the *skp*$_2$ mutant were not due to proteolytic activity of DegQ, as shown by the results described above. Similarly, we found no increase in porin levels or the accumulation of unfolded porins when we inactivated in the *skp*$_2$ mutant the genes for two other periplasmic proteases, i.e., NMB1332, which is a *prc*/*tsp* homolog (37), and NMB1433, an *spr* homolog (12) (data not shown). To test whether the expression of *porB* might be affected by the absence of Skp, we constructed a single-copy P$_{porB}$-*lacZ* translational fusion in the chromosomal *hrtA* locus in strain HB-1 and generated a *skp*$_2$ mutation in this strain. A control strain containing a promoterless *lacZ* gene did not generate detectable β-galactosidase activity. In contrast, the P$_{porB}$-*lacZ* parent strain produced high levels of β-galactosidase activity: 7,299 ± 244 Miller units. In cells lacking Skp, the β-galactosidase activity dropped by 44%, to 4,154 ± 38 Miller units, demonstrating that the lower PorB levels in the *skp*$_2$ mutant are caused at least in part by decreased production of the protein.

Cross-complementation analysis. *E. coli*-derived Skp was reported to bind the neisserial autotransporter NalP with high affinity *in vitro* (29). To determine whether *E. coli* Skp can substitute for *N. meningitidis* Skp *in vivo*, we introduced a copy of *E. coli skp* under IPTG control on plasmid pFP10-Skp$_{Ec}$ into the *skp*$_2$ mutant. Porin levels were not restored upon induction of the expression of the *E. coli skp* gene, whereas this was clearly the case when neisserial *skp* was present on the plasmid (Fig. 4A). Immunoblot analysis of cell envelopes confirmed

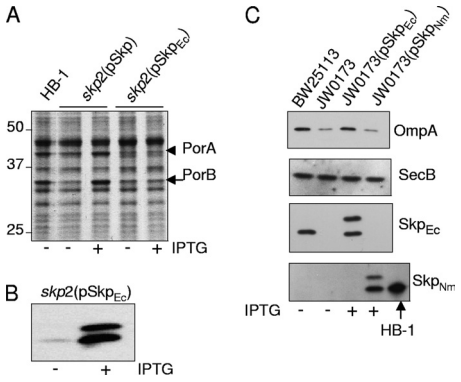

FIG. 4. Lack of cross-complementation of *E. coli* and *N. meningitidis* Skp. (A) The indicated *N. meningitidis* strains were grown in the presence or absence of 1 mM IPTG as specified below the gel and analyzed as cell lysates by denaturing SDS-PAGE and Coomassie brilliant blue staining. The positions of PorA and PorB are indicated. (B) Synthesis of *E. coli* Skp in *N. meningitidis*. Cell envelopes of HB-1 carrying pFP10-Skp$_{Ec}$ grown in the presence or absence of 1 mM IPTG were analyzed by denaturing SDS-PAGE and immunoblotting with antiserum directed against *E. coli* Skp. (C) The indicated *E. coli* strains were grown in M9 medium with or without 0.5 mM IPTG as indicated. Cell lysates were processed for immunoblots and probed with antisera against the proteins indicated on the right. SecB levels served as loading controls. The lower panel shows an extra lane containing cell envelopes from HB-1 to indicate the position in the gel of fully processed Skp.

that *E. coli* Skp was indeed produced upon IPTG induction, albeit with somewhat incomplete processing as suggested by the two bands detected with the antiserum (Fig. 4B). These bands probably correspond to the precursor and mature forms of Skp. Thus, *E. coli* Skp is not able to functionally replace *N. meningitidis* Skp. For the reverse experiment, we introduced pFP10-Skp_{Nm} and pFP10-Skp_{Ec} into an *E. coli skp* mutant. As reported before (6), OmpA levels decreased in the absence of Skp and were restored by expressing a plasmid copy of the *E. coli*-derived *skp* (Fig. 4C, top panel). However, the expression of neisserial *skp* did not restore OmpA levels (Fig. 4C, top panel), even though the Skp protein was produced and for the most part processed (Fig. 4C, bottom panel). Thus, the Skp proteins of *E. coli* and *N. meningitidis* cannot replace each other *in vivo*, at least not in maintaining wild-type OMP levels.

DISCUSSION

In this work, we investigated the effect of Skp, SurA, and DegQ deficiencies on OMP biogenesis in *N. meningitidis*. Individual and double deficiencies had surprisingly little effect on OMP biogenesis. Only the absence of Skp resulted in a clear defect, i.e., lower cellular levels of the major OMPs, the porins PorA and PorB. The levels of other, minor OMPs, such as PilQ, NspA, Omp85/BamA, and Imp/LptD, were not affected by the absence of Skp. As the levels of another highly expressed OMP, FrpB, were also not diminished, it appears that the absence of Skp specifically affected the porins. In *E. coli*, the absence of Skp also results in diminished cellular levels of major OMPs, such as OmpA (Fig. 4C) (6), which is, at least in part, due to DegP-mediated degradation of unfolded OmpA, since aggregates of unfolded OmpA accumulate in the periplasm of an *skp degP* double mutant (36). *N. meningitidis*, which naturally lacks DegP, is apparently different in this respect, since we did not find any evidence for the presence of unfolded porins in the *skp* mutant. We also found no indications that other periplasmic proteases, i.e., DegQ, NMB1332, or NMB1433, were responsible for the removal of unfolded porins. However, we cannot exclude a possible role for other periplasmic proteases that we did not investigate.

Using a P_{porB}-*lacZ* translational fusion, we observed that the expression of *porB* was reduced at the transcriptional and/or translational level in the *skp* mutant, suggesting that a feedback system is operating when Skp is absent. In *E. coli*, a number of signaling systems exist that respond to extracytoplasmic stress (31). The σ^E pathway that responds to the accumulation of unfolded OMPs in the periplasm of *E. coli* is absent in *N. meningitidis* (5). Possibly other pathways, such as the Cpx or Bae two-component systems, which were shown to respond to disorders in OMP biogenesis in *E. coli* (30), play similar roles in *N. meningitidis*. Meningococci contain only four two-component systems, which are not yet well characterized; one of them may be functionally homologous to the Cpx or Bae system in sensing OMP assembly defects and may respond by repressing the transcription of porin genes. Of note, however, unfolded OMPs accumulate in meningococcal strains with a defective Bam complex (47, 48), suggesting that under those conditions, the feedback inhibition system is not triggered. In *E. coli*, Skp was reported to bind OMPs while they are still engaged with the Sec machinery (13) and to stimulate their release from the IM (36). Possibly, the occupancy of the Sec system is sensed in a *skp* mutant of *N. meningitidis*, resulting in the feedback inhibition of the synthesis of the major exported proteins, the porins.

A surprising result of our study is the apparent absence of a role for SurA in OMP biogenesis. SurA was proposed to be the major periplasmic chaperone involved in OMP biogenesis in *E. coli*, whereas Skp would act in a parallel rescue pathway that deals only with substrates that fall off from the SurA pathway (38). Also, the absence of synthetic lethality of the *skp* and *surA* mutations in *N. meningitidis* constitutes another difference from the situation in *E. coli* but is consistent with the fact that we did not observe any phenotype of the *surA* mutation. The different function of SurA in *N. meningitidis* compared to that in *E. coli* might be reflected in its different structure. *N. meningitidis* SurA lacks one of the two PPIase domains found in *E. coli* SurA. Based on sequence alignments (data not shown), the PPIase 1 domain, which together with the N- and C-terminal parts forms the core of the protein in the crystal structure of *E. coli* SurA (2), is missing. Although the PPIase domains are dispensable for function (1), the PPIase 1 domain of *E. coli* SurA was shown to selectively bind peptides that are rich in aromatic residues and characteristic for OMPs (14, 54). Thus, SurA of *N. meningitidis* may fail to specifically recognize OMP substrates because it lacks the PPIase 1 domain. Interestingly, the SurA proteins of several other bacteria, such as those of *Haemophilus influenzae* and *Pasteurella multocida*, also lack the PPIase 1 domain (2), and we speculate that in these bacteria also, SurA has no general role in OMP biogenesis.

We found no evidence for a DegP-like function of neisserial DegQ as a chaperone or as a protease in the degradation of unassembled OMPs, although the *E. coli* DegQ can functionally replace DegP when overproduced (50). Recent structural studies have shed new light on the functional mechanisms of DegP. DegP appears to exist in several different conformations: in solution, inactive hexameric forms can transform into huge, cagelike 12- and 24-mers upon binding of substrates, which then become enclosed within the cage, where they are stabilized in a folded conformation or are degraded (16, 21). Interestingly, the neisserial DegQ protein is likely anchored to the inner or outer membrane by a lipid tail, since the C terminus of its signal sequence shows a characteristic lipobox sequence, LAGC. It seems impossible that a membrane-anchored lipoprotein could form cagelike assemblies similar to those of DegP, reinforcing the notion that neisserial DegQ is not a functional homolog of *E. coli* DegP.

The role of periplasmic chaperones in autotransporter biogenesis has been explored in only a few studies. The autotransporters IcsA of *Shigella flexneri* and EspP of *E. coli* require DegP, Skp, and SurA for proper cell surface presentation (28, 34, 49), whereas the biogenesis of Hbp in *E. coli* is affected by the absence of SurA but not by that of DegP or Skp (35). We found no defects in the assembly of the trimeric autotransporter NhhA or in the levels of the secreted monomeric autotransporter IgA protease in the neisserial chaperone mutants; apparently, chaperone requirements may be species and autotransporter specific.

Outer membrane-destined lipoproteins are chaperoned through the periplasm by the LolA protein (55). In *E. coli*, these lipoproteins are anchored in the inner leaflet of the

OM; however, in *N. meningitidis* and in many other pathogens, lipoproteins anchored in the outer leaflet of the OM are also found. It is completely unknown how these lipoproteins reach the cell surface. As we did not find any defect in the cell surface exposure of the lipoprotein fHbp in our chaperone mutants, we conclude that SurA, Skp, and DegQ are required neither directly for the biogenesis of such lipoproteins nor indirectly by playing an essential role in the assembly of a putative lipoprotein transfer system.

Interestingly, we found no cross-species complementation of *E. coli* and *N. meningitidis skp*. Skp does not demonstrate much species specificity *in vitro*, as *E. coli*-derived Skp was reported to bind a neisserial OMP (29). Since, nevertheless, *E. coli* Skp could not functionally replace its homolog in *N. meningitidis* and vice versa, it appears that Skp interacts with another component of the OMP biogenesis machinery and that such interaction possibly happens in a species-specific manner.

In conclusion, we found no general role for Skp, SurA, or DegQ as an OMP chaperone in *N. meningitidis.* However, Skp is involved in the biogenesis of porins by affecting protein production, presumably through a feedback system that remains to be identified.

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