The Omp85 protein of *Neisseria meningitidis* is required for lipid export to the outer membrane

Stéphanie Genevrois[1,2], Liana Steeghs[3], Paul Roholl[4], Jean-Jacques Letesson[1] and Peter van der Ley[3]

[1]Research Unit in Molecular Biology (URBM), University of Namur (FUNDP), Rue de Bruxelles, 61, 5000 Namur, Belgium, [3]Laboratory of Vaccine Research (LVR) and [4]Laboratory of Pathology and Immunobiology (LPI), National Institute of Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, PO Box 1, 3720 BA Bilthoven, The Netherlands

[2]Corresponding author
e-mail: stephanie.genevrois@fundp.ac.be

S.Genevrois and L.Steeghs contributed equally to this work

In Gram-negative bacteria, lipopolysaccharide and phospholipid biosynthesis takes place at the inner membrane. How the completed lipid molecules are subsequently transported to the outer membrane remains unknown. Omp85 of *Neisseria meningitidis* is representative for a family of outer membrane proteins conserved among Gram-negative bacteria. We first demonstrated that the *omp85* gene is co-transcribed with genes involved in lipid biosynthesis, suggesting an involvement in lipid assembly. A meningococcal strain was constructed in which Omp85 expression could be switched on or off through a *tac* promoter-controlled *omp85* gene. We demonstrated that the presence of Omp85 is essential for viability. Depletion of Omp85 leads to accumulation of electron-dense amorphous material and vesicular structures in the periplasm. We demonstrated, by fractionation of inner and outer membranes, that lipopolysaccharide and phospholipids mostly disappeared from the outer membrane and instead accumulated in the inner membrane, upon depletion of Omp85. Omp85 depletion did not affect localization of integral outer membrane proteins PorA and Opa. These results provide compelling evidence for a role for Omp85 in lipid transport to the outer membrane.
Keywords: lipopolysaccharide and phospholipid export/*Neisseria meningitidis*/Omp85/outer membrane

Introduction

The envelope of Gram-negative bacteria consists of an inner membrane (IM), the peptidoglycan layer and an outer membrane (OM) (Nikaido, 1996). The latter is an asymmetrical bilayer with phospholipids (PLs) in the inner leaflet and lipid A, the hydrophobic anchor of lipopolysaccharide (LPS) in the outer leaflet. LPS has a cell surface-exposed oligosaccharide chain, which in many organisms is linked further to a longer O antigen chain composed of repeating sugar subunits (Raetz and Whitfield, 2002). LPS and PL biosynthesis takes place in both the cytoplasm and IM of the bacterial cell. The enzymes responsible for the lipid A biosynthesis pathway have been well characterized in *Escherichia coli* (Raetz and Whitfield, 2002) and are targets for the design of novel antibacterial agents (Onishi *et al*., 1996; McMurry *et al*., 1998).

During the past 20 years, both genetic and biochemical studies have led to the description of LPS composition, biosynthesis and regulation. The formation of LPS is a complex process involving the synthesis of activated precursors in the cytoplasm, followed by the assembly of the lipid A core at the IM (Raetz and Whitfield, 2002). In *E.coli*, the lipid A biosynthetic pathway has been elucidated entirely. Conditionally lethal mutants have been used to isolate and characterize genes involved in the early steps of lipid A biosynthesis. These genes (*lpxA*, *lpxC* and *lpxD*) were shown to be essential for viability (Beall and Lutkenhaus, 1987; Galloway and Raetz, 1990; Kelly *et al*., 1993). However, in *Neisseria meningitidis*, it is possible to make a knockout mutant of *lpxA*, encoding UDP-GlcNAc acyltransferase required for the first step of lipid A biosynthesis (Galloway and Raetz, 1990), while maintaining cell viability (Steeghs *et al*., 1998). This mutant still possesses an OM in which the PL composition is altered in comparison with the wild-type strain (Steeghs *et al*., 2001).

In *E.coli*, the *lpxA* gene belongs to a largely conserved cluster of genes, present in almost all Gram-negative bacteria for which genome sequences are available. Figure 1A shows the chromosomal arrangement of this locus in several Gram-negative bacteria. In addition to the lipid A biosynthesis genes *lpxA*, *lpxD* and *lpxB*, this cluster also includes *fabZ* and *cdsA* required for fatty acid and phospholipid synthesis, respectively (Raetz and Dowhan, 1990; Mohan *et al*., 1994), as well as *yaeM* and *yaeL* which are known to be involved in membrane biogenesis in *E.coli*: *yaeM* encodes 1-deoxy-D-xylulose 5-phosphate reductoisomerase involved in the synthesis of isoprenoids (Takahashi *et al*., 1998), which have been characterized in many diverse organisms and have been shown to serve as pigments, defensive agents, constituents of membranes or components of signal transduction networks (Sacchettini and Poulter, 1997). *yaeL* encodes the essential EcfE IM protease, which is involved in regulation of the heat shock response in *E.coli* (Dartigalongue *et al*., 2001). EcfE modulates the level of expression of both RpoH and RpoE σ factors, which control protein folding and degradation in the cytoplasm and extracytoplasm, respectively. Moreover, RpoE regulates lipid biogenesis in response to environmental stress (Raivio and Silhavy, 2001). The *yaeT* gene, encoding a putative outer membrane protein (OMP) of unknown function, is always found upstream of *lpxD*, in most cases associated with *hlpA*, which encodes a periplasmic chaperone (Chen and Henning, 1996;
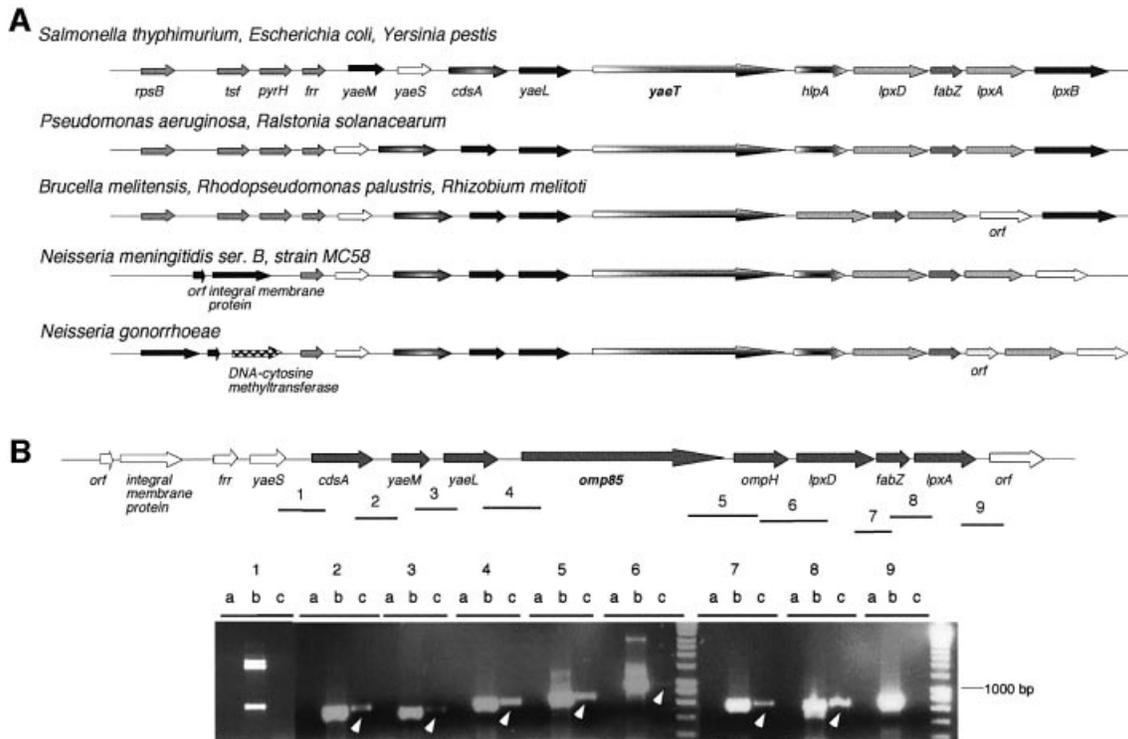
Fig. 1. (**A**) Schematic representation of the *lpx* locus of different Gram-negative bacteria. Arrows of the same shadings represent homologous genes. (**B**) Results of RT–PCR experiments. Top: transcriptional organization of the neisserial *lpx* locus showing the regions amplified by the primer pairs 1–9. Bottom: agarose gel of the RT–PCR amplification products. For each primer pair, three lanes are shown [a, negative control using RNA as template (no RT); b, positive control using genomic DNA as template; c, RT–PCR]. The *cdsA-lpxA* transcript is indicated with grey arrows.

Missiakas *et al.*, 1996). The presence of a gene encoding a highly conserved OMP in a cluster dedicated to LPS and PL biosynthesis suggests a possible involvement in lipid transport.

The way in which completed LPS and PL are translocated to the cell surface represents the major unresolved question in their biosynthesis. Recently, a clue to the mechanism of lipid A core and PL transport within the IM has emerged from the discovery that a mutation in *msbA*, encoding an essential ABC transporter, results in a defect in lipid export (Doerrler *et al.*, 2001). The MsbA protein is thought to function as an IM flippase for PL and lipid A core transport to the periplasm. To complete the LPS biosynthesis, lipid A core is attached further to the O-polysaccharide chain once both molecules are in the periplasm. The three currently known pathways for O-polysaccharide biosynthesis are distinguished by their export mechanisms (Raetz and Whitfield, 2002). The pathways are called Wzy-dependent, ABC transporter-dependent and synthase-dependent. The first two processes are widespread, while the last one has limited distribution in O-polysaccharide biosynthesis. Despite the export differences, the initiation reactions and ligation to lipid A core are similar. The molecular mechanism underlying the transport of the completed LPS molecules to the OM is still unknown. As for LPS, the mechanisms of PL transport from the IM to OM are unknown. Several hypotheses have been proposed, such as PL and LPS translocation through membrane adhesion zones (Bayer's junctions) (Bayer, 1968), but it is still not clear whether

or not these structures are physiologically relevant (Kellenberger, 1990). Another possibility is that specific proteins may serve as a shuttle for lipid transfer to the OM, but this has never been demonstrated.

Neisseria meningitidis is, to date, the only Gram-negative bacterium in which a completely LPS-deficient but still viable mutant can be obtained (Steeghs *et al.*, 1998). This makes it a uniquely suitable organism for the study of LPS and PL transport. In the present study, we have investigated the involvement of Omp85, the neisserial YaeT homologue, in the export of lipids to the OM. We first showed that *omp85*, the gene encoding Omp85, is part of a transcriptional unit that starts with *cdsA* and ends with *lpxA*. We further demonstrated that Omp85 is essential for the viability of *N.meningitidis* and is required for LPS and PL transport in the OM. We also showed that Omp85 is not directly involved in the transport of integral OMPs such as PorA and Opa, though the depletion of Omp85 leads to increased amounts of their degradation products in the OM.

Results

The omp85 gene is transcriptionally linked to genes involved in lipid A, fatty acid and phospholipid biosynthesis

The *lpx* locus is highly conserved among different Gram-negative bacteria (Figure 1A), containing genes involved in lipid A, fatty acid and PL biosynthesis together with genes implicated in membrane biogenesis. In order to
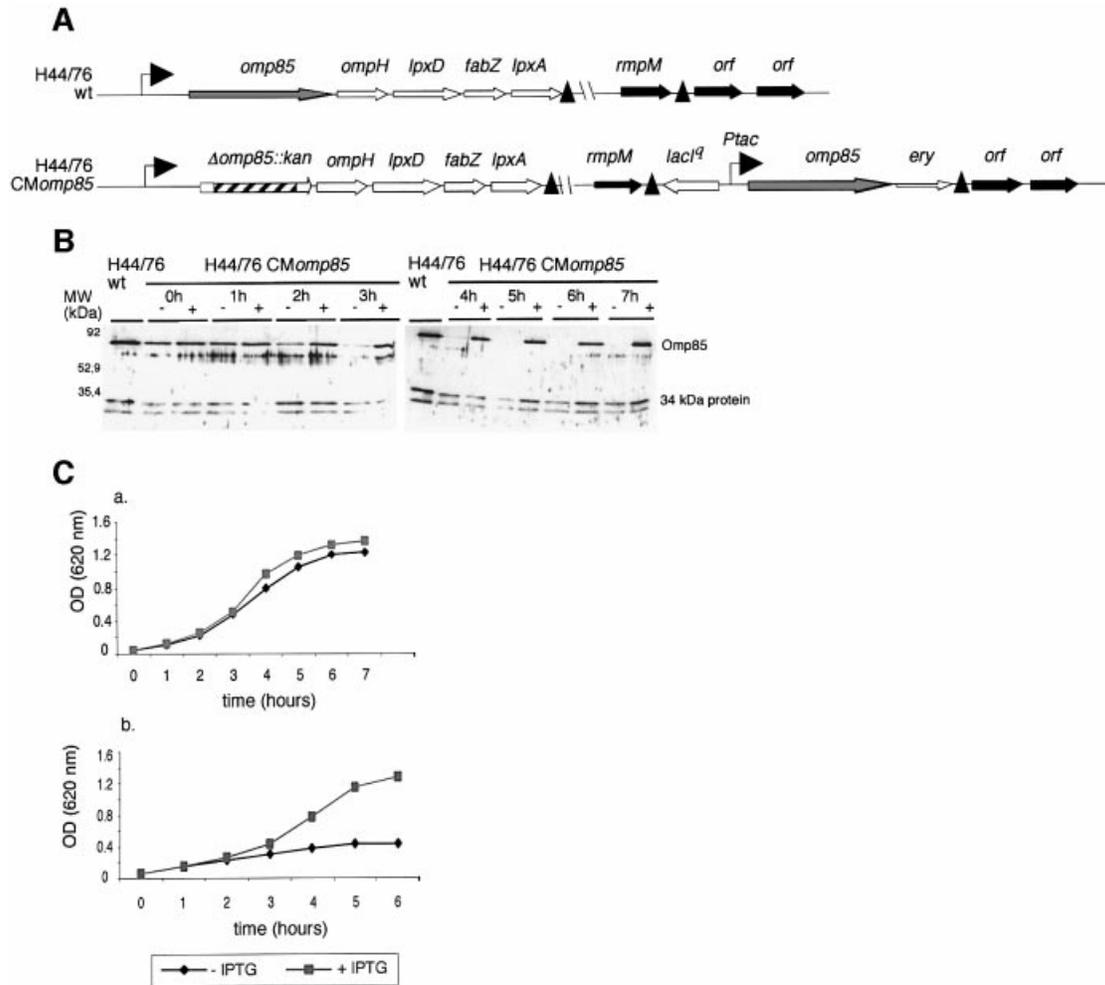
Fig. 2. Omp85 is essential for viability of *N.meningitidis*. Construction of a meningococcal strain in which Omp85 expression is under the control of the *tac* promoter. (**A**) Chromosomal arrangement of the H44/76 wild-type and CM*omp85* strains. Transcriptional terminators are indicated by black triangles. Details of cloning are explained in Materials and methods. (**B**) Western blot on whole-cell extracts of the H44/76 CM*omp85* strain grown without (–) and with (+) 0.05 mM IPTG, and the H44/76 wild-type strain as control, at different time points of the first 7 h of growth. The antibody used is a rabbit polyclonal Omp85 antiserum. (**C**) Growth curves of the *lacI^q-Ptac*-regulated *omp85* strain in the absence (black diamonds) and presence of 0.05 mM IPTG (grey squares). In this panel, two growth curves are shown: in (a), colonies were picked up from IPTG-containing plates, washed and grown for 7 h. After 4 h of growth without IPTG, bacteria were diluted into new fresh medium without IPTG and grown further for 6 h. This is shown in (b).

determine if *omp85* is co-transcribed with these genes in *N.meningitidis*, RT–PCR assays were performed (Figure 1B). A negative control, consisting of DNase-treated RNA, and a positive control, consisting of genomic DNA, were included in the experiment. Using cDNA as template, we could amplify overlapping regions between genes of the cluster, from *cdsA* to *lpxA*. This result demonstrates that *omp85* is part of a transcriptional unit that contains eight genes, starting with *cdsA* and ending with *lpxA*, which could reflect the fact that they encode proteins or enzymes involved in a common pathway.

Omp85 is essential for viability of N.meningitidis

To test directly the involvement of Omp85 in LPS and PL transport, we first attempted to make an *omp85* knockout mutant in *N.meningitidis* wild-type strain H44/76 and its LPS-deficient *lpxA* mutant by allelic replacement with constructs containing either a kanamycin or chloramphenicol marker in *omp85*, but without any success.

Moreover, we tried to delete *omp85* in the HA3003 strain in which LPS expression could be switched on or off through a *tac* promoter-controlled *lpxA* gene (Steeghs *et al.*, 2001). Again, no double crossover recombination events leading to the inactivation of the *omp85* gene could be obtained, whether HA3003 expressed LPS or not, indicating an essential role for Omp85 in the viability of both the wild-type and LPS-deficient mutant.

To study the function of Omp85 in spite of this limitation, we next constructed a meningococcal strain in which Omp85 expression is regulated from the *tac* promoter (Figure 2A). The *lacI^q-Ptac*-regulated *omp85* DNA fragment was first introduced at the *rmpM* locus. Secondly, the *omp85* gene was deleted at its own locus using the kanamycin cassette, which does not contain a transcriptional terminator. The latter insertion did not affect expression of downstream *lpx* genes in the resulting *omp85* conditional mutant (termed CM*omp85*), as was checked by RT–PCR (results not shown). We could
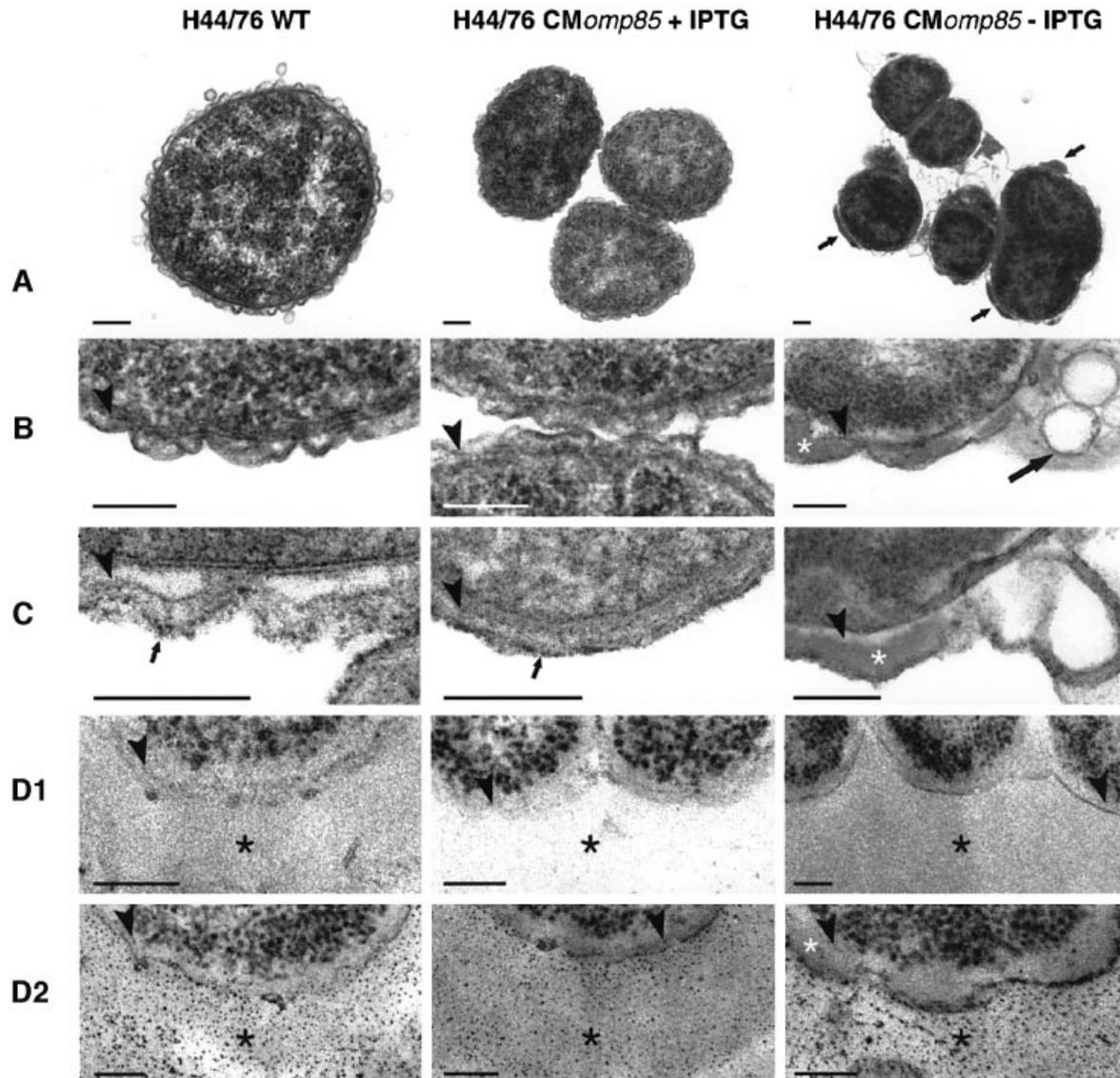
Fig. 3. Electron micrographs of ultrathin sections of H44/76 wild-type and CM*omp85* grown in the presence and absence of 0.05 mM IPTG. (**A**) Overview and (**B–D**) high magnification of the cell envelope of the different strains. Bacteria were fixed in a Karnovsky solution and contrasted according to standard procedures (A and B), or they were fixed in a combined glutaraldehyde and OsO₄ solution to retain lipids and contrasted to standard procedures (C), and fixed in PFA, followed directly by embedding without contrasting (D1/2). Sections in (D1) were not post-stained and all the contrast was imparted by the embedding medium. A cytochemical reaction for the presence of PAS-positive material on ultrathin sections is illustrated in (D2). Depletion of Omp85 leads to accumulation of electron-dense amorphous material (small arrows in A, white asterisk in B) and to vesicular structures (large arrow in B) in the periplasmic space. This amorphous material is not lipophilic or cytochemically stained for polysaccharides (white asterisk in C and D2). The small arrows in (C) mark the osmiophilic granules outside the OM. The black asterisks in (D1) and (D2) denote a layer of PAS-positive material outside the cells, presumably the capsular polysaccharide. Arrowheads in (B–D) mark the peptidoglycan layer. The bars represent 200 µm in (A) and 100 µm in (B–D).

only select kanamycin-resistant clones in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), again demonstrating that *omp85* expression is essential for bacterial survival. Using this mutant, the expression of Omp85 could be regulated by the presence or absence of IPTG in the medium. Depletion of Omp85 was checked every hour of growth by western blot on whole-cell extracts using a rabbit polyclonal antiserum directed against Omp85 (Figure 2B). After 3 h of growth without IPTG, depletion of Omp85 became visible, and was apparently complete after 5 h of growth. Upon induction with IPTG, *omp85* is transcribed and cells are viable (Figure 2C). When no IPTG was added in the medium, cell

growth was affected. The effect of depletion of Omp85 on bacterial growth was more pronounced when, after 4 h of incubation without IPTG, the culture was diluted further into fresh medium without IPTG (see Figure 2C, panel b). This clearly demonstrates that Omp85 is essential for the viability of *N.meningitidis*.

Analysis of the ultrastructure of the cell envelope upon depletion of Omp85

The ultrastructure of the H44/76 CM*omp85* strain grown in the presence and absence of IPTG, and its parental strain was examined by transmission electron microscopy (Figure 3). We did not observe any ultrastructural
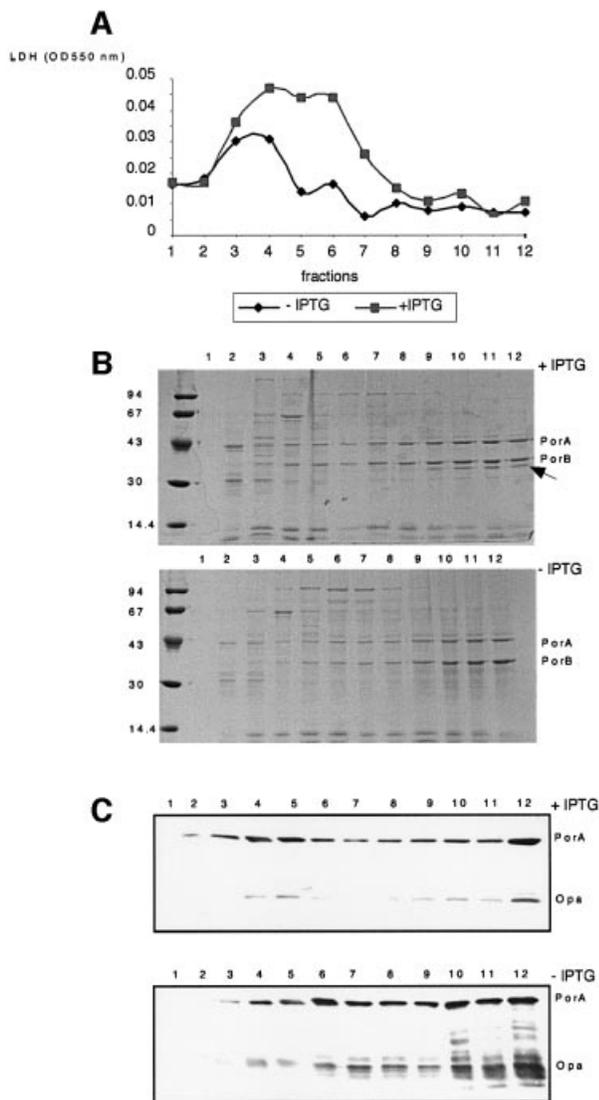
A

LDH (OD550 nm)

B

C

Fig. 4. Analysis of fractions 1–12, collected after isopycnic sucrose gradient centrifugation, to separate the membranes of the H44/76 CM*omp85* strain grown for 6 h in the presence and absence of 0.05 mM IPTG. (**A**) LDH activity per 5 µg of proteins as a marker for the IM. (**B**) SDS–PAGE analysis of the fractions. The positions of molecular weight standard proteins are indicated on the left in kDa; the positions of the porins PorA and PorB are indicated on the right. The black arrow shows the 35 kDa band. (**C**) Western blot using PorA (MN5C11G) and Opa (15-1-P5.5 + αD2) monoclonal antibodies as markers for the OM.

difference in the cell envelope between the CM*omp85* strain grown with IPTG and the wild-type strain. In contrast, cells of H44/76 CM*omp85* grown without IPTG were more heterogeneous in size, and a significant proportion of cells showed signs of lysis (Figure 3A and B). A clear accumulation of both electron-dense material and vesicular structures in the periplasm was observed for these latter cells, suggesting that the depletion of Omp85 leads to a defect in transport of some component(s) that can no longer reach the OM. We further analysed ultrastructurally whether this accumulating material consists of lipids or polysaccharides. Therefore, bacteria were fixed in glutaraldehyde/osmium tetroxide solution to retain lipids and in a paraformaldehyde (PFA) solution to

perform the periodic acid–Schift (PAS) reaction. H44/76 wild-type and IPTG-stimulated CM*omp85* bacteria showed small lipophilic granules at the outside of the OM, whereas CM*omp85* bacteria grown without IPTG did not show these lipophilic granules. The accumulating material in these cells was also not reactive with osmium and is therefore considered as not lipophilic (Figure 3C). A cytochemical reaction for polysaccharides revealed many granules in a broad band directly outside these cells, presumably representing the capsule. No reaction was seen within the periplasmic space (Figure 3D). These findings suggest that the accumulating electron-dense material in the periplasm is of a proteinaceous nature.

IM and OM separation of the H44/76 CMomp85 strain

The cell envelope composition of the H44/76 CM*omp85* strain, grown for 4 and 6 h in the presence and absence of IPTG, was examined by IM and OM separation through isopycnic sucrose gradient centrifugation (Steeghs *et al.*, 2001). We will only present the data on the collected fractions after 6 h of growth. Gradient fractions of 1 ml were collected, protein quantified and analysed for the presence of lactate dehydrogenase (LDH) activity as a marker for the IM (Osborn *et al.*, 1972), and for the presence of PorA and Opa as markers for the OM (Figure 4). LDH activity peaked in fractions 3 and 4 when the H44/76 CM*omp85* strain was grown without IPTG (Figure 4A). In contrast, in the presence of IPTG, LDH activity was higher and peaked in fractions 3–6. In both cases, no LDH activity was detected in fractions 8–12, which are therefore regarded as pure OM fractions. We analysed the protein content on each collected fraction by SDS–PAGE followed by Coomassie Blue staining (Figure 4B). The protein profile for these fractions is the same for bacteria grown in the presence or absence of IPTG, with the exception of a band (marked with a black arrow) at a molecular weight of 35 kDa that disappears when bacteria are not induced with IPTG. We performed a western blot experiment using monoclonal antibodies directed against PorA and Opa, two integral OMPs (Figure 4C). PorA and Opa proteins are visible in almost all the fractions, but with strong enrichment of these proteins in the OM fractions. This was observed previously using the same procedures for the IM and OM separation of the wild-type strain and the LPS-deficient mutant (Steeghs *et al.*, 2001). The Opa protein remains present in the OM fractions of the strain grown without IPTG, suggesting that the 35 kDa band that disappears upon depletion of Omp85 does not correspond to Opa. Moreover, depletion of Omp85 did not lead to accumulation of PorA and Opa in the IM fractions, indicating that Omp85 is not directly involved in the transport of these proteins to the OM. The only effect is a somewhat increased amount of PorA and Opa degradation products in the OM fractions. Degradation of OMPs has also been reported in the LPS-deficient mutant (Steeghs *et al.*, 2001).

Omp85 is required for LPS transport to the OM

To study the effect of Omp85 depletion on LPS localization, the LPS content of each proteinase K-treated fraction was analysed by Tricine-SDS–PAGE followed by silver staining (Lesse *et al.*, 1990) (Figure 5). When Omp85

Fig. 5. Omp85 is required for LPS export to the OM. Silver-stained Tricine–SDS–polyacrylamide gel showing the LPS pattern of proteinase K-treated fractions containing 5 µg of protein of the H44/76 CM*omp85* strain grown in the presence (top) and absence (bottom) of 0.05 mM IPTG.

was present, LPS was observed mainly in the OM fractions. In contrast, when Omp85 was depleted, LPS mostly disappeared from the OM fractions and instead accumulated in the IM fractions. Moreover, a two band pattern was observed, suggesting that depletion of Omp85 in addition leads to the relative increase in a modified form of LPS with slightly lower gel mobility. After 4 h of growth without IPTG, the same result was observed, though the effect of depletion of Omp85 on LPS accumulation in IM fractions was less marked, suggesting that LPS accumulation in the IM occurs directly and progressively upon depletion of Omp85.

Omp85 is required for PL transport to the OM

The results shown in Figure 5 indicate a role for Omp85 in LPS export. However, as a knockout *omp85* mutant could not be isolated in the LPS-deficient mutant, Omp85 might also be required for another function in membrane biogenesis. Therefore, we examined the effect of Omp85 depletion on PL transport to the OM (Figure 6). Bacteria were grown for 6 h as previously described, but in the presence of [1,2-^{14}C]acetic acid. Bacterial cell fractionation was performed by another method, based on the lysis of lysozyme–EDTA spheroblasts (Osborn *et al.*, 1972). IMs and OMs were separated on a sucrose gradient. Each fraction was analysed for the presence of PorA as a marker for the OM (Figure 6A). Compared with the previous membrane separation, we observed a slightly different distribution of OM fractions in this experiment: PorA is distributed in fractions 4–10. This might be explained by the use of lysozyme, which degrades the peptidoglycan layer, leading to a shift of OM to the lower density fractions of the gradient. This has been observed previously in the sucrose gradient fractionation of membranes from *Salmonella typhimurium* using the same procedures (Osborn *et al.*, 1972). The *N.meningitidis* OM localizes in the same density region of the gradient (38–43% sucrose) as the 'H' band representing the *S.typhimurium* OM. PLs were extracted and analysed for their c.p.m. value as shown in Figure 6B. We calculated the relative percentage of each PL extract compared with the sum of the c.p.m. of all extracts. The distribution of PLs can also be visualized after their separation on thin-layer chromatography (TLC) as shown in Figure 6C. Compared with the strain grown with IPTG, PLs are decreased in the OM (fractions 6–10) and instead accumulate in the IM (particularly in fraction 2) after depletion of Omp85 (–IPTG). These results demonstrate that Omp85 is also required for PL transport to the OM. Moreover, this might explain why no *omp85*
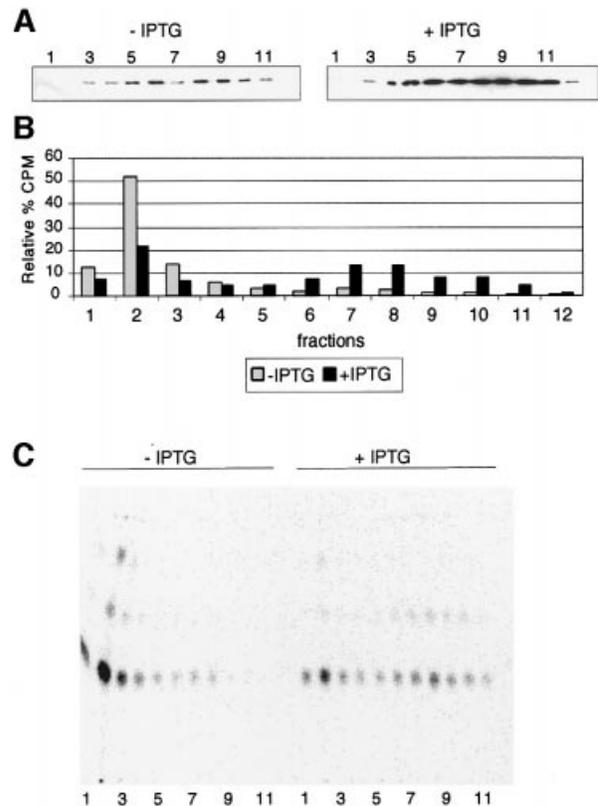

Fig. 6. Omp85 is required for PL export to the OM. The H44/76 CM*omp85* strain was grown in the presence of [1,2-^{14}C]acetic acid for 6 h in the presence and absence of 0.05 mM IPTG. (**A**) Collected fractions 1–12 were analysed by western blot using PorA monoclonal antibody as a marker for the OM. (**B**) Relative percentage of the c.p.m. value in each fraction [(c.p.m. value FX/total c.p.m. value) × 100]. (**C**) TLC separation of PLs. PLs were extracted using the method of Bligh and Dyer (1959) and separated on TLC plates as explained in Materials and methods.

knockout mutant could be isolated in the LPS-deficient mutant.

Discussion

In the present study, we have investigated the potential involvement of Omp85 in transport of lipids to the OM of *N.meningitidis*. In contrast to most Gram-negative bacteria, *N.meningitidis* is viable without LPS, making it a uniquely suitable organism to study lipid transport.

The *omp85* gene has a conserved location in all Gram-negative bacterial genomes sequenced to date. We demonstrated that, in *N.meningitidis*, *omp85* is part of a transcriptional unit that starts with *cdsA* and ends with *lpxA*. The association of *omp85* with genes encoding proteins involved in LPS and PL biosynthesis and cell envelope assembly suggested a common involvement in lipid biosynthesis and membrane biogenesis pathways.

The *omp85* gene is essential for survival of *N.meningitidis* wild-type and its LPS-deficient mutant. We constructed a derivative strain of H44/76, designated H44/76 CM*omp85*, which carries an IPTG-inducible *omp85* gene on the chromosome, allowing us to study the function of Omp85 after its gradual depletion. The analysis of the ultrastructure of the bacterial envelope by electron microscopy revealed that together with depletion
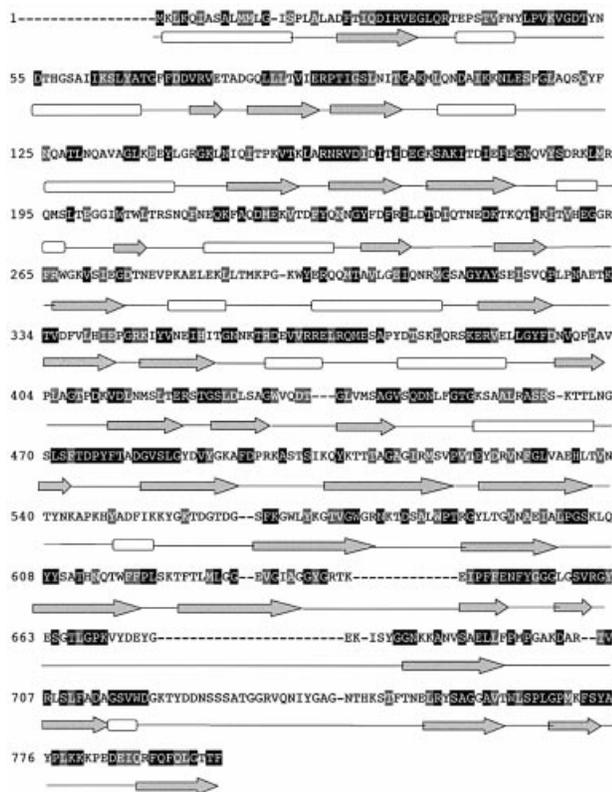
Fig. 7. Secondary structure predictions for Omp85 in *N.meningitidis* and its homologues in other Gram-negative bacteria (*N.gonorrhoeae*, *S.enterica*, *E.coli*, *H.influenzae*, *P.multocida* and *B.abortu*s). Multiple sequence alignments were performed using the ClustalW program. Shown is the *N.meningitidis* Omp85 sequence. Black and grey squares represent identical and similar residues in the alignment, respectively. Predicted β-strands and α-helices are represented by grey arrows and white rectangles, respectively.

of Omp85, an electron-dense amorphous material, as well as vesicular structures are accumulating between the IM and OM. Different staining techniques were used to demonstrate that the electron-dense material probably consists of protein. The vesicular structures are reminiscent of the IM reduplications seen in the *E.coli msbA* mutant (Doerrler *et al.*, 2001), and suggest a block in lipid export. IM and OM separation of the CM*omp85* strain, grown in the presence and absence of IPTG, demonstrated that Omp85 is required for LPS transport to the OM. Analysis of the LPS content in the IM and OM revealed a more pronounced two-band pattern in the mutant, indicating that depletion of Omp85 leads to some modification of LPS. This could be explained by increased sialylation of LPS in the mutant. In *Neisseria*, sialylation is catalysed by an α-2,3-sialyltransferase (Lst) that monosialylates the terminal galactose of LOS (for lipooligosaccharide) by using 5′-cytidine monophospho-*N*-acetylneuraminic acid as donor (Mandrell *et al.*, 1990). Recently, it has been shown that Lst is a surface-exposed OMP with its active site possibly facing the periplasm (Shell *et al.*, 2002). In the CM*omp85* strain grown without IPTG, LPS is accumulating in a compartment where it could be subjected to increased sialylation by Lst. To check whether the accumulating upper band in the non-induced cells corresponds to intensive sialylation of LPS, these samples were subjected to neuraminidase treatment. The

upper band is disappearing following this treatment, indicating that this band corresponds to a sialylated form of LPS (results not shown).

Transport of the integral OMPs PorA and Opa to the OM is not impaired when Omp85 is depleted, though an increased amount of PorA and Opa degradation products is observed in the OM fractions. Various studies have shown that in *E.coli*, LPS is required for the correct assembly of OMP (de Cock and Tommassen, 1996). In contrast, in *N.meningitidis*, the trimerization of porins is not affected in the LPS-deficient mutant (Steeghs *et al.*, 2001), though some degradation products of these OMPs were observed.

We have shown that in the absence of Omp85, LPS accumulates in the IM; this is probably toxic for the bacteria and would explain why an *omp85* knockout mutant could not be isolated. The isolation of an *omp85* knockout mutant in the LPS-deficient mutant would have demonstrated that Omp85 is only involved in LPS transport to the OM. Using different constructs containing either a kanamycin or chloramphenicol marker inserted into *omp85*, it was not possible to isolate such a mutant, suggesting that Omp85 might have an additional function. We therefore checked if depletion of Omp85 also has an effect on PL transport to the OM. This turned out to be the case, explaining why we could not isolate a double *omp85-lpxA* mutant. Consistently, homologues of Omp85 have also been found in the genomes of *Treponema pallidum* and *Borrelia burgdorferi* (Fraser *et al.*, 1997, 1998), which are spirochetes that do not contain LPS in their OM (Hardy and Levin, 1983). In these genomes, no *lpx* genes are found and the *omp85* homologues are associated with *ompH* encoding a periplasmic chaperone. Nevertheless, other glycolipids as constituents of the OM of LPS-lacking bacteria have been described (Schultz *et al.*, 1998), and it would be interesting to study the probable involvement in lipid transport of the Omp85 homologues in these organisms.

Under Omp85 depletion conditions, growth is affected and the bacteria will eventually start to lyse. This makes it critical to find the right timing for analysing membrane composition. If bacteria are growing for too short a time without IPTG, the Omp85 depletion is not complete; if the depletion time is too long, bacteria start to lyse, making membrane fractionation difficult to achieve. We used different growth conditions in this work: after 4 h of growth without IPTG, we could see an effect on LPS localization (data not shown), but it was less marked than after 6 h of growth. However, after longer depletion times, the OM in particular became difficult to isolate, presumably due to its disintegration when the lipid content becomes too low (results not shown). These disadvantages are inherent with mutants with regulated expression, and can only be circumvented by using a temperature-sensitive mutant of Omp85, which may, however, be difficult to isolate. This strategy has been successful in the characterization of the MsbA protein involved in the transport of lipids through the IM (Doerrler *et al.*, 2001).

During the revision of our manuscript, another study on the role of meningococcal Omp85 in OM biogenesis was published (Voulhoux *et al.*, 2003). These authors reported that Omp85 appeared to be essential for viability, and that unassembled forms of various OMPs accumulated upon Omp85 depletion. However, they analysed the effect

of Omp85 depletion after 20 h of incubation under non-induced conditions, and by this time the observed defect in OMP assembly could very well be a secondary result due to a block in lipid export to the OM. Indeed, they report a reduction in LPS content of cell envelopes of 60% upon Omp85 depletion. The authors argue against this possibility by quoting our previous work on the LPS-deficient meningococcal *lpxA* mutant, which demonstrated that LPS is not required for OMP export and assembly in this organism (Steeghs *et al.*, 2001). However, our present work shows a role for Omp85 as a more general lipid transporter and, if no new OM lipid bilayer can be formed due to Omp85 depletion, insertion of new OMPs is bound to be affected at some point. Our results with OM and IM separation at earlier time points (4 and 6 h) demonstrate that the effect on lipid export precedes any effect on OMP localization.

A topological model of Omp85 was elaborated to investigate how an OMP could be involved in lipid transport. We performed ClustalW alignment between Omp85 and its homologues in *Neisseria gonorrhoeae*, *Salmonella enterica*, *E.coli*, *Haemophilus influenzae*, *Pasteurella multocida* and *Brucella abortus* (Higgins *et al.*, 1996). Figure 7 shows the results for the Omp85 sequence of *N.meningitidis*, with black boxes for conserved identical residues and grey boxes for conserved similar residues. Conservation in amino acid sequences is observed throughout, with some defined boxes corresponding to conserved secondary structures in the N-terminal part of the protein. Secondary structure prediction methods, such as PsiPred (Jones, 1999) and PHD (Rost and Sander, 1993), predict at least two domains. The N-terminal domain consists of β-strands and α-helices and is very well conserved among all Omp85 homologues. According to secondary structure predictions, a motif of three β-strands and two α-helices is repeated five times in the N-terminal domain. The C-terminal domain mainly consists of β-strands and could form a β-barrel for anchorage in the OM. This model thus reveals features characteristic of a transporter, as described for other OMPs belonging to the family of iron transporters, FepA (Buchanan *et al.*, 1999) and FhuA (Ferguson *et al.*, 1998). Conceivably, the C-terminal β-barrel domain could be involved in transport while the N-terminal domain interacts with other proteins in the periplasm. Indeed, Manning *et al.* (1998) reported surface cross-linking experiments that suggest that Omp85 is surface exposed and interacts with other proteins. Among them is a 34 kDa protein, homologous to IAP34, a GTP-binding protein belonging to the chloroplast import-associated protein (IAP) complex (Schnell *et al.*, 1994).

In conclusion, we have demonstrated that Omp85 is required for LPS and PL transport to the OM. In the same way that MsbA functions in the IM to transport lipid A core and PL actively to the periplasm (Chang and Roth, 2001; Doerrler *et al.*, 2001), Omp85 could be a general lipid transporter in the OM. LPS transport to the OM is likely to involve two steps: first, from the periplasm to the inner leaflet of the OM and, secondly, from the inner leaflet of the OM to the outer surface of the OM (Raetz and Whitfield, 2002). Our data strongly suggest an essential role for Omp85 in this process. Whether Omp85 directly functions as a transporter, or has a more indirect effect due to its interaction with other components required for this export pathway, remains to be established. However, its nature as an OMP potentially forming a β-barrel is consistent with a transport function within the OM, i.e. the last step in the pathway mentioned above. The present work demonstrates its essential role in lipid transport and, subsequently, opens the door to further elucidation of this pathway. In addition, because Omp85 is a surface-exposed and highly conserved protein essential for viability, it will be a good target for the design of new antibiotics.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli NM522 (Promega), used for propagation of plasmids, was grown in LB medium containing ampicillin (100 μg/ml), kanamycin (100 μg/ml) and erythromycin (200 μg/ml) at 37°C. *Neisseria meningitidis* strains were grown overnight at 37°C on GC medium base (Difco Laboratories) supplemented with IsoVitaleX (Becton Dickinson) in a humid atmosphere containing 5% CO_2. *Neisseria meningitidis* H44/76 CM*omp85* strain was grown on GC plates in the same conditions but with addition of 0.01 mM IPTG (Roche). For the characterization of the CM*omp85* mutant, bacteria were grown in Meningococcal Medium (van der Ley *et al.*, 1993) without IPTG to impose depletion of Omp85. As control, bacteria were grown with IPTG at a concentration of 0.05 mM. Bacteria were grown for 4 or 6 h (log phase) and inactivated by incubation for 2 h with 200 μg/ml tetracycline in 2% ethanol (final concentration) at 37°C under aeration. For PL characterization, bacteria were grown for 6 h in the presence of 1 μCi/ml [1,2-^{14}C]acetic acid (specific activity 50–120 mCi/mmol, ICN Biochemicals). Transformation of *N.meningitidis* was done as described previously (van der Ley and Poolman, 1992) with selection of erythromycin (5 μg/ml) or kanamycin (100 μg/ml).

RNA isolation and RT–PCR assays

Neisseria meningitidis cultures (100 ml) were grown to mid-exponential growth phase in TSB (Difco Laboratories). Bacterial cells were harvested by centrifugation for 15 min at 2500 r.p.m., washed and resuspended in 2 ml of TE (Tris 10 mM, EDTA 1 mM pH 8) containing 1% SDS and proteinase K (200 μg/ml final concentration) and incubated for 1 h at 37°C. Total RNA was then extracted using the RNeasy Total RNA Midi kit (Qiagen). Contaminating DNA was digested with RQ1 DNase I (Promega). This enzyme was later eliminated by extraction twice with phenol/chloroform/isoamylalcohol (25:24:1) followed by ethanol precipitation. RNA was resuspended in 20 μl of RNase-free water. RNaseOUT ribonuclease inhibitor (Life Technologies) and random primers were added to 2 μl of this DNase-treated RNA and denatured by heating to 70°C for 10 min. Reverse transcription was performed with Superscript II reverse transcriptase (Life Technologies), in a final volume of 20 μl containing 10 mM dithiothreitol, 1 mM dNTP (Eurogentec), 50 U of reverse transcriptase and the manufacturer's buffer (provided with the enzyme). This mixture was incubated at 42°C for 50 min and the enzyme was inactivated by heating to 70°C for 15 min. A control reaction containing the same components but no reverse transcriptase was included to check for DNA contamination. The cDNA products (2 μl) were then used in a PCR performed in a final volume of 50 μl containing 1 U of Biotools DNA polymerase (B&M Labs SA), 1 mM dNTP, manufacturer's buffer (provided with the enzyme) and 10 pmol of each primer. A positive control in which *N.meningitidis* genomic DNA was used as a template for PCR was included. The amplification programme consisted of one cycle of 5 min at 95°C followed by 30 cycles of 45 s at 95°C, 45 s at annealing temperature (depending on the primer used), 1 min at 72°C and a final step of 10 min at 72°C.

Construction of plasmids

Plasmids were constructed by using standard recombinant DNA techniques (Sambrook and Russel, 2001). Plasmid DNA was isolated using the Wizard kit (Promega). Plasmid pGEM-Teasy-*omp85* is a pGEM-T easy derivative (Promega) containing the *omp85* gene amplified with Expand High Fidelity polymerase (Roche) with primers *omp85*F (5′-ctcatgatgctgatgatggc-3′) and *omp85*R.3 (5′-aaacgggtcatggtaaaactcc-3′). This plasmid was the basis for the construction of plasmid pCF3ery-CM*omp85* which was used to make the conditional *omp85* mutant. The

omp85 gene was cloned from pGEM-Teasy-*omp85* in pJF118EH in *Eco*RI sites, in such a way so as to place the *omp85* gene under the control of the *tac* promoter (Furste *et al.*, 1986). In parallel, an 85mer linker (5′-cgatgatgtgaaacaaacccccgcttttgcggggtttgtttttttgctcgcgagtagcccaagcttgg-gaaccgctcgagcggat-3′) containing *Nru*I, *Hin*dIII and *Xho*I sites (under-lined) possesses *Cla*I overhangs and contains the 42 bp transcriptional terminator of the *rmpM* gene. It was cloned in the *Cla*I site of the pCF3 plasmid (van der Ley *et al.*, 1995), downstream of the *rmpM* gene and its transcriptional terminator. The orientation of the linker in pCF3 was checked by PCR and sequence analysis with an Applied Biosystems automatic sequencer 310 on double-stranded plasmid DNA templates with a cycle sequencing protocol and primer hybridizing with the *rmpM* gene. The erythromycin cassette was cloned in *Hin*dIII–*Xho*I sites to obtain the pCF3ery plasmid. Subsequently, the *lacI^q-tac-omp85* region released from plasmid pJF118EH*omp85* by an *Nru*I–*Hin*dIII digestion was inserted into the unique *Nru*I–*Hin*dIII sites of the pCF3ery plasmid, resulting in plasmid pCF3ery-CM*omp85*. The orientation of the *lacI^q-tac-omp85* region was checked by restriction analysis with *Kpn*I, which is present both in the original plasmid and in the *omp85* gene. Plasmid pGEM-Teasy-Δ*omp85*-Kan was used to delete *omp85* at its own locus. We first amplified upstream (439 bp) and downstream (539 bp) parts of *omp85* with primers *omp85*F2SB (5′-gttcctgcaggcggatccgctggatggt-gaagtcggcaa-3′) and *orf*R (5′-aacgcctccgtcagccatat-3′) for the upstream part, and primers *omp85*R2SB (5′-gcggatccgcctgcaggaacgaagacgaaatc-caacgctt-3′) and *ompH*F.1 (5′-aatgacatcgtaaccttcctgt-3′) for the down-stream part. Primers *omp85*F2SB and *omp85*R2SB contain *Bam*HI sites at their 5′ ends and region of 20 bp that anneal to each other. We used a site-directed mutagenesis approach to amplify and clone both fragments together in the pGEM-Teasy vector. Subsequently, we cloned in the *Bam*HI site the Kan cassette released by *Bam*HI restriction from pUC4K. The orientation of the Kan cassette was checked by *Cla*I restriction analysis. We first transformed *N.meningitidis* H44/76 with pCF3ery-CM*omp85* plasmid. Erythromycin-resistant clones were transformed with pGEM-Teasy-Δ*omp85* and selected on kanamycin only upon addition of IPTG to the media. The strain obtained was called *N.meningitidis* H44/76 CM*omp85*.

We used plasmid pGEM-T-easy-Δ*lpxA*-ery to delete *lpxA* at its own locus. We used the same strategy as explained above for the deletion of *omp85* to delete *lpxA*. The upstream part of *lpxA* was amplified using primers *lpxA*.2 (5′-cggtccgcacgccgtcatcaac-3′) and *lpxA*-UP (5′-aattccccgggaagcttccggtaacgtgtccggcaa-3′) containing *Ava*I and *Hin*dIII sites (underlined). The downstream part of *lpxA* was amplified using primers *lpxA*com.2US (5′-ccgatgccgtctgaaacgtacggtcagcggat-gatgccg-3′) containing the uptake sequence (underlined), and *lpxA*-DOWN (5′-ggaagcttccccgggggaattccaattctgccgcatcggc-3′) containing *Hin*dIII and *Ava*I sites (underlined).

The resulting clone allowed us to transform with pGEM-T-easy-Δ*omp85*-Kan in an assay to isolate double mutant Δ*lpxA*-Δ*omp85*.

Electron microscopy
Meningococci were grown with or without 0.05 mM IPTG for 6 h, harvested, fixed in three different solutions, washed in cacodylate buffer, pelleted for 5 min at 2000 *g* at 20°C, taken up in agar [2% (w/v)] and embedded in epoxy resin glycidether 100 (Merck). The following three procedures were used. (i) Bacteria were fixed in Karnovsky solution (2% PFA and 2.5% glutaraldehyde in 0.1 M cacodylate/HCl pH 7.4) and post-fixed in osmium tetroxide (OsO$_4$) [1% (w/v)] with 1.5% ferrocyanide in 0.1 M cacodylate buffer. Ultrathin sections were contrasted with aqueous uranyl acetate [2% (w/v)] and lead citrate (1% w/v). (ii) A procedure in which lipids were retained: bacteria were fixed in freshly prepared ice-cold glutaraldehyde [2% (w/v)] with OsO$_4$ [1% (w/v)] in 0.1 M cacodylate/HCl pH 7.4 buffer (Hayat, 1981). Sections were contrasted with aqueous uranyl acetate [2% (w/v)] and lead citrate [1% (w/v)]. (iii) A procedure in which polysaccharides were detected cytochemically: bacteria were fixed in PFA [4% (w/v)] in 0.1 M cacodylate/HCl pH 7.4. Ultrathin sections were stained cytochemically for polysaccharides (Ainsworth *et al.*, 1972). Ultrathin sections were examined in a Philips TECHNAI 12 electron microscope (Philips/FEI, Eindhoven, The Netherlands). Images were digitally acquired with an SIS MegaView II CCD-camera and processed with SIS NT-DOCU TEM analysis software (Soft Imaging System, Münster, Germany).

Membrane separation
For LPS and OMP characterization, IMs and OMs were isolated and separated by sucrose density gradient centrifugation as described (Steeghs *et al.*, 2001).

For PL characterization, bacterial cell fractionation was performed by another method, based on the lysis of lysozyme–EDTA spheroblasts (Osborn *et al.*, 1972). The lysed bacteria were loaded directly on to the gradient without a sonication step.

Lactate dehydrogenase activity assay
LDH activity was measured as described (Osborn *et al.*, 1972).

SDS–PAGE and western blotting
Prior to electrophoresis, whole-cell extracts or fractions were incubated in sample loading buffer (Lugtenberg *et al.*, 1975) containing 2% SDS (final concentration). Incubations were performed for 10 min at 100°C. SDS–polyacrylamide gels were prepared according to Lugtenberg *et al.* (1975). Western blots were performed as described (Towbin *et al.*, 1979). The PorA-specific monoclonal antibody was MN5C11G (P1.16). The Opa-specific monoclonal antibodies were 15-1-P5.5 and αD2. The Omp85 rabbit polyclonal antiserum is a generous gift from Dr E.Wedege and Dr J.Holst (Fredriksen *et al.*, 1994).

LPS analysis
Tricine-SDS–PAGE was performed in 4% stacking and 16% separating gels as described (Lesse *et al.*, 1990). Proteinase K-treated and boiled bacterial cells were used as samples (Apicella *et al.*, 1994). The gel was run for 16–17 h at a constant current of 20 mA, and silver stained (Tsai and Frasch, 1982).

PL analysis
PLs were extracted from protein quantified fractions according to the method of Bligh and Dyer (1959). The amount of ^{14}C-labelled PL was determined by liquid scintillation counting of the PL extracts. PLs were separated by TLC on silica gel 60 plates (Merck) using CHCl$_3$/MeOH/HAc, 65:25:10 (by vol.) as eluent. The radioactive spots were analysed with an Instant Imager (Packard Bioscience).

Acknowledgements

References

Ainsworth,S.K., Ito,S. and Karnovsky,M.J. (1972) Alkaline bismuth reagent for high resolution ultrastructural demonstration of periodate-reactive sites. *J. Histochem. Cytochem.*, **20**, 995–1005.

Apicella,M.A., Griffiss,J.M. and Schneider,H. (1994) Isolation and characterization of lipopolysaccharides, lipooligosaccharides and lipid A. *Methods Enzymol.*, **235**, 242–252.

Bayer,M.E. (1968) Areas of adhesion between wall and membrane of *Escherichia coli*. *J. Gen. Microbiol.*, **53**, 395–404.

Beall,B. and Lutkenhaus,J. (1987) Sequence analysis, transcriptional organization and insertional mutagenesis of the *envA* gene of *Escherichia coli*. *J. Bacteriol.*, **169**, 5408–5415.

Bligh,E.G. and Dyer,W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911–917.

Buchanan,S.K., Smith,B.S., Venkatramani,L., Xia,D., Esser,L., Palnitkar,M., Chakraborty,R., van der Helm,D. and Deisenhofer,J. (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat. Struct. Biol.*, **6**, 56–63.

Chang,G. and Roth,C.B. (2001) Structure of MsbA from *E.coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science*, **293**, 1793–1800.

Chen,R. and Henning,U. (1996) A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane protein. *Mol. Microbiol.*, **19**, 1287–1294.

Dartigalongue,C., Loferer,H. and Raina,S. (2001) EcfE, a new essential inner membrane protease: its role in the regulation of heat shock response in *Escherichia coli*. *EMBO J.*, **20**, 5908–5918.

de Cock,H. and Tommassen,J. (1996) Lipopolysaccharides and divalent cations are involved in the formation of an assembly-competent

intermediate of outer-membrane protein PhoE of *E.coli*. *EMBO J.*, **15**, 5567–5573.

Doerrler,W.T., Reedy,M.C. and Raetz,C.R. (2001) An *Escherichia coli* mutant defective in lipid export. *J. Biol. Chem.*, **276**, 11461–11464.

Ferguson,A.D., Hofmann,E., Coulton,J.W., Diederichs,K. and Welte,W. (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharides. *Science*, **282**, 2215–2220.

Fraser,C.M. *et al.* (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature*, **390**, 580–586.

Fraser,C.M. *et al.* (1998) Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science*, **281**, 375–388.

Fredriksen,J.H., Griffiths,E., Grotterod,E.M., Hoiby,E.A., Rosenqvist,E., Stevenson,P. and Wedege,E. (1994) Characterization of high molecular weight components in MenB-vaccine 'Folkehelsa'; an outer membrane vesicle vaccine against group B meningococcal disease. In Conde-Glez,C.J., Morse,S., Rice,P., Sparling,F. and Calderon,E. (eds), *Pathobiology and Immunobiology of Neisseriaceae. Proceedings of the VIII International Pathogenic Neisseria Conference*. ISBN: 968-6502-13-0, pp. 818–824.

Furste,J.P., Pansegrau,W., Frank,R., Blocker,H., Scholz,P., Bagdasarian,M. and Lanka,E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. *Gene*, **48**, 119–131.

Galloway,S.M. and Raetz,C.R. (1990) A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. *J. Biol. Chem.*, **265**, 6394–6402.

Hardy,P.H.,Jr and Levin,J. (1983) Lack of endotoxin in *Borrelia hispanica* and *Treponema pallidum*. *Proc. Soc. Exp. Biol. Med.*, **174**, 47–52.

Hayat,M. (1981) *Fixation for Electron Microscopy*. Academic Press, New York.

Higgins,D.G., Thompson,J.D. and Gibson,T.J. (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.*, **266**, 383–402.

Jones,D.T. (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.*, **292**, 195–202.

Kellenberger,E. (1990) The 'Bayer bridges' confronted with results from improved electron microscopy methods. *Mol. Microbiol.*, **4**, 697–705.

Kelly,T.M., Stachula,S.A., Raetz,C.R. and Anderson,M.S. (1993) The *firA* gene of *Escherichia coli* encodes UDP-3-*O*-(R-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase. The third step of endotoxin biosynthesis. *J. Biol. Chem.*, **268**, 19866–19874.

Lesse,A.J., Campagnari,A.A., Bittner,W.E. and Apicella,M.A. (1990) Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *J. Immunol. Methods*, **126**, 109–117.

Lugtenberg,B., Meijers,J., Peters,R., van der Hoek,P. and van Alphen,L. (1975) Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. *FEBS Lett.*, **58**, 254–258.

Mandrell,R.E. *et al.* (1990) *In vitro* and *in vivo* modification of *Neisseria gonorrhoeae* lipooligosaccharide epitope structure by sialylation. *J. Exp. Med.*, **171**, 1649–1664.

Manning,D., Reschke,D. and Judd,R. (1998) Omp85 proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis* are similar to *Haemophilus influenza* D15 antigen and *Pasteurella multocida* Oma87. *Microb. Pathog.*, **25**, 11–21.

McMurry,L.M., Oethinger,M. and Levy,S.B. (1998) Triclosan targets lipid synthesis. *Nature*, **394**, 531–532.

Missiakas,D., Betton,J.M. and Raina,S. (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol. Microbiol.*, **21**, 871–884.

Mohan,S., Kelly,T.M., Eveland,S.S., Raetz,C.R. and Anderson,M.S. (1994) An *Escherichia coli* gene (*FabZ*) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to *fabA* and suppression of mutations in lipid A biosynthesis. *J. Biol. Chem.*, **269**, 32896–32903.

Nikaido,H. (1996) The outer membrane. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella: Cellular and Molecular Biology*. ASM Press, Washington, DC, pp. 29–47.

Onishi,H.R. *et al.* (1996) Antibacterial agents that inhibit lipid A biosynthesis. *Science*, **274**, 980–982.

Osborn,M.J., Gander,J.E., Parisi,E. and Carson,J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J. Biol. Chem.*, **247**, 3962–3972.

Raetz,C.R. and Dowhan,W. (1990) Biosynthesis and function of phospholipids in *Escherichia coli*. *J. Biol. Chem.*, **265**, 1235–1238.

Raetz,C.R. and Whitfield,C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.*, **71**, 635–700.

Raivio,T.L. and Silhavy,T.J. (2001) Periplasmic stress and ECF σ factors. *Annu. Rev. Microbiol.*, **55**, 591–624.

Rost,B. and Sander,C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.*, **232**, 584–599.

Sacchettini,J.C. and Poulter,C.D. (1997) Creating isoprenoid diversity. *Science*, **277**, 1788–1789.

Sambrook,J. and Russel,D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schnell,D.J., Kessler,F. and Blobel,G. (1994) Isolation of components of the chloroplast protein import machinery. *Science*, **266**, 1007–1012.

Schultz,C.P., Wolf,V., Lange,R., Mertens,E., Wecke,J., Naumann,D. and Zahringer,U. (1998) Evidence for a new type of outer membrane lipid in oral spirochete *Treponema denticola*. Functioning permeation barrier without lipopolysaccharides. *J. Biol. Chem.*, **273**, 15661–15666.

Shell,D.M., Chiles,L., Judd,R.C., Seal,S. and Rest,R.F. (2002) The *Neisseria* lipooligosaccharide-specific α-2,3-sialyltransferase is a surface-exposed outer membrane protein. *Infect. Immun.*, **70**, 3744–3751.

Steeghs,L., den Hartog,R., den Boer,A., Zomer,B., Roholl,P. and van der Ley,P. (1998) Meningitis bacterium is viable without endotoxin. *Nature*, **392**, 449–450.

Steeghs,L., de Cock,H., Evers,E., Zomer,B., Tommassen,J. and van Der Ley,P. (2001) Outer membrane composition of a lipopolysaccharide-deficient *Neisseria meningitidis* mutant. *EMBO J.*, **20**, 6937–6945.

Takahashi,S., Kuzuyama,T., Watanabe,H. and Seto,H. (1998) A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl Acad. Sci. USA*, **95**, 9879–9884.

Towbin,H., Staehelin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.

Tsai,C.M. and Frasch,C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.*, **119**, 115–119.

van der Ley,P. and Poolman,J.T. (1992) Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. *Infect. Immun.*, **60**, 3156–3161.

van der Ley,P., van der Biezen,J., Hohenstein,P., Peeters,C. and Poolman,J.T. (1993) Use of transformation to construct antigenic hybrids of the class 1 outer membrane protein in *Neisseria meningitidis*. *Infect. Immun.*, **61**, 4217–4224.

van der Ley,P., van der Biezen,J. and Poolman,J.T. (1995) Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *porA* gene for use in the production of a multivalent outer membrane vesicle vaccine. *Vaccine*, **13**, 401–407.

Vieira,J. and Messing,J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene*, **19**, 259–268.

Voulhoux,R., Bos,M.P., Geurtsen,J., Mols,M. and Tommassen,J. (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science*, **299**, 262–265.