Editor’s Choice

Dissection of the function of the RmpM periplasmic protein from Neisseria meningitidis

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RmpM is a periplasmic protein from Neisseria meningitidis that comprises an N-terminal domain (residues 1–47) and a separate globular C-terminal domain (residues 65–219) responsible for binding to peptidoglycan. Here we show, through the use of size exclusion chromatography and pull-down assays, that a recombinant N-terminal fragment of RmpM binds to both the major outer membrane porins, PorA and PorB. Analysis by semi-native SDS-PAGE established that both recombinant full-length RmpM and an N-terminal fragment, but not the C-terminal peptidoglycan-binding domain, were sufficient to stabilize the PorA and PorB oligomeric complexes. Evidence from binding assays indicated that the meso-diaminopimelate moiety plays an important role in peptidoglycan recognition by RmpM. Site-directed mutagenesis showed that two highly conserved residues, Asp120 and Arg135, play an important role in peptidoglycan binding. The yield of outer membrane vesicles, which have been used extensively as a vaccine against N. meningitidis, was considerably higher in an N. meningitidis strain expressing a truncated N-terminal fragment of RmpM (ΔC-term rmpM) than in the WT strain. The native oligomeric state of the PorA/PorB complexes was maintained in this strain. We conclude that the dual functions of RmpM are independent, and that it is possible to use this knowledge to engineer a strain with higher yield of outer membrane vesicles, whilst preserving PorA and PorB, which are key protective antigens, in their native oligomeric state.

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Neisseria meningitidis is a fastidious, encapsulated, aerobic Gram-negative diplococcus. It colonizes the human nasopharynx and is carried asymptotically by 10–15 % of healthy people (Yazdankhah & Caugant, 2004). Occasionally a commensal strain from the nasopharynx penetrates the mucosal layer and invades the blood stream, causing invasive meningococcal disease. This results in an acute meningitis and rapidly progressive fulminant septicaemia. Several vaccines have been developed against meningococcal disease using outer membrane vesicles (OMVs), which were prepared by detergent-based extraction (Granoff, 2010). PorA is a porin protein in the outer membrane (OM), and a substantial body of work has established that it is a major protective antigen (Feavers & Pizza, 2009; Granoff, 2010). Unfortunately, PorA is subject to a high degree of antigenic variation, particularly within externally exposed regions, which severely limits the coverage of any OMV vaccine derived from a single strain. Attempts have been made to extend coverage, for example through the use of strains that express multiple PorA variants (Kaaijk et al., 2013).

PorA, and the structurally related porin PorB, constitute the most highly expressed outer membrane proteins (OMPs) found in the N. meningitidis OM (Frasch et al., 1985). The crystal structure of PorB shows the canonical 16-stranded transmembrane β-barrel structure characteristic of many bacterial porins (Tanabe et al., 2010). PorA is likely to form a similar fold, given its sequence homology with PorB (Derrick et al., 1999). In common with many other porins (Koebnik et al., 2000), PorA and PorB have the potential to form trimers, either as individual homotrimers or mixed PorA/PorB heterotrimers (Derrick et al., 1999; Minetti et al., 1997). The implications of trimer formation for antigen presentation are unclear, but it is possible that the conformational epitopes that induce bactericidal antibodies require formation of trimeric complexes.

RmpM is a periplasmic protein from N. meningitidis and is thought to provide a structural function in linking the peptidoglycan layer to the OM. It comprises two parts, a

Abbreviations: DAP, meso-diaminopimelate; LDAO, lauryldimethyl amine-N-oxide; OM, outer membrane; OMP, outer membrane protein; OMV, outer membrane vesicles; PGN, peptidoglycan; SEC, size exclusion chromatography.

Three supplementary figures are available with the online Supplementary Material.
short 47 residue N-terminal segment (RMn) and a 154 residue C-terminal domain (RMc), separated by an 18 residue proline-rich hinge region (Grizot & Buchanan, 2004). The RMC domain belongs to a family that shares sequence and structural similarity with the C-terminal domain of the OmpA protein from *Escherichia coli*, with a common peptidoglycan (PGN)-binding function. OmpA and many proteins included in this family, such as Pal and bacterial flagellum motor proteins like MotB (Roujeinikova, 2008), are also associated with the OM. Although they all share a common PGN-binding domain, other domains in these proteins differ: the Pal lipoprotein is anchored to the OM through an N-terminal lipid group, for example (Parsons et al., 2006). The flagellar MotB proteins, by contrast, play a role in anchoring the inner membrane to PGN, to immobilize the stator ring of the bacterial flagellar motor (De Mot & Vanderleyden, 1994; Roujeinikova, 2008). RMn is too short to form a transmembrane β-barrel in a similar manner to the N-terminal domain of *E. coli* OmpA, which forms an eight-stranded, 171 residue transmembrane β-barrel (Pautsch & Schulz, 2000). Unlike Pal, RmpM does not contain an N-terminal lipid group for anchoring to the OM. In some cases the protein contains a PGN-binding domain and a separate domain that interacts with porins in the OM, thereby anchoring the OM to PGN (Zeth et al., 2000). RmpM is therefore hypothesized to anchor the OM to the PGN layer in a similar manner (Grizot & Buchanan, 2004; Prinz & Tommassen, 2000). Studies on porin complexes isolated from meningococcal OMVs using Blue Native PAGE (Marzoa et al., 2009a), cross-linking of OM preparations (Sanchez et al., 2006), and semi-native SDS-PAGE (Jansen et al., 2000) have shown the presence of RmpM in hetero-oligomeric porin complexes containing PorA and PorB. These investigations were carried out using OM preparations derived from WT *N. meningitidis* expressing both PorA and PorB. It is not clear, however, if the presence of RmpM in the porin complexes is due to a specific interaction between RmpM and PorA or PorB, or some form of indirect interaction. Moreover, since PorA and PorB can form a heterotrimer, it could not be ascertained from these studies if RmpM binds to PorA, PorB or both. Here we dissect the functions of RmpM in greater detail and show how this information can be used to produce a strain of *N. meningitidis* with higher OMV yield but that retains PorA/PorB oligomeric assembly, and is therefore of potential value in vaccine production.

**METHODS**

**Cloning, expression and purification of RmpM and its fragments.** Coding sequences for the full-length RmpM, the N terminus (RMn) and the C terminus of RmpM (RMc) were amplified from meningococcal genomic DNA (H44/76, NIBSC strain no. 2851) using the primers listed in Table 1. The amplified full-length RmpM coding sequence and pET28a vector (Novagen) were digested with restriction enzymes Ncol and Xhol, purified and ligated to generate pET28a_RmpM. The RMc amplicon was initially cloned into the TA vector (Invitrogen), and digested, and the purified insert was ligated into Ncol and Xhol-treated pET22b +, to generate pET22b_RMc. The RMc amplicon was ligated at a ligation-independent cloning site (LIC) into pET30EkLIC (Novagen, Merck Biosciences) to generate pET30EkLIC_RMc. For expression, all recombinant plasmids were transformed into competent BL21 Origami E. coli cells (Novagen, Merck Biosciences). Two or three colonies were inoculated into 50 ml 2 × YT medium (Sigma Aldrich) containing appropriate antibiotics (50 μg ml⁻¹ ampicillin for pET22b_RMn, pET30EkLIC_RMc and 50 μg ml⁻¹ kanamycin for pET28a_RmpM). The cells were grown for 14 h at 37 °C to generate a ‘starter’ culture, which was then diluted into 2 l fresh 2 × YT medium, containing appropriate antibiotics, and the cells were grown to mid-exponential phase (OD₆₀₀ 0.8–1.0) at 37 °C. For expression of RmpM (pET28a_RmpM) and RMc (pET30EkLIC_RMc), the cells were induced by addition of IPTG to 0.1 mM and grown for 16 h at 18 °C. For RMn (pET22b_RMn) expression, the cells were induced with 1 mM IPTG for 4 h at 37 °C.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Host vector</th>
<th>Restriction site</th>
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</thead>
<tbody>
<tr>
<td>RmpM-Frd</td>
<td>GGCACGTGCTCATATGGGCGAGGCGGTCCG</td>
<td>pET28a</td>
<td>NdeI</td>
</tr>
<tr>
<td>RmpM-Rev</td>
<td>GCAGAATATTGCCTGAGTTAAGGTTTG</td>
<td>pET28a</td>
<td>Xhol</td>
</tr>
<tr>
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<td>pET22b +</td>
<td>Ncol</td>
</tr>
<tr>
<td>RMn-Rev</td>
<td>CTCAGAGCCCTGCTCCCAACAG</td>
<td>pET22b +</td>
<td>Xhol</td>
</tr>
<tr>
<td>RMc-Frd</td>
<td>GAGGACGACACAGATGAAATGATGATGAAACCATTCTCCCTG</td>
<td>pET32EkLIC</td>
<td>NA</td>
</tr>
<tr>
<td>RMc-Rev</td>
<td>GACAGAACACAGATGAAATGATGATGAAACCATTCTCCCTG</td>
<td>pET32EkLIC</td>
<td>NA</td>
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<tr>
<td>D120N RMc Frd</td>
<td>GCCGACGACACAGATGAAATGATGATGAAACCATTCTCCCTG</td>
<td>pET32EkLIC</td>
<td>NA</td>
</tr>
<tr>
<td>D120N RMc-Rev</td>
<td>GCCGACGACACAGATGAAATGATGATGAAACCATTCTCCCTG</td>
<td>pET32EkLIC</td>
<td>NA</td>
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<tr>
<td>R135K RMc Frd</td>
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<td>pET32EkLIC</td>
<td>NA</td>
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<tr>
<td>R135K RMc-Rev</td>
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<td>pUC19</td>
<td>Xmal</td>
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<td>pUC19</td>
<td>XbaI</td>
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<td>ATGCCGTCAGAATGTGTTCTCTGTATCTGTGCT</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Downstream R1</td>
<td>AAGCTTGGCTCAATGCCGCTAGACTGAC</td>
<td>NA</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

NA, Not applicable. *Underlined sequence denotes restriction site.
The induced cells were centrifuged and the pellet was resuspended in 50 ml Tris buffer (50 mM Tris/HCl pH 7.9, 200 mM NaCl) containing 0.3 mg DNase, 40 mg lysozyme (Sigma Aldrich) and 1 x complete protease inhibitor (EDTA-free; Roche). The resuspended cells were lysed by sonication (TT13/FZ; Bandelin Sonopuls HD3200) at 35 % amplitude for 4 min with pulses of 10 s on and 5 s off. The lysate was centrifuged for 30 min at 15 000 g and the supernatant was passed through a 0.45 μm filter (Merck Millipore) to remove debris. The filtered supernatant was added to 2 ml Ni-NTA resin (Qiagen), pre-equilibrated in 50 mM Tris/HCl pH 7.9, 200 mM NaCl; the suspension was incubated at 4 °C for 1 h. The resin was packed into an empty column (ThermoFisher) and washed with 6 ml wash buffer (50 mM Tris/HCl, 200 mM NaCl, 10 mM imidazole, pH 7.9). The protein was eluted with 6 ml elution buffer (50 mM Tris/HCl pH 7.9, 200 mM NaCl, 200 mM imidazole). The eluted fractions were concentrated to 2 ml and further purified using size exclusion chromatography (SEC; following section). Site-directed mutant RmpM proteins were prepared using the same protocol.

**SEC.** SEC was used for protein purification and for studying the interaction between PorA, PorB and RMn. Before injecting the sample, the column was pre-equilibrated with the appropriate buffer in which the protein was purified. A 0.5 ml aliquot of sample was injected and separated using either a Superdex 75 10/300 GL (GE Healthcare) SEC column. The flow rate was set at 0.5 ml min⁻¹ and 0.5 or 0.25 ml fractions were collected.

**SDS-PAGE.** For sample preparation for semi-native PAGE under denaturing conditions, the protein samples were incubated at 100 °C with loading buffer containing 1 % SDS with 5 % 2-mercaptoethanol. For sample preparation under non-denaturing conditions, the samples were mixed with loading buffer containing 0.1 % (w/v) SDS at 25 °C in the absence of a reducing agent. The samples were run on 12 % Novex Tris-Glycine or Bis-Tris precast gels (Life Technologies) using Tris-glycine or MOPS running buffer containing 1 % SDS.

**Production of OMVs.** *N. meningitidis* (H44/76 NIBSC strain no. 2851) from a frozen stock culture was inoculated onto a blood agar plate and incubated overnight at 37 °C in a 5 % CO₂ atmosphere. Colonies from the overnight plate were subcultured onto another blood agar plate and grown at 37 °C in a 5 % CO₂ atmosphere for 7 h. Colonies from this plate were inoculated into 50 ml Mueller–Hinton broth (Oxoid) and grown with orbital shaking at 150 r.p.m. overnight at 37 °C. The cells were harvested when the OD₆₀₀ reached 1.0. Bacterial cells were sedimented by centrifugation at 5500 g for 1 h at 4 °C.

Prior to extraction of OMVs released into the culture medium, a viable bacteria count of the culture at OD₆₀₀ was estimated by serial 10-fold dilutions, followed by inoculation into Mueller–Hinton blood agar plates (Oxoid) and overnight incubation at 37 °C in a CO₂ incubator. The colonies were counted and the total c.f.u. ml⁻¹ was calculated.

For extraction of OMVs secreted into the culture supernatant, the supernatant from the first, low-speed centrifugation step was ultra-centrifuged at 185 500 g at 4 °C for 2 h. The supernatant was discarded and the pellet resuspended in distilled water. The suspension was ultracentrifuged at 185 500 g at 4 °C for 2 h and the supernatant discarded. The washing step was repeated and, finally, the pellet was resuspended in 500 μl distilled water plus 0.02 % (w/v) sodium azide.

For production of native OMVs using EDTA, the supernatant from the first, low-speed centrifugation step was discarded, the pellet, containing bacterial cells, was resuspended in 5 ml Tris/EDTA buffer (50 mM Tris/HCl pH 7.9, 150 mM NaCl, 10 mM EDTA) and stirred for 30 min to extract the vesicles. The suspension was centrifuged at 19 900 g for 30 min at 4 °C, the supernatant retained, the pellet resuspended in 5 ml Tris/EDTA buffer and the extraction repeated. The supernatants were pooled and passed through a 0.22 μm filter. The filtrate was ultracentrifuged at 110 000 g at 4 °C for 2 h to sediment the OMVs. The supernatant was discarded and the OMV pellet resuspended in distilled water. The suspension was ultracentrifuged again at 110 000 g at 4 °C for 2 h and the supernatant discarded. This washing step was repeated once more. Finally, the OMV pellet was resuspended in 500 μl water plus 0.02 % (w/v) sodium azide.

**RmpM and RMn binding to PorA and PorB measured by SEC.** OMVs were solubilized in 50 mM Tris/HCl pH 7.9, 200 mM NaCl, 0.1 % lauryldimethylamine-N-oxide (LDAO) and centrifuged at 17 000 g to remove insoluble material. For interaction studies, 0.3 mg RmpM or 0.6 mg RMn was incubated with 0.5 ml solubilized ∆porB/∆RmpM OMV (5 mg ml⁻¹) or ∆porA/∆RmpM OMV (3 mg ml⁻¹) preparations. Following incubation, 0.5 ml was injected onto a Superdex 200 10/30 column (GE Healthcare), with 50 mM Tris/HCl pH 7.9, 200 mM NaCl, 0.1 % LDAO running buffer, at a flow rate of 0.5 ml min⁻¹.

**Isolation of *N. meningitidis* PGN.** *N. meningitidis* PGN was isolated using an adaptation of a previously published method (Glauer, 1988). *N. meningitidis* strain H44/76 was grown for 14 h in 1 ml Mueller–Hinton broth at 37 °C; cells were centrifuged at 6000 g and washed with 500 ml of 50 mM Tris/HCl pH 7.9, 200 mM NaCl. The washed pellet was resuspended in 25 ml of the same buffer and added drop-wise to 25 ml boiling 8 % (w/v) SDS in the same Tris buffer. The solution was incubated at 100 °C for 30 min, and left at 25 °C for 2 h to allow the suspension to cool. Subsequently, the suspension was ultracentrifuged at 185 000 g for 1 h. Following ultracentrifugation, the supernatant was discarded and the PGN-containing pellet washed four times with 50 mM Tris/HCl pH 7.9, 200 mM NaCl to remove the SDS. The PGN pellet was resuspended in 10 ml 50 mM Tris/HCl pH 7.9, 10 mM NaCl and 20 mM MgCl₂ by sonication for 1 h. The suspension was preheated to 60 °C, and added to the PGN mixture to 200 μg ml⁻¹, and the mixture was incubated at 60 °C for 2 h. The mixture was then added drop-wise to 8 % (w/v) SDS in 50 mM Tris/HCl pH 7.9, 200 mM NaCl and incubated at 100 °C for 30 min. After cooling, the PGN mixture was left at 25 °C for 2 h, ultracentrifuged at 185 000 g and washed as before. The final PGN pellet was resuspended in 4 ml 50 mM Tris/HCl pH 7.9, 200 mM NaCl and stored at −80 °C.

**PGN-binding assay.** The assay used is an adaptation of those described previously (Persson et al., 2007; Yao et al., 2012). Recombinant RMc or RMn protein (20 μg 0.5 mg ml⁻¹) was added to 50 μl purified PGN and incubated at 4 °C for 1 h, with gentle shaking. The suspension was centrifuged at 17 000 g for 15 min to separate soluble and insoluble fractions. The supernatant was removed and the pellet washed twice with 75 μl 50 mM Tris/HCl pH 7.9 and 200 mM NaCl. Finally, the pellet was resuspended in the same buffer, but with SDS added to 4 % (w/v), and incubated, with shaking, at 25 °C for 15 min.

**Magnetic beads pull-down assay.** The assay was performed using a Dynabeads Histag Isolation kit (Life Technologies). Briefly, 50 μl Dynabeads were sedimented with a magnet, the supernatant was aspirated and the beads were washed with 300 μl Tris buffer (20 mM Tris/HCl pH 7.9, 150 mM NaCl, 0.1 % LDAO). Fifty microlitres of either RMn or RMc at 0.25 mg ml⁻¹ was added, mixed and incubated on a roller at 6 °C for 20 min. The tube was placed on a magnet for 2 min and the supernatant was discarded. The pellet was washed four times with 300 μl wash buffer (20 mM Tris/HCl pH 7.9, 150 mM NaCl, 0.1 % LDAO, 5 mM imidazole) by placing the tube on a magnet for 2 min and removing the supernatant after each wash step. For the pull-down assay, 100 μl ∆porA/∆RmpM OMV
Site-directed mutagenesis. Site-directed mutagenesis was carried out using the QuikChange Mutagenesis kit (Agilent Technologies); the primers employed are listed in Table 1. Mutations were confirmed by DNA sequencing.

Construction of N. meningitidis mutants lacking rmpM and porA or porB. The strains NIBSC 3116 (ΔrmpM), NIBSC 3050 (Δpora) and NIBSC 3103 (ΔporB) were obtained from NIBSC strain collections, and were used for creating ΔrmpM/Δpora, ΔrmpM/ΔporB strains. These are isogenic mutant strains of H44/76 N. meningitidis (NIBSC strain no. 2851). The ΔrmpM mutant strain contains an erythromycin resistance cassette (ermC) in place of the rmpM gene. Both Δpora and ΔporB mutants possess a kapamycin resistance (kapB) cassette replacing porA and porB genes. In order to knock out the rmpM gene from the Δpora and ΔporB mutant strains, the strains were transformed using genomic DNA purified from the ΔrmpM mutant. Uptake of the DNA and subsequent homologous recombination led to the rmpM sequence being replaced with the ermC cassette. Consequently, a transformed strain will contain both ermC and kanR resistance genes. Successful transformants were selected by growing cells in media containing both erythromycin and kanamycin. To confirm the absence of the rmpM sequence and presence of erythromycin cassette in transformed clones, genomic DNA was isolated and sequenced.

Construction of N. meningitidis mutant strain (ΔC-term rmpM) expressing the N terminus of RmpM. The RmM sequence encoding residues 1–67 and a part of the sequence upstream of the RmM gene was amplified using the primers rmpM upstream F1 and N-term rev XbaI, and the insert cloned into the same sites in a pUC19/rampl vector. This recombinant plasmid had been previously constructed in our laboratory to insert a kanamycin cassette, a truncated rmpM gene and a part of the sequence downstream of rmpM. Thus the truncated rmpM sequence in the pUC19/rampl vector was replaced with the RmM sequence. The resulting construct was named pUC19/rampl/N-term. The complete cassette, including both RmM and kanR sequences, was then amplified from this plasmid using the primers rmpM DNA Frd (containing the neisseria DNA uptake sequence at the 5’ end) and Downstream R1 (Table 1). Subsequently, the PCR product was transformed into the H44/76 N. meningitidis strain (NIBSC strain no. 2851). Successful clones were verified by PCR and DNA sequencing and the strain named ΔC-term rmpM. Expression of the RmM fragment in the cell lysate was confirmed by MS (see below).

MS. Protein bands of interest were excised from the relevant SDS-PAGE gel and identified by peptide sequencing using LC-MS/MS. Gel slices were destained with 50 % acetonitrile in 50 mM ammonium bicarbonate until colourless, followed by repeated alternate washes of 100 % 50 mM ammonium bicarbonate, 50 % acetonitrile in 50 mM ammonium bicarbonate and 100 % acetonitrile. Each gel slice was then incubated with 0.1 % RapiGest (a cleavable detergent to fully denature the protein complex in the gel; Waters) at 37 °C for 10 min. Gel slices were then dehydrated using acetonitrile, before in-gel digestion with trypsin overnight at room temperature. Peptides were extracted using ultrasound in a sonic bath with sequential steps of 1 % trifluoroacetic acid (once), 50 % acetonitrile in 0.2 % trifluoroacetic acid (twice) and 100 % acetonitrile (once). The pooled extracts were dried in a vacuum centrifuge and resuspended in 100 μl 5 % acetonitrile plus 0.1 % formic acid.

MS analysis was performed using an LTQ-Orbitrap Discovery mass spectrometer, coupled with an Ultimate 3000 nano-LC system (Thermo Fisher Scientific). The digests were separated in a PepMap C18 reversed-phase nano-column (3 μm, 100 Å, 15 cm length) under a column flow rate of 0.3 μl min⁻¹ using a linear gradient from 5 to 70 % of 95 % acetonitrile and 0.1 % formic acid for 180 min. MS scan and MS-MS fragmentation were carried out in Orbitrap and LTQ, respectively, using top 5 data-dependent acquisition with dynamic exclusion mode enabled. The total cycle time was approximately 30 ms. Data analysis, including MS processing and database searching, was carried out using Thermo Proteome Discoverer 1.4 with built-in Sequest, using the UniProt FASTA database containing the N. meningitidis MC58 and H44/76 strain genome sequences. Initial mass tolerances for MS were set to 10 p.p.m. Up to two missed tryptic cleavages were considered and methionine oxidation was set as dynamic modification. Peptides at rank 1 with high confidence are considered to be unambiguously sequenced.

RESULTS

Binding of RmpM and RMn to PorA and PorB: examination by SEC

Previous work has demonstrated the presence of native RmpM within hetero-oligomeric porin complexes containing PorA and PorB (Jansen et al., 2000; Marzoa et al., 2009a; Sánchez et al., 2006). We wanted to investigate whether we could reproduce the binding of recombinant RmpM to either porin; accordingly, an expression construct was generated for full-length RmpM (see Methods). To examine the role of the N- and C-terminal domains of RmpM in porin binding, two other recombinant proteins were also generated; the first (termed RMn), for the RmpM N terminus, comprised the first 63 residues, and was expressed and purified in a similar way. The second (termed RMc) covered residues 65 to 219 in RmpM, and covered the C-terminal region, which is predicted to bind to peptidoglycan. All three proteins were expressed and purified to homogeneity, and their identities were confirmed by MS (Fig. S1, available in the online Supplementary Material).

The binding of purified RmpM, RMn and RMc to LDAO-solubilized OMV preparations derived from ΔporA/ΔrmpM and ΔporB/ΔrmpM strains, in which the major porin was either PorB or PorA, respectively, was examined. Following incubation of RmpM or RMn with each solubilized OMV preparation, the components were separated by SEC. PorA and PorB eluted at approximately 10 ml from the SEC column (Figs 1a and 2a); in each case, the identities of PorA and PorB were confirmed by MALDI-TOF MS. Other major protein components of OMVs were also identified by MS, including Omp85, at ~85 kDa, and NspA, at ~17 kDa. Recombinant RmpM or RMn co-eluted with either PorA or PorB, consistent with specific recognition (Figs 1b, c and 2b, c). RmpM alone eluted from

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the SEC column at 15 ml and had an apparent mass of ~26 kDa, suggesting that it forms a monomer under these conditions (Fig. 1e). Similarly, RMn alone eluted at 18 ml, reflecting its lower molecular mass of ~8 kDa (Fig. 1f). It should be noted that RmpM and RMn migrate at higher apparent masses on SDS-PAGE, a feature that may be connected with the relatively high proline content in the N-terminal region of RmpM. Some dissociation of

**Fig. 1.** Binding of RmpM, RMc and RMn to PorA measured by SEC. OMVs from the ΔporB/ΔrmpM (a) strain were solubilized in LDAO and incubated with either recombinant RmpM (b), RMn (c) or RMc (d). Gels (e) and (f) show RmpM and RMn alone, respectively. Samples were injected onto a Superdex 200 10/30 SEC column (GE Healthcare), and eluted fractions analysed by SDS-PAGE. Fractions (0.25 ml) were collected, starting at lane 1 (8 ml elution volume). The migration positions of molecular mass standards are indicated on the left of each gel. The identities of individual protein bands, indicated by roman capital numbers, were examined by MS. Major components were identified as follows: I, Omp85; II, PorA; III, RmpM; IV, NspA; V, RMn (RmpM).
both RmpM and RMn from either porin seems to occur during passage down the SEC column, probably reflecting a significant off-rate of either protein from its complex. The potential for binding of RMc, the peptidoglycan-binding domain, was also analysed; in both cases no bands for RMc were observed co-eluting with either PorA or PorB in SDS-PAGE, indicating that RMc failed to bind (Figs 1d and 2d).

Examination of the binding of RMn and RMc to PorA and PorB by magnetic bead pull-down assay

We sought to verify the observation that the N-terminal domain of RmpM was responsible for binding to PorA and PorB. Preparations of purified recombinant RMn or the C-terminal domain, RMc, were coupled to magnetic beads via their hexahistidine tags and incubated with LDAO-solubilized OMV preparations from the ΔporA/ΔrmpM or ΔporB/ΔrmpM strains. Unbound material from the supernatant was recovered; the beads were washed twice before magnetic field capture and recovery of bound protein. Protein present in the supernatant, the two wash fractions and the pellet was analysed by SDS-PAGE (Fig. 3). PorA and PorB fail to bind to immobilized RMc, as they are identified in the supernatant but not in any of the other fractions (Fig. 3a). Binding was detected, however, of both porins to beads coated with recombinant RMn; recovery of bound PorA appeared to be better than that of PorB, but both were detected in the pellet fraction (Fig. 3b).

The N terminus of RmpM stabilizes hetero-oligomeric PorA/B complexes

The previous two experiments provided evidence that recombinant RmpM and RMn bind to PorA and PorB. These porin proteins form mixed trimers which can be detected using semi-native SDS-PAGE of OM preparations (Jansen et al., 2000). We therefore proceeded to examine whether recombinant RmpM and its fragments could influence the oligomeric state of PorA/B using this method. OMV samples were assayed in either a native or denatured form; the latter were generated by heat treatment in SDS. Denatured PorA and PorB migrated at approximately 40 and 34 kDa, respectively (Fig. 4,

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**Fig. 2.** Binding of RmpM, RMc and RMn to PorB measured by SEC. OMVs from the ΔporA/ΔrmpM (a) strain were solubilized in LDAO and incubated with either recombinant RmpM (b), RMn (c) or RMc (d). Identities of major protein bands [indicated by arrows (I), (II) and (III)] were verified by MS analysis. Major components identified by MS were as follows: I, PorB; II, RmpM; III, RMn (RmpM). Other details are as for Fig. 1.
lane 1). Under native conditions, however, PorA/B formed a hetero-oligomeric complex with RmpM, which migrated more slowly, at a position equivalent to 110 kDa (Fig. 4, lane 2). The identity of PorA, PorB and RmpM in this complex, formed from OMVs from a WT strain, was confirmed by MS. In the equivalent experiment carried out on OMVs from a \( \Delta rmpM \) strain, this high molecular mass complex was formed to a much lesser extent (Fig. 4, lanes 3 and 4). This observation suggests that RmpM is required for maintaining the hetero-oligomeric complex. Given that the previous experiments had established that it is the N terminus of RmpM that appears to bind to each porin, we tested this hypothesis by addition of recombinant RMn: most, although not all, PorA and PorB was incorporated into the hetero-oligomeric complex (Fig. 4, lanes 5 and 6). RMn co-migrated with another OmpA, NspA: the band at 16 kDa in lane 1 was confirmed as NspA by MS analysis. On addition of RMn, this band was enhanced (lanes 5 and 6), and the presence of both proteins was confirmed by MS. Critically, the hetero-oligomeric complex also formed in a \( \Delta C\text{-term } rmpM \) strain, where the presence of PorA, PorB and peptides from the remaining RmpM N-terminal fragment were confirmed by MS (lanes 7 and 8).

### Influence of RmpM on OMV yield

The observation that the PorA/B hetero-oligomeric complex is preserved in the \( \Delta C\text{-term } rmpM \) mutant suggested that this strain might have an advantage for OMV-based vaccine production. Deletion of \( rmpM \) is known to increase OMV recovery (van de Waterbeemd et al., 2010). It is possible, therefore, that the \( \Delta C\text{-term} \) \( rmpM \) mutant shows a similarly high recovery of OMVs, but also retains stability of the PorA/B hetero-oligomeric complex. OMV recovery from all three strains was therefore quantified. Bacterial cultures for the three strains were grown overnight and viable bacterial counts were found to be approximately equivalent at \( 10^{10} \) c.f.u. ml\(^{-1}\). The OMVs released into the culture medium supernatant were extracted and the OMV total protein content was determined. Compared with WT, the \( \Delta rmpM \) and \( \Delta C\text{-term } rmpM \) showed more than three times higher OMV yield (Fig. 5).

### Recognition of PGN by RmpM

Earlier work, in the form of a pull-down assay, demonstrated that \( N.\ meningitidis \) RmpM binds to PGN (Grizot & Buchanan, 2004). To explore this recognition in more detail, a similar binding assay was carried out, in which recombinant RmpM, RMc or RMn were added to insoluble PGN that had been purified from \( N.\ meningitidis \). Soluble and insoluble fractions were separated by centrifugation, and the supernatant, wash and pellet fractions were subjected to SDS-PAGE analysis. The results showed that RmpM and RMc associated exclusively with the \( N.\ meningitidis \) PGN pellet, whereas RMn failed to bind and remained in the supernatant fraction (Fig. 6a).
Fig. 4. Semi-native SDS-PAGE of OMV preparations. The solubilized OMV preparations from WT, ΔrmpM and ΔC-term rmpM strains were run on semi-native SDS-PAGE, under both denaturing (d) and non-denaturing (n) conditions. The migration positions of molecular mass standards are indicated. The individual protein bands, indicated by roman capital numbers, were examined by MS. Major components were identified as follows: I, PorA, PorB, Omp85 and RmpM; II, PorA, PorB and Omp85; III, PorA, PorB, Omp85 and RmpM; IV, PorA, PorB, Omp85 and RmpM; V, NspA; VI, NspA and RmpM.

Fig. 5. Comparison of amount of OMV produced by WT and mutant N. meningitidis strains. The yield of native OMVs formed in 100 ml (10^{12} cells) culture medium supernatant is shown, for WT, ΔrmpM and ΔC-term rmpM strains. Results are means of duplicate experiments. Error bars represent SDs from duplicate experiments.
OmpA-like proteins are found exclusively in bacteria with *meso*-diaminopimelate (DAP)-type PGN (De Mot & Van-derleyden, 1994), which implies a degree of specificity for DAP. To examine the DAP-specific binding of RmpM, pull-down assays were performed using DAP-type PGN from *Bacillus subtilis* and *E. coli*, and the lysine-type PGN from *Staphylococcus aureus* (Fig. 6b). RmpM binds to *E. coli* PGN with high affinity, but with less avidity to PGN from *B. subtilis*, where it is distributed between the supernatant and pellet fractions. There was no measurable affinity of RmpM for the lysine-type PGN from *S. aureus*, suggesting that DAP plays an important role in recognition of PGN. Soluble *E. coli* PGN was unable to compete effectively for binding of RmpM to insoluble *N. meningitidis* PGN (not shown). This observation implies that other components of the *N. meningitidis* PGN structure, in addition to DAP, are important for conferring higher binding affinity.

We proceeded to test DAP recognition by RmpM further using site-directed mutagenesis. Multiple amino acid sequence alignment showed that the residues D120 and R135 are well conserved among the OmpA-like proteins (Fig. S2). The equivalent residues in OmpA-like domains in *Acinetobacter baumannii* and *Mycobacterium tuberculosis* have been shown to recognize DAP; mutation of these amino acids resulted in loss of binding to DAP (Park *et al.*, 2012; Yao *et al.*, 2012). Binding of D120N and R135K RMc to *N. meningitidis* PGN was significantly reduced, but not eliminated, relative to WT (Fig. S3a). Binding to *B. subtilis* PGN, which was only partial by this assay for RMc, was eliminated by both the D120N and R135K RMc mutations (Fig. S3b).

**DISCUSSION**

The experiments reported above have extended our current knowledge of the function of *N. meningitidis* RmpM. Separation of the functions into the N- and C-terminal portions of the protein has shown that the N-terminal domain is responsible for binding to both PorA and PorB. This is consistent with previous studies that reported complexes of RmpM with PorA/B in OM preparations studied using semi-native SDS-PAGE or high resolution clear native PAGE (Jansen *et al.*, 2000; Marzoa *et al.*, 2009, 2010). In a crystal structure of PorB, purified from an OM preparation of *Neisseria gonorrhoeae*, electron density was attributed to a bound peptide from the N terminus of RmpG, a homologue of RmpM (Zeth *et al.*, 2013). However, the resolution of the PorB–RmpG peptide complex was not sufficient to trace the side-chains of the peptide and therefore it was modelled as a polyalanine sequence. The RmpG peptide binds on the periplasmic side of PorB, around the three-fold noncrystallographic symmetry axis (Fig. 7). The stoichiometry of binding is unclear, i.e. whether it forms a 1 : 1 or 1 : 3 RmpG : PorB ratio. A 1 : 3 ratio would explain the role of RmpM in stabilizing trimer formation: a single RmpG or RmpM chain could bind to three porin monomers simultaneously, thus promoting stability of the trimer. The RmpG peptide makes contacts with residues 2 to 4 in PorB, which form part of the first β-strand in the barrel. This sequence, VTL, is VSL in PorA, and well conserved; it is therefore plausible that the RmpM interaction is preserved in the complex with PorA. This would also provide an explanation for how RmpM binds to mixed heterotrimers of PorA and PorB and stabilizes the heterotrimeric complex.

The observation that the N-terminal region of RmpM was sufficient to stabilize the trimeric PorA/PorB complexes suggested that this function might be preserved in the ΔC-term *rmpM* mutant where the C terminus was deleted. Semi-native SDS-PAGE of OMV preparations from the ΔC-term *rmpM* *N. meningitidis* strain demonstrated that stable oligomeric PorA/PorB complexes were formed in a similar fashion to those from the WT strain. The yield of supernatant OMVs was increased in the ΔrmpM *N. meningitidis* strain as compared with WT strain, confirming earlier
reports that deletion of rmpM generates a phenotype in which OMV production proliferates (van de Waterbeemd et al., 2010). In addition, the OMV yield from the ΔC-term rmpM strain was further increased above the ΔrmpM strain.

There is evidence that RmpM interacts with iron-regulated and other OMPs. Prinz & Tommassen (2000) demonstrated that the transferrin and lactoferrin-binding proteins TbpA and LpbA migrated with a molecular mass of ~170 kDa on non-denaturing SDS-PAGE gels. Similarly, the iron transporter FrpB (also known as FetA) was detected as a 170 kDa complex. Comparison of these complexes in OMV preparations from an rmpM mutant showed increased mobility, indicative of a loss of mass due to removal of RmpM. Significantly, the proportion of each OM that migrated as denatured monomers increased in the rmpM mutant in each case, an observation that parallels those made in Fig. 4. Subsequent determination of the crystal structure of the TbpA–transferrin complex indicated that it is monomeric, however (Noinaj et al., 2012). Crystal forms of FrpB/FetA have been reported in both trimeric and monomeric states (Saleem et al., 2013). The way in which RmpM stabilizes oligomers of this group of iron-regulated OMPs is unclear at present. In addition, there is evidence that RmpM associates with the Omp85 complex, although it is unclear whether this is attributable to a direct interaction between the two proteins (Volokhina et al., 2009).

Here, we have shown that recombinant RmpM was able to bind to PGNs from N. meningitidis, E. coli and B. subtilis, which contain DAP, and was found to not bind to the lysine-containing PGN from S. aureus. We infer from this observation that the DAP component plays an important role in PGN recognition by RmpM. Park et al. (2012) reported the crystal structure of an OmpA-like domain from A. baumannii bound to DAP; the ligand binds in a crevice on the protein surface. Comparison with the structure of RMc suggests that the site is more open (Fig. 8a), although it should be noted that the neisserial protein was crystallized without a ligand in this binding site and structural changes could occur on complex formation. Recognition of DAP is mediated by a charged interaction between a well conserved Arg residue (number 135 in RmpM) and one of the two carboxylates in DAP. We have shown that mutation of Arg135, and also Asp120, which also lies close to the proposed DAP-binding site in RmpM, considerably reduces PGN binding (Figs 8b and S2). The amidation of this carboxylate group in B. subtilis (Atrihi et al., 1999) is probably responsible for reduced affinity of RmpM for B. subtilis PGN. These observations all point to the conclusion that N. meningitidis...
RmpM recognizes DAP in a similar manner to the OmpA-like protein from *A. baumannii*. It is likely, however, that the PGN-binding site extends further than the immediate, predicted, contact site with DAP, to a wider surface. Mapping sequence conservation of RmpM onto a molecular surface demonstrates a wider extent of conserved residues (Fig. 8c), which would explain the higher affinity of RmpM for insoluble, more complex PGN (Fig. 6).

We have established that the two binding functions of RmpM – to porins and PGN – are separable. The current model for RmpM function is that it stabilizes porin oligomers, although it is not clear why this is important, nor why a link between porin stabilization and PGN binding is required. Here the observation that OMV production is greater in a ΔrmpM mutant might be instructive. There may be some value in OMV production, in contributing to colonization, for example (Unal et al., 2011). However, excessive OMV production could lead to overproduction of endotoxin, with deleterious consequences for the bacterium, at least in a commensal state. In *E. coli*, the structural role of OmpA has been shown to have implications for its function *in vivo* (Wang & Kim, 2002). A newly identified OmpA-like protein from *N. gonorrhoeae* has been shown to bind human epithelial cells and facilitate *in vivo* colonization (Serino et al., 2007). The prime function of RmpM may therefore lie in regulating OMV release, which could have implications for the immunological response to *N. meningitidis* during carriage and disease.

Our observation that RmpM maintains stable PorA/B oligomeric complexes suggests that it could function by ensuring proper assembly of these porins. This would suggest that RmpM helps to maintain functional porin trimers and therefore that mutation of RmpM would result in a phenotype of reduced PorA or PorB function. However, the laboratory cultures do not show any obvious difference in growth rate between WT and ΔrmpM strains (Klagman et al., 1989). Use of highly enriched culture media may account for this lack of visible difference in laboratory culture. The effects of impaired porin function on growth may be more readily manifest under nutrient-deprived conditions *in vivo*.

The maintenance of the PorA trimer could affect the presentation of antigens from the two hypervariable loop regions (VRs) in the porin (Derrick et al., 1999; van der Ley et al., 1991). Modelling, based on the PorB crystal structures (Tanabe et al., 2010; Zeth et al., 2013), suggests that the VRs from adjacent monomer chains lie close in space in the assembled trimer. The effect of RmpM mutation may therefore be more subtle, through indirectly influencing antigen presentation by PorA.

We have shown that a *N. meningitidis* strain that only expresses the N-terminal portion of RmpM shows increased vesiculation, but also maintains stable PorA/PorB complexes. This observation suggests that such a mutation could be employed in the development of vaccines based on naturally secreted OMVs. This could therefore provide a viable alternative to detergent extraction, cysteine depletion and associated oxidative stress (van de Waterbeemd et al., 2013) for increasing OMV release for vaccine production.

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**References**


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