Isolation of Neisseria meningitidis Mutants Deficient in Class 1 (PorA) and Class 3 (PorB) Outer Membrane Proteins

JAN TOMMASSEN,1,2* PAUL VERMEIL,1 MARLIES STRUYVÉ,1 ROLAND BENZ,3 AND JAN T. POOLMAN4

Department of Molecular Cell Biology1 and Institute of Molecular Biology and Medical Biotechnology,2 State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands; Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-7750 Würzburg, Federal Republic of Germany; and National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven, The Netherlands

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The class 1 major outer membrane protein of Neisseria meningitidis is a serious candidate for a meningococcal vaccine. To facilitate studies on the function of this protein, mutants were isolated that lacked this protein or the structurally related class 3 protein. These mutants were obtained by using the antibody-dependent bactericidal action of the complement system. The class 1 protein-deficient strain grew normally in vitro, whereas growth of the class 3 protein-deficient strain was slightly retarded. The class 3 protein-deficient strain displayed increased resistance to the antibiotics tetracycline and cefsulodin, which is consistent with the proposed role of the protein as a pore-forming protein. The class 1 protein was purified to homogeneity from the class 3 protein-deficient strain. Lipid bilayer experiments revealed that this protein also formed pores. The class 1 protein pores were cation selective.

Disease caused by Neisseria meningitidis is a public health problem because of its epidemic nature and high mortality rate. A vaccine based on purified capsular polysaccharides from meningococcal serogroups A, C, Y, and W135 provides protection against these serogroups in adults but not in very young children (14, 23). In contrast, the capsular polysaccharide of group B meningococcus is poorly immunogenic even in adults (34), probably because it cross-reacts with polysialic antigens present in human tissues during embryonal development (10). Therefore, the use of outer membrane proteins (OMPs) as vaccine components is currently being evaluated (7, 11, 12, 36).

Meningococci contain four classes of major OMPs, i.e., class 1, 2/3, 4, and 5 proteins. The class 5 OMPs appear to be unattractive vaccine candidates, since they exhibit extremely weak antigenic heterogeneity (25). Also the class 4 OMP seems to be unsuitable. This protein is related to protein III of Neisseria gonorrhoeae (18). This protein III induces antibodies capable of blocking the bactericidal activity of human immune serum against serum-resistant gonococci (28). All meningococci contain either a class 2 or a class 3 OMP. These proteins are probably the equivalents of the gonococcal protein I, which forms anion-selective pores in the outer membrane through which small hydrophilic solutes can pass in a diffusionlike process (8, 17, 20). The function of the class 1 OMP is unknown. The structural gene for this protein has recently been cloned and sequenced (3), and it appears that the protein is structurally related to the gonococcal porins and to class 2 OMP (20). Therefore, also the class 1 OMP may have a pore function. Monoclonal antibodies have been raised against the class 1 and the class 2/3 OMPs; in both cases, antibodies with bactericidal activity have been obtained. However, in an animal model system, only the bactericidal antibodies against the class 1 OMP were highly protective against bacterial challenge (29), making this protein the prime vaccine candidate for meningococcus group B.

To construct an efficient and broadly protective vaccine, nonprotective antigens should be removed from OMP preparations. However, class 1 and class 2/3 OMPs are hard to separate (27). A possible solution to this problem would be the isolation of meningococcal strains lacking the class 2/3 OMP. To this end, and to study the function of the class 1 OMP, we isolated mutant strains lacking these major OMPs.

MATERIALS AND METHODS

Bacteria, mutant isolation, and growth conditions. N. meningitidis H44/76 was a generous gift of E. Holten, Norway. The strain was serosubtyped as B:15:P1.16 (13). Mutants from this strain that lack the class 1 or class 3 OMP were isolated by using the antibody-dependent bactericidal activity of the complement system as described previously (32, 33), except that guinea pig instead of rat complement was used. In these experiments, we used the monoclonal antibodies Mn5C11G and Mn15A14H6, which recognize cell-surface-exposed epitopes of class 1 and class 3 OMPs, respectively (24). Routinely, to 90 µl of cells in Veronal-buffered saline (optical density at 660 nm [OD660], 0.5) were added 10 µl of ascites fluid diluted 1:20 in Veronal-buffered saline and guinea pig serum to a final concentration of 25%. After incubation for 30 min at 37°C, additional guinea pig serum was added to a final concentration of 50%. After another 30 min, cells were plated. To increase the bactericidal activity in case of the class 3 OMP-specific antibody Mn15A14H6, the ascites fluid was only diluted 1:10, and the final concentrations of guinea-pig serum during the subsequent incubations were increased to 40 and 80%, respectively.

Cells were grown at 37°C with shaking in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) or, where indicated, in synthetic NDM medium as described previously (2). For plating, the medium was solidified with 1.5% (wt/vol) agar (BBL). Plates were incubated overnight in a candle jar at 37°C.

Growth curves and sensitivity to antibiotics. Growth curves were determined by diluting overnight cultures 1:20 in fresh
medium. During subsequent incubation at 37°C, growth, represented by an increase in turbidity, was measured in a Klett-Summerson photometer at 660 nm.

Sensitivity of strains to antibiotics was determined by diluting overnight cultures 1:20 in fresh medium to which different concentrations of antibiotics were added. After overnight growth, the OD₆₆₀ of the cultures was determined.

**Isolation and characterization of outer membrane complexes.** Outer membrane complexes were isolated from meningococci with the LiCl-EDTA method described previously (25). Protein profiles of the outer membrane complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15) and Western immunoblotting (1).

**Enzyme-linked immunosorbent assay.** After inactivation of bacteria for 30 min at 56°C, enzyme-linked immunosorbent assays with whole cells as the immobilized antigen were performed (31).

**Purification of class 1 OMP.** Class 1 protein was purified from the class 3 protein-deficient strain CE2001 as described previously (27). CaCl₂-Zwittergent 3-14 extraction followed by diafiltration and DEAE-Sepharose and Sephacryl S300 chromatography, all as described previously (27), enabled us to purify class 1 OMP to near homogeneity, with approximately 5% (wt/wt) lipopolysaccharide in the final preparation as determined by 2-keto-3-deoxyoctulosonic acid analysis.

**Lipid bilayer experiments.** The methods used for black lipid bilayer experiments have been described previously (4). The apparatus consisted of a Teflon chamber with two aqueous compartments connected by a circular hole with a surface area of 0.1 mm² for single-channel experiments or 1 mm² for selectivity measurements. The membranes were formed from a 1% (wt/vol) solution of diphytanyl glycerophosphocholine (Avanti Biochemicals, Birmingham, Ala.) in n-decane across the holes. After membrane formation, class 1 OMP was added from the concentrated stock solution to the aqueous phase consisting of 1 M KCl. The current through the membranes was measured after application of a transmembrane potential of 10 mV with two calomel electrodes switched in series with a voltage source and a current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart. Zero-current membrane potentials were the result of a 2.6-fold KCl gradient (50 versus 130 mM) across a membrane in which between 100 and 500 pores were reconstituted (5). The membrane potential, which reached its steady-state value within 5 to 10 min, was measured with a Keithley 610 C electrometer.

**RESULTS**

**Selection of mutants lacking class 1 or class 3 OMP.** To select meningococcal mutants that lack class 1 OMP, cells of strain H44/76 were incubated with the class 1 protein-specific monoclonal antibody Mn5C11G and complement as described in Materials and Methods. The incubation resulted in an over 10,000-fold reduction in the number of viable (colony-forming) cells. In control experiments, where the monoclonal antibody was omitted during the incubations, only a 10-fold reduction in the number of viable cells was observed. Thus, the observed bactericidal activity is largely dependent on the presence of the monoclonal antibody. Colonies of surviving cells were subcultured and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western immunoblotting, and whole-cell enzyme-linked immunosorbent assays. Out of 60 colonies tested, 11 contained reduced amounts of class 1 protein or did not produce this protein at all. One of these strains, designated CE2001, was characterized in detail. This strain did not bind Mn5C11G in whole-cell enzyme-linked immunosorbent assays. When protein profiles of whole cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western immunoblotting, no band reacting with Mn5C11G was observed (data not shown). The protein profile of the outer membrane complexes of this strain is shown in Fig. 1, lane 2.

When monoclonal antibody Mn15A14H6 was used in a similar procedure, no class 3 protein-deficient mutants were obtained. However, by slightly modifying the procedure (as described in Materials and Methods), the bactericidal activity could be increased. The modified procedure resulted in a 10⁶-fold reduction in the number of viable cells. Sixty colonies of surviving cells were further analyzed. Two of them, designated CE2002 and CE2003, did not reveal any class 3 OMP when whole cells or outer membrane complexes (Fig. 1, lanes 3 and 4) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These two strains did not bind Mn15A14H6 in whole-cell enzyme-linked immunosorbent assays. Interestingly, class 4 OMP, which was already a weak band in the parent strain (Fig. 1, lane 1), was not detected in the OMP profiles of the two mutants (Fig. 1, lanes 3 and 4). However, in Western immunoblotting experiments class 4 OMP could still be detected, showing that expression of this protein is not completely abolished in the mutants (data not shown).

**Characterization of the OMP mutants.** The growth curves of the parent and mutant strains were determined in Trypticase soy broth and in synthetic NDM medium. In both
media, the growth curves of H44/76 and of the class 1 protein-deficient mutant CE2001 were indistinguishable, whereas growth of strain CE2002, which lacked class 3 OMP, was slightly retarded. In spite of this slight growth defect, reversion of the porB mutation during culture was never observed. After overnight incubation all strains reached similar final ODs (data not shown).

Hydrophilic antibiotics can pass the permeability barrier of the outer membrane via water-filled channels constituted by pore-forming proteins. Thus, mutants that lack one or more of these pore-forming proteins may exhibit increased resistance to these antibiotics. Consistent with the proposed role of class 3 OMP as a pore-forming protein (8), cells lacking this protein were more resistant than the parent strain to the antibiotics tetracycline and cefsulodin (Table 1). No differences in sensitivity to chloramphenicol and cephaloridine were observed (data not shown), suggesting that these antibiotics use a pathway other than that formed by the class 3 OMP to permeate the outer membrane. No significant differences were observed in the sensitivity of the class 1 OMP-less mutant CE2001 and the parent strain to tetracycline and cefsulodin (Table 1) and to chloramphenicol and cephaloridine (data not shown). Thus, from these experiments no evidence for pore-forming activity of the class 1 protein could be obtained.

Purification of class 1 OMP. The purification of the class 1 OMP is in general a difficult task, because this protein is difficult to separate from class 2/3 OMP (27). The absence of the class 3 OMP and the reduced amounts of class 4 OMP in the outer membrane complexes of strain CE2002 (Fig. 1, lane 3) make this strain very suited for the purification of class 1 OMP. The methodology of CaCl₂-Zwittergent 3-14 extraction, diafiltration, DEAE-Sepharose, and S300 Sepharcl chromatography allows for the purification of the meningococcal OMPs directly from whole cells. In strain CE2002, this procedure resulted in a pure class 1 OMP preparation. A limited amount of lipopolysaccharide (approximately 5% on a weight basis) was present in the preparation as revealed by 2-keto-3-deoxyoctulosonic acid analysis.

Demonstration of pore function of class 1 OMP in vitro. To study the possible pore function of class 1 OMP, lipid bilayer experiments were performed. The addition of small amounts of purified class 1 OMP to membranes after application of a transmembrane potential of 10 mV resulted in step increases in membrane conductance. These steps indicated that the class 1 OMP formed defined structures similar to the OmpF, OmpC, and PhoE porins of Escherichia coli K-12 (6). The step increases of conductance were assumed to represent the incorporation of one conductive unit. The single-channel record demonstrated that the conductance fluctuations were directed exclusively upward. No closing events were observed, which indicates that the lifetime of the pores was long, usually exceeding 10 min. Even when the transmembrane potential was increased up to 100 mV, no closing events were observed. This shows that the class 1 OMP pores are not voltage sensitive at physiological potentials, in contrast to the results obtained with gonococcal porin (19).

Figure 2 shows a histogram of the conductance increments measured at a transmembrane potential of 10 mV. The average single-channel conductance was 1.8 nS. For comparison, the average single-channel conductances of the E. coli K-12 pores OmpF, OmpC, and PhoE, measured under identical conditions, have been reported to be 1.9, 1.5, and 1.8 nS, respectively (6).

The selectivity of the pores was measured by zero-current membrane potential measurements in the presence of KCl salt gradients. KCl was chosen in these experiments because the cation and the anion have approximately equal mobility in the aqueous phase (9). The potential was always positive on the more dilute side of the membrane. These results indicate a preferential movement of the cations over the anions through the pores. The zero-current membrane potential for the class 1 protein pores in the presence of a 2.6-fold KCl gradient was 16 mV. The ratio of the cation permeability to anion permeability, calculated from the Goldman-Hodgkin-Katz equation (5), was 6.4. Thus, potassium ions have a six- to sevenfold higher permeability through the class 1 protein pores than chloride ions, despite the same mobility of the ions in the aqueous phase. For comparison, cation/anion permeability ratios of KCl for the OmpF, OmpC, and PhoE porins have been reported to be 3.6, 26, and 0.3, respectively (6).

**DISCUSSION**

The meningococcal class 2/3 OMP has been proposed to be the analog of the gonococcal protein 1, which forms anion-selective pores in the outer membrane (8, 17). Here, we describe the isolation of mutants lacking this protein. As discussed by Nikaido and Vaara (21), the best way to

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**TABLE 1. Sensitivity of meningococcal strains to antibiotics**

<table>
<thead>
<tr>
<th>Drug concn (µg/ml)</th>
<th>Tetracycline</th>
<th>Cefsulodin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>H44/76</td>
<td>CE2001</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
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<td>13</td>
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<tr>
<td>0.2</td>
<td>11</td>
<td>11</td>
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<tr>
<td>0.3</td>
<td>13</td>
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<tr>
<td>0.4</td>
<td>13</td>
<td>11</td>
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<tr>
<td>0.5</td>
<td>11</td>
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</tbody>
</table>

* The OD₅₆₀ of cultures, grown overnight in the presence of antibiotics at the indicated concentrations, were measured. The ODs of the control cultures, grown in the absence of antibiotics, were set at 100%, and the relative ODs of the other cultures are given in percentages. The data represent the averages of two independent experiments.
characterize pore function in intact cells is to measure the permeation of β-lactam antibiotics as described by Zimmer-
man and Rosselet (35). However, for this assay a β-lacta-
mase has to be present in the periplasm, and this is not the
case in meningococci. Since meningococcal disease can be
treated efficiently with β-lactams, we did not want to create a
β-lactamase-producing meningococcal strain. Neverthe-
less, the observation that the class 3 OMP-deficient strain
exhibited increased resistance to tetracycline and cefsulodin
is consistent with the proposed pore function. On the other
hand, the observation that this strain is as sensitive to
chloramphenicol and cephaloridine as the parent strain may
be considered as an indication of the existence of another
diffusion pathway in the outer membrane which is at least as
efficient as the class 3 pores for the permeation of these
antibiotics. This alternative diffusion pathway may be
formed by the class 1 OMP, for which we could demonstrate a
pore function in vitro. However, the class 1 protein pores
are not the exclusive permeation pathway for chlorampheni-
col and cephaloridine, since the class 1 OMP-deficient strain
was as sensitive to these antibiotics as the parent strain.
Probably the class 1 and 3 pores are mutually exchangeable
with respect to the permeation of these antibiotics.

The observation that the primary structure of the class 1
OMP is very homologous to the gonococcal protein I and
meningococcal class 2 OMP (3, 20) suggested that this protein
could also be a pore-forming protein. Although the assays for sensitivity to antibiotics gave no indication for
such a function, this could indeed be demonstrated in vitro.
With respect to apparent diameter and slight cation selectiv-
ity, the characteristics of the class 1 protein pores are very
similar to those reported for OmpF protein of E. coli K-12
(6).

Class 1 OMP is being considered as a potential vaccine
candidate for meningococcus group B (29). Our results show
that it is possible to isolate meningococcal strains that lack
the class 3 OMP by mutation. These mutant strains also
appear to contain reduced amounts of the class 4 protein. In
E. coli, the mutational removal of a major OMP is usually
compensated by increased production of other OMPs (16).
Unfortunately, this does not appear to be the case in the
meningococcal mutants. Nevertheless, the isolation of class
3 protein-deficient strains like CE2002 will facilitate the
purification of class 1 OMPs, free of contamination with
inactive antigens.

Preferentially, a vaccine should consist of an antigen that
is essential for the viability of a virulent organism, to prevent
the selection of “escape mutants.” The mutational removal
of class 1 OMP did not have any adverse effect on the growth
of the bacteria in vitro. Apparently, the presence of the class
3 protein pores is sufficient to maintain normal growth rates
in vitro. Similarly, E. coli K-12 produces two pore-forming
proteins when grown under standard laboratory conditions,
i.e., OmpF and OmpC (6, 16, 21). This does not seem to be
essential for growth of E. coli even in vivo, since other E.
coli isolates, such as strains B and C, produce only one of
these proteins. However, growth of E. coli under conditions
of phosphate starvation results in the synthesis of an addi-
tional pore-forming protein, PhoE (22, 30). Like OmpF and
OmpC, PhoE forms general diffusion pores, which are anion
selective, in contrast to OmpF and OmpC, which are cation
selective (6). Recently, we demonstrated the existence of a
phiE gene in 35 of 35 tested E. coli and Shigella isolates by
using a phoE-specific DNA probe (29a). Thus, the existence
of one anion-selective pore and (at least) one cation-selective
pore seems to be conserved in E. coli and may therefore be
essential for growth in vivo. Similarly, the functions of the
class 1 and 3 protein pores seem to be complementary,
rather than identical, with the class 1 OMP pores being
cation selective and the class 3 OMP pores, by analogy to the
gonococcal porin, probably being anion selective. Exper-
iments in animal models to determine whether the class 1
OMP is, indeed, essential for growth of meningococci in vivo
are in progress.

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