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Mutagenesis of the *Neisseria gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness

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Summary

Porin (PorB), the major outer membrane protein of *Neisseria gonorrhoeae*, has been implicated in pathogenesis previously. However, the fact that porin deletion mutants are not viable has complicated investigations. Here, we describe a method of manipulating the porin gene site-specifically. *N. gonorrhoeae* MS11, which harbours the $porB_{1B}$ (P.1B) porin allele, was used to generate mutants carrying deletions in the surface loops 1 and 5. An 11-amino-acid deletion in loop 1 impaired Opa_{50}-dependent invasion into human Chang epithelial cells, whereas loop 5 deletion exhibited no apparent phenotype. In a second approach, the complete gonococcal $porB_{1B}$ was replaced by the $porB_{Nla}$ gene of *Neisseria lactamica*. Such mutants were unable to induce efficient uptake by epithelial cells but induced an enhanced respiratory response in HL60 phagocytic cells. The increased respiratory burst was accompanied by an enhanced phagocytic uptake of the mutant compared with the wild-type strain. Our data extend previous evidence for multiple central functions of PorB in the infection process.

Introduction

Neisseria gonorrhoeae (*Ngo*) is an obligate human pathogen and the causative agent of the name-giving disease gonorrhoea. This Gram-negative diplococcus preferentially infects mucosal surfaces of the male urethra and the female cervix. However, other tissues can be infected, and complications may arise as a result of inflammation and dissemination. The usually strong inflammatory response leads to the typical purulent discharge consisting of polymorphonuclear leucocytes (PMNs) filled with seemingly intact diplococci.

Several factors playing crucial roles during gonococcal infection have been identified previously. A prerequisite for the infection *in vivo* is the synthesis of pili (Kellogg *et al*., 1968). These hair-like structures protruding from the gonococcal surface mediate the initial attachment of the bacteria to epithelial cells (McGee *et al*., 1981). In a subsequent step, integral outer membrane proteins, including the phase-variable opacity-associated (Opa) proteins, mediate intimate adherence and invasion of epithelial cells. Distinct members of this protein family, e.g. Opa_{50}, interact with heparan sulphate-containing proteoglycans (HSPGs) (Makino *et al*., 1991; Chen *et al*., 1995; van Putten and Paul, 1995), while the majority of Opa proteins (e.g. Opa_{52}) bind to a variable extent to members of the CD66 receptor family (Kupsch *et al*., 1993; Bos *et al*., 1997; Gray-Owen *et al*., 1997a), mediating phagocytic uptake and invasion and transcytosis of epithelial cells (Chen and Gotschlich, 1996; Virji *et al*., 1996; Gray-Owen *et al*., 1997b; Wang *et al*., 1998).

Several pathways have been recognized that function specifically in Opa_{50}/HSPG-dependent invasion (for review, see Dehio *et al*., 1998a). In human Chang epithelial cells, invasion normally proceeds in the absence of serum factors (Grassmé *et al*., 1997), while serum factors such as vitronectin and fibronectin have the potential to increase invasion in several other epithelial cell lines via integrin-dependent mechanisms (Gomez-Duarte *et al*., 1997; Duensing and van Putten, 1997; Dehio *et al*., 1998b). Expression of Opa_{50} in *E. coli* is not sufficient to confer efficient invasion (Simon and Rest, 1992; Kupsch *et al*., 1993). However, the ligation of HSPG receptors confers uptake of antibody-coated latex beads, and this uptake can be enhanced in the presence of vitronectin (Dehio *et al*., 1998b).

In addition to the Opa-mediated processes, other invasion pathways have been described including the lutropin receptor-dependent, contact-inducible infection of Hec1B cells (Spence *et al*., 1997). Recently, the invasion of Chang epithelial cells has been reported to occur independently of Opa proteins in medium devoid of phosphate (van Putten *et al*., 1998). In this case, the invasive potential has been attributed to the P.1A allele of the gonococcal porin (PorB).

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PorB is the major outer membrane protein of *Ngo* comprising a hydrophilic pore that allows the passage of small nutrients and waste products across the outer membrane of Gram-negative bacteria up to a size of 1.4 kDa (Nikaido, 1994; Benz *et al*., 1995; Jeanteur *et al*., 1995). The pores are formed by a trimeric configuration of the porin monomer, which spans the membrane in amphipathic β-sheets exposing surface loops of different lengths (Weiss *et al*., 1991; Weiss and Schulz, 1992; Cowan *et al*., 1992; Schirmer *et al*., 1995). The three-dimensional structure of neisserial PorB has not been resolved yet, but several topological models have been proposed (Jeanteur *et al*., 1991; 1995; van der Ley *et al*., 1991; Ward *et al*., 1992). Accordingly, 16 membrane-spanning regions with eight surface loops are predicted. In contrast to certain lipopolysaccharide (LPS) epitopes, Opa and the pilin subunits PilC and PilE, PorB is not a phase-variable protein and is encoded by a single gene locus. The amino acid sequences of the membrane-spanning domains are well preserved within the genus *Neisseria*, but the surface-exposed loops vary between different strains constituting two major porin alleles in *Ngo*, i.e. P.1A and P.1B (Jeanteur *et al*., 1991; 1995; van der Ley *et al*., 1991; Ward *et al*., 1992). As genome sequencing recently revealed a homologue of the unrelated meningococcal PorA in *Ngo* (www.genome.ou.edu; D. Günther, A. Kahrs and T. F. Meyer, unpublished) and the gonococcal P.1A and P.1B alleles represent homologues of the meningococcal PorB porins (Hitchcock, 1989), we refer to these PorB homologues here as $PorB_{1A}$ and $PorB_{1B}$.

An intriguing feature of the pathogenic *Neisseria* porins is their ability to translocate into artificial membranes (Lynch *et al*., 1984) as well as into eukaryotic cell membranes (Blake and Gotschlich, 1987; Weel and van Putten, 1991; Weel *et al*., 1991; Rudel *et al*., 1996). PorB, but not PorA, porins of pathogenic and potentially pathogenic *Neisseria* species have been shown to interact with nucleotide triphosphates that downregulate the pore size (Rudel *et al*., 1996). Furthermore, the nucleotide-binding PorB alleles exhibited a voltage-dependent channel activity, which is strikingly reminiscent of eukaryotic porins.

During infection, *Ngo* encounters the bactericidal action of polymorphonuclear neutrophils (PMNs). Purified gonococcal porin selectively interferes with the signalling machinery of PMNs (Haines *et al*., 1988; Bjerknes *et al*., 1995), thereby inhibiting degranulation (Haines *et al*., 1988). The addition of purified PorB to phagocytes was further shown to downregulate actin polymerization, opsonin receptor expression and phagocytosis of *Ngo* (Bjerknes *et al*., 1995). Moreover, in line with these observations, porin has recently been shown to interfere with phagosome maturation, suggesting an important role for this factor in the intracellular accommodation of *Ngo* (Mosleh *et al*., 1998).

PorB is essential for the viability of *Ngo* and is therefore not accessible to knockout mutagenesis that would allow studies on its role in pathogenesis. Previously, a method has been reported that permits the generation of hybrid porin genes and allelic gene replacements (Carbonetti *et al*., 1988; 1990). Here, we describe a novel method for generating *porB* mutants synthesizing site-specifically mutated *porB*, or porin genes of commensal *Neisseria* species instead of wild-type *porB*. The mutants constructed showed a significant decrease in their potential to invade epithelial cells and evoked a stronger oxidative burst and an enhanced uptake by professional phagocytes.

Results

Strategy of porB *mutagenesis*

The cloning of an intact *porB* gene in *E. coli* has been hampered by the strong expression of its native promoter, and *Ngo porB* deletion mutants are not viable. Therefore, in order to clone and mutate *porB*, we chose an *in vitro* approach that depends entirely on polymerase chain reaction (PCR). As outlined in Fig. 1, the strategy is based on the *in vitro* generation of a *por* fragment flanked by two resistance markers amplified from chromosomal DNA (donor). The fragment serves as a template for PCR-based mutagenesis. Subsequently, the mutated *por* gene fragment is transformed directly into a *Ngo* recipient strain in which it replaces the wild-type $porB_{1B}$ by homologous recombination between flanking resistance genes (Fig. 1). That is to say, the insertion of mutated *porB* results in the repair of a non-functional erythromycin resistance gene (*ermC3′*) and the restoration of erythromycin resistance to the mutant.

Identification of a permissive integration site downstream of the porB *locus*

In order to generate donor and recipient strains, permissive integration sites for resistance markers had to be identified flanking the genomic $porB_{1B}$ gene. A genomic library was screened with the oligonucleotides TR67 and TR46, which hybridized to the 5′ and 3′ ends of the $porB_{1B}$ locus in *Ngo* MS11 respectively. Surprisingly, nine independent clones of the *porB* 3′ end and downstream region were isolated, but no clone of the *porB* upstream region.

One of the *porB* 3′ clones, pMS9, containing a 2.6 kb insert, was mutagenized with the Tn*Max1* mini-transposon, which confers chloramphenicol (Cm) resistance in both *E. coli* and *Ngo* (Haas *et al*., 1993). Derivatives of pMS9 harbouring a transposon directly downstream of the $porB_{1B}$ terminator (pMS10a, Fig. 2), approximately 500 bp (pMS10b) or approximately 1000 bp (pMS10c) downstream of $porB_{1B}$, respectively, were used to transform *Ngo* MS11 N137. As determined by PCR, transformants

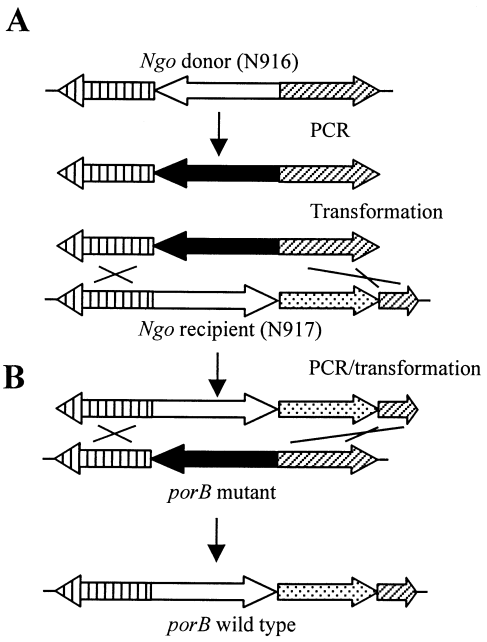

Fig. 1. Construction of PorB mutants.
A. Site-directed mutagenesis of *porB*. Two overlapping PCR fragments were amplified from chromosomal DNA of the donor strain. The resulting fragments were fused in a subsequent PCR to give a 4.2 kb fragment comprising cat_{GC}, $porB_{\Delta loop1}$ and *ermC′*. Using the same procedure, a DNA fragment was constructed containing cat_{GC}, $porB_{\Delta loop5}$ and *ermC′*. In order to exchange *porB* for the porin gene of *Nla*, a fragment was amplified comprising cat_{GC}, $PorB_{Nla}$ and *ermC′*. The PCR fragment was transformed directly into a *Ngo* recipient strain, where it recombined as indicated by the crossed lines.
B. Introduction of *porB* into the mutants. White arrows, *porB* wild type; black arrows, mutated *porin* genes; vertical stripes, cat_{GC}; diagonal stripes, *ermC′*; dotted, kan_{GC}. The crossed lines indicate the site of recombination upon transformation of the PCR construct into *Ngo*.

contained the Cm marker at the correct site downstream of $porB_{1B}$ and gave rise to Cm-resistant colonies. A pMS10a-transformed *Ngo* clone was used for further constructions.

Integration of resistance cassettes upstream of porB

As we were unable to isolate the $porB_{IB}$ promoter from the genomic library, a direct cloning strategy was chosen. *Ngo* chromosomal DNA was digested with several restriction enzymes, and the fragments were probed with pTR67 by Southern hybridization. A 1.5 kb fragment was identified in the *Mbo*I-digested DNA. As the $porB_{1B}$ gene harbours an *Mbo*I site at position 706 (Carbonetti *et al*., 1988), the 1.5 kb fragment contained around 800 bp of the $porB_{1B}$ upstream region. *Mbo*I-digested chromosomal fragments were size fractionated and inserted into the *Bam*HI site of the Bluescript KS+ vector. A PCR fragment of the $porB_{1B}$ upstream region was generated from the ligated DNA using the M13 forward primer and MAS5, a primer that corresponds to a sequence upstream of $porB_{1B}$ (Table 1). The resulting fragment was cloned as a tandem duplication separated by *ermC′* ($>P_{porB}<ermC'>P_{porB}$) in plasmid pMS19a (Fig. 2).

Construction of Ngo *recipient and donor strains for* porB *mutagenesis*

The pMS10a-transformed *Ngo* clone was transformed with pMS19a to generate the donor strain N916. N916 carried the *ermC′* gene in the centre of a $porB_{1B}$ promoter duplication (Fig. 2) and a cat_{GC} gene downstream of $porB_{1B}$. The next step was the construction of a recipient strain that could be used to integrate the *in vitro*-mutated *porB* genes. To exclude undesired recombination events, the mutated *porB* gene was integrated in the opposite direction to the endogenous $porB_{1B}$. Therefore, the *ermC′* of pMS19a was exchanged for a cat_{GC} gene in pSH14 (Fig. 2). The cat_{GC} gene of pSH24, which carried the pMS10a insert in a Bluescript vector, was exchanged for the *apha*-3 gene conferring kanamycin resistance (Kan^r) and a truncated *ermC′* gene (*ermC3′*) to yield pFJB1. pSH14 and pFJB1 were subsequently used to transform MS11 N137. This resulted in the Cm^r and Kan^r strain N917, which now harboured cat_{GC} upstream and *apha*-3-*ermC3′* downstream of $porB_{1B}$ ($cat_{GC}<>porB_{1B}>apha$-3-*ermC3′*). The cat_{GC} gene and the truncated *ermC′* gene served as recombination sites for the PCR-generated fragments derived from N916.

Table 1. Oligonucleotides used in this study.

Oligo	Sequence (5′ → 3′)	Locus/remarks
IS3	TTATGTCGACCCGGGCCCGAGGTCACAAAAAATAGGTACACG	3′*ermC′*
FJB4	GCTTGCCGTCTGAATTACGCCCCGCCCTGCCACTCATGC	3′cat_{GC}/uptake signal (Elkins *et al*., 1991)
FJB10	TACACATGCCTGAAACCGGCAGCGAAA	$porB_{1B}$/Δloop1, *Bgl*II
FJB11	GCCGGTTTCAGATCTGTAAGTTTGTA	$porB_{1B}$/Δloop1, *Bgl*II
FJB14	ATACGGCGAATTCGTTGAAAAACTGCAA	$porB_{1B}$/Δloop5, *Eco*RI
FJB15	TTTCAACGAAATTCGCCGTATCTTTGGAA	$porB_{1B}$
FJB5	TAAGATCTAAAAAAGGAATACAGCAAT 5′	$porB_{1B}$/*Bgl*II
MAS5	ATAAGCTTAGATCTAAGCAGGCCGGGCTTTCC	$porB_{1B}$ promoter
TR46	CAGGAAACAGCTATGACCAAGCTTTGCAGATTAGAATTTG	3′*porB*

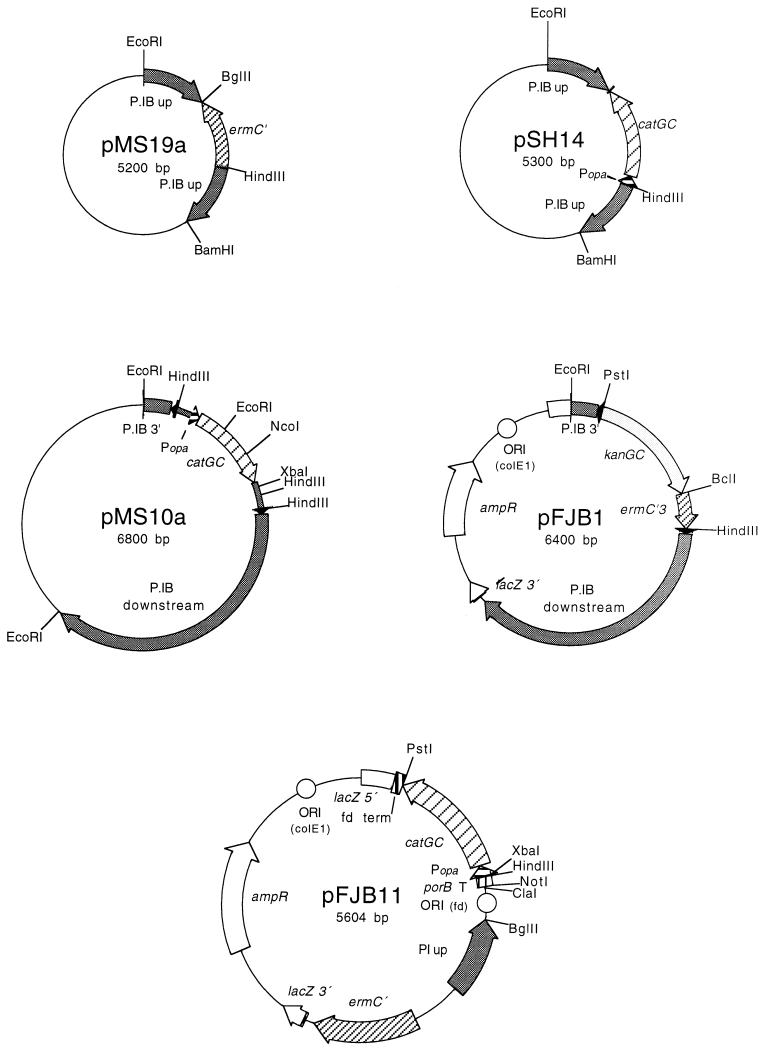

Fig. 2. Constructs used for the generation of donor and recipient strains. Constructs used for the generation of the donor strain N916 (pMS10a and pMS19a) and the recipient strain N917 (pSH14 and pFJB1) are shown. pFJB11 used for the expression of the porin gene of *Nla* harbours the PI upstream (PI up) region including the $porB_{1B}$ promoter and the $porB_{1B}$ terminator (*porB* T).

Mutagenesis of $porB_{1B}$

Via PCR, two overlapping chromosomal fragments were amplified from the donor strain N916, one containing the *ermC′* cassette and the 5′ part of *porB*, and the other carrying the cat_{GC} cassette and the 3′ end of *porB*. The oligonucleotides were designed in such a way that a restriction site was introduced into a deletion in *porB*. The two fragments were combined via overlap extension by PCR (Higuchi *et al*., 1988; Ho *et al*., 1989) and transformed directly into recipient N917. The wild-type $porB_{1B}$ was exchanged for the mutated *porB* by double crossover between the *ermC′* and the cat_{GC} cassettes, resulting in the deletion of kan_{GC} and the complementation of *ermC′* (Fig. 1A). Transformants were thus rendered sensitive to Kan and resistant to Erm and Cm.

Applying this method, we tried to delete short peptides in putative loop regions of PorB corresponding to amino acids 18–28 in loop 1, 109–116 and 117–124 in loop 3 and 186–202 in loop 5 of the mature PorB of MS11. However, only bacteria transformed with deletions of loop 1 (N919) or loop 5 (N918), but not loop 3, were isolated on Erm- and Cm-containing plates. The presumptive mutants were first tested by restriction analysis of amplified *porB*. To confirm that no secondary mutations were introduced, the mutant *porB* genes were re-amplified from chromosomal DNA of the recombinant strains and sequenced directly. PorB molecules derived from mutants were slightly smaller compared with the native PorB, as expected (Fig. 2).

Exchange of $porB_{1B}$ *by* $porB_{Nla}$ *from* N. lactamica

In order to exchange the MS11 $porB_{1B}$ allele for corresponding *porB* alleles from non-pathogenic *Neisseria* species, the vector pFJB11 was constructed, which carried cat_{GC}, the *porB* promoter, the *porB* terminator and *ermC′* in the vector pIC20R (Fig. 2). *Not*I and *Bgl*II sites between

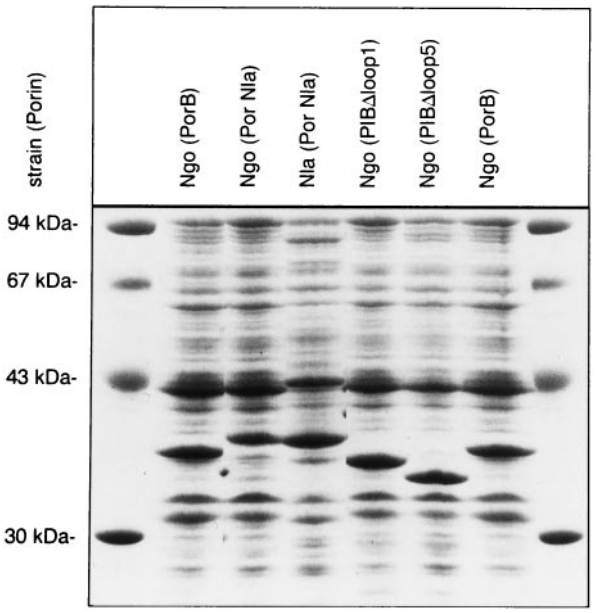

Fig. 3. SDS–PAGE with total-cell lysates of gonococcal PorB mutants and the *Ngo* and *Nla* wild-type strains. The gel shows the *Ngo* strains PorB (N916), $PorB_{Nla}$ (N922), $PorB_{\Delta loop1}$ (N919) and $PorB_{\Delta loop5}$ (N918). The size of $PorB_{\Delta loop1}$ (35 kDa) and $PorB_{\Delta loop5}$ (34 kDa) is slightly smaller compared with wild-type PorB. The molecular mass is given in kDa.

the promoter and the terminator allowed the insertion of porin genes. The porin gene of *Nla*-N348 ($porB_{Nla}$) was PCR amplified from chromosomal DNA, ligated into pFJB11 and re-amplified from the ligation reaction, generating a fragment comprising a $cat_{GC} < porB_{Nla} < > ermC'$ fragment. This fragment was used to transform the recipient strain N917, and the transformants were tested as described. The resulting strain N922 produced a new protein of 37 kDa corresponding to the $PorB_{Nla}$, instead of the original PorB of 36 kDa. (Fig. 3).

As the mutation or the exchange of PorB could affect the vitality of the porin mutants, the growth of mutants in liquid culture was compared with the wild-type strain. All the mutants showed a similar generation time to the wild type, suggesting that these mutations are well tolerated by the gonococci (data not shown).

Epithelial cell invasion of porin mutants

In order to analyse the phenotype of *porB* mutations with regard to the infection process, several modifications were necessary. As the lack of pili significantly increases the level of epithelial cell invasion under *in vitro* conditions (Makino *et al*., 1991), non-piliated (P^-) colonies were selected, and the lack of PilE expression was confirmed by Western blot analysis (data not shown). Furthermore, a constitutively expressed gene encoding Opa_{50} was introduced into the strain expressing wild-type porin and the mutants by conjugation (Kupsch *et al*., 1996), and production of the plasmid-encoded Opa_{50} was confirmed by immunoblot analysis (data not shown). Again, the growth of all Opa_{50}-expressing strains was similar in liquid culture.

With this set of recombinant porin mutants, the efficiency of epithelial cell invasion in the human Chang conjunctiva infection model was determined using the gentamicin survival assay for selective detection of intracellular bacteria (Makino *et al*., 1991). The recombinant *Ngo* N932 expressing the $PorB_{\Delta loop5}$ survived the gentamicin treatment at a similar rate to the wild-type strain N948. However, strains N949 and N950 producing the $PorB_{Nla}$ or $PorB_{\Delta loop1}$, respectively, survived in this assay about 100-fold less efficiently (Fig. 4). The survival of N949 and N950 was about 10-fold higher compared with their Opa^- progenitors N922 and N919 that, however, adhered 1000-fold less than the Opa_{50} derivatives (data not shown). As the adherence of all Opa_{50} strains to Chang epithelial cells was similar, the observed differences in survival could be caused either by a decreased uptake of the mutants by epithelial cells or a by diminished survival of the mutants inside cells.

To address this question, the numbers of bacteria internalized during a 6 h invasion period were determined by microscopic evaluation. Intracellular gonococci are known to co-localize with the human lysosome-associated membrane protein h-lamp-1, which allows the selective quantification of intracellular bacteria by double immunofluorescence staining and confocal laser scanning microscopy of infected Chang conjunctiva cells (Hauck and

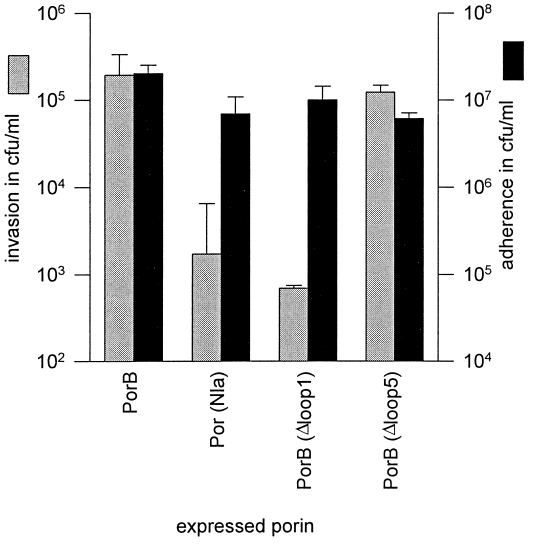

Fig. 4. Opa_{50}-mediated adherence to, and invasion of, Chang cells by *Ngo* strains expressing different porins. The assays were performed in triplicate on three separate occasions with the *Ngo* strains $PorB_{MS11}$ (N948), $PorB_{Nla}$ (N949), $PorB_{\Delta loop1}$ (N950) and $PorB_{\Delta loop5}$ (N932). The results are expressed as gentamicin-surviving colony-forming units (cfu ml^{-1}).

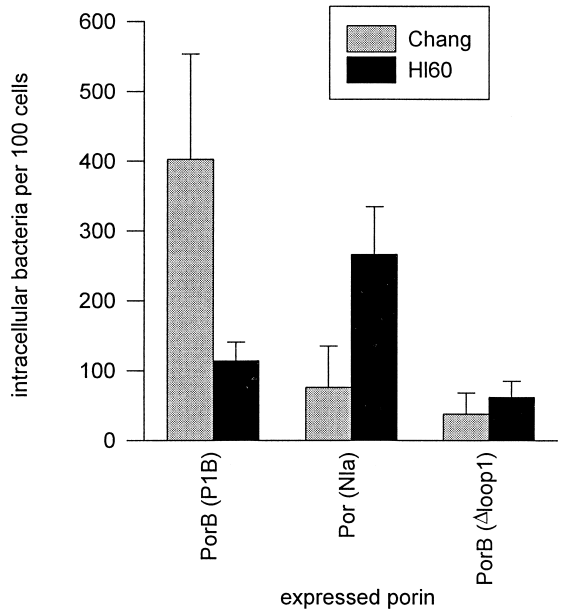

Fig. 5. Microscopical evaluation of intracellular gonococci in Chang cells after 6 h and in differentiated HL60 cells after 2 h. The experiments were performed on three separate occasions with the *Ngo* strains PorB (N948), $PorB_{Nla}$ (N949) and $PorB_{\Delta loop1}$ (N950).

Meyer, 1997). These experiments revealed that recombinant gonococci expressing $PorB_{Nla}$ or $PorB_{\Delta loop1}$ were taken up less efficiently by Chang conjunctiva cells compared with the wild-type strain (Fig. 5).

The protein and the LPS profiles of the wild-type strain and the mutants analysed on silver-stained SDS–PAGE were identical, except for the changes in PorB (not shown). To demonstrate that the reduced invasion of mutants results from a defect in the porin and not in any other variable factor, wild-type $porB_{1B}$ was re-introduced into the mutants. This was accomplished by transforming a PCR fragment carrying selection markers and the wild-type $porB_{1B}$ derived from recipient strain N917 (Fig. 1B). Kan^r transformants expressed the wild-type PorB and consistently showed the same invasion rate as the wild type (data not shown).

$PorB_{Nla}$ induces an increased oxidative burst in human neutrophils

Purified porin inhibits degranulation of fMLP-stimulated human neutrophils without affecting superoxide anion generation (Haines *et al*., 1988). We examined the potential of the PorB mutants to induce degranulation in human phagocytic cells. The luminol-dependent chemiluminescence (LmCL) depends on both the generation of superoxide anions by activated NADPH oxidase and the release of myeloperoxidase (MPO) (Edwards, 1987; Faulkner and Fridovich, 1993). As MPO is stored in azurophilic granules of neutrophils and is released during the respiratory burst, luminol-dependent chemiluminescence (LmCL) is a powerful technique for studying degranulation. Differentiated HL60 cells were incubated with luminol and stimulated with non-piliated Opa_{50}-expressing PorB mutants. The Por_{Nla}-expressing gonococci induced a significantly stronger LmCL compared with the wild-type strain and the $PorB_{\Delta loop1}$ mutant (Fig. 6).

$PorB_{Nla}$ leads to an increased gonococcal phagocytosis by human neutrophils

To address the question whether the enhanced LmCL of the $PorB_{Nla}$ mutant was associated with an altered efficiency of the phagocytic uptake of *Ngo*, differentiated HL60 cells were infected for 2 h with the different porin mutants. Internalized diplococci were determined by double immunofluorescence staining and confocal microscopy (Fig. 5). The data indicate that the $PorB_{Nla}$ variant was phagocytosed more efficiently than the PorB wild type, while the number of intracellular $PorB_{\Delta loop1}$-expressing gonococci was only slightly lower than the wild type. The increased respiratory response was thus associated with an increased phagocytic uptake of *Ngo* in this cell line.

Discussion

Previous efforts at generating *porB* mutants have allowed the construction of hybrid *porB* genes and the mutual exchange of the $porB_{1A}$ and $porB_{1B}$ alleles in *Ngo* (Carbonetti *et al*., 1988; 1990). However, an assessment of the

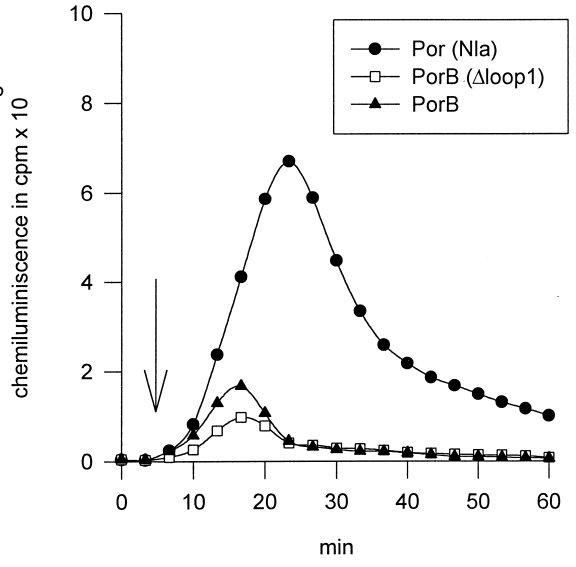

Fig. 6. LmCL of differentiated HL60 cells stimulated by gonococci expressing different porins. The figure shows one representative experiment out of five performed with the strains *Ngo* PorB (N948), *Ngo* $PorB_{Nla}$ (N949) and *Ngo* $PorB_{\Delta loop1}$ (N950). (↓) time point at which bacteria were added.

possible role of gonococcal porin in pathogenesis has been hampered because the porin gene was not accessible to site-specific manipulation. Here, we have applied a novel method that allows the complete exchange of the chromosomal *porB* gene in *Ngo* by mutant *porB* genes or porin genes from other *Neisseria* species. The generation of a 'donor' DNA fragment solely by PCR and the subsequent direct transformation of a gonococcal recipient circumvented the cloning of the complete *porB* gene in *E. coli*, which causes toxic effects. Furthermore, the strategy applied allows the sequential generation of point mutations, as any mutant strain can serve as donors for a subsequent round of PCR mutagenesis.

We used this strategy to introduce small deletions in the loop regions of PorB. Modifying the loop regions of porins is usually well tolerated, because it does not affect the native trimeric structure, whereas deletions in membrane-spanning segments prevent proper assembly of the porin trimer (Benson *et al*., 1988; Bauer *et al*., 1989; Klebba *et al*., 1994). The fact that deletions of amino acids 18–28 or 186–202 of PorB were well tolerated supports the hypothesis that these sequences are located within loop regions. This result is in agreement with the proposed topological models of PorB that assign these regions to the external loops 1 and 5 (Jeanteur *et al*., 1991, 1995; van der Ley *et al*., 1991; Ward *et al*., 1992). In contrast, the deletion of amino acids 109–116 and 117–124, putatively located in loop 3, appeared to affect the viability of the bacteria, as no transformants were isolated. In the OmpC-type porins, loop 3 has been shown to extend across the channel, thus diminishing the size of the pore and contributing to the stability of the barrel structure and the trimer (Rocque and McGroarty, 1990; Cowan *et al*., 1992; Weiss *et al*., 1991; Fourel *et al*., 1994). It is therefore possible that the removal of the amino acids 109–116 and 117–124 interferes with the proper assembly of PorB, thereby affecting the viability of such mutants.

The results presented here suggest that PorB is involved in the Opa_{50}-mediated invasion of epithelial cells. The exchange of the wild-type $PorB_{1B}$ for the corresponding porin of the commensal strain *N. lactamica* or a small deletion in surface loop 1 of PorB led to a dramatic reduction in epithelial cell uptake via heparan sulphate proteoglycan receptors (HSPGs). The ligation of HSPGs was shown to induce the phagocytic uptake of latex beads in Chang and HeLa epithelial cells (Dehio *et al*., 1998b). However, vitronectin, which interacts with the $\alpha_v\beta_5$ integrin receptor, further enhanced the uptake in HeLa cells (Duensing and van Putten, 1997; Gomez-Duarte *et al*., 1997; Dehio *et al*., 1998a,b). These observations suggest that the interaction of Opa_{50} with HSPGs alone is not fully sufficient for an efficient epithelial cell invasion. Consistent with this notion is the observation that the expression of Opa_{50} in *E. coli* conferred attachment to, but not uptake by, epithelial cells (Kupsch *et al*., 1993; Grassme *et al*., 1996). In contrast, the co-expression of Opa and PorB in *E. coli* enhanced invasion, while the expression of PorB alone was ineffective (see Jerse and Rest, 1997). These findings contrast with the Opa_{52}-mediated invasion of HeLa cells transfected with receptors of the CD66 family. In this case, the expression of CD66-specific Opa proteins alone (e.g. Opa_{52}) enables *E. coli* to invade and traverse epithelial cells (Chen and Gotschlich, 1996; Gray-Owen *et al*., 1997a,b; Bos *et al*., 1997; Wang *et al*., 1998). This suggests that PorB might be of particular relevance as a co-stimulatory factor for Opa_{50}-mediated invasion.

We have shown recently that Opa_{50}-mediated invasion of Chang cells requires the activation of acidic sphingomyelinase and the formation of ceramide (Grassme *et al*., 1997), while the invasion of other epithelial cell lines involves the binding of *Ngo* to HSPG and integrin receptors via vitronectin and a stimulation of protein kinase C (PKC) (Duensing and Van Putten, 1997; Gomez-Duarte *et al*., 1997; Dehio *et al*., 1998b). Interestingly, purified PorB induces a strong transient Ca^{2+} flux in epithelial cells and phagocytes (Müller *et al*., 1999). The Ca^{2+} flux may activate PKC directly and thus stimulate epithelial cell invasion by *Ngo*. Recently, van Putten *et al*. (1998) observed that the P.1A allele of PorB can, in the absence of phosphate, trigger gonococcal uptake in Chang cells even independently of Opa_{50}, supporting the notion that PorB provides at least a co-stimulatory function for epithelial cell invasion. In the case of Opa-independent invasion, an unknown gonococcal determinant may, however, substitute for the adhesin function of Opa.

The invasion deficiency of the porin mutants measured by the gentamicin survival assay is approximately 100-fold lower compared with the wild-type strain, while four to 10 times fewer mutants were observed inside the cells under microscopic evaluation. The discrepancy between the data from the two assays might well reflect a reduced survival of the mutants after host cell invasion. Although this assumption would fit the role of PorB in the intraphagosomal accommodation and survival of *Ngo* nicely (Haines *et al*., 1988; 1991; Bjerknes *et al*., 1995; Mosleh *et al*., 1998), one has to be careful with this interpretation. The numbers of gentamicin survivors are generally lower than the number of bacteria detectable microscopically inside cells (Makino *et al*., 1991). Thus, the gentamicin assay gives only a rough estimate of the real invasion and survival, and other factors, such as growth or interbacterial adhesion, are also important criteria. As we compared isogenic *Ngo* derivatives in this assay that were identical in their growth and clumping, reduced survival of mutants has to be considered and investigated further in future.

While the expression of $PorB_{Nla}$ exerts an inhibitory effect on epithelial cell invasion, this porin appears to promote phagocytic uptake by professional phagocytes. The

exchange of PorB for $PorB_{Nla}$ also resulted in an increased LmCL in differentiated HL60 cells. This is in agreement with previous work showing that purified PorB inhibits the receptor-mediated degranulation of neutrophils without affecting superoxide anion generation (Haines *et al*., 1988) and downregulates the phagocytic uptake of the bacteria (Bjerknes *et al*., 1995). The deletion of loop 1 of PorB did not affect the interaction with professional phagocytes, although it resulted in a decreased invasion in epithelial cells. This puzzling observation probably reveals distinct biological effects of the porin that may depend on specific epitopes. Loop1 might be important for the bacterial uptake by epithelial cells, while other loops may be involved in functions relating to CD66-mediated phagocytosis.

Little is known about the mode of interaction of the gonococcal porin with the target cells. PorB is reported to translocate into target cell membranes (Lynch *et al*., 1984; Blake and Gotschlich, 1987; Weel and van Putten, 1991; Rudel *et al*., 1996), where the porin channel is regulated by the membrane potential and nucleotides (Rudel *et al*., 1996). Using a random shuttle mutagenesis approach, we identified previously a substantial number of genes mostly encoding secretory proteins that appeared to be involved in Opa_{50}-mediated invasion (Kahrs *et al*., 1994; Fussenegger *et al*., 1996a). We speculate that these secreted proteins include factors that modulate the properties of the gonococcal cell surface in such a way that the translocation of PorB to target cell membranes is affected. In order to address this question, it will be necessary to study the mechanism of porin translocation in more detail.

Experimental procedures

Bacterial strains and plasmids

All *Ngo* strains used in this study are derived from MS11 (Meyer *et al*., 1982; Carbonetti *et al*., 1988) and are listed in Table 2. They were grown on GC agar base (Becton Dickinson) supplemented with 1% vitamin mix at 37°C in 5% CO_2. The antibiotic resistance genes cat_{GC}, *ermC′* and kan_{GC} have been described previously (Projan *et al*., 1987; Haas *et al*., 1993; Fussenegger *et al*., 1996b). Plasmid pFJB11 harbours cat_{GC}, the *porB* promoter, the *porB* terminator and *ermC′* in the vector pIC20R (Marsh *et al*., 1984). A *Not*I and a *Bgl*II site between the promoter and the terminator allows the insertion of *porin* genes. *N. lactamica* was from Dr U. Berger and was obtained via the Reference Laboratory for Bacterial Meningitides (Amsterdam).

Mutagenesis of PorB

Two overlapping PCR fragments were amplified from the chromosomal DNA of the donor strain N916 with the oligonucleotides FJB4/FJB10 and IS3/FJB11 (Table 1). The resulting fragments were fused in a subsequent PCR with the oligos FJB4 and IS3 to give a 4.2 kb fragment comprising cat_{GC}, $porB_{\Delta loop1}$ *and ermC′*. Using the same procedure, a DNA fragment was constructed using oligos FJB4/FJB14 and IS3/FJB15 containing cat_{GC}, $porB_{\Delta loop5}$ *and ermC′*. In order to exchange *porB* for the porin gene of a commensal strain, $porB_{Nla}$ was amplified with FJB5 and TR46 and ligated via *Not*I and *Bgl*II into pFJB11. A fragment was amplified comprising cat_{GC}, $porB_{Nla}$ and *ermC′*. The donor N917 was transformed according to a protocol described by Rudel *et al*. (1992) with these fragments, and transformants were selected for erythromycin resistance and checked for sensitivity to kanamycin. For restriction and sequence analysis, the *porin* loci of the transformants were amplified with FJB5 and TR46.

Deletion of pilE *and expression of PilC and* Opa_{50}

The mutants were selected for P^- phenotype. Deletion of *pilE* (Rudel *et al*., 1995a) and expression of *pilC* were confirmed by immunoblotting (Rudel *et al*., 1995b). pTH6a harbouring opa_{50} was mobilized from N352 into the mutants, and expression was detected by immunoblotting (Kupsch *et al*., 1993).

Epithelial cell invasion and adherence

The invasion and adherence experiments were performed as described previously (Kahrs *et al*., 1994) with the human Chang conjunctiva cell line (ATCC CCL20.2) derived from Flow Laboratories. Briefly, the cells were cultured in RPMI containing 5% fetal calf serum (FCS) at 37°C and 5% CO_2 in 24-well plates (Costar) before use in a survival assay. Approximately

Table 2. Strains used in this study.

Strain	Relevant genotype and orientation of genes in the *porB* locus[a]; plasmid	Relevant phenotype
N352	pTH6 (opa_{50})	
N916	cat_{GC}< >*porB*< >*ermC*	PorB, P^+, Opa^-, $PilC^+$
N917	*catGC*< >*porB*>*kanGC–ermC*(3′)	PorB, P^+, Opa^-, $PilC^+$
N918	cat_{GC}< >$porB_{\Delta loop5}$>*ermC*	$PorB_{\Delta loop5}$, P^+, Opa^-, $PilC^+$
N919	cat_{GC}< >$porB_{\Delta loop1}$>*ermC*	$PorB_{\Delta loop1}$, P^+, Opa^-, $PilC^+$
N922	cat_{GC}< >$porB_{Nla}$>*ermC*	$PorB_{Nla}$, P^+, Opa^-, $PilC^+$
N932	cat_{GC}< >$porB_{\Delta loop5}$>*ermC*; pTH6 (opa_{50})	$PorB_{\Delta loop5}$, P^-, Opa_{50}, $PilC^+$
N948	cat_{GC} < >*porB*>*ermC*; pTH6 (opa_{50})	PorB, P^-, Opa_{50}, $PilC^+$
N949	cat_{GC}< >$porB_{Nla}$>*ermC*; pTH6 (opa_{50})	$PorB_{Nla,}$ P^-, Opa_{50}, $PilC^+$
N950	cat_{GC} < >$porB_{\Delta loop1}$>*ermC*, pTH6 (opa_{50})	$PorB_{\Delta loop5}$, P^-, Opa_{50}, $PilC^+$

a. Arrowheads indicate 5′end (> or <) and 5′ to 3′ orientation (>) of genes.

3×10^5 cells were infected with 5×10^6 bacteria in RPMI-1640 (Gibco BRL) containing 5% FCS and 1 U of benzonase (Boehringer Mannheim) and incubated for 6 h. Then, non-adherent bacteria were removed by repeated washings with PBS. In order to determine the amount of adherent bacteria, cells were lysed with 1% saponin in PBS, and appropriate dilutions were plated on GC agar. Intracellular bacteria were counted by killing extracellular bacteria with a 2 h treatment of the infected cells with RPMI containing 50 μg ml^{-1} gentamicin before plating out. For the microscopical evaluation of invasion, cells were grown on glass coverslips. After infection and the removal of non-adherent bacteria, the probe was fixed by treatment with 3% paraformaldehyde in PBS for 30 min.

Infection of HL60 cells

The cells were grown as suspensions in RPMI supplemented with 10% FCS at 37°C and 5% CO_2. HL60 cells were differentiated 5–7 days before infection by adding 1.25% DMSO to the culture medium. Approximately 1×10^6 differentiated HL60 cells suspended in PBS containing 1 mM Mg^{2+}, 1 mM Ca^{2+} and 0.1% glucose were infected with *N. gonorrhoeae* at a multiplicity of infection (MOI) of 100 for 2 h at 37°C. Non-ingested gonococci were separated from the cells by repeated centrifugation at 150 × *g* for 5 min and washing with PBS. Cells were centrifuged on glass coverslips in 24-well plates and fixed with 3% paraformaldehyde.

Microscopic evaluation

After two washes with PBS, probes were incubated for 10 min in PBS, 0.1% Triton X-100 in order to permeabilize the cells. The two washes were repeated, and non-specific binding sites were blocked by 5 min treatment with PBS, 0.2% BSA. Suitable dilutions of polyclonal rabbit anti-*N. gonorrhoeae* (1:100; AK93) and monoclonal mouse anti-h-lamp-1 (1:40; clone H4A3; obtained from DSHB, University of Iowa, USA) in PBS were added for 1 h. Samples were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies (Sigma ImmunoChemicals) for 1 h. After three washes, coverslips were mounted in glycerol medium (Sigma ImmunoChemicals), sealed with nail polish and viewed with a Leica TCS 4D confocal laser scanning microscope (Leica Lasertechnik) equipped with an argon–krypton mixed gas laser. Images were taken serially using appropriate excitation and emission filters for the fluorescent dyes used.

Chemiluminescence assay

Approximately 1×10^6 differentiated cells in 100 μl of PBS were infected for 1 h at 37°C with gonococci (MOI 100) in 400 μl of PBS containing 0.1% glucose and 100 μM luminol. Chemiluminescence was detected with a six-channel Bioluminat LB9505 (Berthold).

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References

Bauer, K., Struyve, M., Bosch, D., Benz, R., and Tommassen, J. (1989) One single lysine residue is responsible for the special interaction between polyphosphate and the outer membrane porin pho-e of *Escherichia coli*. *J Biol Chem* **264:** 16393–16398.

Benson, S.A., Occi, J.L.L., and Sampson, B.A. (1988) Mutations that alter the pore function of the ompf porin of *Escherichia coli* K12. *J Mol Biol* **203:** 961–970.

Benz, R., Ghuysen, J.M., Hakenbeck, R. (1995) Uptake of solutes through bacterial outer membranes. In *Bacterial Cell Wall*. Ghuysen, J., and Hakenbeck, R. (eds). Amsterdam: Elsevier Science Publishers, pp. 397–423.

Bjerknes, R., Guttormsen, H., Solberg, C.O., and Wetzler, L.M. (1995) Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but prime the neutrophils to increase their oxidative burst. *Infect Immun* **63:** 160–167.

Blake, M.S., and Gotschlich, E.C. (1987) Functional and immunogenic properties of pathogenic *Neisseria* surface proteins. In *Bacterial Outer Membranes as Model Systems*. Inouye, M. (ed.). New York: John Wiley and Sons, pp. 377–400.

Bos, M.P., Grunert, F., and Belland, R.J. (1997) Differential recognition of members of the carcinoembryonic antigen family by Opa variants of *Neisseria gonorrhoeae*. *Infect Immun* **65:** 2353–2361.

Carbonetti, N.H., Simnad, V.I., Seifert, H.S., So, M., and Sparling, P.F. (1988) Genetics of protein i of *Neisseria gonorrhoeae* construction of hybrid porins. *Proc Natl Acad Sci USA* **85:** 6841–6845.

Carbonetti, N., Simnad, V., Elkins, C., and Sparling, P.F. (1990) Construction of isogenic gonococci with variable porin structure: effects on susceptibility to human serum and antibiotics. *Mol Microbiol* **4:** 1009–1018.

Chen, T., and Gotschlich, E.C. (1996) CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. *Proc Natl Acad Sci USA* **93:** 14851–14856.

Chen, T., Belland, R.J., Wilson, J., and Swanson, J. (1995) Adherence of pilus$^-$ Opa$^+$ gonococci to epithelial cells *in vitro* involves heparan sulfate. *J Exp Med* **182:** 511–517.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., *et al*. (1992) Crystal structures explain functional properties of two *Escherichia coli* porins. *Nature* **358:** 727–733.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (1998a) The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol* **6:** 489–495.

Dehio, M., Gomez Duarte, O.G., Dehio, C., and Meyer, T.F. (1998b) Vitronectin-dependent invasion of epithelial cells by *Neisseria gonorrhoeae* involves α_v integrin receptors. *FEBS Lett* **424:** 84–88.

Duensing, T.D., and Van Putten, J.P.M. (1997) Vitronectin mediates internalization of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. *Infect Immun* **65:** 964–970.

Edwards, S.W. (1987) Luminol- and lucigenin-dependent chemiluminescence of neutrophils: role of degranulation. *J Clin Lab Immunol* **22:** 35–39.

Elkins, C., Thomas, C.E., Seifert, H.S., and Sparling, P.F. (1991) Species-specific uptake of DNA by gonococci is

mediated by a 10-base-pair sequence. *J Bacteriol* **173:** 3911–3913.

Faulkner, K., and Fridovich, I. (1993) Luminol and lucigenin as detectors for superoxide. *Free Radical Biol Med* **15:** 447–451.

Fourel, D., Bernadac, A., and Pages, J.M. (1994) Involvement of exposed polypeptide loops in trimeric stability and membrane insertion of *Escherichia coli* OmpF porin. *Eur J Biochem* **222:** 625–630.

Fussenegger, M., Kahrs, A.F., Facius, D., and Meyer, T.F. (1996a) Tetrapac (tpc), a novel genotype of *Neisseria gonorrhoeae* affecting epithelial cell invasion, natural transformation competence and cell separation. *Mol Microbiol* **19:** 1357–1372.

Fussenegger, M., Facius, D., Meier, J., and Meyer, T.F. (1996b) A novel peptidoglycan-linked lipoprotein (ComL) that functions in natural transformation competence of *Neisseria gonorrhoeae*. *Mol Microbiol* **19:** 1095–1105.

Gomez-Duarte, O.G., Dehio, M., Guzman, C.A., Chhatwal, G.S., Dehio, C., and Meyer, T.F. (1997) Binding of vitronectin to Opa-expressing *Neisseria gonorrhoeae* mediates invasion of HeLa cells. *Infect Immun* **65:** 3857–3866.

Grassmé, H.U., Ireland, R.M., and van Putten, J.P. (1996) Gonococcal opacity protein promotes bacterial entry-associated rearrangements of the epithelial cell actin cytoskeleton. *Infect Immun* **64:** 1621–1630.

Grassmé, H., Gulbins, E., Brenner, B., Ferlinz, K., Sandhoff, K., Harzer, K., *et al*. (1997) Acidic sphingomyelinase mediates entry of *N. gonorrhoeae* into nonphagocytic cells. *Cell* **91:** 605–615.

Gray-Owen, S.D., Dehio, C., Haude, A., Grunert, F., and Meyer, T.F. (1997a) CD66 carcinoembryonic antigens mediate interactions between Opa- expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes. *EMBO J* **16:** 3435–3445.

Gray-Owen, S.D., Lorenzen, D.R., Haude, A., Meyer, T.F., and Dehio, C. (1997b) Differential Opa specificities for CD66 receptors influence tissue interactions and cellular response to *Neisseria gonorrhoeae*. *Mol Microbiol* **26:** 971–980.

Haas, R., Kahrs, A.F., Facius, D., Allmeier, H., Schmitt, R., and Meyer, T.F. (1993) TnMax – a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. *Gene* **130:** 23–31.

Haines, K.A., Yeh, L., Blake, M.S., Cristello, P., Korchak, H., and Weissmann, G. (1988) Protein I, a translocatable ion channel from *Neisseria gonorrhoeae*, selectively inhibits exocytosis from human neutrophils without inhibiting O2-generation. *J Biol Chem* **263:** 945–951.

Haines, K.A., Reibman, J., Tang, X.Y., Blake, M., and Weissmann, G. (1991) Effects of protein I of *Neisseria gonorrhoeae* on neutrophil activation: generation of diacylglycerol from phosphatidylcholine via a specific phospholipase C is associated with exocytosis. *J Cell Biol* **114:** 433–442.

Hauck, C.R., and Meyer, T.F. (1997) The lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of *Neisseria gonorrhoeae*. *FEBS Lett* **405:** 86–90.

Higuchi, R., Krummel, B., and Saiki, R.K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res* **16:** 7351–7367.

Hitchcock, P.J. (1989) Unified nomenclature for pathogenic *Neisseria* species. *Clin Microbiol Rev* **2** (Suppl.): S64–S65.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77:** 51–59.

Jeanteur, D., Lakey, J.H., and Pattus, F. (1991) The bacterial porin superfamily: sequence alignment and structure prediction. *Mol Microbiol* **5:** 2153–2164.

Jeanteur, D., Lakey, J.H., and Pattus, F. (1995) The porin superfamily: diversity and common features. In *Bacterial Cell Wall*. Ghuysen, J., and Hakenbeck, R. (eds). Amsterdam: Elsevier Science Publishers, pp. 363–380.

Jerse, A.E., and Rest, R.F. (1997) Adhesion and invasion by the pathogenic neisseria. *Trends Microbiol* **5:** 217–221.

Kahrs, A.F., Bihlmaier, A., Facius, D., and Meyer, T.F. (1994) Generalized transposon shuttle mutagenesis in *Neisseria gonorrhoeae*: a method for isolating epithelial cell invasion-defective mutants. *Mol Microbiol* **12:** 819–831.

Kellogg, D.S., Cohen, I.R., Norins, L.C., Schoeter, A.L., and Reising, G. (1968) *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 month *in vitro*. *J Bacteriol* **96:** 596–605.

Klebba, P.E., Hofnung, M., and Charbit, A. (1994) A model of maltodextrin transport though the sugar-specific porin, LamB, based on deletion analysis. *EMBO J* **13:** 4670–4675.

Kupsch, E.M., Aubel, D., Gibbs, C.P., Kahrs, A.F., Rudel, T., and Meyer, T.F. (1996) Construction of Hermes shuttle vectors: a versatile system useful for genetic complementation of transformable and non-transformable *Neisseria* mutants. *Mol Gen Genet* **250:** 558–569.

Kupsch, E.M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T.F. (1993) Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *EMBO J* **12:** 641–650.

van der Ley, P., Heckels, J.E., Virji, M., Hoogerhout, P., and Poolman, J.T. (1991) Topology of outer membrane porins in pathogenic *Neisseria spp*. *Infect Immun* **59:** 2963–2971.

Lynch, E.C., Blake, M.S., Gotschlich, E.C., and Mauro, A. (1984) Studies on porins spontaneously transferred from whole cells and from proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Biophys J* **45:** 104–107.

McGee, Z.A., Johnson, A.P., and Taylor-Robinson, D. (1981) Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *J Infect Dis* **143:** 413–422.

Makino, S., van Putten, J.P., and Meyer, T.F. (1991) Phase variation of the opacity outer membrane protein controls invasion by *Neisseria gonorrhoeae* into human epithelial cells. *EMBO J* **10:** 1307–1315.

Marsh, J.L., Erfle, M., and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32:** 481–485.

Meyer, T.F., Mlawer, N., and So, M. (1982) Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell* **30:** 45–52.

Mosleh, I.M., Huber, L.A., Steinlein, P., Pasquali, C., Günther, D., and Meyer, T.F. (1998) *Neisseria gonorrhoeae* porin modulates phagosome maturation. *J Biol Chem* **237:** 35332–35338.

Müller, A., Günther, D., Düx, F., Naumann, M., Meyer, T.F., and Rudel, T. (1999) Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. *EMBO J* **18:** (in press).

Nikaido, H. (1994) Porins and specific diffusion channels in bacterial outer membranes. *J Biol Chem* **269:** 3905–3908.

Projan, S.J., Monod, M., Narayanan, C.S., and Dubnau, D. (1987) Replication properties of pIM13, a naturally occurring plasmid found in *Bacillus subtilis*, and of its close relative pE5, a plasmid native to *Staphylococcus aureus*. *J Bacteriol* **169:** 5131–5139.

van Putten, J.P., and Paul, S.M. (1995) Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *EMBO J* **14:** 2144–2154.

van Putten, J.P.M., Duensing, T.D., and Carlson, J. (1998) Gonococcal invasion of epithelial cells driven by P.IA, a bacterial ion channel with GTP binding properties. *J Exp Med* **188:** 941–952.

Rocque, W.J., and McGroarty, E.J. (1990) Structure and function of an OmpC deletion mutant porin from *Escherichia coli* K-12. *Biochemistry* **29:** 5344–5351.

Rudel, T., Van Putten, J.P.M., Gibbs, C.P., Haas, R., Meyer, T.F. (1992) Interaction of two variable proteins PilE and PilC required for pilus-mediated adherence of *Neisseria gonorrhoea*e to human epithelial cells. *Mol Microbiol* **6:** 3439–3450.

Rudel, T., Boxberger, H.J., and Meyer, T.F. (1995a) Pilus biogenesis and epithelial cell adherence of *Neisseria gonorrhoeae pilC* double knock-out mutants. *Mol Microbiol* **17:** 1057–1071.

Rudel, T., Scheuerpflug, I., and Meyer, T.F. (1995b) Neisseria PilC protein identified as type-4 pilus tip-located adhesin. *Nature* **373:** 357–359.

Rudel, T., Schmid, A., Benz, R., Kolb, H.A., Lang, F., and Meyer, T.F. (1996) Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. *Cell* **85:** 391–402.

Schirmer, T., Keller, T.A., Wang, Y.F., and Rosenbusch, J.P. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution. *Science* **267:** 512–514.

Simon, D., and Rest, R.F. (1992) *Escherichia coli* expressing a *Neisseria gonorrhoeae* opacity-associated outer membrane protein invade human cervical and endometrial epithelial cell lines. *Proc Natl Acad Sci USA* **89:** 5512–5516.

Spence, J.M., Chen, J.C., and Clark, V.L. (1997) A proposed role for the lutropin receptor in contact-inducible gonococcal invasion of Hec1B cells. *Infect Immun* **65:** 3736–3742.

Virji, M., Makepeace, K., Ferguson, D.J.P., and Watt, S.M. (1996) Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol Microbiol* **22:** 941–950.

Wang, J., Gray-Owen, S.D., Knorre, A., Meyer, T.F., and Dehio, C. (1998) Cellular CD66 receptors mediate traversal of polarised T84 epithelial cell monolayers. *Mol Microbiol* **30:** 657–671.

Ward, M.J., Lambden, P.R., and Heckels, J.E. (1992) Sequence analysis and relationships between meningococcal class 3 serotype proteins and other porins from pathogenic and non-pathogenic Neisseria species. *FEMS Microbiol Lett* **73:** 283–289.

Weel, J.F., and van Putten, J.P. (1991) Fate of the major outer membrane protein P.IA in early and late events of gonococcal infection of epithelial cells. *Res Microbiol* **142:** 985–993.

Weel, J.F., Hopman, C.T., and van Putten, J.P. (1991) Bacterial entry and intracellular processing of *Neisseria gonorrhoeae* in epithelial cells: immunomorphological evidence for alterations in the major outer membrane protein P.IB. *J Exp Med* **174:** 705–715.

Weiss, M.S., and Schulz, G.E. (1992) Structure of porin refined at 1.8 Å resolution. *J Mol Biol* **227:** 493–509.

Weiss, M.S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., and Schulz, G.E. (1991) Molecular architecture and electrostatic properties of a bacterial porin. *Science* **254:** 1627–1630.