Gonococcal Resistance to β-Lactams and Tetracycline Involves Mutation in Loop 3 of the Porin Encoded at the *penB* Locus

M. J. GILL,[1]* S. SIMJEE,[1] K. AL-HATTAWI,[2] B. D. ROBERTSON,[2] C. S. F. EASMON,[2] AND C. A. ISON[2]

Department of Infection, University of Birmingham Medical School, Birmingham,[1] and Department of Infectious Diseases and Microbiology, Imperial College School of Medicine, St. Mary's Campus, London,[2] United Kingdom

penB is a chromosomal mutation that confers resistance to β-lactams and tetracyclines and reduced susceptibility to quinolones in *Neisseria gonorrhoeae*. It is linked to the porin gene (*por*) and requires the increased expression of an efflux pump due to *mtr*. Transformation of a susceptible gonococcus (strain H1) with chromosomal DNA from strain FA140 (*penA mtr penB*; porin serovar IB1) and conjugal transfer of a β-lactamase-expressing plasmid was used to produce isogenic strains for determination of equilibrium periplasmic penicillin concentrations by the method of Zimmermann and Rosselet (W. Zimmermann and A. Rosselet, Antimicrob. Agents Chemother. 12:368–372, 1977). In transformants with the Mtr and PenB phenotypes, equilibrium concentrations of penicillin were reduced. DNA sequence analysis of *por* from isogenic *penB* and *penB*⁺ transformants revealed 14 sequence differences; nine of these differences resulted in amino acid changes. Three amino acid changes were found in the putative gonococcal equivalent of the pore-constricting loop 3 of *Escherichia coli* OmpF. Two of these changes (Gly-101–Ala-102→Asp-Asp) result in an increased negative charge at this position in *por* loop 3. PCR products comprising the complete *por* gene from strain FA140 were transformed into strain H1-2 (*penA mtr*; porin serovar IB-3), with the resulting transformants having the antibiotic susceptibility phenotype associated with *penB*. penB-like mutations were found in loop 3 of clinical isolates of gonococci with chromosomally mediated resistance to penicillin. We conclude that *penB* is a mutation in loop 3 of *por* that reduces porin permeability to hydrophilic antibiotics and plays an important role in the development of chromosomally mediated resistance to penicillin and tetracycline in gonococci.

Low-level chromosomally mediated resistance to penicillin and tetracycline in *Neisseria gonorrhoeae* has been shown in laboratory mutants (29) and clinical isolates (15) to be due to three mutations: *penA*, *mtr*, and *penB*. *penA* results in decreased binding of penicillin to PBP 2 (8) and results from the insertion of an aspartate codon (10). *mtr* (formerly *ery* [29]) results in increased resistance to a range of hydrophilic and hydrophobic substances including antibiotics. The *mtr* phenotype results in increased expression of the MtrCDE efflux pump (14, 27).

penB increases the level of resistance to both penicillin and tetracycline (29). It is apparent only in strains with the Mtr phenotype. Subsequent study of strains exhibiting the *penB* phenotype showed that the molecular weight of a major outer membrane protein was altered with acquisition of the *penB*-associated antibiotic phenotype (3, 13). The locus for this "new membrane protein" (*nmp*; now known as *por*) cotransformed with *penB* at a frequency of 98%. Using clinical isolates, Bygdeman et al. (2) found 100% cotransformation between a locus for low-level penicillin resistance and that for a IB (WII/WIII) Por serogroup specificity.

The gonococcus has only one major porin (Por; formerly protein I or PI). Its different forms are alleles of a single gene, *por* (4). Liposome swelling assays (9), electrophysical ion conductivity experiments (31), and the predicted amino acid sequence hydrophobicity plot of Por (4) indicate that it has a structure similar to those of *Escherichia coli* OmpC and OmpF. However, its anion selectivity is more similar to that of *E. coli* PhoE. A single gonococcal strain will express one structurally and immunologically invariant form of Por. However, the amino acid sequence of Por shows considerable diversity. This diversity has allowed Por to be used as the basis of serotyping by coagglutination with a panel of monoclonal antibodies (21).

Porins are well recognized as allowing the diffusion across the outer membrane of hydrophilic molecules including antibiotics (24). We have investigated the possibility that *penB* is a mutation in *por* by determining the antibiotic susceptibilities, serotypes, equilibrium penicillin concentrations, and *por* sequences of isogenic transformants produced by using the chromosomal DNA of strain FA140 (*penA mtr*, *penB*; porin serovar IB1). Transformation to the PenB antibiotic phenotype was also attempted with PCR-derived *por* from FA140. Given the diversity of Por, to help us ascribe functional significance to differences in Por amino acid sequence, we used isogenic strains of gonococci of the same serovar. In addition, we have compared the sequence of loop 3 of *por* from clinical isolates of gonococci susceptible to or with chromosomally mediated resistance to penicillin.

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MATERIALS AND METHODS

Gonococcal strains. The four isogenic strains FA19 (wild type), FA102 (*penA*), FA136 (*penA mtr*), and FA140 (*penA mtr penB*) (29) and strain H1, a clinical isolate (15), were used. Gonococcal strains were grown on GC agar base (36 g/ liter; Difco Laboratories, West Molesey, Surrey, United Kingdom) supplement-

* Corresponding author. Mailing address: Department of Infection, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT., United Kingdom. Phone: 44 121 414 3436. Fax: 44 121 414 3454. E-mail: m.j.gill@bham.ac.uk.

TABLE 1. Serovars and antibiotic susceptibilities of isogenic strains of *N. gonorrhoeae*

| Strain | Genotype | Serovar | MIC (mg/liter)[a] | | | | | | |
			Pen	Cxm	Tet	NA	Cip	CV	Ery
FA19	Wild type	IA-2	0.015	0.03	0.5	1	0.004	1	0.25
FA102	*penA*	IA-2	0.12	0.12	0.5	1	0.004	1	0.25
FA136	*penA mtr*	IA-2	0.25	0.25	0.5	1	0.008	4	2
FA140	*penA mtr penB*	IB-1	1	0.5	1	2	0.015	4	2
H1	Wild type	IB-3	0.03	0.06	0.25	1	0.008	2	0.5
H1-1	*penA*	IB-3	0.12	0.12	0.25	1	0.008	2	0.5
H1-2	*penA mtr*	IB-3	0.25	0.25	0.5	1	0.008	4	2
H1-3	*penA mtr penB*	IB-3	1	0.5	1	2	0.015	4	2

[a] Antibiotic abbreviations: Pen, penicillin; Cxm, cefuroxime; Tet, tetracycline; NA, nalidixic acid; Cip, ciprofloxacin; CV, crystal violet; Ery, erythromycin.

ed with 1% IsoVitaleX (Becton Dickinson, Cowley, Oxford, United Kingdom) at 36°C in 5% carbon dioxide. Derivatives of H1 (H1-1, H1-2, and H1-3) were constructed by transformation with chromosomal DNA from FA140 (15). Erythromycin was used to select for *mtr* transformants; penicillin was used for all other selections. The concentration of antibiotic used to select for transformants was equal to or four times the MIC of that antibiotic for the recipient.

Clinical isolates of gonococci susceptible to penicillin or with chromosomally mediated resistance to penicillin were from the collection at the Imperial College School of Medicine and were originally isolated from male patients attending clinics in Dubai, United Arab Emirates. Strain BL1066 was a β-lactamase-producing clinical isolate containing a 36-kb conjugative plasmid and a 7.2-kb β-lactamase-expressing plasmid (17).

Phenotypic characterization of strains. Susceptibility to a range of antibiotics including, penicillin, tetracycline, ciprofloxacin, and erythromycin was tested by an agar dilution method (15). Suspensions for susceptibility testing were prepared from overnight growth of the test strain on GC agar base supplemented with 1% IsoVitaleX incubated at 36°C in 5% CO_2. Antibiotic-containing medium (GC agar base supplemented with 1% IsoVitaleX) was inoculated with a multipoint inoculator to give a final inoculum of 10^5 CFU per spot. The inoculated plates were incubated at 36°C in 5% carbon dioxide for 24 h. The MIC was read as the lowest concentration of the antibiotic to give complete inhibition of growth.

Transformants were also serotyped by coagglutination with the Genetic Systems panel of 12 monoclonal antibodies to Por (Syva, Palo Alto, Calif.) (21). To confirm the Mtr phenotype in H1 derivatives, outer membrane proteins were prepared by Sarkosyl extraction (13) and were analyzed for the presence of the *mtr*-associated proteins (14) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

Equilibrium periplasmic penicillin concentration assay. Equilibrium periplasmic penicillin concentrations in β-lactamase-producing derivatives of the test strains were calculated by the method of Zimmermann and Rosselet (32). Spontaneous rifampin-resistant mutants of the test strains were used as recipients. Conjugations were performed (16) with rifampin-sensitive strain BL1066 as the donor. Selection was with penicillin and rifampin. To ensure that transconjugants were derived from the recipient strains, both transconjugants and recipients were serotyped, tested for β-lactamase production by using nitrocefin (26), and tested for susceptibility to cefuroxime, tetracycline, nalidixic acid, ciprofloxacin, crystal violet, and erythromycin. All the rifampin-resistant β-lactamase-producing transconjugants used as test strains showed no changes in serovar or susceptibilities to these antibiotics when compared to the serovars and susceptibilities of their corresponding isogenic recipients.

The β-lactamase-producing test strains to be tested were grown overnight on GC agar base (36 g/liter; Difco) containing 5 mg of penicillin per liter. They were then grown to the mid-logarithmic phase in 1.5% proteose peptone broth (Difco) containing 1% IsoVitaleX, harvested by centrifugation, washed three times with assay buffer (10 mM magnesium chloride, 10 mM sodium phosphate [pH 7]), and resuspended in assay buffer. Measurements were made with three different preparations of the same aliquot of cells: intact cells, cells disrupted by freezing-thawing (three cycles of placement in liquid nitrogen for 10 min and exposure to 37°C for 10 min), and the supernatant of intact cells was collected by filtration (0.45-μm-pore-size nitrocellulose filter) to correct for the extracellular β-lactamase that leaked during cell harvesting. The final concentration of cells in the assay mixture was equivalent to a cell optical density (OD) of 0.01 at 540 nm. The microiodometric method (25) of measuring β-lactamase was used with a penicillin concentration of 250 μM at 37°C in 10 mM magnesium chloride–10 mM sodium phosphate (pH 7) in a reaction volume of 1 ml. To control for thermal hydrolysis of penicillin and nonspecific reduction of the color developer, a control tube was run simultaneously with the test samples. In addition to the constituents of the other tubes, the control tube contained sodium tungstate and acetic acid to inhibit β-lactamase. Assay conditions allowed calculation of rates of hydrolysis by linear regression from duplicate samples at five time points during the linear time course. The hydrolysis by intact and disrupted cells was measured over a 10-min time course (time points of 2, 4, 6, 8, and 10 min). Hydrolysis by the cell supernatant was measured over 20 min (time points of 2,

5, 10, 15, and 20 min). All measurements for intact cells were made within 40 min of their final wash. To stop penicillin hydrolysis at the various time points, 0.5 ml of 0.5 M sodium tungstate in 1 M acetic acid was added with vigorous mixing. Detection of the penicilloic acid resulting from penicillin hydrolysis was by the decolorization of 0.5 ml of the starch-iodide color developer (final iodine concentration, 40 μM) (25) for at least 20 min. The ODs of the test samples were measured at 620 nm in a split beam with reference to the control sample. The change in OD with time was proportional to the rate of penicillin hydrolysis. The "hydrolysis ratio" was the ratio of the rate of penicillin hydrolysis by intact cells (corrected for the β-lactamase that leaked into the supernatant) to the rate of penicillin hydrolysis by an identical aliquot of cells that had been disrupted. The Michaelis-Menten constant (K_m) was calculated from hydrolysis assays performed with enzyme liberated by freezing-thawing. Under these assay conditions the K_m was 64 μM. This value of K_m and the hydrolysis ratio were used to calculate the equilibrium concentration of penicillin in the periplasm (28, 32) with an extracellular concentration of 250 μM penicillin.

***por* sequencing.** For sequencing, *por* PCR (11) was performed with whole cells as the DNA source. Briefly, sequencing was performed with the transformants of H1 made by using chromosomal DNA from strain FA140 (H1-2, *penA mtr*; H1-3, *penA mtr penB*). PCR products (PCRPs) were purified by electrophoresis in low-melting-point agarose. Sequencing reactions were performed with this purified PCRP as a template for dideoxynucleotide sequencing (Sequenase kit; Amersham). Both strands of *por* were sequenced, and additional primers were prepared as necessary. Sequence differences between strains H1-2 and H1-3 were confirmed by at least two independent sequencing reactions in each direction performed by at least two different PCRs. The region of *por* thought to encode for the gonococcal equivalent of loop 3 of *E. coli* OmpF (18, 19) was sequenced by amplification of the complete *por* gene (11) followed by cycle sequencing with two internal primers (Kal1, 5′-^{251}TTGGAACAAGGTGCCCTCCGT^{270}-3′; Kal2, 5′-^{600}TGTGCGAAGAAGCCGCTGTT^{581}-3′; sequence numbers correspond to the *por* sequence published by Butt et al. [1]). Cycle sequencing was performed with an ABI 310 Genetic Analyzer and by fluorescent dye terminator cycle sequencing chemistry (PE Applied Biosystems, Warrington, United Kingdom).

Transformation of *por* PCR products. *por* PCR (11) was performed with whole cells added directly to the reaction mixture. This PCR amplified the complete *por* gene including the leader sequence (12) was ligated into the *Eco*RV site of pBluescript II KS− (Stratagene) to which dTTP had been added. This ligation was transformed into competent *E. coli* XL-1 Blue (Stratagene). PCRPs derived from the strain FA140 *por* (por_{FA140}-PCRPs) were generated with four different clones of *por* in *E. coli* as the source of target DNA. The por_{FA140}-PCRPs generated by these four separate PCRs were pooled. Hence, por_{FA140}-PCRPs uncontaminated by FA140 chromosomal DNA were produced for transformation into H1-2.

To facilitate PCRP transformation, the 10-bp "uptake sequence" associated with transformation (12) was attached to the por_{FA140}-PCRPs. This construct was then methylated. In order to do this, a complementary pair of primers (UTS1 and UTS2) was synthesized to allow the formation of the uptake sequence: UTS1 (sense strand), <u>CTGCAG</u>CCGTCTGAA<u>TTC</u>; UTS2 (antisense strand), <u>GAAT</u>TCAGACGGC<u>TGCAG</u> (underlined regions indicate linker sequences). For ligation to *por*-PCRP, UTS1 and UTS2 were combined in equimolar amounts, boiled for 5 min, and allowed to cool to room temperature. The resultant double-stranded uptake sequence was then digested with *Pst*I. por_{FA140}-PCRPs from the equivalent of 500 μl of the PCR mixture was digested with *Pst*I. The PCRPs and 1 nmol of the uptake sequence were ligated; the 5′ linker of the PCRP was removed by *Hin*dIII digestion. This construct was then sequentially methylated in 20-μl volumes with 2 U of M*Sss*I (New England Biolabs) and 5 U of M*Hae*III (New England Biolabs) and was then used for transformation (15). Selection for transformants was at two times the MIC of penicillin for the recipient strain (H1-2). The following negative controls were used to exclude the possibility of spontaneous mutation: a recipient with no DNA and a recipient with donor DNA pretreated with DNase. To exclude the possibility of transformation by host or vector DNA when transformation was done with PCRPs derived from subcloned *por*, purified, methylated pBluescript (100 ng) and *E. coli* XL-1 Blue chromo-

TABLE 2. Hydrolysis ratios and equilibrium penicillin concentrations in the periplasm achieved at an extracellular penicillin concentration of 250 μM in isogenic strains of *N. gonorrhoeae*[a]

Strain	Genotype	Mean ± SD hydrolysis ratio[b] (n^c)	Mean ± SD equilibrium penicillin concn (μM) in periplasm[d] (n)
FA19	Wild type	0.669 ± 0.033 (9)	72 ± 2 (9)*
FA102	*penA*	0.643 ± 0.051 (6)	67 ± 3 (6)*,**
FA136	*penA mtr*	0.516 ± 0.047 (7)	44 ± 3 (7)**,***
FA140	*penA mtr penB*	0.231 ± 0.058 (6)	14 ± 3 (6)***
H1	Wild type	0.718 ± 0.050 (8)	85 ± 3 (8)†
H1-1	*penA*	0.747 ± 0.034 (4)	90 ± 2 (4)†,‡
H1-2	*penA mtr*	0.635 ± 0.059 (4)	65 ± 3 (4)‡,††
H1-3	*penA mtr penB*	0.420 ± 0.048 (4)	31 ± 3 (4)††

[a] Hydrolysis ratios and penicillin concentrations were determined by the method of Zimmermann and Rosselet (32). The strains were β-lactamase-producing, spontaneous rifampin-resistant mutants.
[b] Rate of hydrolysis of penicillin by intact cells: rate of hydrolysis by disrupted cells.
[c] n, number of determinations.
[d] P values from unpaired Student's t test between equilibrium periplasmic penicillin concentrations for the indicated pairs of strains: *, $P = 0.1$; **, $P = 0.002$; ***, $P < 0.0001$; †, $P = 0.37$; ‡, $P = 0.013$; ††, $P = 0.002$.

somal DNA (1 μg) were used. Transformation with the *bla* marker (β-lactamase) of pBluescript was excluded by testing transformants for nitrocefin hydrolysis (26).

RESULTS

Transformation experiments with FA140 chromosomal DNA. A series of transformants with successive acquisition of *penA*, *mtr*, and *penB* were made in strain H1. The changes in the susceptibilities of these transformants to a range of antibiotics closely followed that of the FA series of strains as they acquired each of the *penA*, *mtr*, and *penB* mutations (Table 1). In both series of strains acquisition of *mtr* resulted in increased levels of resistance to penicillin, cefuroxime, crystal violet, and erythromycin. In addition, outer membrane protein analysis showed increased production of efflux proteins of approximately 46 kDa in *mtr* strains (data not shown). Acquisition of *penB* was associated with increased levels of resistance to penicillin, cefuroxime, tetracycline, nalidixic acid, and ciprofloxacin (Table 1). When H1-2 was transformed with FA140 DNA to the PenB phenotype, its serovar remained IB-3. The serovar of FA136 changed from IA-2 to IB-1 when it was transformed to FA140 DNA (Table 1).

Equilibrium penicillin concentrations in isogenic transformants. Equilibrium periplasmic concentrations did not show significant changes when the wild-type strains (H1 and FA19) acquired *penA* (H1-1 and FA102, respectively; Table 2). However, the acquisition of *mtr* (H1-2 and FA136, respectively) and *penB* (H1-3 and FA140, respectively) did result in significant reductions in equilibrium penicillin concentrations. The effect was cooperative such that the presence of both mutations (*mtr penB*) led to lower levels of penicillin in the periplasm than those from the presence of *mtr* alone.

Sequencing of *por* from isogenic strains. DNA sequencing of the complete *por* gene of the isogenic strains H1-2 and H1-3 revealed 14 differences in sequence, of which 9 resulted in amino acid differences between the two strains (Table 3). Three of the amino acid sequence differences were found in the Por equivalent of *E. coli* OmpF and PhoE loop 3 (18, 19);

TABLE 3. Por sequence differences between *N. gonorrhoeae* H1-2 and H1-3

Amino acid no.	Amino acid[a] H1-2	Amino acid[a] H1-3	Putative position in Por[b]
22	Lys	Asp	Loop 1
101	Gly	Asp	Loop 3
102	Ala	Asp	Loop 3
126	Gly	Glu	Loop 3
128	Leu	Arg	Transmembrane region 6
224	Ser	Cys	Transmembrane region 11
238	Arg	Met	Loop 6
240	Ala	Gly	Loop 6
255	Phe	Tyr	Transmembrane region 12

[a] Amino acids are numbered from the start of the mature protein.
[b] The putative positions of amino acids in Por are based upon alignments of Jeanteur et al. (18, 19).

one sequence difference (amino acid 128) was found close to the carboxyl end of loop 3 (Table 3).

Transformation experiments with *por*$_{FA140}$-PCRP. Strain H1-2 (*penA mtr*) was transformed with *por*$_{FA140}$-PCRP, and transformants were selected on GC agar containing 0.5 mg of penicillin per ml (twice the MIC for H1-2). No transformants of H1-2 were obtained with pBluescript II KS− DNA, *E. coli* XL-1 Blue DNA or DNase-treated *por*$_{FA140}$-PCRP. Twenty transformants and the recipient (H1-2) from each of four independent transformation experiments were tested for their susceptibilities to penicillin, tetracycline, and nalidixic acid. All of the transformants produced by *por*$_{FA140}$-PCRP had the same antibiotic susceptibility and serotype as H1-3 (*penA mtr penB*). No transformants produced detectable β-lactamase.

Loop 3 sequence of Por in clinical isolates. The susceptibilities of the clinical isolates to penicillin, erythromycin, and Triton X-100 are detailed in Table 4. In these isolates the *por* gene was sequenced from the 80 bases at the 5′ end of the putative loop 3 through to the 25 bases from the 3′ end of loop 3. This sequencing identified only two amino acid differences in loop 3. In the sensitive group of isolates, amino acids 101 and 102 were both arginine; in the resistant group of isolates, these amino acids were glutamine and glycine (Table 4).

DISCUSSION

Low-level resistance to structurally diverse hydrophilic antibiotics associated with *penB* and cotransformation of *penB* with *por* (3, 13) suggested a role for porin in the PenB phenotype. Our use of the method of Zimmermann and Rosselet (32) for measuring outer membrane permeability to penicillin further implicates Por in this phenotype. This method is a sensitive and specific means of measuring equilibrium β-lactam concentrations. Such concentrations are the net result of influx and efflux of a β-lactam antibiotic. Two findings support the validity of

TABLE 4. Antibiotic susceptibilities and Por loop 3 sequence differences in clinical isolates of *N. gonorrhoeae*

Strain group	No. of isolates	MIC (mg/liter) Penicillin	MIC (mg/liter) Erythromycin	MIC (mg/liter) Triton X-100	Loop 3 sequence[a] AA101	Loop 3 sequence[a] AA102
Sensitive	3	<0.008–0.5	0.5–4	62.5–125	Arg	Arg
Resistant	6	4	4	>8,000	Gln	Gly

[a] Amino acids (AAs) are numbered from the start of the mature protein.

this method for determination of equilibrium concentrations in our isogenic strains. First, the equilibrium concentrations were reduced in association with *mtr*. This reduction is consistent with the recognized increased efflux of drugs resulting from derepression of the MtrCDE efflux pump with *mtr* (14, 27). The second finding supporting this method is the lack of significant changes in equilibrium concentrations in association with a change in penicillin-binding proteins alone (*penA*). In our measurements of equilibrium penicillin concentrations in two sets of isogenic transformants, we have shown that *penB* results in such concentrations being significantly reduced. These findings suggest that the PenB phenotype may result from a reduction in the total porin permeability of the outer membrane due either to changes in the amount of Por expressed or to changes in the structure of Por that influence its function. When FA136 (*penA mtr*; serovar IA-2) was transformed to FA140 (*penA mtr penB*; serovar IB-3), the porin serovar changed from IA to IB, indicating the possibility that a structural change in Por is important in *penB*. However, we have shown that *penB* may be transformed into H1-2 (serovar IB-3) without any change in serovar. Thus, if structural changes in Por are relevant to the PenB phenotype, they are probably in regions not associated with serovar-specific epitopes.

We were able to transform H1-2 to the *penB* phenotype with the *por* PCRP from strain FA140. The transformation of H1-2 to the *penB* phenotype with por_{FA140}-PCRP indicates that it is changes in the structure of the porin itself that may be responsible for this phenotype. Sequence analysis of *por* from the isogenic strains H1-2 and H1-3 made by transformation with FA140 DNA demonstrated a number of amino acid differences.

No porin structural data are available for gonococcal Por that would allow the precise location of the differences in Por between H1-2 and H1-3 to be identified. However, inferences may be drawn from data relating to other porins. Alignments of Por with other members of the porin superfamily by using the long alignment of the poorly conserved central region proposed by Jeanteur et al. (18, 19) revealed three mutations associated with *penB* in a region equivalent to loop 3 of *E. coli* OmpF and PhoE. A fourth mutation (Leu-128→Arg) was found close to the carboxyl end of loop 3. Crystal structures of *E. coli* OmpF and PhoE show that although this loop is on the external surface of the porin, it falls into the lumen of the pore, thereby constricting it (7). Several studies have demonstrated the importance of loop 3 in OmpF function. A colicin-resistant *E. coli* mutant with a single mutation (Gly-119→Asp) in loop 3 of OmpF has been shown to have this area of constriction altered. The consequences of this alteration are a reduction in channel conductance, decreased sugar permeation, and probably, decreased colicin diffusion across the outer membrane (20). In addition, this mutant was shown to have reduced cephalosporin susceptibility, depending on the charge and structure of the drug (23).

Our attempts at modeling gonococcal Por on OmpF have been unsuccessful due to the gaps in the alignments of Por with other porins (18, 19). The contribution of other mutations in Por to the PenB phenotype are therefore unknown. However, by analogy with *E. coli* OmpF, it is likely that the *penB*-associated changes in strains H1-2 and H1-3 in loop 3 are responsible for the decreased levels of entry of penicillin, tetracycline, and other hydrophilic antibiotics into the cell. The mutation Gly-101–Ala-102→Asp-Asp results in an increase in negative charge at this point in loop 3. No significant alterations in charge result from the combined mutations Gly-126→Glu and Leu-128→Arg. It may be that the mutation Gly-101–Ala-102→Asp-Asp alone is responsible for reduced porin permeability to antibiotics such as penicillin and tetracycline which have a net

negative charge at pH 7. The changes in Por that result from mutations at amino acids 101 and 102 may alter permeability only to a small degree. Reduced diffusion in a *penB* porin might therefore become manifest only when the MtrCDE efflux pump functions. This explains the early observation of Sparling et al. (29) that the PenB phenotype requires the Mtr phenotype.

Asp-Asp at positions 101 and 102 of Por have been found in strain MS11 (5) and a number of other strains sequenced (EMBL accession no. AF044790, four strains; EMBL accession no. AF044793, one strain). This illustrates the diversity in amino acid sequence found in Por, but insufficient antibiotic susceptibility data are available to us to allow us to assess whether these strains have the PenB phenotype. However, when Carbonetti et al. (6) introduced MS11 Por (IB) into a derivative of FA136 with a IA Por, resistance to penicillin and tetracycline increased. When, in the same study, a derivative of FA136 with a greater proportion of the 5′ end of *por* from a IA strain was constructed, this increase in resistance was not seen, possibly because Por loop 3 from MS11 was not present.

Chromosomally mediated resistance to penicillin and tetracycline is associated with gonococci of IB porin serovars (30). Clinical isolates resistant to these drugs contain three mutations: *penA*, *mtr*, and *penB* (15). We sequenced loop 3 of the *por* genes from clinical isolates to ascertain if *penB*-like mutations were present. It is possible that *penB* mutations that are not phenotypically manifest due to the absence of *mtr* may be present in clinical isolates. To allow division into clinical *penB*⁺ and *penB* groups of strains, they were categorized by antibiotic phenotype. The sensitive group of isolates had reduced susceptibility to erythromycin and Triton X-100 combined with penicillin susceptibility, which may suggest that *mtr* was expressed while *penB*-like mutations were not present. By contrast, the antibiotic susceptibilities of the resistant group of isolates indicated that they were likely to express *mtr* and *penB*. The only differences in the loop 3 amino acid sequence between these two groups was at Por positions 101 and 102. As in the isogenic laboratory strains, the differences (Arg-101–Arg-102→Gln-Gly) result in an increase in the negative charge at this position. No differences comparable to those in the laboratory strains H1-2 and H1-3 (Gly-126→Glu and Leu-128→Arg) were found between the two groups. This further implicates amino acids 101 and 102 as being of importance in the *penB* phenotype.

In conclusion, it appears that *penB* is associated with mutations in loop 3 of the gonococcal porin that are also found in clinical isolates. These mutations increase the negative charge at amino acid positions 101 and 102, with a consequent reduction in porin permeability to negatively charged solutes in the presence of an efflux pump. The gonococcus has demonstrated that, like other bacteria (24), it can develop the efflux and permeability changes which, working in concert, allow it to resist antibiotics of structurally different classes.

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