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Author Manuscript

*Mol Microbiol.* Author manuscript; available in PMC 2009 April 27.

Published in final edited form as:

*Mol Microbiol.* 2005 September ; 57(5): 1238–1251. doi:10.1111/j.1365-2958.2005.04752.x.

## The *penC* mutation conferring antibiotic resistance in *Neisseria gonorrhoeae* arises from a mutation in the PilQ secretin that interferes with multimer stability

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### Abstract

The *penC* resistance gene was previously characterized in a FA19 *penA mtrR penB* gonococcal strain (PR100) as a spontaneous mutation that increased resistance to penicillin and tetracycline. We show here that antibiotic resistance mediated by *penC* is the result of a Glu-666 to Lys missense mutation in the *pilQ* gene that interferes with the formation of the SDS-resistant high-molecular-mass PilQ secretin complex, disrupts piliation, and decreases transformation frequency by 50-fold. Deletion of *pilQ* in PR100 confers the same level of antibiotic resistance as the *penC* mutation, but increased resistance was observed only in strains containing the *mtrR* and *penB* resistance determinants. Site-saturation mutagenesis of Glu-666 revealed that only acidic or amidated amino acids at this position preserved PilQ function. Consistent with early studies suggesting the importance of cysteine residues on stability of the PilQ multimer, mutation of either of the two cysteine residues in FA19 PilQ led to a similar phenotype as *penC*: increased antibiotic resistance, loss of piliation, intermediate levels of transformation competence, and absence of SDS-resistant PilQ oligomers. These data show that a functional secretin complex can enhance the entry of antibiotics into the cell and suggest that the PilQ oligomer forms a pore in the outer membrane through which antibiotics diffuse into the periplasm.

### INTRODUCTION

For nearly 30 years, sexually transmitted infections caused by *Neisseria gonorrhoeae* were routinely treated with penicillin. During that time, however, clinical isolates gradually became more resistant until treatment failure occurred and penicillin was discontinued as a therapeutic agent. Resistance to penicillin in the gonococci occurs by two routes: the plasmid-encoded production of a TEM-1-like  $\beta$ -lactamase, and multiple chromosomally mediated mutations that increase resistance in a stepwise manner (Cannon and Sparling, 1984). As shown in an elegant series of studies by Sparling and colleagues, chromosomally mediated resistance genes can be transferred from a high-level resistant strain (e.g. FA6140) to a susceptible strain (e.g. FA19) by transformation and homologous recombination (Maness and Sparling, 1973; Sarubbi *et al.*, 1974).

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The mechanisms underlying chromosomally mediated penicillin resistance in the gonococci are complex and not fully understood. Multiple gene mutations are required for high-level resistance, but none of these mutations increases resistance dramatically. Only when these mutations are combined do they increase resistance to levels above that achieved clinically. There are five gene mutations known to contribute to high-level penicillin resistance (Minimum Inhibitory Concentration (MIC)  $\geq 2$   $\mu\text{g/ml}$ ) in chromosomally mediated resistant *N. gonorrhoeae* (CMRNG) strains (Ropp *et al.*, 2002), and these genes are transferred from a resistant strain to a susceptible strain in a specific order. The first resistance determinant transferred is *penA*, which encodes altered forms of penicillin-binding protein 2 (PBP 2) with decreased rates of acylation by penicillin (Brannigan *et al.*, 1990; Dowson *et al.*, 1989). The second resistance locus transferred is *mtrR*, which carries a mutation in the promoter region of the MtrR repressor that abolishes transcription of the *mtrR* gene and results in overexpression of the MtrC-MtrD-MtrE efflux pump (Hagman *et al.*, 1995; Pan and Spratt, 1994). Overexpression of the MtrC-MtrD-MtrE efflux pump increases penicillin resistance only slightly, but is required for further increases in resistance mediated by subsequent resistance genes (Hagman *et al.*, 1997; Veal *et al.*, 2002). The third resistance determinant transferred, designated *penB*, corresponds to porin alleles carrying mutations on loop 3 that decrease the concentration of antibiotics in the periplasmic space (Gill *et al.*, 1998; Olesky *et al.*, 2002).

Transformation of a third-level resistant strain (FA19 *penA mtrR penB*) to a higher level of resistance is very difficult to achieve in the laboratory (Dougherty, 1986; Faruki and Sparling, 1986). We previously investigated the role of the *ponA* gene encoding PBP 1, the other essential PBP in *N. gonorrhoeae*, in high-level penicillin resistance (Ropp and Nicholas, 1997; Ropp *et al.*, 2002). The *ponA* gene from high-level penicillin-resistant strains contains a single base mutation that decreases the rate of acylation of PBP 1 by 3- to 4-fold. Reversion of the mutant *ponA* allele (*ponA1*) back to the wild type sequence in the CMRNG strain FA6140 decreased the MIC for penicillin by 2-fold, conclusively demonstrating that *ponA1* is involved in high level resistance. However, additional determinants are required to enable *ponA1* to increase resistance, since no increase in resistance was observed when *ponA1* was transferred to PR100 (FA19 *penA mtrR penB*), a third-level transformant strain.

In a search for additional mutations that would increase resistance above that conferred by the first three determinants, we identified a mutation, termed *penC*, that increased resistance to both penicillin and tetracycline (Ropp *et al.*, 2002). Resistance due to *penC* is the result of a spontaneous mutation and is readily transferable between strains. Surprisingly, introduction of the *ponA1* allele into PR100 increased resistance two-fold, indicating that the *penC* allele conferred the ability to increase resistance to *ponA1* strains. However, the absence of *penC* in CMRNG strains suggests that clinical isolates use another as yet undetermined mechanism to further increase resistance levels.

In this report, we show that the increased resistance due to *penC* is the result of a point mutation in the *pilQ* gene (*pilQ2*). PilQ (previously known as Outer Membrane Protein-Macromolecular Complex or OMP-MC), a member of the secretin family of proteins, is a major component of the gonococcal outer membrane (Newhall *et al.*, 1980). PilQ is required for formation of the Type IV pilus, which is involved in twitching motility, cell attachment and invasion of epithelia, and DNA transformation (Tonjum and Koomey, 1997). PilQ forms a ring-shaped multimeric complex, most likely a dodecamer (Collins *et al.*, 2001), with the pilus formed by a repeating polymer of pilin subunits protruding from the central cavity (Collins *et al.*, 2003; Collins *et al.*, 2004). The *pilQ2* mutation disrupts the formation of an SDS-resistant multimeric complex, causes a loss of piliation, and decreases transformation competence by 50-fold. Increased resistance to a wide range of antibiotics due to acquisition of *pilQ2* is observed only in strains containing the *mtrR* and *penB* resistance determinants. The increase in MICs of a variety of

antibiotics in these strains highlights the importance of a functional PilQ multimer in the entry of antibiotics into the cell.

## RESULTS

### Antibiotic resistance due to *penC* is the result of a point mutation in *pilQ*

We previously reported the isolation of spontaneously arising colonies of PR100 (FA19 *penA mtrR penB*; see Table 1) having 2-fold increases in the MICs for both penicillin and tetracycline (Ropp *et al.*, 2002). This mutation, termed *penC*, was not due to additional mutations in the *penA*, *mtrR*, or *penB* genes. Because genomic DNA from PR100 *penC* transformed PR100 to increased penicillin resistance with high frequency, we cloned the *penC* gene by generating a size-fractionated *Cl*I plasmid library of genomic DNA from one of the PR100 *penC* colonies and identifying plasmids capable of transforming PR100 to increased penicillin resistance (see Materials and Methods for more details). Three individual clones were identified that harbored a plasmid with an identical 10.6 kb insert. Transformation experiments with PCR fragments amplified from the DNA insert localized the *penC* mutation to the *pilQ* gene, and sequencing of the *pilQ* gene revealed that the *penC* phenotype was the result of a single G to A mutation at nucleotide 1996 in the *pilQ* gene that changes Glu-666 to Lys (numbering from the initiating methionine). We refer to the *penC* allele hereafter as *pilQ2*.

### Effect of *pilQ2* on pilus formation and transformation efficiency

Transformation of PR100 with pUC18-*pilQ2* plasmid DNA to increased penicillin resistance resulted in a nonpiliated colony morphology, but how *pilQ2* influences pilus formation was unclear. Thus, we generated isogenic strains of RM11.2 *recA6*, a defined pilin variant of strain FA1090 (Long *et al.*, 2001) with a regulatable *recA* gene that allows for IPTG control of pilin antigenic variation (Seifert, 1997), containing either the FA19 *pilQ* allele (*pilQ*<sub>FA19</sub>; FA19 and FA1090 PilQ differ by two amino acids in the C-terminal domain) or the *pilQ2* allele. We examined the piliation state of these strains by immunogold transmission electron microscopy with an anti-pilin peptide antibody (Long *et al.*, 2003). All of the RM11.2 *recA6* derivatives were confirmed to have identical *pilE* sequences, which assured that differences in piliation were not due to pilin variation. Approximately 50% of RM11.2 *recA6* or RM11.2 *recA6 pilQ*<sub>FA19</sub> cells had pili and the majority of pili were in bundles (Fig. 1A, left and middle panels). In contrast, two independently derived RM11.2 *recA6 pilQ2* mutants had no observable distinct pili on 955 cells examined (Fig. 1A, right panel). However, in a small number of RM11.2 *recA6 pilQ2* cells (~0.6%), we observed short, membrane-attached projections that interacted specifically with the anti-pilin antibody (Fig. 1B). These projections did not have the same morphology as pili (Fig. 1A) and therefore represent an alternative expression of the pilin protein on the cell surface of *pilQ2* strains.

We also examined the effect of the *pilQ2* mutation on transformation competence. The transformation frequency of a *gyrB* allele encoding resistance to nalidixic acid (Stein *et al.*, 1991) was determined with SZ3 (RM11.2 *recA6 penA mtrR penB*; see Table 1) alone and SZ3 harboring either the *pilQ2*,  $\Delta$ *pilE*, *pilT::erm*, or *pilQ:: $\Omega$*  alleles. As shown in Fig. 2, the *pilQ2* allele resulted in a 50-fold reduction in transformation frequency, whereas the *pilT*, *pilE*, and *pilQ:: $\Omega$*  alleles reduced the transformation frequency to  $\leq 1 \times 10^{-6}$ . Thus, although the *pilQ2* mutation appears to disrupt piliation based on electron microscopy, the mutation has a much less dramatic effect on transformation frequency. Taken together, these data suggest that the *pilQ2* mutation results in PilQ complexes that are not functional for pilus assembly, but retain the partial ability to facilitate DNA transformation. It is possible that the pilin-expressing membraneous projections provide a means for transforming DNA to enter the bacterial cell.

### Effect of the *pilQ2* mutation on PilQ oligomerization

To understand the effects of the *pilQ2* mutation on PilQ, we analyzed cell lysates from PR100 and PR100 *pilQ2* by SDS-PAGE and Western blotting with a PilQ antibody. As shown in Fig. 3A, PilQ from PR100 migrated at two positions: at the top of the gel, which corresponds to an SDS-resistant high-molecular-mass (HMM) multimer (Drake *et al.*, 1997) (most likely a dodecamer (Collins *et al.*, 2001; Collins *et al.*, 2004)), and at 75 kDa, which corresponds to a PilQ monomer. In contrast, PilQ from PR100 *pilQ2* showed no evidence of the SDS-resistant multimer, but instead migrated as a band at 75 kDa with increased amounts of PilQ breakdown products. PR100 with a disrupted *pilQ* gene (*pilQ::Ω*), in which the Ω fragment encoding spectinomycin resistance (Prentki and Krisch, 1984) replaced codons 266 to 367 of the *pilQ* gene (Chen *et al.*, 2004), showed a complete loss of PilQ protein (Fig. 3A). We also tested the effects of temperature on the capacity of the mutant PilQ to resist denaturation by SDS. However, no HMM PilQ multimer was observed regardless of the temperature at which the samples in SDS-PAGE loading buffer were incubated before electrophoresis (Fig. 3B).

Next, we investigated whether the *pilQ2* mutation interfered with transport of PilQ subunits to the outer membrane, where it is assembled into SDS-resistant HMM oligomers, or whether it interfered with oligomer assembly directly. Outer membranes were isolated (Heckels, 1977) and subjected to SDS-PAGE and Western blotting. As shown in Fig. 3C, a banding pattern for PilQ essentially identical to whole cell lysates was observed in each strain, suggesting that the PilQ mutant protein is transported to the outer membrane but is not present as an SDS-resistant HMM oligomer.

To determine in more detail the effects of the *pilQ2* mutation on the oligomeric structure of *pilQ* in the bacterial envelope, we extracted total membranes from FA19 and FA19 *pilQ2* with the zwitterionic detergent SB-10 and fractionated the extracts by gel filtration. The levels of PilQ oligomer (FA19) and monomer (FA19 *pilQ2*) from individual fractions were assessed by Western blotting and densitometry (Fig. 3D). In the wild type cells, the SDS-resistant PilQ oligomer eluted as a broad peak at a position consistent with its predicted molecular mass (~1 MDa). Interestingly, PilQ protein from FA19 *pilQ2* was found in two distinct peaks: the first peak eluted at the same position as the PilQ oligomer, whilst the second more prominent peak eluted with an approximate molecular mass of ~200 kDa. Although larger than the mass of a PilQ monomer (75 kDa), this latter peak eluted at the same position as the SDS-sensitive PilQ monomer present in wild type cells (data not shown). This peak potentially represents the trimer structure of meningococcal PilQ reported by Collins *et al.* (Collins *et al.*, 2004). These results strongly suggest that a fraction of the Glu-666 to Lys mutant of PilQ exists in the membrane in an oligomeric form, but the mutation prevents its maturation into an SDS-resistant form.

Finally, because we did not observe any pilin fibers in the *pilQ2* or *pilQ::Ω* strains, we examined the level of pilin proteins in whole cells by Western blot analysis (Fig. 3E). The total amounts of pilin in RM11.2 *recA6*, RM11.2 *recA6 pilQ*<sub>FA19</sub>, RM11.2 *recA6 pilQ2*, and RM11.2 *recA6 pilQ::cat* were very similar. The biggest difference between the individual strains was observed in the band corresponding to the proteolytic breakdown product, S-pilin. Compared to RM11.2 *recA6*, the small portion of pilin migrating as S-pilin was ~2-fold higher in RM11.2 *recA6* FA19 *pilQ*, whereas the amount of S-pilin in RM11.2 *recA6 penC* and RM11.2 *recA6 pilQ::cat* was ~2-fold lower (Fig 3E). Thus, the *pilQ2* mutation had no significant effects on the total levels of pilin, but appeared to cause a modest decrease in the levels of the minority S-pilin form.

### Effect of *pilQ2* on antibiotic resistance

To further investigate the effect of *pilQ2* on gonococcal antibiotic resistance, we generated strains with various combinations of resistance genes in concert with either the *pilQ2* or *pilQ::Ω* allele. When either *pilQ2* or *pilQ::Ω* was introduced into FA19, FA19 *penA*, or FA19 *penA mtrR*, no increase in the MIC of penicillin or tetracycline was observed (Fig. 4, left panels). In contrast, introduction of either of the two *pilQ* alleles into PR100 (FA19 *penA mtrR penB*) or FA6140, a high level penicillin-resistant CMRNG isolate (Danielsson *et al.*, 1986), conferred a 2- to 3-fold increase in the MICs for penicillin and tetracycline (Fig. 4, middle panels). The levels of resistance conferred by *pilQ2* were essentially identical to those conferred by *pilQ::Ω*. Acquisition of *pilQ2* by strains with only one of the two resistance determinants *mtrR* or *penB* was not sufficient to confer resistance to penicillin, consistent with previous data demonstrating that *penB* is unable to increase resistance to penicillin in the absence of *mtrR* (Olesky *et al.*, submitted; Sparling *et al.*, 1975; Veal *et al.*, 2002). In contrast, acquisition of *pilQ2* by a strain harboring both *mtrR* and *penB* resulted in increases in both penicillin and tetracycline resistance (Fig. 4, right panels).

To examine the specificity of the effect of PilQ on the influx of antibiotics, we determined the MICs of a variety of antibiotics for PR100 and PR100 *pilQ2* (data not shown). The MICs of ampicillin, ceftriaxone, and ciprofloxacin each increased ~1.5- to 2-fold in PR100 *pilQ2* compared to PR100, suggesting that these antibiotics readily enter the cell in a PilQ-dependent manner. In contrast, rifampin, chloramphenicol, and erythromycin showed much more modest increases (~1.2-fold) in their MICs for PR100 *pilQ2* compared to PR100. Thus, PilQ promotes the entry of a range of antibiotics into the bacterial cell, although there is some preference for hydrophilic antibiotics.

### The role of Type IV Pilus accessory subunits in conferring penicillin resistance

Since the only known role for PilQ in the gonococci is for pilus assembly, we investigated the role of several accessory proteins involved in type IV pilus biogenesis on antibiotic sensitivity. We introduced deletions of *pilT*, *pilE*, and *pilP* into SZ3 (RM11.2 *penA mtrR penB*), which has a normal *pilQ* allele, and determined the MIC of penicillin in each of these strains (Fig. 5). Pilin proteins form the pilus fiber that requires PilQ for expression outside of the bacterial cell (Wolfgang *et al.*, 2000), and we examined whether the presence of pilin fibers might influence the entry of penicillin into the periplasm. A  $\Delta$ *pilE* mutant showed only a small increase in penicillin resistance, indicating that pilin fibers neither interfere nor assist with antibiotic diffusion into the periplasm. We also examined whether the pilus retraction-associated ATPase, PilT (Maier *et al.*, 2002; Merz *et al.*, 2000), was involved in helping penicillin enter the cell. When the *pilT::erm* allele was introduced into SZ3, no change in resistance was observed, demonstrating that pilus retraction is not involved in the entry of penicillin. Finally, we examined the role of PilP, which has been reported to influence PilQ multimerization (Drake *et al.*, 1997), in altering antibiotic resistance. Deletion of the *pilP* gene in SZ3 resulted in decreased levels of multimeric PilQ in whole-cell lysates by Western blotting, while the levels of PilQ monomer were similar to those of the parent strain (data not shown). The MIC of penicillin for SZ3  $\Delta$ *pilP* increased, but the level of resistance was lower than that observed for SZ3 *pilQ2* or SZ3 *pilQ::Ω* (Fig. 5). These data confirm that deletion of PilP decreases the amount of the SDS-resistant PilQ complex in the outer membrane, and, similar to the *pilQ2* point mutation, results in a decrease in penicillin entry and a subsequent increase in resistance.

### Random mutagenesis of Glu-666

To investigate in more detail the importance of Glu-666 in formation of an SDS-resistant PilQ oligomer, we randomized the codon for Glu-666 in the wild type *pilQ* gene and transformed SZ3 to increased penicillin resistance. The *pilQ* gene was amplified from resistant colonies, and those genes with a silent *NsiI* site, which was present only in the randomized clones, were



chosen for further analysis (Table 2). Sequencing of the *pilQ* genes from these colonies and subsequent Western blotting (data not shown) showed that substitution of Glu-666 with almost any uncharged polar or nonpolar amino acid resulted in the same phenotype as *pilQ2*: i.e. increased penicillin resistance (to the same level as *pilQ2*; Table 2) and inhibition of formation of SDS-resistant PilQ oligomers (data not shown). To our surprise, we did not identify the original Glu-666 to Lys mutation of *pilQ2* in our random screening, nor did we identify any acidic, amide, or other basic residues. To investigate whether these three classes of amino acids were capable of substituting for Glu-666, we employed site-directed mutagenesis to change Glu-666 to Asp, Arg, or Gln. Transformation of SZ3 with the individual mutant plasmids revealed that only the Glu-666 to Arg mutation gave rise to penicillin-resistant colonies with frequencies above controls. The Glu-666 to Arg mutation also interfered with formation of the HMM PilQ multimer as determined by Western blotting (data not shown). These data demonstrate that only acidic or amidated amino acids are capable of replacing Glu-666 and allowing formation of the SDS-resistant PilQ multimer. Moreover, alignment of *N. gonorrhoeae* PilQ near the region of Glu-666 with several of the most homologous PilQ secretins from other bacterial species revealed that the amino acid in the same position as Glu-666 was either Glu, Asp, or Asn (Fig. S1), suggesting that this residue may play an important role in other secretins as well.

### Importance of Cys-644 and Cys-653 in PilQ oligomerization

Early studies on PilQ (then named OMC or OMP-MC) revealed that the high molecular mass complex of PilQ obtained on SDS-PAGE could be converted quantitatively to a single 75 kDa subunit on SDS-PAGE following reduction and alkylation (Hansen and Wilde, 1984; Newhall *et al.*, 1980). There are only two cysteine residues in FA1090 and FA19 PilQ, Cys-644 and Cys-653, both of which are relatively close to the site of the *pilQ2* mutation described herein. To determine whether mutation of these two cysteine residues would also alter PilQ properties, we mutated the codons for the two cysteine residues of PilQ to serine either individually or together and transformed the mutant genes into strain SZ3. Strikingly, strains containing *pilQ* mutations of either cysteine (or both) resulted in a very similar phenotype as SZ3 *pilQ2*. Western blots of PilQ showed the complete absence of the high molecular mass dodecamer, increased levels of the monomer subunit (Fig. 6A), and identical MICs of penicillin compared to SZ3 *pilQ2* (Fig. 6B). Transformation competence of SZ3 containing the *pilQ* cysteine mutants also was decreased to a similar extent as SZ3 *pilQ2* (data not shown). As expected, strains harboring the mutant cysteine *pilQ* alleles displayed a non-piliated colony morphology. These data indicate that cysteine mutations prevent formation of an SDS-resistant PilQ complex and affect antibiotic resistance to the same extent as *pilQ2*.

## DISCUSSION

In the search for genes involved in high-level penicillin resistance ( $\text{MIC} \geq 2 \mu\text{g/ml}$ ), we identified in PR100 a spontaneously arising resistance determinant, termed *penC*, that conferred an increase in both penicillin and tetracycline resistance, suggesting that *penC* alters the general permeability of antibiotics (Ropp *et al.*, 2002). We show here that *penC* results from a point mutation in the *pilQ* gene (*pilQ2*), which changes Glu-666 to Lys and prevents formation of an SDS-resistant high-molecular-mass multimer of PilQ without altering the levels of PilQ in the cell. These observations suggest that the PilQ secretin can facilitate the entry of small molecules into the bacterial cell in addition to its more well-defined role in pilin biogenesis and DNA transformation.

Two major models can account for our results. The most straightforward model is that antibiotics enter the periplasm through the pore made by the PilQ multimer and that the *pilQ2* mutation blocks entry by destabilizing the pore. This model is consistent with reports

that the pIV and PulD secretins function as gated pores when reconstituted into planar lipid bilayers (Marciano *et al.*, 1999; Nouwen *et al.*, 1999). In addition, the structure of meningococcal PilQ obtained by cryo-transmission electron microscopy (Collins *et al.*, 2003) reveals a central cavity 65 Å in diameter at the largest end that tapers to a closed point at the other end, which needs to open to allow the pilus to transverse the outer membrane. This structure is consistent with the idea that PilQ, much like pIV and PulD, can function as a pore through which antibiotics diffuse into the periplasm.

The second major model is that the presence of a nonfunctional PilQ in the outer membrane alters the function of porin or the MtrC-MtrD-MtrE efflux pump, leading to a decrease in antibiotic entry or an increase in antibiotic efflux respectively. However, we believe this second model is unlikely for several reasons. First, both the *pilQ2* and *pilQ::Ω* alleles had identical effects on penicillin resistance when introduced into PR100 or SZ3, even though in a *pilQ2* strain the protein is still present in the outer membrane while in a *pilQ::Ω* strain it is absent. Secondly, neither *pilQ2* nor *pilQ::Ω* had any effect on penicillin or tetracycline resistance when introduced into strains containing the *mtrR* or *penB* gene alone (Fig. 4). Finally, the *pilQ2* mutation appears to increase resistance to hydrophilic antibiotics more than to hydrophobic antibiotics, which is opposite of what would be expected if the *pilQ2* mutation increased the activity of the MtrC-MtrD-MtrE efflux pump (the pump shows preferences for hydrophobic agents and antibiotics; (Hagman *et al.*, 1995)). Taken together, our data are most consistent with the idea that the PilQ secretin forms a pore across the outer membrane that allows the diffusion of antibiotics into the periplasm.

The model of PilQ as a pore-forming protein is also consistent with a previous study investigating heme import into *N. gonorrhoeae* (Chen *et al.*, 2004). In that study, the authors identified several classes of spontaneously arising colonies of FA1090 harboring a deletion in the hemoglobin receptor protein HpuA that were capable of utilizing hemoglobin as the sole source of iron and porphyrins. One class of such mutants contained a F595L mutation in the *pilQ* gene (*pilQ1*), which appeared to alter the pore formed by the PilQ multimer to allow the diffusion of free heme released from hemoglobin into the cell. The *pilQ1* mutation also increased antibiotic sensitivities to several antibiotics and Triton X-100, reinforcing the idea that a measurable influx of antibiotics can occur through the PilQ secretin complex. Interestingly, the *pilQ1* mutation required the pilus-associated ATPase, PilT, for growth on hemoglobin, while we have shown that increase of penicillin resistance conferred by the *pilQ2* mutation is PilT-independent (Fig. 6). This difference may reflect different processes, however, as the heme must enter into the cytoplasm to support growth, whereas penicillin need only diffuse into the periplasm. Indeed, Chen *et al.* (2004) showed that increased sensitivity to ampicillin in the *pilQ1* strain was not dependent on PilT.

Other studies have documented the effects of mutations and/or insertions in secretins on outer membrane permeability. For example, Wall *et al.* (Wall *et al.*, 1999) characterized a mutant allele of *Myxococcus xanthus pilQ*, which is involved in pilin biogenesis and social motility in this organism. The mutant allele contained two missense mutations (G741S and N762G) in its C-terminus that conferred hypersensitivity to vancomycin. Similarly, Russel (Russel, 1994) identified mutants in the pIV secretin involved in export of the filamentous phage f1 that conferred hypersensitivity to both vancomycin and deoxycholate. These *pilQ* mutants therefore resemble the F595L mutation characterized by Chen *et al.* (Chen *et al.*, 2004) in that the alterations increased the susceptibility of the organism to antibiotics and deoxycholate, presumably by increasing the size of the PilQ channel or by changing the conformation of a region that constricts the channel. Interestingly, alignment of the PilQ secretins from *M. xanthus* and *N. gonorrhoeae* shows that one of the two mutations in the mutant *M. xanthus pilQ* allele (N762G) maps to within seven residues of Phe-595 of gonococcal *pilQ1* (Chen *et al.*, 2004), whereas the other mutation is ~25 amino acids distant (data not shown). The similar

location of these mutants in PilQ homologues suggests that this area is involved in defining the size of the PilQ channel.

In contrast to these other studies, in which mutations increased the sensitivity of the strain to antibiotics and detergents, the mutations in gonococcal PilQ characterized here increase resistance provided that at least *mtrR* and *penB* are present. The *pilQ2* mutation also prevents formation of an SDS-resistant PilQ oligomer, but the direct cause and effect of these two observations has yet to be established. To our knowledge, this is the first report of a mutation in PilQ from *Neisseria* that interferes with formation of an SDS-resistant HMM multimer, although a recent report showed that deletion of the presumed chaperone protein, PilW, also resulted in the loss of the SDS-resistant PilQ multimer (Carbonnelle *et al.*, 2005). While the actual effect of the *pilQ2* mutation on PilQ structure is unclear, gel filtration of wild-type and mutant PilQ proteins (Fig. 3D) suggests that at least a portion of the PilQ protein in the *pilQ2* mutant exists as an oligomeric species (presumably, like the wild-type, a dodecamer). These results suggest that the pilin-expressing membraneous projections in FA19 *pilQ2* observed by immunoelectron microscopy (Fig. 1B) may be the result of this oligomeric PilQ interacting with pili and could explain the high residual transformation competence of the *pilQ2* strain (Fig. 2). However, if present these PilQ complexes are unable to facilitate the diffusion of antibiotics across the outer membrane. More knowledge about how *pilQ* assembles and interacts with the pilus assembly proteins will be required before a detailed molecular understanding of the effect of these mutations on the entry of DNA and antibiotics into the bacterial cell is known.

Type IV pilin biogenesis in *N. gonorrhoeae* requires multiple accessory subunits in addition to pilin and PilQ (Wolfgang *et al.*, 1998; Wolfgang *et al.*, 2000). Thus, we also examined the role of pilin, PilT, and PilP in antibiotic resistance by creating deletion mutants in SZ3, a 3<sup>rd</sup> level transformant of RM11.2 *recA6* with a wild type *pilQ* allele (Fig. 5). The lack of a substantial increase in penicillin resistance in SZ3 following deletion of the *pilE* or *pilT* gene shows that neither assembly or disassembly of the pilus fiber alters the ability of PilQ to enable penicillin entry, even though structural modeling suggests that a pilin fiber would fully occupy the central cavity of PilQ (Collins *et al.*, 2003). In contrast, penicillin resistance in SZ3  $\Delta$ *pilP* increased significantly, although not to the same level as the *pilQ2* mutation. Because deletion of PilP decreased the expression of the multimeric form of PilQ without changing the levels of the monomeric form (data not shown; (Drake *et al.*, 1997)), our data suggest that the intermediate increase in resistance is the result of a decrease in the number of stable PilQ multimers in the outer membrane. The simplest model to account for all of these observations is that only some of the PilQ complexes are dedicated to expressing pili, but that all PilQ complexes are influenced by expression of PilP. This model predicts that different complexes of *pilQ* are involved in different functions in the outer membrane and that the decreased multimeric form detected in gonococcal membranes of a *pilP* strain may explain the differential phenotypes of the *pilQ2* mutation.

One of the intriguing aspects of chromosomally mediated resistance is that the resistance determinants must be transferred in a specific order. For example, our results demonstrate that *pilQ2* does not increase the MIC of penicillin or tetracycline unless other resistance determinants are present (Fig. 4). Of the previously described resistance determinants, only *mtrR* and *penB* are necessary for *pilQ2* to increase resistance to penicillin or tetracycline (Fig. 4, right panels). The *penA* gene further increases resistance, but its presence is not required for the phenotypic increase in resistance mediated by *pilQ2* (Fig. 4). The fact that *pilQ2* has no influence on the MIC unless other resistance determinants are present that reduce the concentration of antibiotics in the periplasm (*mtrR* and *penB*) suggests that influx of antibiotics through the PilQ multimer in susceptible strains is only a small fraction of the antibiotic influx compared to that through porin. Only when *mtrR* and *penB* combine to decrease influx through



porins does the influx of antibiotics through PilQ become significant, thereby increasing resistance when PilQ is deleted or mutated (see Figure 8). Because this mutation disrupts normal piliation, increases in resistance as a result of *pilQ* mutations are unlikely to be observed in the clinical setting owing to the importance of pili in mucosal infections (Hamrick *et al.*, 2001; Swanson, 1973), although *pilQ* mutations may have a role in resistance in disseminated infections in which the role of pili is not well established. Nonetheless, our results underscore the role of the PilQ secretin in the diffusion of antibiotics and other molecules into the periplasm of the bacterium.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

FA19 (Maness and Sparling, 1973) is a penicillin-susceptible laboratory strain. FA6140 is a high-level chromosomally mediated resistant strain of *N. gonorrhoeae* isolated in Durham in the 1970's (Danielsson *et al.*, 1986). RM11.2 *recA6* (Seifert, 1997) is a derivative of FA1090 (Dempsey *et al.*, 1991) containing the *recA* gene under regulation of the lac operator/promoter. Other strains used in this work are described in Table 1. Transformations with FA19 strains were carried out exactly as described previously (Ropp *et al.*, 2002). Transformations of RM11.2 *recA6* were similar except that 1 mM IPTG was added to the cells before exposure to DNA to allow for the expression of the *recA* gene product required for homologous recombination (Seifert, 1997). Selection for the *penA4*, *mtrR*, and *penB5* stepwise transformants in RM11.2 *recA6* was carried out on GCB plates with 0.04 µg/ml penicillin G, 2 µg/ml erythromycin, and 0.28 µg/ml penicillin G respectively. SZ3  $\Delta$ *pilE* was constructed by transforming SZ3 with genomic DNA from a strain of FA1090 with a complete deletion in the *pilE* gene (kindly provided by Dr. Janne Cannon, UNC-Chapel Hill) and selecting for nonpiliated colonies.

In order to generate cell lines harboring the *pilQ2* mutation, we constructed a plasmid (pUC18-*pilQ2*-kan) containing the last 864 bp of FA19 *pilQ2* (encoding residues 445-731), the kanamycin phosphotransferase gene inserted 20 bp past the *pilQ* stop codon, and 280 bp of additional downstream sequence, and used this plasmid to transform the various recipient strains to kanamycin resistance. A silent *NsiI* site was created by changing T to G at nucleotide 1959 of the FA19 *pilQ2* gene to aid in identifying transformants containing the *pilQ2* mutation. To generate strains with a disrupted *pilQ* allele (*pilQ*:: $\Omega$ ), strains were transformed with pUNCH290 (Chen *et al.*, 2004). pUNCH290 comprises nucleotides 434 of the FA1090 *pilQ* gene to 152 bp downstream of the stop codon, in which an internal *HincII* fragment was replaced with the  $\Omega$  fragment conferring spectinomycin resistance (Prentki and Krisch, 1984). All strains were verified by PCR amplification and sequencing, and by Western blotting to verify the absence of the high molecular mass PilQ oligomer (*pilQ2* strains) or PilQ reactivity (*pilQ*:: $\Omega$ ).

### Growth conditions and MIC determinations

*N. gonorrhoeae* were grown on GCB plates with Supplements I and II (Kellogg *et al.*, 1963) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, and *E. coli* were grown on LB plates or in 2X YT media (Sambrook *et al.*, 1989) as described (Ropp *et al.*, 2002). MICs were determined by the spot method as described (Ropp *et al.*, 2002). Briefly, cells were resuspended at a density of  $1 \times 10^7$  cells/ml and 5 µl (50,000 colonies) were spotted onto GCB agar plates containing increasing concentrations of the appropriate antibiotics. The MIC was defined as the lowest concentration at which no more than 5 colonies grew following incubation for 24 hr. MICs were determined for multiple transformants and represent the average  $\pm$  S.E.M of at least three independent experiments.

### Cloning the *pilQ2* gene

Chromosomal DNA from PR100 *penC* (Ropp *et al.*, 2002) was digested with *ClaI*, separated on a 0.7% agarose gel, and ten fractions ranging in size from 12 kb to 300 bp were tested for their ability to transform PR100 to penicillin resistance on GCB with 1.2 µg/ml penicillin. Fraction 2, comprising fragments from ~ 8 to 10 kb, was enriched in the *pilQ2* gene and was used to create a mini-library in *ClaI*-digested pBeloBac 11 bacmid vector (Kim *et al.*, 1996). Initial attempts to clone the gene into the medium copy-number plasmid pACYC184 were unsuccessful, necessitating the use of the single copy-number bacmid vector. Three clones were found to transform PR100 to penicillin resistance with high frequency. Each clone harbored an identical 10.6 kb insert containing seven genes: the 3'-end of *pilQ*, *aroK*, *aroB*, *yafJ*, and three hypothetical genes of unknown function. The location of the *penC* gene was narrowed down by amplifying each gene and testing the fragments in the transformation assay. Only the fragments containing the *pilQ* gene transformed PR100 to higher penicillin resistance with high frequency, demonstrating that the *penC* mutation maps to the 3'-end of the *pilQ* gene (bp 1333-2196 encoding residues 445-731). All three clones revealed the identical basepair mutation.

### Immunoelectron microscopy of pilin fibers

Analysis of the piliation states of gonococcal strains was determined exactly as described previously (Chen *et al.*, 2004). The C-terminal domain of FA19 PilQ differs from FA1090 PilQ by two amino acid differences (G523R and N620S). To ensure that sequences of PilQ had no bearing on the piliation state of the gonococci, the C-terminal domain of *pilQ* from RM11.2 *recA6* (an FA1090 derivative) was also exchanged with the corresponding region from FA19. A plasmid identical to the one used to transfer the *pilQ2* allele into strains (see above), except that it contained the wild type sequence from FA19, was used to construct RM11.2 *recA6 pilQ*<sub>FA19</sub>.

### Transformation efficiency

Strains were passaged on GCB agar plates, resuspended in GCB with supplements I and II, 20 mM NaHCO<sub>3</sub>, 10 mM MgCl<sub>2</sub> and 1 mM IPTG (GCB+), and diluted to OD<sub>600</sub> = 0.18 in the same medium. Twenty µl of cells and 100 ng of pSY6 were added to 180 µl of GCB+ and incubated at 37°C in 5% CO<sub>2</sub>/95% air for 15 min. GCB+ (1.8 ml) was then added and the cells were incubated for a further 5 hrs, followed by serial 10-fold dilutions in GCB+. A portion (50 µl) of the appropriate dilution was plated in duplicate on GCB + 2 µg/ml nalidixic acid (NAL) plates. For determination of total CFU, 50 µl of a 1 × 10<sup>4</sup> dilution were plated on GCB plates.

### Electrophoresis and Western blotting

For SDS-PAGE and Western blotting of whole cell lysates, cells were swabbed from GCB plates, resuspended in GCB+ at an OD<sub>560</sub> of 0.18, and 1 ml was removed to a microfuge tube. After pelleting the cells and removal of the medium, 100 µl of SDS-PAGE loading buffer was added and the cells were dispersed. Three to ten µl of the samples were loaded onto SDS-polyacrylamide gels (8 or 10%) and separated by electrophoresis. The proteins in the gel were then electrotransferred to nitrocellulose filters overnight in 25 mM Tris, 192 mM glycine, and 10% methanol. The lower methanol appeared to increase the efficiency of transfer of the high molecular mass PilQ oligomer. Following transfer, the filter was blocked with 5% dry milk in phosphate-buffered saline with 0.1% Tween (PBS-T), then incubated with a 1:10,000 dilution of a PilQ antibody (kindly provided by Charles E. Wilde III, Indiana University School of Medicine) for 2 hrs at 25°C. The filter was washed 3 times with PBS-T, and incubated for another 1 hr with HRP-conjugated mouse anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ). After washing the filter, PilQ bands were visualized with SuperSignal West Pico (Pierce Chemical Co., Rockford, IL).

For pilin blots, cells were prepared as described above, checked for protein levels to ensure equal loading, and separated on 15% SDS-polyacrylamide gels. Proteins were transferred from gels to PVDF membranes, and membranes were blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween. The membranes were probed with the pilin monoclonal antibody 1E8/G8 (gift from M. Koomey and M. Blake), washed, incubated with HRP-conjugated goat anti-mouse secondary antibody (Roche Molecular Biochemicals), and washed again. The ECL Plus Western Blot kit (Amersham Biosciences) was used to detect pilin bands.

### Gel Filtration of PilQ proteins

Liter cultures of FA19 (wild-type PilQ) and FA19 *pilQ2* (PilQ-E666K) were grown in GCB broth with Supplements B, I and II, and 10 mM sodium bicarbonate. Cells were pelleted, lysed with an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada), and membranes were isolated by centrifugation at 100,000g. The membrane pellets were dounce-homogenized in Buffer A (20 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 8.0), mixed with an equal volume of 40 mM Tris, 4% SB-10, 3 M NaCl, 2 mM EDTA, pH 8, and incubated on ice for 1 h. The membranes were pelleted at 15,000g for 30 min, and the supernatants containing the extracted PilQ proteins were filtered through 0.45µm filters. Aliquots (300 µl) were submitted to gel filtration on a Sephacryl S-500 column (16 mm × 70 cm) in 20 mM Tris, pH 8, 500 mM NaCl, 1 mM EDTA, 1.2% SB-10. Twenty µl from the indicated fractions were processed for SDS-PAGE and Western blotting, and the resulting bands were imaged on a Bio-Rad Fluoro-S system and quantitated with QuantityOne software (Bio-Rad, Hercules, CA).

### Site-directed and site-saturation mutagenesis of *pilQ*

To generate a library of *pilQ* fragments in which the codon for Glu-666 was randomized, we utilized overlap-extension PCR (Ho *et al.*, 1989). The sense mutagenic primer (5'-TGAATACGCAGGCTATGGTTNNNAACGGCGGCACTTTGATTG-3') comprised 20 bp at the 5' end of codon-666, three random bases, and 19 bp at the 3' end of codon-666, while the antisense mutagenic primer (5'-AACCATAGCCTGCGTATTCA-3') comprised the reverse complement of the first 20 bp of the sense primer. The template was a plasmid vector (pUC18) containing a portion of the *pilQ* gene starting at 1333 bp to 300 bp downstream of the stop codon. In the first set of reactions, a sense (relative to the direction of *pilQ*) vector primer and the antisense mutagenic primer or the sense mutagenic primer and an antisense vector primer were used to amplify the same template in separate amplifications. The resulting fragments were isolated, combined, and the full-length *pilQ* gene with a randomized codon at position-666 was generated in a second PCR reaction with only the vector primers. The mutagenized PCR fragments were purified on a Marligen (Gaithersburg, MD) PCR purification column and used to transform PR100 to penicillin resistance. Resistant colonies were passaged, examined by western blotting to determine clones expressing only the monomeric PilQ subunit, and the *pilQ* gene from those clones were amplified from genomic DNA and sequenced to determine the identity of the mutation. Site-directed mutagenesis was carried out with the same template described above using the QuickChange kit from Stratagene (La Jolla, CA).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### ACKNOWLEDGEMENTS

This work was supported by NIH grants R01 AI36901 (RAN) and R01 AI055977 (HSS). We thank Sohban Nandi and Josh Tomberg for helpful discussions. We also thank Janne Cannon, Fred Sparling, and Ching-ju Chen for generous access to reagents and stimulating discussions.

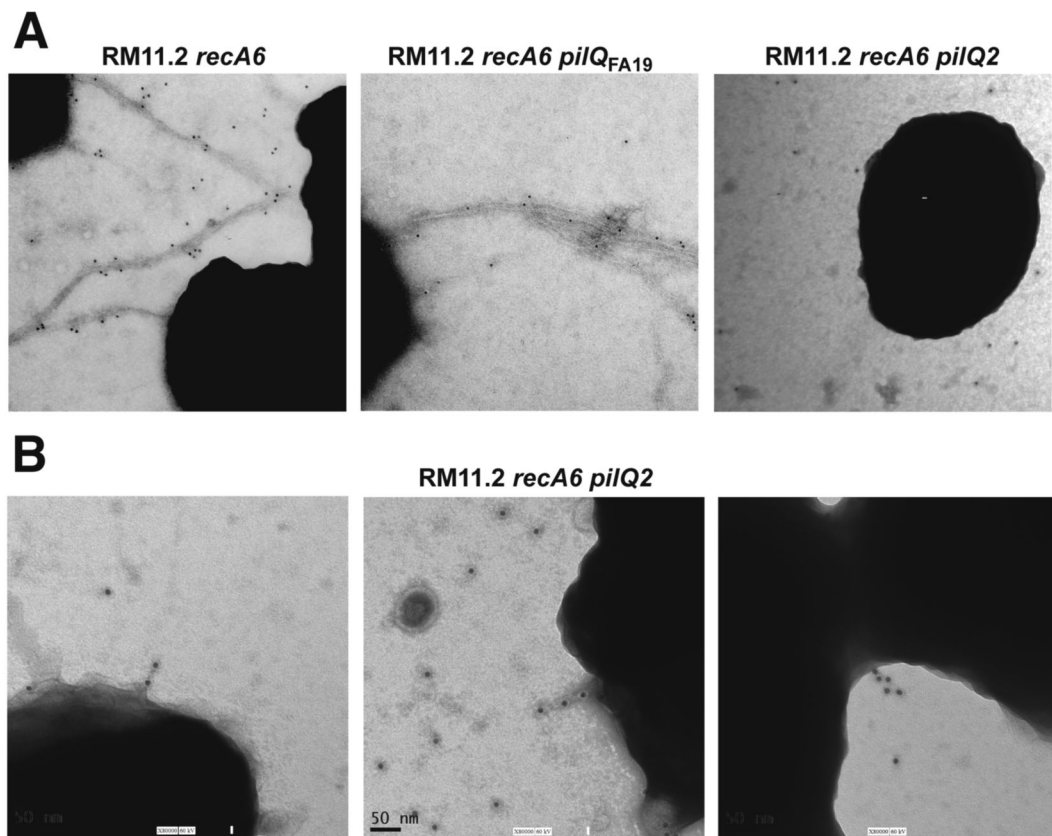
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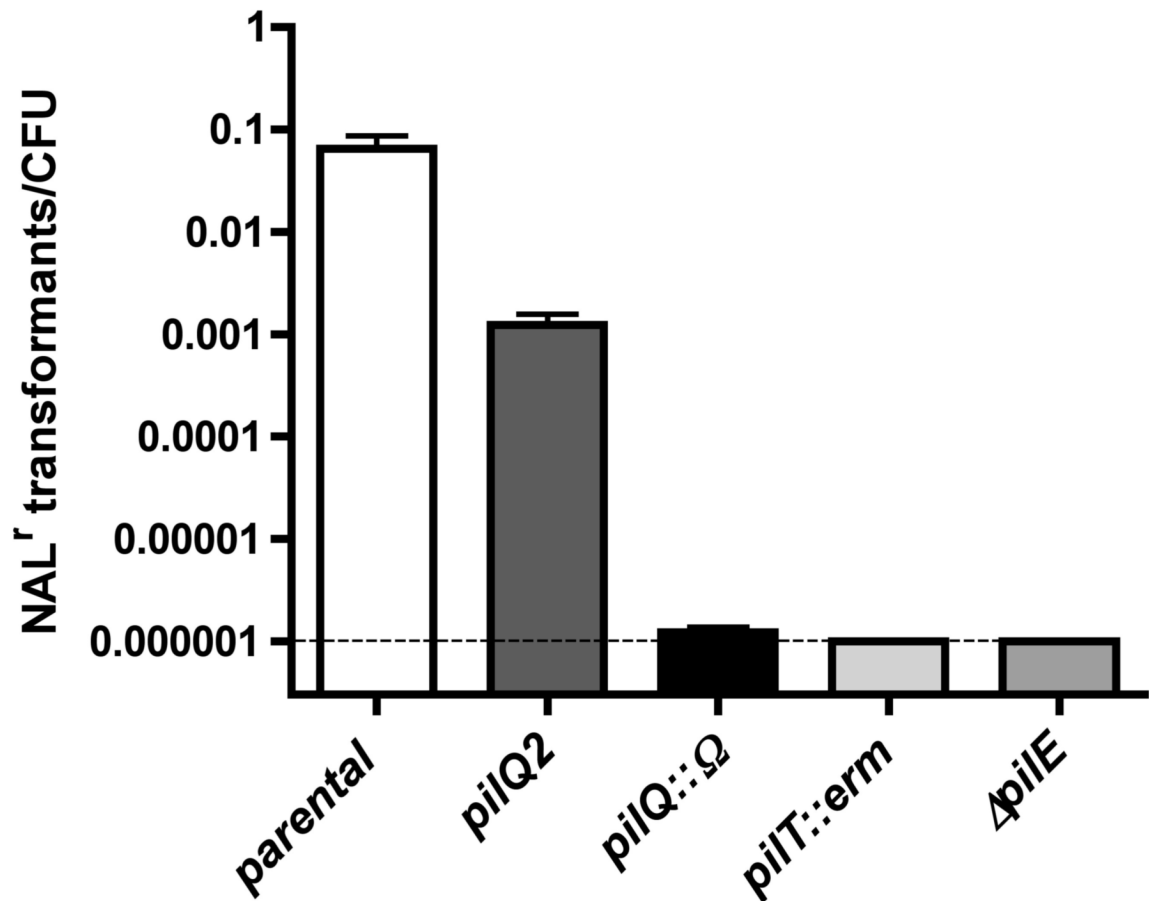


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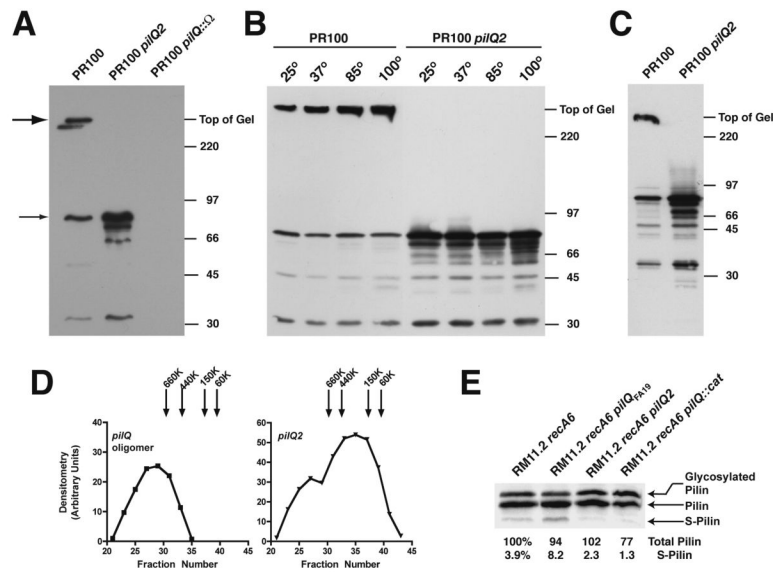
**Figure 1. Electron micrographs of immunogold-labeled pili from strain RM11.2 *recA6* with different *pilQ* alleles**

(A) RM11.2 *recA6* (left panel), RM11.2 *recA6 pilQ*<sub>FA19</sub> (middle panel), and RM11.2 *recA6 pilQ2* (right panel) were constructed as described in Materials and Methods. Strains were grown on GCB plates and colonies were lifted directly onto Formavar-coated grids. Pili were detected with an anti-pilin antibody and detected with a gold-conjugated secondary antibody as described previously (Long *et al.*, 2003). Whereas most of the RM11.2 *recA6 pilQ2* cells were as shown in the right panel of (A), a small number of cells had small projections that were labeled with the anti-pilin antibody (B).



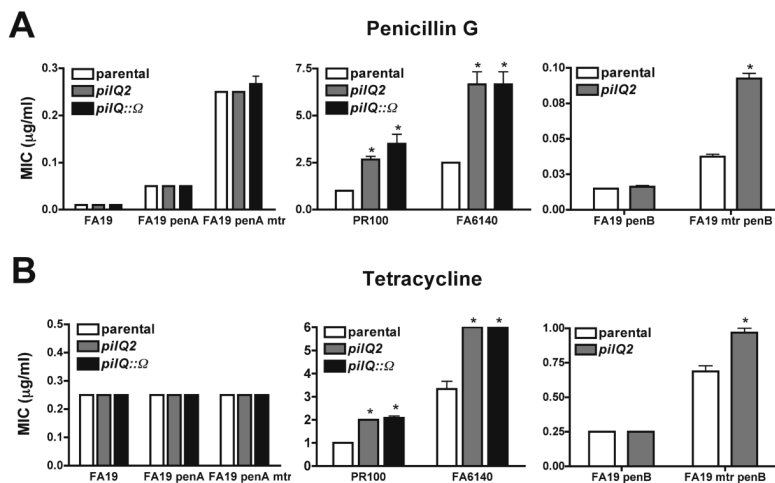
**Figure 2. Transformation competence of SZ3 (RM11.2 *recA6 penA mtrR penB*) and SZ3 with *pilQ2*, *pilQ::Ω*, *pilE*, or *pilT::erm* alleles**

The indicated strains were prepared as described in Materials and Methods and incubated with 100 ng of pSY6, which confers resistance to nalidixic acid (NAL<sup>r</sup>), for 5 hrs before plating. The transformation efficiency is expressed as the number of NAL<sup>r</sup> transformants per CFU. The dotted line represents the lowest transformation efficiency that was measurable. The average  $\pm$  S.D. for three independent experiments is shown.



**Figure 3. Western blots of PilQ from strains with wild type and *pilQ2* alleles**

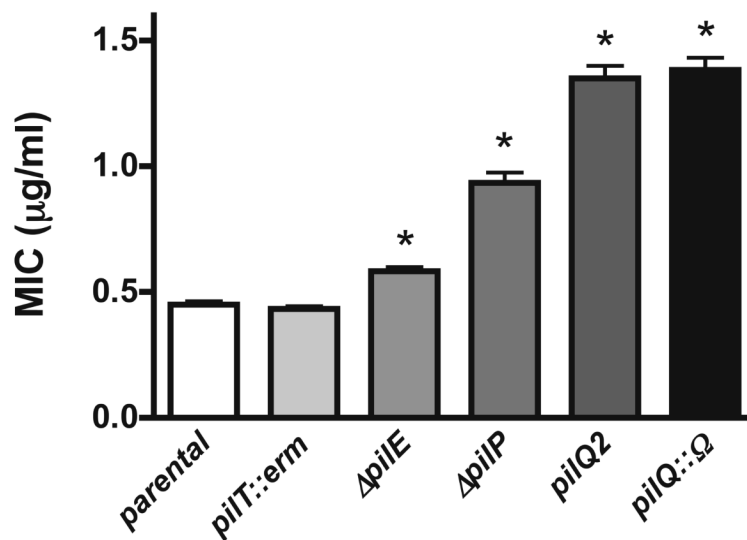
Western blots of whole cell lysates (A, B) or outer membranes (C) probed with a polyclonal antibody against PilQ. The large arrow indicates the SDS-resistant high-molecular-mass PilQ multimer, whereas the small arrow indicates the PilQ monomer. In panels (A) and (C), samples in SDS-loading buffer were incubated at 100°C for 5 min before loading, while in panel (B), samples were treated for 5 min at the indicated temperature before loading. The outer membranes in (C) were prepared as described by Heckels (1977). In panel (D), proteins from FA19 (left) or FA19 *pilQ2* (right) membranes extracted into SB-10 were submitted to gel filtration on Sephacryl S-500. The elution of PilQ oligomer (left graph) and total PilQ (right graph) was assessed by quantitation of Western blots as described in Materials and Methods. The arrows at the top denote the positions of protein standards (MWs are listed above the arrows). In panel (E), Western blots of whole cell lysates were probed with a monoclonal antibody directed against the pilin protein. Percentage of total pilin protein (RM11.2 *recA6* taken as 100%) and S-pilin (reported as the % of total pilin in that lane) were determined by densitometry and are listed below each lane.



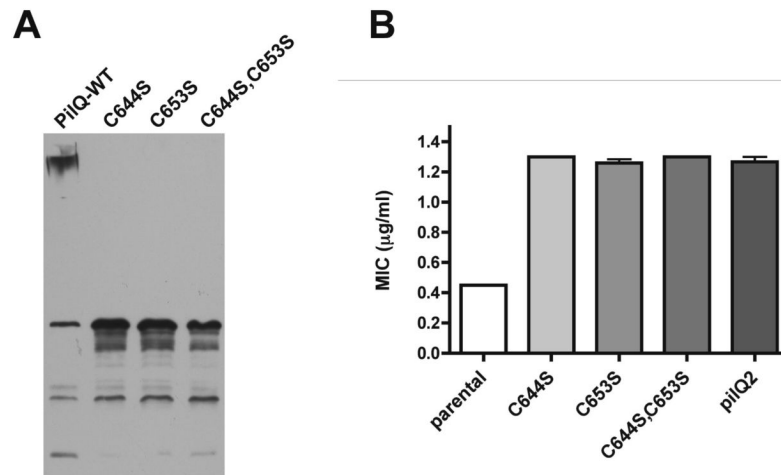
**Figure 4. Penicillin and tetracycline resistance of strains containing wild type, *pilQ2*, and *pilQ::Ω* alleles**

FA19 harboring various combinations of the *penA* (mutations in PBP 2), *mtrR* (increased expression of the MtrC-MtrD-MtrE efflux pump) and *penB* (mutations in porin; see Table 1 for more information) resistance determinants was transformed with plasmids encoding mutant (*pilQ2*) and inactivated *pilQ* (*pilQ::Ω*) alleles and the MICs of penicillin G and tetracycline for the resulting strains were determined as described in Materials and Methods. *pilQ2* and *pilQ::Ω* were also introduced into FA6140, a chromosomally mediated penicillin-resistant clinical isolate (Danielsson *et al.*, 1986). \**P*<0.01 relative to the parental strain.

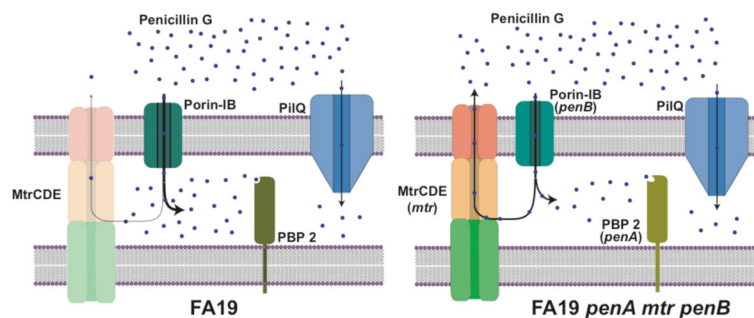




**Figure 5. MICs of penicillin for SZ3 with deletions in Type IV pilus-associated genes** SZ3 (RM11.2 *recA6 penA4 mtrR penB5*; see Table 1) was transformed with plasmids or genomic DNA containing inactivated or deleted alleles of *pilT*, *pilE* and *pilP*, and the MICs of penicillin for multiple colonies from each transformation and for SZ3 *pilQ2* and SZ3 *pilQ::Ω* were determined as described in Materials and Methods. \* $P < 0.01$  relative to the parental strain.



**Figure 6. Effects of Cys to Ala mutations in PilQ when expressed in SZ3**  
Cys-643 and Cys-654 were changed individually or together to Ser and the mutant alleles were transformed into SZ3. (A) Western blots of PilQ in whole cell lysates. (B) MICs of penicillin for SZ3 with wild type (parental) and the indicated mutant *pilQ* alleles.



**Figure 7. Model showing why the *pilQ2* and *pilQ::Ω* alleles increase resistance only in strains also harboring *mtrR* and *penB* mutations**

The two panels show a schematic representation of the cell envelope of *N. gonorrhoeae*. *Left panel*, FA19. In this strain the entry of penicillin through porin is much greater than the entry through the PilQ complex. The low level of the MtrC-MtrD-MtrE efflux pump is represented by light shading. *Right panel*, PR100. This strain has acquired *penA*, *mtrR*, and *penB* resistance determinants, resulting in the expression of altered forms of PBP 2 and porin IB and overexpression of the MtrC-MtrD-MtrE efflux pump. The *mtrR* and *penB* determinants function to lower the influx of penicillin through porin such that entry of penicillin through the PilQ complex becomes a significant portion of the total influx. Only in PR100 will functional deletion of the PilQ complex (due to *pilQ2* or *pilQ::Ω*) lead to increase resistance to penicillin. See text for details.

Table 1

## Strains and plasmids used in this study

| Strain or plasmid                        | Description <sup>a</sup>   | Reference                         |
|--|--|-----------------------------------|
| pUC18- <i>pilQ2</i> -kan                 | Plasmid containing bp 1333-2196 of <i>pilQ2</i> , a kan resistance cassette, and an additional 300 bp of downstream sequence                         | This study                        |
| pUC18- <i>pilQ</i> <sub>FA19</sub> -kan  | Same as above without the mutation at Glu-666  | This study                        |
| pUNCH290                                 | Plasmid containing <i>pilQ</i> disrupted by the $\Omega$ fragment  | (Chen <i>et al.</i> , 2004)       |
| pMO/ <i>porIB</i> -G120K-erm             | Plasmid containing the mature coding sequence of PIB with a G120K mutation, an erm resistance cassette, and additional 300 bp of downstream sequence | (Olesky <i>et al.</i> , 2002)     |
| pSY6                                     | Plasmid containing gonococcal DNA fragment encoding nalidixic acid resistance  | (Stein <i>et al.</i> , 1991)      |
| FA19                                     | Penicillin-susceptible laboratory strain   | (Maness and Sparling, 1973)       |
| FA6140                                   | Penicillin-resistant clinical isolate  | (Danielsson <i>et al.</i> , 1986) |
| FA19 <i>penA4</i>                        | FA19 × FA6140 DNA (0.03 µg/ml pen <sup>b</sup> )   | (Ropp <i>et al.</i> , 2002)       |
| FA19 <i>penA4 pilQ2</i>                  | FA19 <i>penA4</i> X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)  | This study                        |
| FA19 <i>penA4 pilQ::Ω</i>                | FA19 <i>penA4</i> X pUNCH290 (100 µg/ml spt)   | This study                        |
| FA19 <i>penA4 mtrR</i>                   | FA19 <i>penA4</i> X FA6140 DNA (1 mg/ml Triton X-100)  | (Ropp <i>et al.</i> , 2002)       |
| FA19 <i>penA mtrRpilQ2</i>               | FA19 <i>penA4 mtrR</i> X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)   | This study                        |
| FA19 <i>penA mtrR pilQ::Ω</i>            | FA19 <i>penA4 mtrR</i> X pUNCH290 (100 µg/ml spt)  | This study                        |
| FA19 <i>penB</i>                         | FA19 X pMO/ <i>porIB</i> -G120K-erm (6 µg/ml erm)  | This study                        |
| FA19 <i>penB pilQ2</i>                   | FA19 <i>penB</i> X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)   | This study                        |
| FA19 <i>mtrR penB</i>                    | FA19 <i>mtrR</i> X <i>porIB</i> -G120K-erm (6 µg/ml erm)   | This study                        |
| FA19 <i>mtrR penB pilQ2</i>              | FA19 <i>mtrR penB</i> X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)  | This study                        |
| PR100                                    | FA19 <i>penA4 mtrR penB5</i>   | (Ropp <i>et al.</i> , 2002)       |
| PR100 <i>pilQ2</i>                       | PR100 X pUC18- <i>penC</i> -kan (50 µg/ml kan)   | This study                        |
| PR100 <i>pilQ::Ω</i>                     | PR100 X pUNCH290 (100 µg/ml spt)   | This study                        |
| FA6140 <i>pilQ2</i>                      | FA6140 X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)   | This study                        |
| FA6140 <i>pilQ::Ω</i>                    | FA6140 X pUNCH290 (100 µg/ml spt)  | This study                        |
| RM11.2 <i>recA6</i>                      | FA1090 with IPTG-regulatable <i>recA</i> allele  | (Long <i>et al.</i> , 1998)       |
| RM11.2 <i>recA6 pilQ</i> <sub>FA19</sub> | RM11.2 <i>recA6</i> X pUC18- <i>pilQ</i> <sub>FA19</sub> -kan (50 µg/ml kan)   | This study                        |
| RM11.2 <i>recA6 pilQ2</i>                | RM11.2 <i>recA6</i> X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)  | This study                        |
| SZ3                                      | RM11.2 <i>recA6 penA4 mtrR penB5</i>   | This study                        |
| SZ3 <i>ApilE</i>                         | <i>pilE</i> deletion strain of SZ3   | This study                        |
| SZ3 <i>pilT::erm</i>                     | SZ3 X RM11.2 <i>recA6 pilT::erm</i> (6 µg/ml erm)  | This study                        |
| SZ3 <i>ApilP</i>                         | SZ3 X pUC18- $\Delta$ <i>pilP</i> (0.5 µg/ml pen)  | This study                        |
| SZ3 <i>pilQ2</i>                         | SZ3 X pUC18- <i>pilQ2</i> -kan (50 µg/ml kan)  | This study                        |
| SZ3 <i>pilQ::Ω</i>                       | SZ3 X pUNCH290 (100 µg/ml spt)   | This study                        |

<sup>a</sup>Strains were constructed by homologous recombination as described in Materials and Methods and are shown as parental strain X donor DNA (selection agent). All relevant genotypes were confirmed by DNA sequencing.

<sup>b</sup>The abbreviations are: pen, penicillin; kan, kanamycin; spt, spectinomycin; erm, erythromycin.

**Table 2****Site-saturation mutagenesis of codon-666 of PilQ**

The amino acids at position-666 of PilQ that increased penicillin resistance and disrupted formation of the SDS-resistant multimer.

| Amino Acid at Position-666 | No. Clones Isolated   | MIC <sub>pen</sub> (μg/ml) |
|----------------------------|-----------------------|----------------------------|
| Glutamate                  | <i>pilQ</i> Wild Type | 0.5                        |
| Lysine                     | <i>pilQ2</i>          | 1.2                        |
| Alanine                    | 2                     | 1.2                        |
| Cysteine                   | 6                     | 1.2                        |
| Glycine                    | 7                     | 1.2                        |
| Isoleucine                 | 4                     | 1.2                        |
| Leucine                    | 3                     | 1.2                        |
| Phenylalanine              | 1                     | 1.2                        |
| Serine                     | 1                     | 1.2                        |
| Threonine                  | 3                     | 1.2                        |
| Tryptophan                 | 1                     | 1.2                        |
| Tyrosine                   | 1                     | 1.2                        |
| Valine                     | 4                     | 1.2                        |
| Arginine <sup>a</sup>      | Site-directed         | 1.2                        |
| Aspartic Acid <sup>a</sup> | Site-directed         | (0.5) <sup>b</sup>         |
| Glutamine <sup>a</sup>     | Site-directed         | (0.5) <sup>b</sup>         |

<sup>a</sup>Constructed by site-directed mutagenesis.

<sup>b</sup>Not determined; transformation of SZ3 with pUC18-*pilQ* E666D or pUC18-*pilQ* E666Q did not give rise to resistant colonies above negative controls.