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A Mutant Form of the *Neisseria gonorrhoeae* Pilus Secretin Protein PilQ Allows Increased Entry of Heme and Antimicrobial Compounds†

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A spontaneous point mutation in *pilQ* (*pilQ1*) resulted in phenotypic suppression of a hemoglobin (Hb) receptor mutant (*hpuAB* mutant), allowing gonococci to grow on Hb as the sole source of iron. PilQ, formerly designated OMP-MC, is a member of the secretin family of proteins located in the outer membrane and is required for pilus biogenesis. The *pilQ1* mutant also showed decreased piliation and transformation efficiency. Insertional inactivation of *pilQ1* resulted in the loss of the Hb utilization phenotype and decreased entry of free heme. Despite the ability of the *pilQ1* mutant to use Hb for iron acquisition and porphyrin, there was no demonstrable binding of Hb to the cell surface. The *pilQ1* mutant was more sensitive to the toxic effect of free heme in growth medium and hypersensitive to the detergent Triton X-100 and multiple antibiotics. Double mutation in *pilQ1* and *tonB* had no effect on these phenotypes, but a double *pilQ1 pilT* mutant showed a reduction in Hb-dependent growth and decreased sensitivity to heme and various antimicrobial agents. Insertional inactivation of wild-type *pilQ* also resulted in reduced entry of heme, Triton X-100, and some antibiotics. These results show that PilQ forms a channel that allows entry of heme and certain antimicrobial compounds and that a gain-of function point mutation in *pilQ* results in TonB-independent, PilT-dependent increase of entry.

Mammalian hosts use iron-binding proteins and iron-sequestering compounds to maintain free iron at a level that is too low for the growth of invading pathogens. Many pathogenic bacteria have evolved mechanisms for scavenging essential iron in vivo from transferrin, lactoferrin, hemoglobin (Hb), and other sources. The ability to scavenge iron is important to bacterial growth in vivo and therefore virulence (49). Many bacteria acquire iron by secretion of iron siderophores, but pathogenic neisseriae do not produce siderophores, relying instead on specific receptors for binding host iron compounds, including transferrin, lactoferrin, and Hb (12, 35). Either the transferrin or the lactoferrin receptor is essential for gonococci to cause urethral infection in male volunteers (1, 11). Moreover, the presence of both the transferrin and lactoferrin receptors provides a selective advantage during experimental male urethral infection (1, 11). The phase-variable gonococcal HpuAB Hb receptor apparently is not involved in infection of the male urethra but is selected in vivo in women during the first half of the menstrual cycle (2) and therefore also is important to infection.

Mechanisms of iron uptake from Hb by the pathogenic neisseriae are well studied (5, 6, 7, 21, 22, 23, 24, 41, 42), but many details remain unclear. Meningococci express two Hb receptors: HmbR for binding Hb or HpuAB for binding either Hb alone or Hb bound to its serum binding protein haptoglobin (22). Gonococci express only the HpuAB receptor, although they do possess an *hmbR* pseudogene (42). HpuA is a lipoprotein, and HpuB is an integral outer membrane protein. Both HpuA and -B are essential for use of Hb as an iron source (5). Construction of *hemA* mutants that cannot synthesize heme proved that gonococci which express HpuAB can utilize Hb-bound heme as a source of porphyrins, and thus heme enters the cytoplasm intact (48). The function of the HpuAB receptor is dependent on activation by TonB (3). HpuA facilitates binding of Hb to the receptor and protects free heme released on the outer surface of the bacterium from binding by human serum albumin (HSA) (6). Heme released from Hb bound to the HpuAB receptor apparently traverses a pore within HpuB in a TonB-dependent manner (6) and then crosses the periplasmic space and cytoplasmic membrane by unknown mechanisms.

Gonococci and meningococci also can utilize free heme for growth in the absence of an Hb receptor (16, 44). Gonococcal growth with free heme does not require TonB (3, 7, 44, 48) and, although the existence of a heme receptor has been suggested (19, 20), one has never been clearly identified in either meningococci or gonococci. At present, pathways for entry and transport of free heme in the absence of an Hb receptor are not well understood.

Recently, we utilized an *hpuA* deletion mutant of gonococcal

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† This paper is dedicated to the memory of the late Igor Stojiljkovic.

strain FA1090 to isolate two classes of Hb-utilizing (Hb^+) mutants. One class required TonB and had point mutations in *hpuB* that restored entry of heme from Hb in the absence of HpuA, whereas the other required neither TonB nor HpuAB (6). The HpuAB- and TonB-independent Hb^+ mutants were designated *hgbX*, in recognition of the lack of understanding of the molecular basis for the Hb-utilizing phenotype. The *hgbX* mutants exhibited increased sensitivity to certain antimicrobial agents and grew normally on both Hb and low concentrations of free heme.

We report here that two of the mutants formerly designated *hgbX* are due to a specific point mutation in the *pilQ* gene (*pilQ1*) that allows gonococci to utilize Hb as a source of essential heme iron and porphyrins in the absence of an Hb receptor. Formerly designated the major subunit of outer membrane protein-macromolecular complex (OMP-MC) (18, 47), PilQ is a member of the secretin family of proteins (34), which functions in type IV pilus organelle biogenesis in *Neisseria gonorrhoeae* (13, 52). The type IV pilus is required for twitching motility and DNA transformation competence (30, 50), and the *pilQ1* point mutation allows for both pilus biogenesis and DNA transformation but at slightly reduced levels. We demonstrate that the *pilQ1* point mutation that results in use of Hb as a heme and iron source in the absence of an Hb receptor also results in elevated sensitivities to various antimicrobial compounds. The phenotype is dependent on the expression of the mutant form of PilQ and in part on expression of PilT. Thus, this mutant PilQ allows for increased entry of exogenous compounds into the bacterial cell in addition to its role in pilus fiber extension.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parent strain used in this study was FA1090. Plasmids and gonococcal strains constructed and/or utilized in this study are listed in Table 1. The growth conditions of gonococcal and *Escherichia coli* strains were as described previously (43). Strains unable to utilize Hb as the sole source of iron (Hb^-) were grown on Bacto GC medium base (Difco/Becton Dickinson, Sparks, Md.) plates (GCB plates) containing Kellogg's Supplement I plus 12 μM ferric nitrate. Strains that were able to grow on Hb (Hb^+) were grown on GCB plates or modified GCB plates (Hb-Des plates) containing human Hb at a concentration of 2 μM and desferoxamine mesylate (Desferal) at 100 μM instead of ferric nitrate.

Antibiotics in growth media were used at the indicated concentrations unless otherwise noted: for *E. coli* strains, ampicillin (AMP) at 100 μg/ml, erythromycin (ERY) at 100 μg/ml, chloramphenicol (CHL) at 30 μg/ml, kanamycin (KAN) at 30 μg/ml; for gonococcal strains: erythromycin at 1 μg/ml (ERY^1), chloramphenicol (CHL) at 1 μg/ml (CHL^1), spectinomycin (SPT) at 100 μg/ml (SPT^{100}), and tetracycline (TET) at 2 μg/ml (TET^2). A stock solution of heme was prepared by dissolving 10 mg of bovine hemin chloride in 1 ml of 0.1 N NaOH. A stock solution of Hb was prepared by dissolving 100 mg of human Hb in 10 ml of 10 mM HEPES (pH 7.4). The solution was rocked overnight at 4°C and then filtered through a 0.45-μm-pore size Acrodisc (Pall Gelman Laboratory, Ann Arbor, Mich.).

Reagents, oligonucleotides, and DNA sequencing. All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise indicated. Oligonucleotides were synthesized at the Lineberger Comprehensive Cancer Center DNA Synthesis Facility of the University of North Carolina–Chapel Hill. Sequencing was carried out at the Automated DNA Sequencing Facility of the University of North Carolina–Chapel Hill with an Applied Biosystems model 373 DNA sequencer by use of the *Taq* dye terminator cycle sequencing kit (Applied Biosystems/Perkin-Elmer, Foster City, Calif.) or in the Seifert laboratory on a Beckman Coulter CEQ L system. All oligonucleotides used as primers for PCR or DNA sequencing of *pilQ* are listed in Table 2.

Construction of FA7168, FA7186 and mutagenesis of *tonB* and *hemA*. Construction of *hpuA* deletion (Δ*hpuA*) mutants which express HpuB under the control of the *hpuA* promoter and isolation of FA7168, a Δ*hpuA hpuB*::*cat hgbX* mutant that grew on Hb, were described previously (6). Isolation of the Hb^+ FA7168 from the Hb^- FA7167 (also Δ*hpuA hpuB*::*cat*) prompted us to seek Hb^+ revertants among derivatives of FA7169 (Δ*hpuA hpuB*$^+$) that were unable to grow on Hb. A *hmbR*::Ω derivative of FA7169 that cannot revert to Hb^+ by point mutations in the pseudogene, *hmbR*, was named FA7186. Hb^+ mutants of FA7186 were identified by plating cell suspensions on Hb-Des plates and picking colonies that grew well when passed onto fresh Hb-Des plates.

TonB dependence of Hb^+, *hgbX* mutants was tested by insertional inactivation of *tonB* with the Ω cassette. This procedure utilized the *tonB*::Ω plasmid pUNCH173 (3) to transform various Δ*hpuA* mutants with selection for resistance to SPT. The ability of various mutants to utilize Hb as a heme source was tested by the construction of *hemA* mutants utilizing the *hemA*::Ω plasmid pUNCH1306 for transformation as described by Turner et al. (48).

Cloning the *pilQ1* gene. FA7168 chromosomal DNA fragments of various sizes were obtained after *Sau*3AI partial digestion and agarose gel electrophoresis fractionation (10). Fragments of 0.5 to 4 kb eluted from gel slices were divided into five fractions and transformed into FA7167. The fraction with DNA fragments of 1.6 to 3 kb was enriched in the gene (see below) that transformed FA7167 to Hb^+. A plasmid DNA library of this fraction was prepared by inserting DNA fragments into *Bam*HI-digested, shrimp alkaline phosphatase (Boehringer Mannheim)-treated, pBluescript II KS(+) (Stratagene, La Jolla, Calif.) and cloning them in *E. coli* DH5αMCR (Gibco-BRL, Gaithersburg, Md.). Pools of plasmid DNA were generated and used to individually transform Hb^- FA7167 to Hb^+. Active pools were subdivided and screened again until eventually a single 5.2 kb clone was identified that conferred the ability to utilize Hb. The 2.2-kb gonococcal insert in this clone contained 862 bp of the 3′ end of *pilQ*, and comparison to the gonococcal genomic database (GenBank accession NC_002946) showed that it contained a point mutation 412 bp from the 3′ end of the open reading frame of *pilQ*. This allele was designated *pilQ1*.

Oligonucleotides were designed (Table 2) to amplify and sequence *pilQ* from the genomes of *hgbX* mutants derived from FA7186. The DNA sequences revealed that FA7186H19 had precisely the same *pilQ* point mutation as that of FA7168. Of six mutants with the *hgbX* phenotype, FA7168 carried a *cat* cassette in *hpuB*; the rest were derivatives of FA7186 and carried the Ω cassette in *hmbR*. They were all sensitive to CHL^1 despite expression of the *cat* resistance cassette in the *hpuB* of FA7168.

In order to use the Ω cassette to inactivate *pilQ* and to provide further proof that the point mutation in *pilQ* was responsible for the Hb^+ phenotype, FA7186H19 *pilQ1* was moved by transformation into FA7167 (Δ*hpuA hpuB*::*cat*, Hb^-), with selection for Hb^+. A parallel transformation was done with the chromosomal DNA of FA7168. All Hb^+ transformants from these crosses were SPT^{100} and CHL^1 sensitive (SPT^s CHL^s). Genomic PCR and DNA sequencing confirmed the presence of *pilQ1* point mutation in the Hb^+ transformants. The transformant with chromosomal DNA from FA7168 was named FA7317. Two transformants obtained from two transformations by FA7186H19 DNA were named FA7294 and FA7318.

Mutagenesis of *pilQ*. A shortened version of *pilQ* was made by genomic-PCR with the upstream PCR primer hgbX08, which started 434 bp from the ATG site, and the downstream primer hgbX03, which ends 52 bp after the TGA site. The 1,872-bp PCR product from FA7168 was inserted into the vector PCR2.1-TOPO (Invitrogen) and cloned in *E. coli* DH5αMCR to generate pUNCH 288. A 30-bp segment of pUNCH 288 between two *Hin*cII sites was removed and replaced with a Ω cassette to become pUNCH 290. Replacing the same segment with a *cat* cassette generated pUNCH 291. Both pUNCH 290 and pUNCH291 were used to create *pilQ* insertional knockout mutants. Western blots were performed to confirm loss of PilQ by using a rabbit antiserum against OMP-MC kindly supplied by Charles E. Wilde III (Indiana University School of Medicine).

Construction of gonococcal *pilQ* mutants in RM11.2*recA6* background. The FA1090 derivative RM11.2*recA6* (25, 31) was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and transformed with FA7167 chromosomal DNA, which contains the Δ*hpuA hpuB*::*cat* mutations. CHL^1-resistant (CHL^r) transformants were screened by Southern blot hybridization and PCR analysis, and the *pilE* sequence was determined. The Δ*hpuA hpuB*::*cat* mutant of RM11.2*recA6* was named FA7332. FA7332 was transformed with FA7168 chromosomal DNA that contains *pilQ1*. Transformants with the *pilQ1* point mutation (FA7333) were identified by growth on Hb-Des plates and were authenticated by genomic PCR and DNA sequencing of *pilQ* and *pilE*.

RM11.2*recA6* Δ*pilE* and RM11.2*recA6* Δ*hpuA hpuB*::*cat* Δ*pilE* mutants were generated by induction of *recA* with IPTG and selection of nonpiliated areas of growth on colonies. To delete the *pilE* of FA7333, chromosomal DNA from RM11.2*recA6* Δ*pilE* was used to transform in the deletion. The *pilE* deletions were verified with Southern blot and PCR analysis. DNA sequencing determined

TABLE 1. Plasmids and gonococcal strains constructed and/or used in this study

Plasmid or strain	Genotype and phenotype	Source or reference
Plasmids		
pHP45Ω	Source for Ω (Sp^r Sm^r) cassette	32
pNC40	Source for *cat* (Cm^r) cassette	46
pUNCH173	*tonB*::Ω	3
pUNCH1306	*hemA*::Ω	48
pUNCH288	Partial FA7168 *pilQ1* in pCR2.1-TOPO	This study
pUNCH290	*pilQ1*::Ω of p288	This study
pUNCH291	*pilQ1*::*cat* of p288	This study
Strains		
FA1090	Parent strain; wild type	15
FA1090 H^+	HpuAB phase on variant of FA1090	7
FA7167	Δ*hpuA hpuB*::*cat*; Cm^r Hb^-	6
FA7168	Cm^s Hb^+ revertant of FA7167; *pilQ1*	6
FA7169	Δ*hpuA hpuB*$^+$ derivative of FA7167; Cm^s Hb^-	6
FA7186	*hmbR*::Ω transformant of FA7169; Sp^r Hb^-	6
FA7186H1	*hgbX2*; Sp^r Hb^+ revertant of FA7186	6
FA7186H12	*hgbX3*; Sp^r Hb^+ revertant of FA7186	This study
FA7186H15	*hgbX4*; Sp^r Hb^+ revertant of FA7186	This study
FA7186H19	Sp^r Hb^+ revertant of FA7186; *pilQ1*	This study
FA7186H20	*hgbX6*; Sp^r Hb^+ revertant of FA7186	This study
FA7294	FA7186H19 *pilQ1* transformant of FA7167; Cm^s Sp^s Hb^+	This study
FA7311	*pilQ*::Ω transformant of FA1090 H^+; Sp^r Hb^+	This study
FA7312	*pilQ1*::Ω transformant of FA7294; Sp^r Hb^-	This study
FA7317	FA7168 *pilQ1* transformant of FA7167; Cm^s Sp^s Hb^+	This study
FA7318	FA7186H19 *pilQ1* transformant of FA7167; Cm^s Sp^s Hb^+	This study
FA7319	*pilQ*::Ω transformant of FA7167; Sp^r Hb^-	This study
FA7320	*pilQ1*::Ω transformant of FA7317; Sp^r Hb^-	This study
FA7321	*tonB*::Ω transformant of FA7317; Sp^r Hb^+	This study
FA7322	*hemA*::Ω transformant of FA7317; Sp^r Hb^+	This study
RM11.2*recA6*	Defined P^+ variant of FA1090 with IPTG-regulated *recA*	25
FA7332 (RM7167)	Δ*hpuA hpuB*::*cat* transformant of RM11.2*recA6*; Hb^-	This study
FA7333 (RM7168)	FA7168 *pilQ1* transformant of FA7332; Hb^+	This study
FA7334	*pilT*::*erm* transformant of RM11.2*recA6*	26
FA7335	*pilQ1 pilT*::*erm* transformant of RM11.2*recA6*	This study
FA7336	Δ*pilE* of RM11.2*recA6*	This study
FA7337	Δ*pilE* transformant of FA7332	This study
FA7338	Δ*pilE* transformant of FA7333	This study
FA7339	*pilQ1* transformant of RM11.2*recA6*	This study
FA7340	*pilT*::*erm* transformant of FA7333	This study
FA7341	*pilT*::*erm* transformant of FA1090	This study
FA7342	*pilT*::*erm* transformant t of FA7168	This study
FA7343	*pilT*::*erm* transformant of FA7186H19	This study
FA7344	*pilT*::*erm* transformant of FA7317	This study
FA7345	*pilT*::*erm* transformant of FA7318	This study
FA7348	*pilT*::*tet* transformant of FA1090	This study
FA7349	*pilT*::*tet* transformant of FA7167	This study
FA7350	*pilT*::*tet* transformant of FA7186H19	This study

[a] Sp^r, SPT resistance; Sm^r, streptomycin (100 μg/ml) resistance; Cm^r, CHL resistance.

that the 924-bp deletion spans between upstream sequence and the cys2 sequence in *pilE*, which deletes the *pilE* promoter and ribosomal binding site.

Construction of *pilT* mutants. In order to make *pilT* mutants of strains in the RM11.2*recA6* background, FA7332 and FA7333 were transformed with RM11.2*recA6 pilT*::*erm* (26) chromosomal DNA. ERY^1-resistant transformants of FA7332 and $ERY^{0.05}$-resistant transformants of FA7333 were screened by Southern blot analysis. The insertional inactivation of *pilT* was identified by screening for the characteristic colony morphology differences of *pilT* (*dud1*) mutants (4). These *pilT* mutants carried the parental *pilE* sequence. The *pilT*::*erm* mutants of FA1090, FA7168, FA7186H19, FA7317, and FA7318 were constructed by moving chromosomal DNA of RM11.2*recA6 pilT*::*erm* into the recipients. Transformants of FA1090 were selected on ERY^1, and transformants of the *pilQ1* mutants were selected on $ERY^{0.5}$ plates. In order to evaluate the effect of PilT inactivation on ERY sensitivity, FA1090, FA7167, and FA7186H19 were also transformed with chromosomal DNA of an MS11 mutant carrying an inducible *pilT* promoter. (The mutation was moved into MS11 by Laura Potter of Oregon Health Sciences University from a strain originally constructed in the laboratory of Michael Koomey [50], University of Oslo, Oslo, Norway.) Transformants were selected on TET^2. Preservation of the *hpuA* deletion and *hpuB* insertion in these *pilT* mutants were confirmed by genomic PCR and DNA sequencing.

TABLE 2. Oligonucleotides used in genomic PCR and DNA sequencing of *pilQ*

Oligonucleotide	Sequence
hgbX06	5′-ACCGACGACAGCATCATCCT-3′
hgbX08	5′-CACCGGCAAAACAACAGGCT-3′
hgbX10	5′-TGCGCCAGCAAGGGAACATC-3′
hgbX04	5′-GGCTGGGGCGTGAACTC-3′
hgbX02	5′-CTGAATACGCAGGCTATGGT-3′
hgbX03	3′-CGGGAATGACGGCTCAAAAG-5′
hgbX01	3′-AGCCGTATCCGACAGATTCC-5′

TABLE 3. The *pilQ1* point mutation in FA7168 increased sensitivity to certain antibiotics and the detergent TX-100

Strain	Diam (mm)[a] with:							
	AMP (5 μg)	CHL (1.5 μg)	ERY (0.5 μg)	RIF (0.5 μg)	TX-100 (5 μl of a 1:100 dilution)	KAN (1.5 μg)	NAL (1.5 μg)	SPT (5 μg)
FA1090 wild type, Hb^+	22	26	14	22	8	10	16	16
FA7167 (Δ*hpuA hpuB*::*cat*), Hb^-	22	8	12	24	8	12	16	18
FA7168 (Δ*hpuA hpuB*::*cat pilQ1*), Hb^+	28	16	26	36	22	10	14	16

[a] Values are diameters of the zone of growth inhibition around each 6-mm disk loaded with the indicated amount of antimicrobial agent measured after 24 h. Data were from one representative experiment.

Immunoelectron microscopy. For analysis of gonococcal piliation states, Formvar-coated grids were used to lift cells directly from 18-h-old colonies grown on GCB plates. Grids were fixed for 15 min by floating on a drop of 0.2% glutaraldehyde–4% paraformaldehyde (Fisher Scientific). After fixation, the grids were rinsed three times with Dulbecco phosphate-buffered saline (PBS; Gibco-BRL) with 1% bovine serum albumin (BSA; Sigma) and then incubated in 0.1% gelatin (Aurion, Inc.) in PBS for 30 min. The grids were rinsed once with PBS-BSA and then incubated with a 1:10 dilution of rabbit α-pilin peptide antiserum (26) for 1 h. The antiserum was raised against a synthetic peptide found in the hypervariable loop region of the pilin variant expressed on RM11.2*recA6* (KRDAGAKTGADDVKADGN), synthesized on keyhole limpet hemocyanin resin and used to immunize New Zealand White female rabbits (Research Genetics). Grids were rinsed three times with PBS-BSA and incubated with 0.1% gelatin in PBS for 30 min. The grids were rinsed once with PBS-BSA and incubated with goat α-rabbit immunoglobulin G antibody conjugated to 12-nm gold (1:20 dilution; Jackson Immunolabs) for 1 h. Grids were rinsed five times in water for 3 min each step. Excess water was carefully wicked away, and grids were negatively stained with 1% uranyl acetate for 1 min. All incubations were performed at 25°C. Grids were viewed by using a JEOL JEM-1220 transmission electron microscope at 60 kV.

Transformation competence assays for strains in RM11.2*recA6* background. Gonococci were grown on GCB plates for 18 h, collected with dacron swabs, and suspended to a density of 10^8 CFU/ml in liquid GCB (GCBL). Then, 20 μl of cells was added to 200 μl of GCBL that contained 5 mM $MgSO_4$ (35), Kellogg supplements, 1 mM IPTG, and 50 ng of pSY6 (37). After 15 min at 37°C, DNase I (Promega) was added, and the incubation was continued for 5 min at 37°C to remove exogenous DNA. Then, transformation mixes were diluted into 2 ml of GCBL plus 5 mM $MgSO_4$, Kellogg supplements, and 1 mM IPTG, followed by incubation at 37°C in 5% CO_2 for 4 h without agitation. Transformants were selected on GCB plates containing 2 μg of nalidixic acid (NAL)/ml.

Hb-binding assays. These procedures were as described previously (6). Briefly, the binding was measured by ^{125}I-labeled anti-human Hb antibodies bound to buffer-washed whole cells previously incubated in Chelex-treated defined medium (CDM) supplemented with Hb.

Growth and microbial sensitivity assays. The ability of a gonococcal strain to use Hb or heme for growth was measured in liquid culture with iron-chelated CDM or on GCB-Des agar plates into which defined amounts of Hb or heme were placed into wells cut into the agar, as described previously (6). Disk assays were used to test antimicrobial sensitivities on GCB agar plates. Single, phenotypically piliated colonies of strains to be tested were passed from selection plates to GCB plates for the following day's experiment. Overnight plate-grown gonococci were suspended in GCB to an optical density at 600 nm (OD_{600}) of 0.1. Then, 50 μl of the cell suspension was mixed with 5 ml of liquid GCB agar, and the mixture was poured over a plate containing 15 ml of solidified GC agar. Four filter paper disks (6 mm in diameter; Schleicher & Schuell, Dassel, Germany) were then placed on top of the solidified GCB agar-gonococci mix. The agents tested on disks included AMP at 1 to 5 μg per disk, CHL at 0.25 μg per disk, ERY at 0.1 to 0.5 μg per disk, rifampin (RIF) at 0.05 to 0.5 μg per disk and 2.5 to 10 μl of Triton X-100 (TX-100) at 1:100 dilution.

Plating efficiency and determination of the MIC. The plating efficiency was determined by plating dilutions of cell suspensions at an OD_{600} of 0.18 onto GCB plates or Hb-Des plates. MICs were determined by plating diluted cell suspensions on GCB plates containing doubling dilutions of antimicrobial agents; the MIC was the lowest concentration that prevented growth.

RESULTS

Isolation of novel Hb^+ mutants. We previously reported spontaneously arising Hb-utilizing (Hb^+) colonies from *hpuA* deletion mutants of FA1090, FA7167 (Hb^-, Δ*hpuA hpuB*::*cat*) and its derivative FA7186 (Hb^-, Δ*hpuA hpuB*$^+$ *hmbR*::Ω) (6). Some of them carried a point mutation in *hpuB*, whereas others had mutations originally designated Δ*hpuA hgbX* that mapped outside the *hpuAB* locus (6). All of the *hgbX* mutants grew well on Hb-Des plates and in liquid culture with Hb as the sole source of iron (data not shown). The *hgbX* mutant strain isolated from FA7167, FA7168, retained the *cat* cassette in *hpuB* but became sensitive to CHL[1]. Nevertheless, it showed a much smaller zone of growth inhibition, compared to wild-type FA1090, when tested with disk loaded with CHL (Table 3). Screening of its sensitivities to several other antimicrobial agents revealed that FA7168 was also more sensitive to heme, AMP, ERY, RIF, and TX-100 than FA7167 and FA1090 (Table 3).

In liquid culture with Hb (1 μM) as the sole source of iron, the growth of FA7168 was inhibited by the addition of HSA which binds free heme (16) (Fig. 1). This result is in contrast to the behavior of FA1090, which is able to compete for heme complexed to HSA by the expression of the HpuAB receptor (6).

The Hb^+ phenotype in FA7168 is due to a point mutation in *pilQ*. Transformation of the Hb^- recipient FA7167 with pooled plasmids containing chromosomal DNA from one *hgbX* mutant, FA7168, resulted in isolation of a plasmid that contained a point mutation in *pilQ*. Subsequent studies showed that this point mutation was responsible for the growth on Hb in the

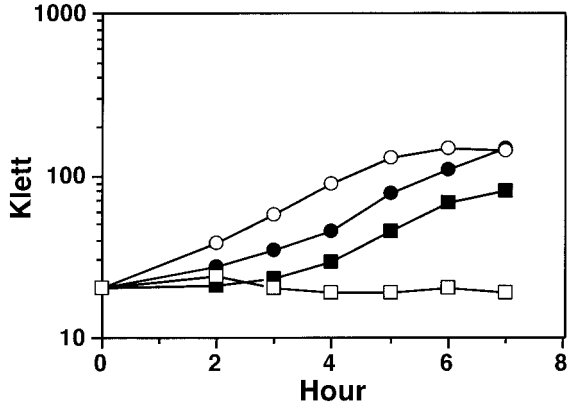

FIG. 1. Representative growth curves of the Δ*hpuA hpuB*::*cat pilQ1* point mutant, FA7168, and HpuAB phase on FA1090 in liquid cultures, as measured by the OD_{600} in the Klett colorimeter. Gonococci were grown in liquid cultures of CDM-Des supplemented with Hb (1 μM) or Hb and HSA (16 μM). Symbols: ●, FA1090 in Hb; ○, FA1090 in Hb-HSA; ■, FA7168 in Hb; □, FA7168 in Hb-HSA.

absence of an Hb receptor and for increased sensitivity to various antimicrobial agents. A T-to-G mutation occurred at base 1761 of FA7168 *pilQ* coding sequence, resulting in an F563L mutation in the predicted mature PilQ protein. Sequence from *pilQ* genomic PCR products from the other five *hgbX* mutants—H1, H12, H15, H19, and H20—derived from FA7186 (Hb^- Δ*hpuA hpuB*$^+$ *hmbR*::Ω) revealed precisely the same point mutation in one, i.e., FA7186H19. The other four *hgbX* mutants had the wild-type *pilQ* sequence. Like FA7168, FA7186H19 showed elevated sensitivities to various antimicrobial agents (data not shown). Therefore, the same F563L mutation was independently isolated in two strain backgrounds and designated *pilQ1.*

Moving FA7168 and FA7186H19 back into FA7167 background generated the Hb^+ transformants FA7317, FA7294, and FA7318 (all Δ*hpuA hpuB*::*cat pilQ1*), supporting the notion that their growth on Hb was dependent on preservation of the *pilQ1* point mutation. The necessity of PilQ for Hb utilization in the Δ*hpuA pilQ1* mutants was also confirmed by the insertional inactivation of *pilQ*. The *pilQ*::Ω transformants of FA7294 and FA7317 were both completely unable to utilize Hb for growth. As a control, a *pilQ*::Ω mutant of FA1090 Hb^+ still grew well on Hb-Des plates, as expected, because of the presence of the HpuAB receptor in FA1090 (data not shown).

The Hb^+ phenotype is independent of TonB and PilQ1 is not an Hb receptor. The HpuAB and HmbR Hb receptors, as well as the transferrin and the lactoferrin receptors, are dependent on TonB for function. To test for possible TonB dependence of the Hb supported growth of *pilQ1* mutant, FA7317 *tonB* were insertionally inactivated with the Ω cassette. Although the previously obtained Δ*hpuA hpuB* point mutants still required TonB to utilize Hb as the sole source of iron (6), the *pilQ1 tonB* double mutants grew on iron-chelated medium containing Hb but not transferrin (data not shown). Thus, the Hb^+ phenotype conferred by *pilQ1* was independent of TonB.

The possibility that the *pilQ1* mutation might restore Hb receptor activity to strains carrying mutations in *hpuAB* was examined by radioimmunoassay. FA7168 exhibited no iron-dependent binding of Hb and very low levels of Hb binding compared to HpuAB phase on FA1090 that expressed a wild-type form of the HpuAB Hb receptor (Table 4). The Hb binding was also low in parent FA7167 (Δ*hpuA hpuB*::*cat pilQ*$^+$) (Table 4). The minor differences between FA7167 and FA7168 were not significant (Student *t* test).

Different classes of Δ*hpuA hgbX* mutants. Compared to their immediate parents, FA7167 and FA7186, the *hgbX* mutants were more sensitive to the same set of antimicrobial compounds and protoporphyrin IX (PPIX) and FePPIX. However, among six mutants identified, the two that were subsequently determined to be *pilQ* mutants, FA7168 and FA7186H19, were relatively less sensitive to TX-100, PPIX, and FePPIX but more sensitive to the antibiotics RIF and ERY (data not shown). The MIC of PPIX was 250 μg/ml for FA1090 compared to ~4 μg/ml for FA7168 and FA7186H19 and <2 μg/ml for the other *hgbX* mutants. These phenotypic differences suggested that there might be different genotypic classes of *hgbX* mutants with a similar Hb^+ phenotype.

According to Drake et al. (14), PilP, a pilus biogenesis lipoprotein, affects the expression of PilQ as a macromolecular multimer. To check for the possibility that a mutation in *pilP* resulted in the Hb^+ phenotype of other *hgbX* mutants, genomic PCR products of the *pilP* gene of all *hgbX* mutants were sequenced. Their sequences were the same as wild-type FA1090. The increased sensitivity to TX-100 and RIF of the *hgbX* mutants prompted an attempt to determine whether the *mtr* CDE-encoded efflux pump might be involved (17). Introduction of *mtr*C::Km, *mtrD*::Km, and *mtrE*::Km mutations (38) by transformation into Hb^- strain FA7167 (Δ*hpuA hpuB*::*cat*) resulted in increased sensitivity to TX-100 and RIF as expected but none of the insertional mutants acquired the ability to grow on Hb-Des plates (data not shown). Thus, it seemed unlikely that the *mtr* genes were involved in the mutations that resulted in ability to grow on Hb in the absence of a Hb receptor. The possible involvement of another efflux pump, NorM (33), that recognizes cationic antimicrobial agents was also studied. Sequencing the gene *norM* also revealed no difference between the *hgbX* mutants and the wild type. At present, the identities of the other *hgbX* mutations are unknown.

TABLE 4. The *pilQ* point mutation does not increase Hb binding to whole cells

Strain	Phenotype	Growth condition	% Total cpm loaded[a] ± SD
FA1090 (HpuAB phase on)	Hb^+	Iron replete	0.24 ± 0.03
		Iron limited	1.51 ± 0.36
FA7167 (Δ*hpuA hpuB*::*cat*)	Hb^-	Iron replete	0.07 ± 0.02
		Iron limited	0.05 ± 0.03
FA7168 (Δ*hpuA hpuB*::*cat pilQ1*)	Hb^+	Iron replete	0.08 ± 0.04
		Iron limited	0.08 ± 0.05

[a] ^{125}I-labeled anti-human Hb antibody bound to gonococcal bound Hb is expressed as a percentage of the total counts per minute added to each well. Data are averages of two experiments, each done in quadruplicate.

Increased entry of free heme due to *pilQ1*. When 5 μg of heme was placed in a well cut into a GCB-Des agar plate previously spread with wild-type strain FA1090 (data not shown) or Δ*hpuA hpuB*::*cat* strain FA7167, these strains exhibited a ring of dense growth surrounding an inner zone of growth inhibition (Fig. 2). In contrast, the *pilQ1* mutants exhibited a thin ring of growth with a larger inner zone of growth inhibition (Fig. 2), indicating increased sensitivity to heme toxicity in *pilQ1* mutants. This result is consistent with these mutants' increased sensitivity to antibiotics and the porphyrins PPIX and FePPIX (40) (Table 3 and data not shown). With reduced amount of heme (2.5 μg) in the central wells, zones of growth inhibition decreased in both the *pilQ*$^+$ and the *pilQ1* strains (Fig. 2). The dose dependence of zone of growth inhibition confirmed that excess free heme was the cause of growth inhibition and that the entry of free heme was augmented in *pilQ1* mutants.

The increased sensitivity to porphyrins of strains with the *pilQ1* point mutation strongly suggested that entry of intact porphyrins was increased in the mutant background. To further address the question of whether the *pilQ1* mutation resulted in entry of heme or merely iron released from heme into the cell, *hemA* mutants were constructed in the relevant strains. Mutation in *hemA* of FA1090 results in inability to grow without exogenous heme source, since the *hemA* mutants are unable to synthesize their own heme (48). The *hemA*::Ω transformant, FA7322, of FA7317 (Hb^+, *pilQ1*) grew well on

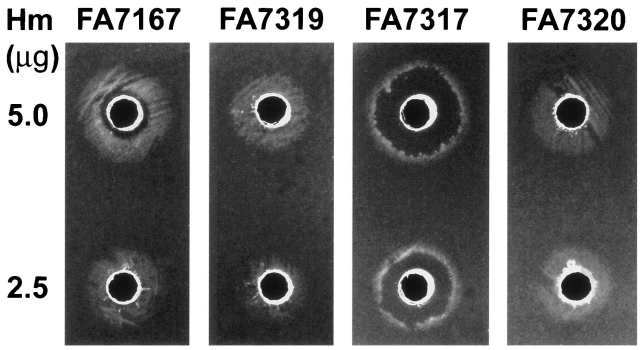

FIG. 2. Free heme supported growth of *hpuAB* mutants and their *pilQ* inactivation mutants. GCB-Des plates were spread with cell suspensions of respective strains: FA7167, Δ*hpuA hpuB*::*cat pilQ*$^+$ parent; FA7319, *pilQ*::Ω transformant of FA7167; FA7317, *pilQ1* transformant of FA7167; FA7320, *pilQ1*::Ω transformant of FA7317. Wells were cut into the agar and loaded with 50 μl of heme at 0.1 or 0.05 μg/μl. Images were obtained after 48 h of incubation. At 5.0 μg of heme per well, FA7167 grew in a wide growth ring, whereas the *pilQ1* mutant, FA7317, showed a thin growth ring with a wide zone of growth inhibition. Decreasing the amount of heme decreased the growth inhibition zones. Insertional inactivation of *pilQ* abolished heme toxicity.

Hb (data not shown), indicating that heme, or at least an intact porphyrin ring, entered the *pilQ1* mutant cells.

Additional evidence for the role of PilQ in the entry of free heme and antimicrobial compounds was provided by analysis of the effects of *pilQ* inactivation on both the *pilQ*$^+$ and the *pilQ1* strains. Inactivation of PilQ completely prevented the zone of heme toxicity in both FA7319 (FA7167 *pilQ*::Ω) and FA7320 (FA7317 *pilQ1*::Ω) (Fig. 2). Reversal of hypersensitivities to AMP, ERY, RIF, and TX-100 was also obvious in FA7320 compared to the parent *pilQ1* mutant (Fig. 3). Slight decrease of sensitivities to ERY, RIF, and TX-100 was also observed in the *pilQ*::Ω mutant of FA1090, FA7311 (Table 5). Moreover, introduction of *pilQ1* by transformation of RM11.2 *recA6* (25) (FA7339) resulted in hypersensitivity to the same agents (data not shown), showing that the effects of *pilQ1* were not dependent on a mutation in the HpuAB receptor. We concluded that PilQ forms an entry channel for free heme and certain other antimicrobial compounds in wild-type gonococci, which is accentuated by the *pilQ1* point mutation. Since there still was growth around wells containing free heme in FA1090 and FA7167 derivatives lacking PilQ, there also must be additional routes for entry of heme that do not depend on PilQ.

FIG. 3. Effects of *pilQ* inactivation on *pilQ1* mutant's sensitivity to antimicrobial agents. Filter paper disks placed on the surface of FA7168 (Δ*hpuA hpuB*::*cat pilQ1*)- and FA7320 (Δ*hpuA hpuB*::*cat pilQ1*::Ω)-coated plates were loaded with AMP (1 μg per disk, top left), ERY (0.25 μg, top right), RIF (0.05 μg, bottom left), and TX-100 (5 μl of a 1:100 dilution, bottom right). Images were obtained after 24 h of incubation.

The *pilQ1* mutation decreases pilus expression and transformation efficiency. The product of *pilQ* is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae* (13). To ascertain the role of the PilQ point mutation in pilus assembly and function, the Δ*hpuA hpuB*::*cat pilQ1* mutation was transformed into RM11.2*recA6*, a defined pilin variant of strain FA1090 with a regulatable *recA* that allows for IPTG control of DNA transformation and pilin antigenic variation (34). All RM11.2*recA6* derivatives were shown to carry identical *pilE* sequences, ensuring that differences in piliation were not due to changes in pilus expression due to pilin variation (data not shown). To directly examine piliation of the RM11.2*recA6* Δ*hpuA hpuB*::*cat* and RM11.2*recA6* Δ*hpuA hpuB*::*cat pilQ1* transformants, FA7332 and FA7333, immunogold electron microscopy with an anti-pilin peptide antibody was performed. Compared to the wild-type RM11.2*recA6*, the number of cells expressing pili was unchanged in FA7332 but was reduced by ~2-fold in the *pilQ1* mutant FA7333 (Fig. 4), which nevertheless retained a piliated colony morphology. The *pilQ1* mutant also showed sevenfold-lower transformation efficiency than FA7332 (Fig. 4), although there is still substantial transformation competence at 10^{-3} transformants/CFU.

To determine the effects of pilus expression on the entry of antimicrobial compounds through PilQ macromolecular complex, Δ*pilE* mutants were made from FA7332 and FA7333 and compared to RM11.2*recA6* Δ*pilE* (FA7336). The transformation efficiencies for each of the three Δ*pilE* mutants were similar and reduced ca. 5 to 6 logs compared to the isogenic Pil$^+$ strains (data not shown). Loss of pilus expression in *pilQ1* mutant had little effect on Hb-supported growth, but the Δ*pilE pilQ1* double mutant, FA7338, showed increased sensitivity to heme toxicity (Fig. 5) and slightly increased sensitivity to AMP and TX-100 (Table 6). Thus, pilus expression might compete with certain molecules for the PilQ macromolecular complex.

PilT is essential for the observed phenotypic effects of the *pilQ1* mutation. The pilus accessory gene *pilT* plays a crucial role in twitching motility and pilus retraction (27, 30), acting as a conditional antagonist of stable pilus fiber formation (50, 51, 52). In order to examine the influence of PilT in a *pilQ1* background, we introduced two kinds of *pilT* loss-of-function mutations into *pilQ* wild-type strains FA1090, FA7167, and *pilQ1* mutants FA7168, FA7186H19, FA7317, and FA7318, some with *pilT*::*erm* and the others with *pilT*::*tet*. The *pilQ1 pilT*::*erm* double mutants showed low plating efficiencies on Hb-Des plates (ca. 5×10^{-3} Hb-Des CFU/GCB CFU). After 48 h of incubation on Hb-Des plates, they exhibited a mixture of a few regular-sized and many tiny colonies. Passage of the regular sized colonies to fresh Hb-Des plates yielded uniformly regular-sized colonies. Passage of tiny colonies on the same media continued to yield the same proportions of tiny and normal-sized colonies. The rates of colony variation suggested possible phase variation of another surface component, but no differences were found in PilC expression (determined by Western blots with anti-PilC polyclonal sera; data not shown).

Similar low plating efficiencies and apparent phase variation

TABLE 5. Insertional inactivation of *pilQ* and *pilT* in FA1090 decreased sensitivities to ERY, RIF, and TX-100

Strain[a]	Mean zone of growth inhibition (mm)[b] ± SD with:			
	AMP (2 μg)	ERY (0.5 μg)	RIF (0.1 μg)	TX-100 (10 μl of a 1:100 dilution)
FA1090 P^+, Hb^+	17.6 ± 1.1	19.8 ± 3.5	18.0 ± 1.0	11.0 ± 1.0
FA1090 P^-, Hb^+	18.2 ± 1.5	20.2 ± 3.3	18.4 ± 1.5	11.0 ± 1.6
FA7311 (FA1090 *pilQ*::Ω), Hb^+	18.0 ± 1.1	18.8 ± 4.3*	16.0 ± 2.1*	9.3 ± 1.0**
FA7348 (FA1090 *pilT*::*tet*), Hb^+	18.3 ± 1.5	18.7 ± 3.0*	16.2 ± 1.0*	9.5 ± 0.8*

[a] For FA1090, phenotypically piliated (P^+) and nonpiliated (P^-) colonies were selectively passed in preparation for the following day's experiment.
[b] Each value is an average of five zones of growth inhibition obtained with the indicated amount of antimicrobial agent measured from five experiments after 48 h of incubation. A paired *t* test showed significant differences (*, $P < 0.05$; **, $P < 0.01$) as indicated compared to FA1090 P^+.

from tiny colonies to large normally growing colonies were noted in earlier studies of double mutants that expressed neither PilQ nor PilT, which was shown to be the result of toxicity from assembled intracellular pilus fibers that could neither be extruded through PilQ, nor broken down due to loss of PilT function (52). In that case, the large-colony variants were due to pilin mutations that resulted in the inability to assemble toxic fibers. To further address whether a similar phenomenon in the *pilQ1 pilT*::*erm* double mutants might account for low plating efficiencies and frequent reversion to large-colony variants on Hb-Des plates, the *pilQ1 pilT*::*erm* mutations were introduced into FA7332 (RM11.2*recA6* Δ*hpuA hpuB*::*cat*), which does not undergo pilin variations due to the *recA6* mutation in the absence of induction of expression of *recA* function by IPTG (36). The results showed that there were similar low plating efficiencies on Hb-Des plates and the appearance of large and small colony variants on these plates (data not shown). This strongly suggested that recombination pathways were not necessary to the reversions to normal growth and therefore that pilin variations were not responsible for the restored growth.

Loss of PilT expression decreased the sensitivity of *pilQ1* mutants to heme toxicity. The *pilQ1 pilT*::*erm* double mutant, FA7340 (in RM11.2*recA6* Δ*hpuA hpuB*::*cat* background), exhibited a heavier ring of growth and a smaller inner zone of growth inhibition around the central well containing heme, compared to the parent strain with intact PilT function (Fig. 5). FA7340 was less sensitive to AMP, RIF, and TX-100 than its parent, FA7333 (Table 6), whereas the *pilT*::*tet* mutant of FA7186H19 (Δ*hpuA hpuB*::*cat pilQ1*), FA7350, demonstrated decreased sensitivity to ERY (data not shown). Loss of PilT also reduced the entry of some antimicrobial compounds in *pilQ* wild-type strains. Although the effect of *pilT* inactivation in FA1090 was small, differences were significant for sensitivities to ERY, RIF, and TX-100 (Table 5). Thus, PilT was required for the susceptibility to antibiotics, detergents, and heme/porphyrins that can utilize both wild-type and PilQ1 macromolecular complex for entry into the bacterial cell.

DISCUSSION

We initiated this study to elucidate the mechanisms responsible for receptor-independent uptake of iron and heme from Hb, based on the previous isolation of gonococcal mutants that were able to grow on Hb despite mutational loss of HpuA, the lipoprotein coreceptor that normally is essential for the function of the HpuAB receptor (6). The *hgbX* mutants fell into two general classes based on differences in their susceptibilities to a variety of antimicrobial agents. Although all were more susceptible to generally hydrophobic antibiotics and TX-100 than the wild type, some were particularly susceptible. One of the two classes of mutants turned out to have a point mutation

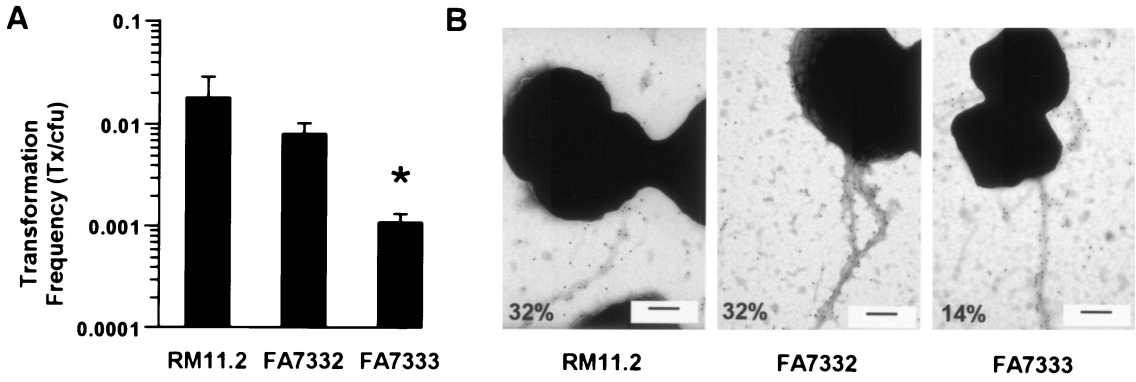

FIG. 4. Bar graph illustrating transformation efficiencies (A) and electron micrographs comparing the piliation (B) of RM11.2*recA6* (wild type), FA7332 (Δ*hpuA hpuB*::*cat*), and FA7333 (Δ*hpuA hpuB*::*cat pilQ1*). The piliation of these three strains is given as the percentage of cells expressing pili, as visualized by immunogold electron microscopy with anti-pilin sera, and the percent piliation is indicated on each electron micrograph. The bar in each electron micrograph is 500 nm. Two grids for each strain were examined. The numbers of cells examined were 216, 346, and 558, respectively. All three strains exhibited predominantly bundled pili. FA7333 showed decreased piliation as well as decreased transformation efficiency. The asterisk in panel A indicates a significant difference by using the Student *t* test ($P < 0.05$) compared to RM11.2 *recA6* and FA7332.

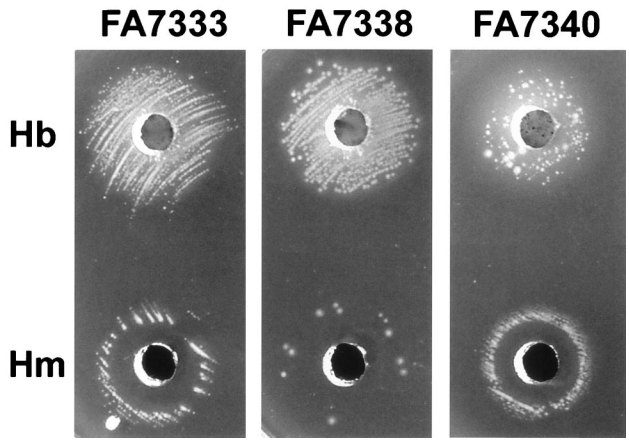

FIG. 5. Effects of *pilE* deletion and *pilT* inactivation on Hb (50 μl at 10 μg/μl) and heme (50 μl at 0.1 μg/μl) supported growth of a *pilQ1* mutant, FA7333 (Δ*hpuA hpuB*::*cat pilQ1*, Hb^+). FA7338 is the Δ*pilE* transformant and FA7340 is the *pilT*::*erm* transformant of FA7333. Images were obtained after 48 h of incubation. The *pilQ1 pilT* double mutant gave only isolated large colonies around the Hb well. The zone of growth inhibition around the heme well was wider in the *pilQ1* Δ*pilE* double mutant but was reduced in the *pilQ1 pilT* double mutant compared to that of FA7333.

near the 3′ end of *pilQ* (*pilQ1*), whereas the other was normal in all tested genes. Efforts to clone the gene(s) responsible for the remaining uncharacterized *hgbX* mutants other than *pilQ* by the same strategy used to isolate the *pilQ1* mutation have been unsuccessful. The results described in this communication showed that the Hb^+ phenotype of the *pilQ1* mutants was neither dependent on HpuAB or TonB nor on Hb binding to whole cells. Rather, growth on Hb was due to the release of free heme from Hb and entry through a channel or channels that did not depend on the Hb receptor. We presume that heme was released from Hb on the cell surface or in the extracellular medium because HSA prevented the entry of heme. So far, there has not been any report of Hb entry in any bacterial species. The relatively large sizes of albumin and Hb (molecular weights of ca. 69,000 and 64,500, respectively) and the funnel-shaped cavity in the PilQ macromolecule (9) make it unlikely that either could enter through the PilQ channel.

Demonstration that the *pilQ1* mutation resulted in increased entry of heme, TX-100, and a variety of antibiotics was surprising, since PilQ is not known to facilitate the entry of exogenous compounds. It is likely that only a few mutations in *pilQ* result in this phenotype, since the same F563L mutation was isolated independently twice. Very recently, an additional and different *pilQ* mutation has been isolated that results in increased resistance to antibiotics, further suggesting that wild-type PilQ allows the entry of a variety of compounds (R. Nicholas et al., unpublished data). Our demonstration that the phenotype was very closely linked to *pilQ1* and was lost by insertional inactivation of *pilQ1* suggests that the effects were mediated by changes within PilQ. We cannot absolutely exclude the possibility that the PilQ F563L mutation provides these phenotypes by affecting membrane integrity or another secondary effect. The simplest hypothesis is that PilQ can directly allow entry of these molecules into the bacterial cell and that the F563L mutation increases the number of molecules that can efficiently utilize this route.

The conclusion that PilQ forms not only a pore for exit of the assembled pilus fibril (13, 52) but also a channel for entry of heme and various antimicrobial agents is strengthened by the additional observation that loss of *pilT* function decreased the effects of the *pilQ1* mutation. PilT is responsible for twitching motility mediated by retraction of the pilus fibril (27, 30). It also is necessary for degradation of the pilus fibril when PilQ is not available for export of the intracellular assembled pilus fibril; in the absence of both PilQ and PilT, cell viability is lost unless other secondary mutations in *pilE* prevent formation of what would otherwise be toxic intracellular pilus fibrils (52). PilT is also required for the transport of DNA into the cell for genetic transformation (48). Our observation that inactivation of *pilT* in strains carrying the *pilQ1* missense mutation resulted in decreased sensitivity to heme, TX-100, and antibiotics argues that PilT normally increases the entry properties of the PilQ macromolecular complex. Perhaps the PilQ-dependent entry of small molecules shares mechanistic properties with pilus retraction, where the assembled pilus is imported back into the cell. It is not clear whether heme and antimicrobial agents bind to pili or to PilQ, but modestly increased sensitivity to heme and certain antimicrobial agents in a Δ*pilE* construct (Fig. 5 and Table 6) suggests that either extruded pili compete with exogenous compounds for PilQ or pili partially impeded passage of these compounds through the PilQ channels.

Many bacteria possess receptors for binding and utilizing heme-carrying compounds (43). The entrance of heme in gonococci is known to be TonB independent (3, 7, 44, 48), and no clear evidence for a heme receptor in the pathogenic *Neisseria* spp. has been produced. Our results demonstrated that some heme enters through a PilT- and PilQ-dependent pathway, but there almost certainly must be an additional mechanism for heme entry as well, since strains lacking both an Hb receptor and PilQ were able to grow on free heme (Fig. 2). It

TABLE 6. Deletion of *pilE* and inactivation of *pilT* changed sensitivities to TX-100 and certain antibiotics in *pilQ1* mutants

Strain	Mean zone of growth inhibition (mm)[a] ± SD with:			
	AMP (1 μg)	ERY (0.1 μg)	RIF (0.05 μg)	TX-100 (5 μl of a 1:100 dilution)
FA7332 (RM11.2*recA6 hpuAB*$^-$), Hb^-	16.3 ± 2.9**	7.7 ± 3.3**	14.3 ± 2.3**	7.0 ± 2.4**
FA7333 (*pilQ1* mutant of FA7332), Hb^+	22.2 ± 1.9	19.5 ± 3.3	30.0 ± 2.3	16.2 ± 2.9
FA7338 (FA7333 Δ*pilE*)	23.7 ± 1.8*	19.1 ± 2.8	30.0 ± 1.8	20.6 ± 1.2*
FA7340 (FA7333 *pilT*::*erm*)	21.3 ± 1.8*	NA	23.4 ± 2.8**	7.4 ± 2.3**

[a] Each value is an average five zones of growth inhibition obtained with the indicated amount of antimicrobial agent measured from five experiments after 48 h of incubation. NA, not applicable. A paired *t* test showed a significant difference (*, $P < 0.05$; **, $P < 0.01$) as indicated compared to FA7333.

was curious that there was no toxicity when Hb was the source of heme, as opposed to adding free heme to the media. This may reflect relatively low concentrations of free heme released by Hb, compared to the amounts added to the wells in the assays used. Heme is known to be toxic for bacteria when in excess (40). Indeed, use of iron regulated receptors for obtaining heme from Hb under physiological circumstances may help to control the amount of heme that enters the cell, thereby avoiding toxicity.

Recent structural studies have utilized electron microscopy to establish proposed quaternary structures for members of the secretin family. According to Collins et al. (8, 9), the assembled meningococcal PilQ product takes the form of a doughnut composed of 12 identical subunits, surrounding a central cavity. When viewed from the side, the assembly is proposed to have a rounded, conical profile. Viewed from the top, the open end of the central channel apparently is just large enough to accommodate the pilus fibril, and the tapered end presumably blocks the channel (9). How the PilQ macromolecular assembly might be gated open so as to allow exit of pilus fibrils has been the subject of speculation involving the propulsive force of the pilus, which is sufficient to cause membrane protrusions in cells lacking PilQ and PilT (52). Such a mechanism might account for the specificity of the channel for pili. The only other function associated with the pilus-PilQ channel has been uptake of transforming DNA, which depends on pili, PilC, PilT, and other members of the complex (51). How DNA enters through this channel remains unclear; however, our data suggest that *pilQ1* changes the specificity of the channel to allow increased entry of other molecules into the cell.

Studies on certain other members of the secretin family (34) have demonstrated gated channel properties. The pIV secretin for the filamentous phage f1 forms an ion conductive pore in planar lipid bilayers, and mutant forms of the protein allow import of molecules in whole cells to which *E. coli* normally is resistant (28). Entry through the pIV channel was decreased by production of phage that could not be released from the cell surface (29). The *Klebsiella oxytoca* secretin PulD also forms ion conductive channels in planar lipid bilayers (31). Each of these secretins has a quaternary shape similar to that proposed for PilQ (8). Future studies on PilQ might be expected to show voltage-dependent ion conductive channels in planar lipid bilayers, and mutants of PilQ such as the one we describe should alter the properties of the channel. These mutants hopefully also will help us to understand how PilQ interacts with other members of the complex (PilT, PilP, and PilC) in the secretion of pili and the mechanisms and physiological roles for entry of exogenous compounds, including heme and antibiotics through PilQ.

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REFERENCES

1. **Anderson, J. E., M. M. Hobbs, G. D. Biswas, and P. F. Sparling.** 2003. Opposing selective forces for expression of the gonococcal lactoferrin receptor. Mol. Microbiol. **48:**1325–1337.
2. **Anderson, J. E., P. A. Leone, W. C. Miller, C.-J. Chen, M. M. Hobbs, and P. F. Sparling.** 2001. Selection of the gonococcal hemoglobin receptor during menses. J. Infect. Dis. **184:**1621–1623.
3. **Biswas, G. D., J. E. Anderson, and P. F. Sparling.** 1997. Cloning and functional characterization of *Neisseria gonorrhoeae tonB*, *exbB*, and *exbD* genes. Mol. Microbiol. **24:**169–179.
4. **Biswas, G. D., S. A Lacks, and P. F. Sparling.** 1989. Transformation-deficient mutants of piliated *Neisseria gonorrhoeae.* J. Bacteriol. **171:**657–664.
5. **Chen, C.-J., C. Elkins, and P. F. Sparling.** 1998. Phase variation of hemoglobin utilization in *Neisseria gonorrhoeae.* Infect. Immun. **66:**987–993.
6. **Chen, C.-J., D. Mclean, C. E. Thomas, J. E. Anderson, and P. F. Sparling.** 2002. Point mutations in HpuB enable gonococcal HpuA deletion mutants to grow on hemoglobin. J. Bacteriol. **184:**420–426.
7. **Chen, C.-J., P. F. Sparling, L. A. Lewis, D. W. Dyer, and C. Elkins.** 1996. Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae.* Infect. Immun. **64:**5008–5014.
8. **Collins, R. F., L. Davidsen, J. P. Derrick, R. C. Ford, and T. Tonjum.** 2001. Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. J. Bacteriol. **183:**3825–3832.
9. **Collins, R. F., R. C. Ford, A. Kimitto, R. O. Olsen, T. Tonjum, and J. P. Derrick.** 2003. Three-dimensional structure of the *Neisseria meningitidis* secretin PilQ determined from negative-stain transmission electron microscopy. J. Bacteriol. **185:**2611–2617.
10. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin binding protein 1 is required for transferrin utilization and is homologous to Ton-B-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
11. **Cornelissen, C. N., M. Kelly, M. M. Hobbs, J. E. Anderson, J. G. Cannon, M. S. Cohen, and P. F. Sparling.** 1998. The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. Mol. Microbiol. **27:**611–616.
12. **Cornelissen, C. N., and P. F. Sparling.** 1994. Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. Mol. Microbiol. **14:**843–850.
13. **Drake, S. L., and M. Koomey.** 1995. The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae.* Mol. Microbiol. **18:**975–986.
14. **Drake, S. L., S. A., Sandstedt, and M. Koomey.** 1997. PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer. Mol. Microbiol. **23:**657–668.
15. **Dumpsey, J. F., W. Litaker, A. Madure, T. L. Snodgrass, and J. G. Cannon.** 1991. Physical map of the chromosome of *Neisseria gonorrhoeae* FA1090 with locations of genetic markers, including *opa* and *pil* genes. J. Bacteriol. **173:**5476–5486.
16. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infect. Immun. **55:**2171–2175.
17. **Hagaman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer.** 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agent is modulated by the *mtrCDE* efflux system. Microbiology **141:**611–622.
18. **Hansen, M. V., and C. E. Wilde III.** 1984. Conservation of peptide structure of outer membrane protein-macromolecular complex from *Neisseria gonorrhoeae.* Infect. Immun. **43:**839–845.
19. **Lee, B. C.** 1992. Isolation of haemin-binding proteins of *Neisseria gonorrhoeae.* J. Med. Microbiol. **36:**121–127.
20. **Lee, B. C., and S. Levesque.** 1997. A monoclonal antibody directed against the 97-kilodalton gonococcal hemin-binding protein inhibits hemin utilization by *Neisseria gonorrhoeae.* Infect. Immun. **65:**2970–2974.
21. **Lewis, L. A., and D. W. Dyer.** 1995. Identification of an iron-regulated outer membrane protein of *Neisseria meningitidis* involved in the utilization of haemoglobin complexed to haptoglobin. J. Bacteriol. **177:**1299–1306.
22. **Lewis, L. A., M. Gipson, K. Hartman, T. Ownbey, J. Vaughn, and D. W. Dyer.** 1999. Phase variation of HpuAB and HmbR, two distinct hemoglobin receptors of *Neisseria meningitidis* DNM2. Mol. Microbiol. **32:**977–989.
23. **Lewis, L. A., E. Gray, Y.-P. Wang, B. A. Roe, and D. W. Dyer.** 1997. Molec-

ular characterization of *hpuAB*, the haemoglobin-haptoglobin-utilization operon of *Neisseria meningitidis.* Mol. Microbiol. **23:**737–749.
24. **Lewis, L. A., M.-H. Sung, M. Gipson, K. Hartman, and D. W. Dyer.** 1998. Transportation of intact porphrin by HpuAB, the hemoglobin-haptoglobin utilization system of *Neisseria meningitidis.* J. Bacteriol. **180:**6043–6047.
25. **Long, C. D., R. N. Madraswala, and H. S. Seifert.** 1998. Comparisons between phase variations of *Neisseria gonorrhoeae* FA1090 and pilus, pilin and S-pilin expression. Infect. Immun. **66:**1918–1927.
26. **Long, C. D., D. M. Tobiason, M. Lazio, K. A. Klin, and H. S. Seifert.** 2003. Low level pilin expression allows for substantial DNA transformation competence in *Neisseria gonorrhoeae.* Infect. Immun. **71:**6279–6291.
27. **Maier, B., L. Potter, M. So, H. S. Seifert, and M. P. Sheetz.** 2002. Single pilus motor forces exceed 100 pN. Proc. Natl. Acad. Sci. USA **99:**16012–16017.
28. **Marciano, D. K., M. Russel, and S. M. Simon.** 1999. An aqueous channel for filamentous phage export. Science **284:**1516–1519.
29. **Marciano, D. K., M. Russel, and S. M. Simon.** 2001. Assembling filamentous phage occlude pIV channels. Proc. Natl. Acad. Sci. USA **98:**9359–9364.
30. **Merz, J. A., M. So, and M. P. Sheetz.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98–102.
31. **Nouwen, N., N. Ranson, H. Saibil, B. Wolpensinger, A. Engel, A. Ghazi, and A. P. Pugsley.** 1999. Secretin PulD: association with pilot PulS, structure, and ion-conducting channel formation. Proc. Natl. Acad. Sci. USA **96:**8173–8177.
32. **Prentki, P., and H. M. Kirsch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
33. **Rouguette-Loughlin, C., S. A. Dunham, M. Kuhn, J. T. Balthazar, and W. M. Shafer.** 2003. The NorM efflux pump of *Neisseria gonorrhoeae* and *Neisseria meningitidis* recognizes antimicrobial cationic compounds. J. Bacteriol. **185:** 1101–1106.
34. **Russel, M.** 1998. Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. J. Mol. Biol. **279:**485–499.
35. **Schryvers, A. B., and I. Stojiljkovic.** 1999. Iron acquisition systems in the pathogenic *Neisseria.* Mol. Microbiol. **32:**1113–1117.
36. **Seifert, H. S.** 1997. Insertionally inactivated and inducible recA6 alleles for use in *Neisseria.* Gene **188:**215–220.
37. **Seifert, H. S., R. S. Ajioka, D. Paruchuri, F. Heffron, and M. So.** 1990. Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutation lower DNA transformation competence. J. Bacteriol. **172:**40–46.
38. **Shafer, W. M., W. L. Veal, E.-H. Lee, L. Zarantonelli, J. T. Balthazar, and C. Rouguette-Loughlin.** 2001. Genetic organization and regulation of antimicrobial efflux systems possessed by *Neisseria gonorrhoeae* and *Neisseria meningitidis.* J. Mol. Microbiol. Biotechnol. **3:**219–224.
39. **Stein, D. C.** 1991. Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. Can. J. Microbiol. **37:**345–349.
40. **Stojiljkovic, I., B. D. Evanvold, and V. Kumar.** 2001. Antimicrobial properties of porphyrins. Expert Opin. Investig. Drugs **10:**309–320.
41. **Stojiljkovic, I., V. Hwa, L. D. S. Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* hemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15:**531–541.
42. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding protein with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.
43. **Stojiljkovic, I., and D. Perkins-Balding.** 2002. Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. **21:**281–295.
44. **Stojiljkovic, I., and N. Srinivasan.** 1997. *Neisseria meningitidis tonB*, *exbB*, and *exbD* genes: Ton-dependent utilization of protein-bound iron in neisseriae. J. Bacteriol. **179:**805–812.
45. **Swanson, J.** 1973. Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. J. Exp. Med. **137:**571–589.
46. **Thomas, C. E., N. H. Carbonetti, and P. F. Saprling.** 1996. Pseudo-transposition of a Tn*5* derivative in *Neisseria gonorrhoeae.* FEMS Microbiol. Lett. **145:**371–376.
47. **Tsai, W.-M., S. H. Larsen, and C. E. Wilde III.** 1989. Cloning and DNA sequence of the *omc* gene encoding the outer membrane protein-macromolecular complex from *Neisseria gonorrhoeae.* Infect. Immun. **57:**2653–2659.
48. **Turner, P. C., C. E. Thomas, C. Elkins, S. Clary, and P. F. Sparling.** 1998. *Neisseria gonorrhoeae* heme biosynthesis mutants utilize heme and hemoglobin as a heme source but fail to grow within epithelial cells. Infect. Immun. **66:**5215–5223.
49. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.
50. **Wolfgang, M., P. Lauer, H. P. Park, L. Brossay, J. Herbert, and M. Koomey.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae.* Mol. Microbiol. **29:**321–330.
51. **Wolfgang, M., H. P. Park, S. F. Hayes, J. P. M. van Putten, S. F. Hayes, and M. Koomey.** 1998. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae.* Proc. Natl. Acad. Sci. USA **95:**14973–14978.
52. **Wolfgang, M. J. P. M. van Putten, S. F. Hayes, D. Dorward, and M. Koomey.** 2000. Component and dynamics of fiber formation define a ubiquitous biogenesis pathway of bacterial pili. EMBO J. **19:**6408–6418.