

A genetic screen identifies genes and sites involved in pilin antigenic variation in *Neisseria gonorrhoeae*

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Summary

It has previously been shown that the frequency of pilin antigenic variation in *Neisseria gonorrhoeae* (the gonococcus, Gc) is regulated by iron availability. To identify factors involved in pilin variation in an iron-dependent or an iron-independent manner, we conducted a genetic screen of transposon-mutated gonococci using a pilus-dependent colony morphology phenotype to detect antigenic variation deficient mutants. Forty-six total mutants representing insertions in 30 different genes were shown to have reduced colony morphology changes resulting from impaired pilin variation. Five mutants exhibited an iron-dependent decrease in pilin variation, while the remaining 41 displayed an iron-independent decrease in pilin variation. Based on the levels of antigenic variation impairment, we defined the genes as being essential for, important for, or involved in antigenic variation. DNA repair and DNA transformation frequencies of each mutant were measured to determine whether other recombination-based processes were also affected in the mutants. Each mutant was placed into one of six classes based on their pilin variation, DNA repair and DNA transformation phenotypes. Among the many genes identified, *recR* is shown to be an additional member of the gonococcal RecF-like recombination pathway. In addition, *recG* and *ruvA* represent the first evidence that the processing of Holliday junctions is required for pilin antigenic variation. Moreover, two independent insertions in a non-coding region upstream of the *pilE* gene suggest that *cis*-acting sequences important for pilin variation are found in that region. Finally, insertions that effect expression of the *thrB* and *thrC* genes suggest that

molecules in the threonine biosynthetic pathway are important for pilin variation. Many of the other genes identified in this genetic screen do not have an obvious role in pilin variation, DNA repair, or DNA transformation.

Introduction

The Gram-negative bacterium *Neisseria gonorrhoeae* (the gonococcus, Gc) is the causative agent of the sexually transmitted disease gonorrhoea. The gonococcal type IV pilus is an important virulence factor that is required for full infectivity (Swanson *et al.*, 1987; Cohen and Cannon, 1999) and mediates multiple functions; including twitching motility (Henrichsen, 1975; Wolfgang *et al.*, 1998), natural DNA transformation (Sparling, 1966; Seifert *et al.*, 1990), and adherence to epithelial cells (Swanson, 1973; Virji *et al.*, 1982; Jonsson *et al.*, 1994).

Although immune responses are directed against gonococci during an infection, protective immunity from subsequent reinfection does not develop, partly because of the extensive variation of bacterial surface-exposed structures including LOS, Opa and the type IV pilus (reviewed in Seifert, 1992). Antigenic variation of the gonococcal pilus arises through changes in the amino acid sequence of the pilin monomer, the predominant component of the Gc pilus. The genome of gonococcal strain FA1090 contains one complete pilin expression locus, *pilE*, and five silent pilin loci, *pilS*, located throughout the chromosome, each containing one to six silent pilin copies (Haas and Meyer, 1986; Hamrick *et al.*, 2001). Pilin variation occurs through unidirectional DNA recombination events that incorporate sequence from any of the 19 silent pilin copies into *pilE*, altering the sequence of the *pilE* gene while the silent *pilS* copy remains unchanged (Hagblom *et al.*, 1985; Haas and Meyer, 1986; Segal *et al.*, 1986). Pilin variation can result in a multitude of possible pilin sequences, because of the many silent pilin copies and the different lengths of sequence that can be incorporated during each recombination reaction.

In Gc, DNA recombination mediates pilin antigenic variation, DNA repair, and natural DNA transformation. The gonococcal *recA* homologue is essential for all homologous recombination (Kooimey and Falkow, 1987; Kooimey *et al.*, 1987). Recently, a second gene has been identified, *recX*, that is necessary for efficient homologous recombination.

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nation in Gc (Stohl and Seifert, 2001). Pilin antigenic variation is mediated by a number of factors. In addition to *recA* and *recX*, the *recJ*, *recO*, *recQ* genes of the RecF-like recombination pathway are also required for efficient pilin variation (Mehr and Seifert, 1998; Hill, 2000; Skaar *et al.*, 2002). The role of the RecBCD recombination pathway in pilin variation is not yet clear, as there are conflicting reports suggesting that the RecBCD pathway either has no role in pilin variation (Mehr and Seifert, 1998), or that RecB and RecD act to inhibit pilin variation (Chaussee *et al.*, 1999; Salvatore *et al.*, 2002). In Gc, DNA lesions are repaired by both the RecBCD pathway (RecA, RecX, RecB, RecC, RecD and RecN) (Kooimey *et al.*, 1987; Mehr and Seifert, 1998; Stohl and Seifert, 2001; Skaar *et al.*, 2002) and by the RecF-like pathway (RecA, RecX, RecJ, RecO and RecQ) (Kooimey *et al.*, 1987; Mehr and Seifert, 1998; Hill, 2000; Stohl and Seifert, 2001; Skaar *et al.*, 2002). DNA transformation is the sole means by which genetic exchange of chromosomal markers has been demonstrated to occur in Gc, and it contributes to strain variation and the acquisition of antibiotic resistance markers. Successful DNA transformation requires the RecBCD pathway, but not the RecF-like pathway (Mehr and Seifert, 1998; Chaussee *et al.*, 1999; Skaar *et al.*, 2002). DNA transformation also requires many genes necessary for pilus biogenesis and pilus function (reviewed in Kline *et al.*, 2003; Chen and Dubnau, 2004), as well as the 10 bp gonococcal DNA uptake sequence (DUS) (Goodman and Scoocca, 1988).

It has previously been shown that iron limitation of gonococcal cultures results in increased frequencies of pilin antigenic variation, DNA repair, and DNA transformation (Serkin and Seifert, 2000), all cellular processes that require DNA recombination and RecA. Further studies to identify factors that mediate the iron-dependent recombination response examined the role of three likely candidates, RecA, RecX and Fur. Comparison of iron-replete and iron-limited gonococci revealed no differences in RecA protein levels (Serkin and Seifert, 2000), and a *recX* null mutant retained the iron-dependent increased recombination phenotype, indicating RecX is not an effector of the enhanced recombination (Stohl and Seifert, 2001). The ferric uptake regulator (Fur) is the only known iron-responsive regulator in Gc (Thomas and Sparling, 1994), but a Gc Fur null mutant was unattainable (Thomas and Sparling, 1996); preventing a *fur* mutant from being used to test the role of *fur* in the iron-dependent elevated recombination frequencies (Serkin and Seifert, 2000).

We conducted a genetic screen designed to identify factors that regulate and/or affect iron-dependent pilin recombination, as well as to identify new factors that are necessary for pilin variation independent of iron availability. Several new genes were identified that are required for pilin variation in both iron-limiting and iron-replete con-

ditions. Additional genes were identified that influence pilin variation only in iron-limiting conditions.

Results

Developing a kinetic colony morphology assay for pilin variation

Previous work describing an iron-dependent increase in pilin variation, DNA repair, and DNA transformation frequencies was performed with Gc grown in liquid medium (Serkin and Seifert, 2000). As a colony morphology-based genetic screen would be more feasible on solid medium, we determined whether an iron-dependent increase in pilin variation could also be observed on solid medium. Piliated gonococci produce colonies with distinct characteristics compared with non-piliated gonococci; such as the size, shape, and colour of the colony (Kellogg *et al.*, 1963; Swanson *et al.*, 1971). Using these characteristics, it is common to measure the subset of gonococcal pilin variation that leads to changes in pilus expression by observing changes in gonococcal colony morphology from that of a piliated (P^+) colony to that of a non-piliated (P^-) colony, or vice versa (Meyer *et al.*, 1982; Segal *et al.*, 1985; Swanson *et al.*, 1985). To examine iron-dependent pilin variation on solid medium, the percentage of Gc colonies that changed morphology from P^+ to P^- was measured after growth on iron-replete or iron-limiting solid medium. We tested many concentrations of the metal chelator, Desferal (from 1 μ M to 100 μ M) and selected a concentration (6 μ M) that supported the best growth rate but provided an iron-limiting environment as measured by transferrin binding protein B (TbpB) expression by Western blot (Fig. 1A). Phase variation frequencies were compared at times when iron-replete and iron-limited colonies contained similar number of colony-forming units (cfu) indicating they had undergone a similar number of generations. We determined that Gc grown on iron-replete media for 20 h had a statistically indistinguishable number of cfu/colony relative to Gc grown on iron-limiting media for 24 h ($P \leq 0.05$ by Student's *T*-test, Fig. 1B). Comparing the percent phase variation frequencies of iron-replete Gc after 20 h and iron-limited Gc after 24 h shows that the iron-limited gonococci displayed a statistically significant nearly 10-fold increase in phase variation as compared with iron-replete gonococci ($P \leq 0.05$ by Student's *T*-test, Fig. 1C). Therefore, Gc grown on iron-limiting solid media exhibit a similar increase in pilin variation compared with Gc grown in liquid media.

As described above, the percent phase variation assay has typically been used to assess the effect of mutations and various environmental conditions on pilin antigenic variation (Meyer *et al.*, 1982; Segal *et al.*, 1985; Swanson *et al.*, 1985; Mehr and Seifert, 1998). However, the

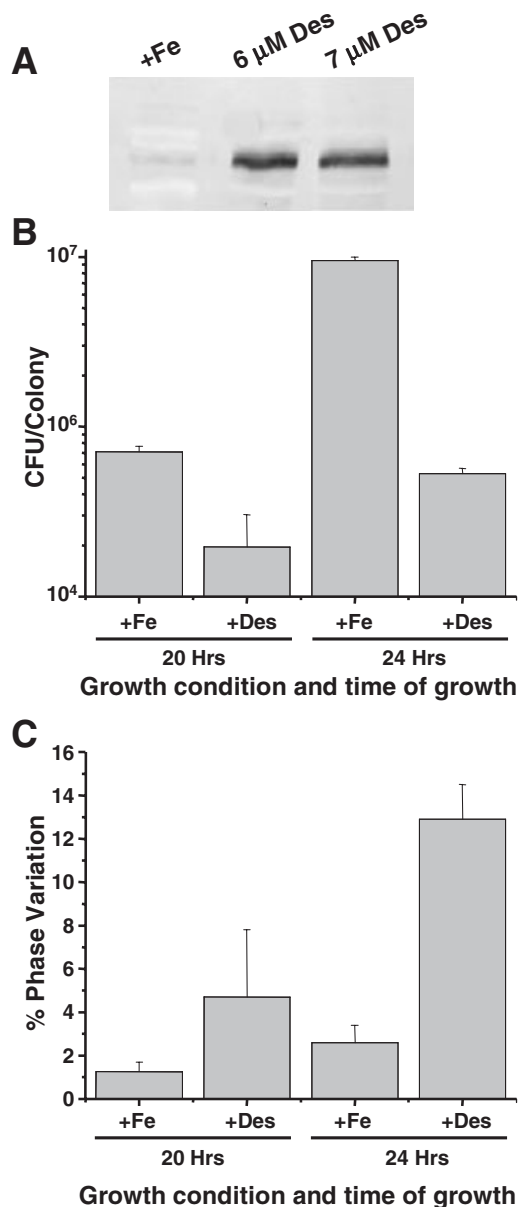


Fig. 1. Iron-limited gonococci exhibit higher frequencies of phase variation after a similar number of generations. A. Western blot of TbpB expression of Gc grown on iron-replete or iron-limiting (6 or 7 μ M Desferal) medium. B. cfu/colony of Gc grown on either iron-replete (+Fe) or iron-limiting (+Des) media after 20 and 24 h of growth. C. Percent of non-piliated colony morphology variants (phase variation) of Gc grown on either iron-replete or iron-limiting media after 20 and 24 h of growth. +Fe indicates iron-replete media. +Des indicates the media contained 6 μ M Desferal.

percent phase variation assay is not practical for use in a genetic screen because of the labour intensive nature of the assay. We therefore sought an alternative method of measuring pilin variation in each of the transformants obtained by the genetic screen. It is well established that strains that do not undergo pilin antigenic variation, such

as *recA* mutants, retain a uniform pilated (P^+) colony morphology during extended growth. Strains that do undergo pilin antigenic variation exhibit alterations in their colony morphology (Koomey *et al.*, 1987; Seifert, 1997). These pilus-dependent colony morphology changes (PDCMC) result from P^- regions that grow out from a P^+ colony where non-piliated regions appear as P^- blebs at the edge of a colony (Fig. 2A). We developed a quantitative method of measuring PDCMC, termed the kinetic PDCMC assay, that records the number of recombination events that lead to observable colony morphology changes (non-piliated blebs) and assigns a score to each colony based on the number of blebs observable per colony (described in *Experimental procedures*). We compared a series of previously characterized Gc strains (*RecA*⁺, *RecA*⁻, *RecJ*⁻, *RecO*⁻ and *RecQ*⁻) to determine whether differences in pilin variation frequencies could be measured using the kinetic PDCMC assay (Fig. 2). We found that the *RecA*⁺ strain displayed a linear progression of colony variation from 22 to 30 h of growth with an average variation score of 1.6 ± 0.11 after 30 h of growth (Fig. 2B). In contrast, a *RecA*⁻ mutant exhibited barely detectable levels of PDCMC and had an average score of 0.05 ± 0.001 after 30 h (Fig. 2B). These results mirror data from the percent phase variation assay in which the *RecA*⁺ strain had a reproducible frequency of phase variation ($4.75 \pm 0.78\%$) and the *RecA*⁻ mutant produced barely detectable levels of phase variation ($0.13 \pm 0.13\%$, Fig. 2C). In both the kinetic PDCMC and percent phase variation assays, *RecJ*⁻, *RecO*⁻, *RecQ*⁻ mutants exhibited a significant decrease in colony variation compared with a *RecA*⁺ strain ($P < 0.05$ by Student's *T*-test). In addition, the frequency of pilin variation of the *RecJ*⁻ and *RecQ*⁻ mutants was statistically different than that of *RecA*⁻ and *RecO*⁻ mutants ($P < 0.05$ by Student's *T*-test, Fig. 2B and C). Thus, the same conclusions can be drawn from the kinetic PDCMC assay as from the percent colony variation assay. As the kinetic PDCMC assay is less labour and time intensive than the percent variation assay, it was used to analyse transformants from the genetic screen for decreases in the frequency of colony variation. In unpublished results from our laboratory, it has been shown that gonococcal variants that have different *pilE* sequences display different frequencies of colony variation (K. Lightfield, K. Kline, and H.S.S. unpubl. results). Therefore, when comparing PDCMC between strains, we insure that they all express the same starting *pilE* sequence.

A genetic screen to identify pilin variation mutants

Gonococcal mutants were generated by *in vitro* transposition of a mini-Tn5 transposon derivative EZ::TN from Epicentre Technologies (mTn5, Goryshin and Reznikoff, 1998) into isolated total Gc genomic DNA and subsequent

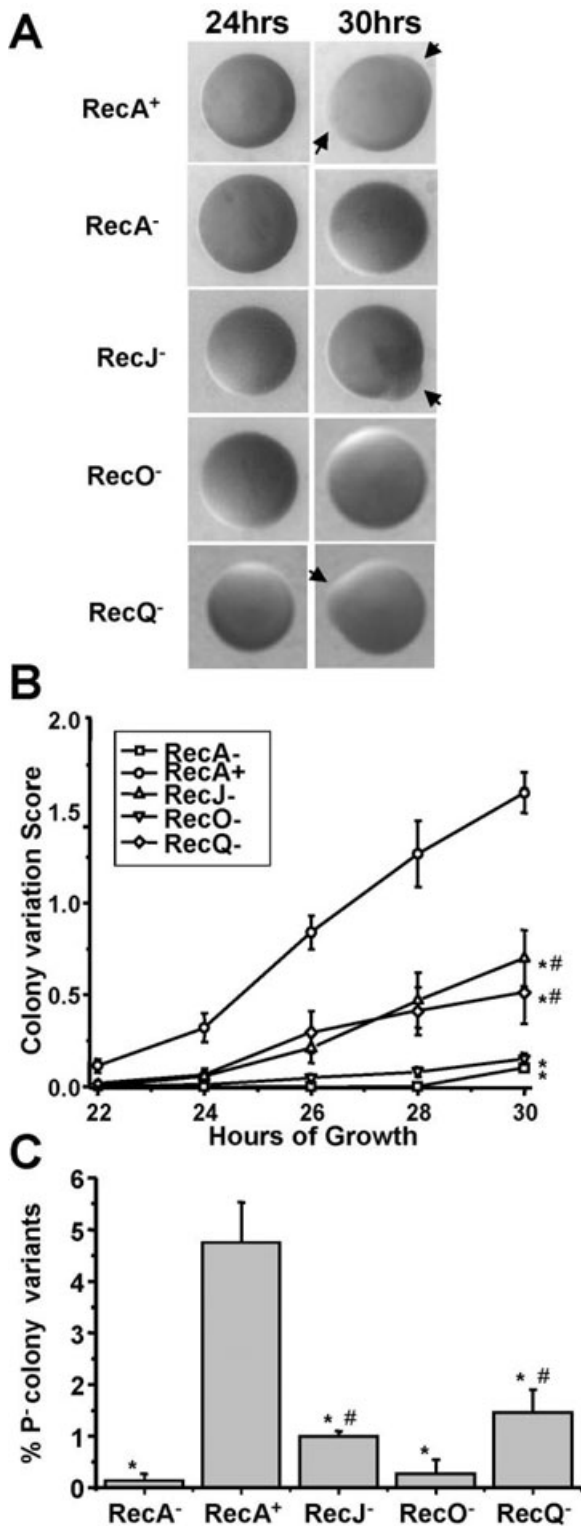


Fig. 2. Comparison of the kinetic PDCMC assay and the percent phase variation assay.

A. Appearance of P⁻ regions in previously characterized Gc strains. Colonies in the left column have been grown for 24 h. Colonies on the right have been grown for 30 h. Black arrows indicate P⁻ regions.

B. Colony variation analysis using the kinetic PDCMC assay. The colony variation score is indicative of the average number of P⁻ blebs each colony exhibited at every time point.

C. Colony variation analysis by percent phase variation measured as the percentage of P⁻ colonies that arise from a P⁺ progenitor. The asterisk indicates a statistically significant difference ($P < 0.05$) compared with RecA⁺. The number sign (#) indicates a statistically significant difference ($P < 0.05$) compared with RecA⁻.

son insertions throughout the chromosome (data not shown). A total of 11 106 transformants generated by eight separate rounds of IVTT were screened on iron-limiting medium for reduced colony variation. The number of transformants screened represents a greater than five-fold over-sampling of the proposed 2182 predicted open reading frames in the gonococcal genome which, by the Poisson distribution, predicts a 99.4% probability that every gene in the genome was disrupted at least once (Daniel, 1999). Of the 11 106 transformants screened, 304 exhibited decreased colony variation (i.e. reduced frequency of P⁻ blebs) after 30 h of growth on iron-limiting medium compared with the majority of the colonies on the primary transformation plates, and these transformants were selected for further analysis.

The kinetic PDCMC assay was used to determine whether the 304 transformants displayed a reproducible decrease in colony variation. Based on the phenotypes of the *recA*, *recJ*, *recO* and *recQ* strains and the 0–4 PDCMC scale, we assigned pilin variation mutants to one of four tiers. Mutants that exhibited a PDCMC score of 0–0.25, similar to *recA* or *recO* mutants, were presumed to have a gene essential for pilin variation inactivated ('----' in Table 1). Mutants that displayed a PDCMC score of 0.25–0.75, similar to *recJ* and *recQ* mutants, were presumed to have a gene important for pilin variation inactivated ('---' in Table 1). Mutants with a PDCMC score of 0.75–1.0 were presumed to have a gene involved in pilin variation inactivated ('--' in Table 1). Finally, mutants showing a PDCMC score higher than 1.0 but lower than the control strain (~1.6) were presumed to have a gene with a minor role in pilin variation inactivated ('+/-' in Table 1). In addition, the kinetic PDCMC assay was performed when Gc were grown on both iron-limiting and iron-replete medium to determine whether the decrease in colony variation of each of the mutants was dependent on iron availability. Mutants that produced a reduced colony variation score only when grown on iron-limited medium were classified as iron dependent (Fig. 3A). Mutants which showed equivalent reductions in colony variation score on either iron-replete or iron-deficient media were classified as iron independent (Fig. 3B).

incorporation of the transposon into the gonococcal chromosome by DNA transformation (in vitro transposition and transformation, IVTT). IVTT resulted in the generation of thousands of transformants with single random transpo-

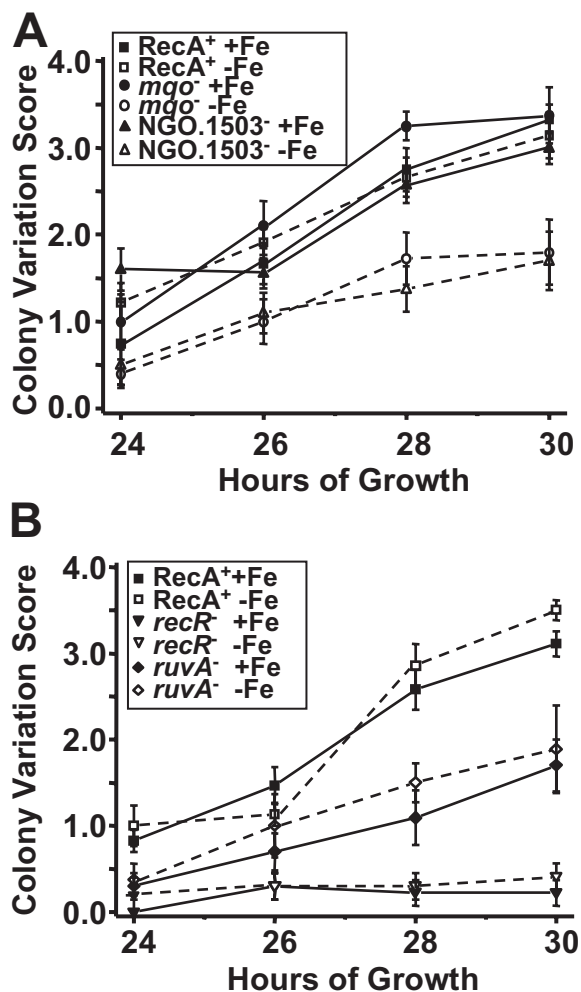


Fig. 3. PDCMC scores of representative mutants. A. Mutants with an iron-dependent phenotype. RecA⁺ control (squares), *mgo*⁻ (circles), NGO.1503 (triangles). B. Mutants with an iron-independent phenotype. RecA⁺ control (squares), *recR*⁻ (triangles), *ruvA*⁻ (diamonds). Data are an average of two separate experiments in which 5–10 colonies were examined per experiment. Solid lines represent growth on iron-replete medium. Dashed lines represent growth on iron-limiting medium.

The 25 transformants that displayed a growth defect on either iron-replete or iron-limiting media were excluded from further analysis, leaving 134 mutants as having a reproducible decrease in colony variation and no growth defect. Of these 134 mutants, 23 exhibited an iron-dependent phenotype while 111 exhibited an iron-independent phenotype. These numbers were reduced to five and 41, respectively, after transformation backcrosses were performed to test whether the colony variation phenotype was directly linked to the transposon. Further inspection of selected mutants whose phenotype did not backcross with the transposon revealed that approximately one-third had *pilE* sequences that differed from the parental strain, indicating that the transformants underwent pilin variation.

Because different pilin variants antigenically vary at different frequencies (K. Lightfield, K. Kline, and H.S.S. unpubl. results), these transformants presumably exhibited lower PDCMC resulting from the variant *pilE* sequence. We are uncertain why the phenotypes of the remaining mutants did not backcross; however, these are most likely because of second site mutations that are not linked to the transposon or the *pilE* gene.

Identification of disrupted genes

To identify the site of transposon insertion in each mutant, two different polymerase chain reaction (PCR)-based approaches were employed (see *Experimental procedures*). For each mutant, the obtained sequence was used in BLASTN searches performed on the entire non-redundant sequence database of the National Center for Biotechnology Information to identify homologous sequences in other organisms with a minimal probability value of E^{-6} used as a cut-off for sequences with significant homology (Altschul *et al.*, 1990). A complete list of genes carrying mTn5 insertions in these mutants is presented in Tables 1 and 2. A preliminary annotated form of the gonococcal genome (provided by D. Dyer) was used to examine the region surrounding each of the disrupted genes for predicted operon structures that were then analysed by reverse transcription polymerase chain reaction (RT-PCR) to define operon structure (data not shown). All potential operons predicted by in silico analysis were found to be transcriptionally linked (Fig. S1). Operons carrying transposon insertions that conferred an essential or important colony variation phenotype ('----' or '---' phenotypes) were subjected to additional transposon mutagenesis to further define the identity of the effecting gene. Mutants with transposon insertions located within operons that conferred less severe colony variation phenotypes were not examined further.

Recombination phenotype analysis of mutants

DNA repair and DNA transformation efficiency assays were performed on each mutant to further assess whether the mutations also affected other homologous recombination-based processes. DNA repair was assayed by measuring resistance to killing by ultraviolet (UV) light, and DNA transformation was assayed by measuring the frequency at which a point mutation in *gyrB* that confers resistance to nalidixic acid was incorporated into the gonococcal chromosome (Stein, 1991). Based on the results of these assays, the mutants were segregated into one of six categories (Tables 1 and 2). All the phenotypes of mutants in Classes 1–4 are iron independent; whereas, the colony variation phenotype of the Class 5 and 6 mutants are iron dependent. Class 1 mutants were those

Table 1. Iron-independent mutants.

Site of insertion				Recombination phenotypes		
Orf no.	Gene/locus	Effector gene	Predicted function	Pilin variation ^a	DNA repair ^b	DNA transformation ^c
Class 1						
1509	<i>recO</i> (3) ^d	<i>recO</i>	Assist in RecA-mediated strand exchange	----	~1000×	+
1510	<i>pheA</i> (8)	<i>recO</i>	Assist in RecA-mediated strand exchange	----	~1000×	+
1722	<i>recQ</i> (2)	<i>recQ</i>	3'→5' helicase	---	~100×	+
414	<i>recJ</i>	<i>recJ</i>	5'→3' single strand exonuclease	---	~1000×	+
767	<i>recR</i> (2)	<i>recR</i>	Assist in RecA-mediated strand exchange	----	~100×	+
117	<i>recG</i> (2)	<i>recG</i>	Branch migration of Holliday junctions	---	~100×	+
1730	<i>ruvA</i>	<i>ruvA</i>	Branch migration of Holliday junctions	---	~100×	+
Class 2						
1800	<i>ecfE/yael</i> (2)	<i>ecfE/yael</i> ^e	Membrane associated protease	----	+	~10×
289	<i>cutE</i>	<i>cutE</i>	Apolipoprotein acyltransferase	---	+	~10×
1695	<i>aroG</i>	ND ^f	Insertion in an operon ^g	--	+	~50×
31	<i>rimI</i>	ND	Insertion in an operon	--	+	~50×
443	<i>dacC</i>	<i>dacC</i>	D-alanyl-D-alanine carboxypeptidase	--	+	~10×
222	NGO.0222	ND	Insertion in an operon	--	+	~10×
1344	<i>asmA</i>	<i>asmA</i>	Outer membrane protein expression	--	+	~5×
1189	<i>yrfL</i> family	<i>yrfL</i>	Heat shock chaperonin	--	+	~5×
Class 3						
1721	<i>trpC</i> (3)	<i>recQ</i>	Tryptophan biosynthesis/helicase	----	+	+
2063	<i>pilE</i> locus (2)	5' of <i>pilE</i>	<i>cis</i> -acting sequence	----	+	+
690	NGO.0690	<i>thrC</i>	Threonine biosynthesis	---	+	+
2075	<i>thrB</i>	<i>thrB</i>	Threonine biosynthesis	---	+	+
^h	23S rRNA	23S rRNA	Large ribosomal subunit component	--	+	+
^h	16S rRNA	16S rRNA	Small ribosomal subunit component	--	+	+
173	<i>rimM</i>	ND	Insertion in an operon	--	+	+
43	<i>prmA</i>	ND	Insertion in an operon	--	+	+
26	NGO.0026	NGO.0026	Endopeptidase	--	+	+
Class 4						
2117	NGO.2117	NGO.2119 ⁱ	ABC transporter	---	~10×	~50×

a. Indicates the frequency of colony variation relative to the parental strain: (----) is a colony variation score of 0–0.5, (---) is a score of 0.5–1, (--) is a score of 1–2, and (– and –/+) is a score of 2–2.5.

b. Relative survival after UV exposure at 80 mJ m⁻² relative to the parental strain.

c. DNA transformation efficiency relative to the parental strain.

d. Number of independent insertions identified.

e. The downstream gene *omp85* is essential and cannot be ruled out as the effector. Insertions in the next gene of the operon, *ompH*, show a growth defect and were not tested for a variation phenotype; however, the *ecfE/yael* mutants do not show a growth defect and is the probable effector.

f. Effector gene of mutants with pilin variation phenotypes of (–) in operons was not determined but are listed in Table S1.

g. Operons were confirmed by RT-PCR analysis.

h. We have not been able to determine which rRNA gene copies were inactivated.

i. Insertions into all three ABC transporter encoding genes (NGO.2117–2119) produced a phenotype but only the last gene in the operon with the phenotype (NGO.2119) has been conclusively shown to be required.

Table 2. Iron-dependent mutants.

Site of insertion			Recombination phenotypes					
Orf no.	Gene/locus name	Predicted function	Pilin variation		DNA repair		DNA transformation	
			+Iron	-Iron	+Iron	-Iron	+Iron	-Iron
Class 5								
1714	<i>surA</i>	peptidyl-prolyl- <i>cis</i> - <i>trans</i> - isomerase	+/-	--	+	+	~5×	~5×
Class 6								
806	<i>cysG</i>	insertion in an operon ^a	+/-	--	+	+	+	+
1980	<i>mgo</i>	malate:quinone oxidoreductase	+/-	--	+	+	+	+
1503	NGO.1503	conserved hypothetical protein	+/-	--	+	+	+	+
1964	NGO.1964	upstream of IS1016-like transposase	+/-	--	+	+	+	+

a. Effector gene not determined because of minimal phenotype.

that have decreased pilin variation and DNA repair capabilities. Class 2 mutants exhibited decreases in pilin variation and DNA transformation. Class 3 mutants were those with a deficit only in pilin variation, and the Class 4 mutant exhibited decreased pilin variation, DNA repair and DNA transformation. The Class 5 mutant exhibited an iron-dependent decrease in pilin variation and an iron-independent decrease in DNA transformation. Class 6 mutants had an iron-dependent decrease in pilin variation and wild-type frequencies of DNA repair and DNA transformation. The essential and important mutants from each class will be discussed below.

Class 1 mutants. Class 1 mutants were defined as having iron-independent decreases in pilin variation and DNA repair capabilities (Table 1). There were seven genes in Class 1 and all are either essential or important for pilin variation based on their colony variation phenotypes (Table 1). All of the Class 1 mutants resulted from insertions that disrupted the expression of genes encoding known or predicted recombination related activities. Among the Class 1 genes are the RecF-like pathway genes *recJ*, *recO* and *recQ*, which have been previously demonstrated to be required for pilin variation and DNA repair in Gc (Mehr and Seifert, 1998; Skaar et al., 2002). Several different insertions were also identified in the *pheA* gene; predicted to encode for corismate mutase, an enzyme involved in phenylalanine biosynthesis. *pheA* is located immediately upstream of *recO* and insertions in *pheA* have been shown to have polar effects on *recO* expression (Mehr and Seifert, 1998). The remaining genes identified in the Class 1 mutants were predicted to encode RecR, RecG and RuvA homologues. Each of these genes represents previously unexamined components of the gonococcal pilin variation and DNA repair machinery. In *Escherichia coli*, the RecR protein acts in a complex with RecO (Umezue and Kolodner, 1994) to stimulate RecA activity (Umezue et al., 1993) and presumably has a similar role in Gc. This predicted cooperation between Gc RecR and RecO is consistent with the pilin variation phenotype of the *recR* mutant which displayed a loss of pilin variation that was indistinguishable from *recO* and *recA* mutants (Fig. 2 and Table 1). These results confirmed previous conclusions that the RecF-like pathway is required for efficient pilin recombination and DNA repair (Mehr and Seifert, 1998; Skaar et al., 2002). The pilin variation phenotypes of Gc *recG* and *ruvA* mutants were indistinguishable from that of *recJ* and *recQ* mutants in that, although clearly deficient in pilin variation, they exhibited higher frequencies of pilin variation than a *recA* mutant (Fig. 2 and Table 1). In *E. coli*, both RecG and RuvA are involved in branch migration of Holliday junctions (Parsons et al., 1992; Lloyd and Sharples, 1993). While the function of RuvA homologues in recombination

is well established, the gonococcal *ruvA* gene appears to be in an operon with five downstream genes. Using IVTT of a defined chromosomal fragment, we disrupted the two genes immediately downstream of *ruvA*, and showed that disruption of either gene downstream of *ruvA* produced parental levels of colony variation (data not shown). Therefore, the loss of RuvA activity was directly responsible for the observed pilin variation phenotype.

Class 2 mutants. Class 2 mutants showed iron-independent decreases in pilin variation and DNA transformation. Based on their PDCMC phenotypes, two Class 2 mutants contained insertions in genes whose products are essential for pilin variation, while the remaining six mutants had inactivated genes that are involved in pilin variation and will not be discussed in detail (Table 1). While the decreased DNA transformation frequencies of the Class 2 mutants ranged from five- to 50-fold lower than that of the parental strain, the phenotypes of the mutants were not as severe as that of a *recA* mutant. These transformation phenotypes were similar to those observed in other DNA transformation mutants, such as disruption of the RecBCD pathway in Gc (Mehr and Seifert, 1998) or the AddA and AddB recombinases of *Bacillus subtilis* (Alonso et al., 1993).

Two insertions were identified in an open reading frame that has homology to the *ecfE/yaeL* gene of *E. coli*. The *E. coli* gene homologue encodes a membrane-associated, Zn-dependent protease (Dartigalongue et al., 2001) that is involved in the heat shock response and the degradation of the antisigma factor RseA (Alba et al., 2002). The gonococcal *ecfE/yaeL* gene is located within a large operon with six downstream genes (Fig. S1A). The severe pilin variation phenotype of these mutants ('----', Table 3) indicates that whichever gene or genes are responsible for the phenotype are essential to pilin variation. To determine whether inactivation of *ecfE/yaeL* or inhibition of gene expression downstream of *ecfE/yaeL* was responsible for the observed loss of pilin variation, cloned PCR products representing the downstream genes were subjected to transposon mutagenesis (*Experimental procedures*). Immediately downstream of *ecfE/yaeL* is the *omp85* gene which is involved in membrane biosynthesis and is an essential gene in *Neisseria meningitidis* (Genevrois et al. 2003; Voulhoux et al., 2003). Neither of the two insertions in *ecfE/yaeL* resulted in a growth defect suggesting that sufficient *omp85* expression was retained in the *ecfE/yaeL* mutants to allow normal growth. No insertions were isolated within *omp85*, confirming its essentiality. While this essentiality prevents us from directly testing for a role of Omp85 in pilin variation, its role in envelope assembly and the lack of a growth phenotype of the *ecfE/yaeL* mutants suggest that *omp85* is not responsible for the phenotypes. Several insertions were isolated

Table 3. Iron-regulation of Class 5 and 6 genes.

Gene	Putative Fur box ^a	No. of matches ^b	Rel. induction ^c
<i>tbpB</i>	<u>AATAAATAAAATAATAATC</u>	14	50
<i>omp3</i>	none ^d	0	0
<i>surA</i>	<u>GGCTTTATTTTCATCATTIT</u>	12	< 2
<i>cysG/N</i>	none	0	0
<i>mgo</i>	<u>ATAAATACTATTTTTATTTTT</u>	13	-2
NGO.1503	<u>CCITCACCGACAACATATTT</u>	10	0
IS1016	none	0	> 2

a. Underlined base pairs indicate matches to the Gc consensus Fur box (GATAATATAATAATTATCTTT).

b. Number of matches to the Gc consensus Fur box.

c. Fold induction of transcript levels in iron-limited Gc relative to iron-replete Gc determined by comparing C_T's using real-time RT-PCR.

d. No Fur box with >9 matches to the 21 bp consensus.

within the next gene in the operon, *ompH*, which encodes the putative periplasmic chaperone SKP (Thome and Muller, 1991). The *ompH* mutants had a severe growth defect, and were not further analysed, although this finding also suggests that inhibition of *ompH* expression was not responsible for the pilin variation phenotype. Five transposon insertions in the two further downstream genes (*lpxD* and *fabZ*) all demonstrated parental levels of pilin variation showing that these genes were not involved in these phenotypes. Based on this evidence, we conclude that direct inactivation of *ecfE/yaeL* is the most likely reason for the phenotypes of the original two Class 2 mutants.

Another Class 2 mutant contained an insertion in an open reading frame that has homology to *cutE* of enteric bacteria. In *E. coli* and *Salmonella typhimurium*, CutE is an essential inner membrane protein that catalyses the transfer of a fatty acid chain from phospholipids to apolipoproteins. Located downstream of Gc *cutE* in the same transcriptional unit is *rpoH* (Fig. S1B), which encodes the RNA polymerase subunit σ^{32} (Laskos *et al.*, 2004). To determine whether loss of *cutE* or *rpoH* was the cause of the measured decrease in pilin variation, the two genes were cloned as one fragment and subjected to transposon mutagenesis. These new *cutE* mutants displayed reduced pilin variation, whereas *rpoH* mutants retained wild-type frequencies of pilin variation (data not shown). These results indicate that inactivation of *cutE* is specifically causing the observed pilin variation phenotype.

Class 3 mutants. The Class 3 mutants exhibited decreased pilin variation but retained frequencies of DNA repair and DNA transformation identical to the parental strain. Of the nine Class 3 genes identified, four were found to be either essential or important for pilin variation, whereas the other five mutant genes conferred phenotypes which suggested that the genes are involved in pilin variation. Three of the nine Class 3 genes are contained within operons as evidenced by both in silico and RT-PCR

analysis (data not shown). Three different insertions were isolated in *trpC*, a gene in the tryptophan biosynthetic pathway (Table 1). Located immediately downstream of *trpC* is *recQ*, which was previously shown to be involved in pilin variation (Mehr and Seifert, 1998); suggesting that the *trpC* mutants were altering pilin variation by effecting *recQ* expression. A functional copy of *recQ*, under *lac* regulatory control, was introduced into an irrelevant intergenic site in the gonococcal chromosome (Mehr and Seifert, 1998). The parental pilin variation phenotype was restored when the *recQ* was expressed in one *trpC*::mTn5 mutant (Fig. 4); proving that the insertions in *trpC* were disrupting expression of RecQ to produce the variation phenotype.

Two Class 3 mutants contained insertions upstream of the *pilE* gene, within 50 bp of each other, both of which resulted in a near total loss of pilin variation (Table 1). The transposon insertions were located upstream of the *pilE*

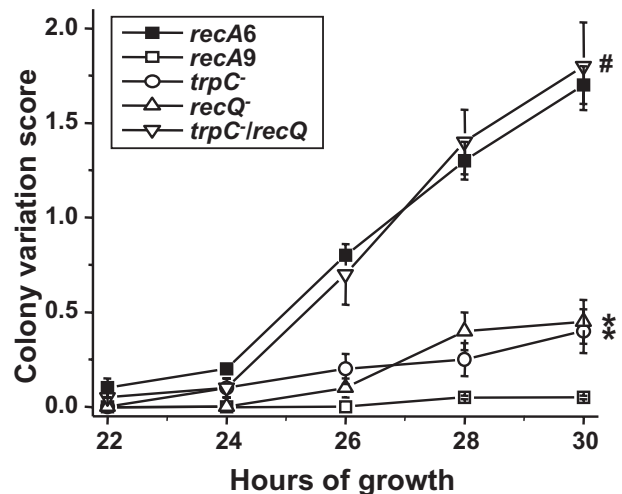


Fig. 4. The *trpC* mutant is complemented by *recQ* at a second site. The PDCMC assay was performed on 5–10 colonies with three replicates. All strains carry the *recA6* allele were grown in the presence of IPTG. The *recQ* complement was introduced into the intergenic nics locus.

promoter (Meyer *et al.*, 1984), but downstream of the upstream silent pilin copy linked to *pilE* and the pilin associated repeats RS1 and RS2 (Haas and Meyer, 1986) (Fig. 5A). These transposon insertions do not lie in a predicted open reading frame and do not alter pilin expression by Western blot analysis (Fig. 5B). Therefore, it is likely that these transposons are affecting *cis*-acting sequences important for pilin antigenic variation.

The two remaining Class 3 mutants carried insertions in genes involved in threonine biosynthesis. One insertion was within the *thrB* coding sequence, which by *in silico* analysis is not part of an operon. The *E. coli thrB* homologue functions as a homoserine kinase and is required for L-threonine biosynthesis (Theze *et al.*, 1974). Expression of the *thrB* gene at an ectopic site proved that the observed phenotype was because of the loss of *thrB* expression (Fig. 6). The second Class 3 mutant had the transposon inserted into NGO.690 which is predicted to encode a *Neisseria*-specific uncharacterized protein. However, *in silico* and RT-PCR analysis showed that NGO.690 was in a four-gene operon with *thrC*, NGO.688 and *fpr* (Fig. S1C and data not shown). Specific transposon mutagenesis showed that insertions within NGO.690 or *thrC* produce pilin variation-deficient cells, whereas insertions within NGO.688 had no effect on pilin variation (data not shown). We conclude that *thrC* is important for pilin antigenic variation, although a role for NGO.690 cannot be ruled out. *thrC* encodes threonine synthetase which acts immediately downstream of *thrB* to produce L-threonine from homoserine. Either levels of L-threonine or

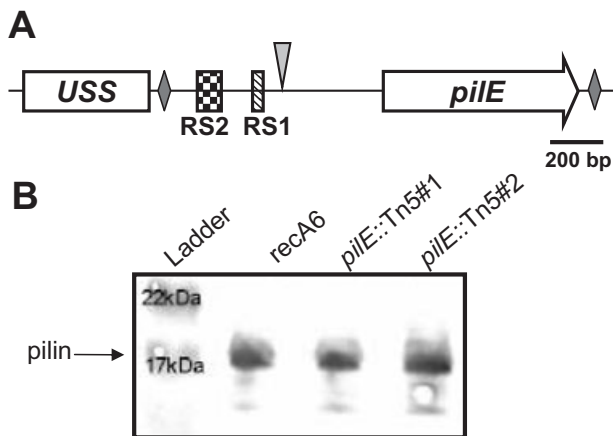


Fig. 5. Class 3 mTn5 insertions upstream of *pilE*.

A. Map of the *pilE* locus. USS shows the upstream silent copy of pilin sequences. Arrow indicates transcription start site. Sites of transposon insertions are indicated by the light grey triangles, the *Sma*/*Cla* repeats are represented by diamonds (Meyer *et al.*, 1984), and repeat sequence 1 is shown by the hatched box while repeat sequence 2 by the checkered box. (Haas and Meyer, 1986).

B. Western blot analysis of pilin expression. Shown are the *recA6*-parental strain and the isogenic *pilE*::Tn5#1 and *pilE*::Tn5#2 Class 3 mutants.

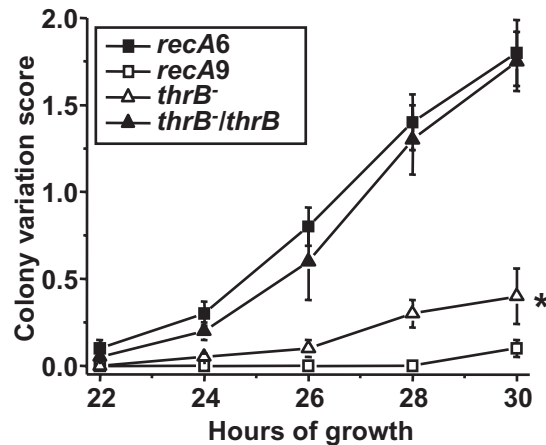


Fig. 6. Complementation of the *thrB* mutant. PDCMC were measured on 5–10 colonies in two separate experiments. A parental copy of the *thrB* gene was expressed in the intergenic *nics* locus. All strains were propagated on media with IPTG to induce RecA expression. The asterisk indicated $P < 0.05$ as compared with the *recA6* strain.

L-homoserine are important for pilin variation, or these enzymes play an unappreciated role in pilin antigenic variation.

The Class 4 mutant. One mutant was identified that exhibited a decrease in all three recombination-based processes examined (Table 1). This mutant had a transposon insertion in a gene encoding for a putative inner membrane component of an ATP-binding cassette (ABC) transporter. Analysis of the surrounding sequence and open reading frames suggested that the transposon insertion resides in the second gene of a possible nine gene operon (Fig. S1D); therefore, the possibility remained that altered expression of a downstream gene may be responsible for the phenotypes of this particular mutant. Downstream of the inner membrane component are genes that encode an outer membrane component and periplasmic solute binding component of the putative ABC transporter. To determine which gene or genes are responsible for the observed decrease in pilin variation, transposon mutagenesis was performed on cloned PCR fragments carrying the entire operon. This analysis revealed that insertions in either the inner membrane component, the outer membrane component, or the periplasmic solute binding component of the putative ABC transporter resulted in a near total loss of pilin variation (data not shown). In contrast, insertions into each of the genes downstream of the ABC transporter components had no effect on pilin variation (data not shown). We can conclude that loss of the periplasmic solute binding protein (NGO.2119) produces the reduction in recombination. We cannot, from these analyses, determine whether the two genes upstream (NGO.2117 and NGO.2118) are also important for recombination because the transposon insertions in these

genes could be acting on NGO.2119 expression. However, as these gene products are all predicted to act together as an ABC transporter, we assume that inactivation of any of the three genes encoding the transport apparatus will alter the recombination abilities of Gc. Our favoured hypothesis is that this ABC transporter is required for the import or export of a molecule important for allowing recombination at normal levels. However, at this time the identity of such a molecule is unknown.

Class 5 and Class 6 mutants. The Class 5 and 6 mutants all exhibited an iron-dependent decrease in colony variation (Table 2). The sole Class 5 mutant also exhibited an iron-independent decrease in DNA transformation competence; whereas, the four Class 6 mutants have no change in their measured DNA repair or DNA transformation capabilities (Table 2). Inactivation of the Class 5 and Class 6 genes showed a minor effect on pilin variation (Table 2) and they will not be discussed in detail except as noted below.

A Class 6 mutant had the transposon insertion upstream of *orf* NGO.1964, a truncated copy of the IS1016 transposase (Fig. 7). The IS1016 insertion sequence was originally identified in *Haemophilus influenzae*, where it borders the capsulation gene cluster and serves as a substrate for *cap* gene amplification (Kroll *et al.*, 1991). To date IS1016 sequences have only been identified in *H. influenzae*, *N. meningitidis* and Gc (Kroll *et al.*, 1991; Thompson and Sparling, 1993). Strain FA1090 contains 23 copies of the IS1016 transposase gene (Gonococcal genome sequencing project, <http://www.genome.ou.edu/gono.html>). Analysis of the DNA sequence surrounding the predicted IS1016 sequence suggests that this copy of IS1016 transposed into a *Neisseria*-specific gene, and since then has diverged such that only the C-terminal half of the transposase could be expressed, because the N-terminal coding sequence carries several stop codons (Fig. 7). The transposon insertion is immediately upstream of the predicted start of the original IS1016 transposase but downstream of the predicted promoter sequence (Fig. 7). It is likely that the transposon insertion is inhibiting expression of the truncated IS1016 transposase and that the iron-dependent pilin variation

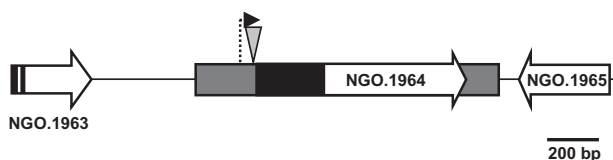


Fig. 7. Transposon insertion near the truncated IS1016. Intact IS1016 C-terminus (NGO.1964), degenerate IS1016 N-terminus (grey box), putative transcription start site of original IS1016 (dashed arrow), transposon insertion (light grey triangle), *Neisseria*-specific gene (dark grey box).

phenotype, albeit minor, results from an unappreciated activity of this truncated transposase.

As each of the Class 5 and Class 6 insertions was identified as an iron-dependent mutant, we sought to determine whether transcription of any of those five genes was iron-regulated. We searched for sequences in the 5'-portion of each gene that were similar to the consensus Gc Fur box (Genco and Desai, 1996). The *surA*, *mgo* and NGO.1503 genes each contained a sequence with homology to the Gc Fur box; whereas, no potential Fur binding sites were identified with the *cysG/N* and IS1016 genes (Table 3). To determine whether any of the genes inactivated in the iron-dependent mutants were iron-regulated, relative, real-time, RT-PCR was used to examine transcript levels of all five genes in both iron-replete and iron-limited grown Gc. The iron-inducible *tbpB* gene and the Fur-independent *omp3* gene were used as controls for iron-responsive genes (Sebastian *et al.*, 2002). We observed a greater than 50-fold increase in *tbpB* message levels in iron-limited Gc compared with iron-replete Gc, and no difference in *omp3* message levels in response to iron limitation (Table 3). When transcript levels of the Class 5 and Class 6 genes were assessed, there was an approximate twofold increase in IS1016 and *surA* transcript levels, a twofold decrease in *mgo* message levels, and no change in transcript levels of either the *cysG/N* or the NGO.1503 genes (Table 3). The level of IS1016 transcript induction may well be larger than what was measured for our inability to specifically examine the mutated IS1016 copy. Therefore some, but not all of the genes mutated in the iron-dependent pilin variation mutants, show changes in transcript level in response to changes in iron-availability. The small changes in gene expression of the truncated IS1016, *surA* and *mgo* genes, coupled with the modest phenotypes of the mutants inactivated for these genes, suggest that none of these genes encode major effectors or regulators of the iron-dependent recombination response.

Discussion

We conducted a genetic screen for factors that are involved in pilin variation in *N. gonorrhoeae*, and identified many distinct mutants with decreased PDCMC. These mutants had insertions into 30 different genes (Tables 1 and 2). Seven of these mutants had inactivated genes essential for pilin variation (PDCMC score of 0–0.25) and seven mutants had insertions in genes important for pilin variation (PDCMC score of 0.25–0.75). The remaining mutants had consistent phenotypes suggesting that the inactivated genes were involved in pilin variation (PDCMC score of 0.75–1), but were not centrally involved in this process. Most of the mutants showed reduced PDCMC regardless of whether they were grown on iron-replete or

iron-limited medium (iron-independent mutants), while five of the mutants only displayed a reduction in PDCMC when grown on iron-limited medium (Table 2). There were 25 genes identified in the iron-independent mutants and five within the iron-dependent mutants. By conducting this genetic screen, many new genes that participate in or influence the process of pilin variation have been identified.

We chose to screen a sufficient number of mutants to provide a fivefold over sampling of all 2182 predicted gonococcal open reading frames and allow for a >99% probability of disrupting each open reading frame at least once. Despite the appropriate scope of the mutant screen, we conclude that this screen was not saturating for mutations that produce reduced frequencies of PDCMC. Of the 30 genes mutated in this screen, eight were identified more than once with distinct insertion sites while three of those eight were identified more than twice. If saturating, we would expect that one third of the mutants would have single insertions per gene, but with almost two thirds of the mutants being single gene insertions, we did not reach statistical saturation. Moreover, there are a few genes we would have predicted to have yielded variation phenotypes that were not isolated. Included in this list is the *recA* gene, which is absolutely required for pilin variation (Kooomey *et al.*, 1987), and the *recX* gene, which aids in pilin variation (Stohl and Seifert, 2001). We also would have expected to have inactivated the *ruvB* gene which should function in conjunction with *ruvA*. The reasons why this screen was not saturating are not known, but it is most likely related to the phenotype used for the screen – changes in the observable frequencies of PDCMC. As the colony-based PDCMC screen depends on growth, any mutation that created a growth defect was not further assayed because it was impossible to separate the growth phenotype from a PDCMC phenotype. Therefore, mutations that altered the expression of an essential gene or a gene that influences growth rate were not assayed (this includes insertions in an operon encoding such a gene). This problem may have been exacerbated by conducting the screen on iron-limited media. Secondly, analysis of the mutants with multiple insertions in the same gene showed they all had a prominent phenotype, whereas all of the single insertion mutants had a weaker phenotype (Tables 1 and 2). It is likely that some mutants with minor reductions in PDCMC were missed in this collection of mutants. Therefore, it is likely that there are genes involved in the process of pilin variation that have not been identified.

This screen was initially undertaken to identify regulators or effectors of the increased recombination frequencies observed when Gc are iron limited. We did not find a mutant that defined an iron-dependent regulator or a necessary effector. Our main hypothesis at this time is that

Fur is the regulator of the iron-dependent recombination response. Subsequent to the conclusion of this screen it was reported that *fur* is not an essential gene in *N. meningitidis*, but that the gene immediately downstream of *fur*, *aat*, which encodes the leucyl, phenylalanyl-tRNA-protein transferase is an essential gene (Delany *et al.*, 2003). Although *fur* is not an essential gene in *N. meningitidis*, the meningococcal *fur* mutant does have a growth defect. We have been unable to create a non-polar *fur* knockout in Gc and therefore have been unable to directly test the role of Fur in regulating recombination in Gc (D. Weinberger, A. Criss, and HSS, unpubl. obs.).

The identification of new genes which are essential or important for pilin variation has yielded the most significant findings of this study. The *recR* mutant displays phenotypes similar to the previously described *recJ*, *recO* and *recQ* mutants (Mehr and Seifert, 1998; Hill, 2000; Skaar *et al.*, 2002). Based on the known association of this gene product with the *E. coli* RecF recombination pathway, we can assign *recR* to the Gc RecF-like recombination pathway (Mehr and Seifert, 1998). Our current hypothesis is that these gene products act to allow RecA to efficiently utilize gapped DNA substrates during pilin variation, although we have no direct evidence for this speculation.

The identification of *recG* and *ruvA* mutants as being pilin variation deficient suggests that Holliday junctions are important for this process. In *E. coli*, both RecG and RuvA have roles in the later stage of recombination by promoting branch migration of Holliday junctions, but these gene products play separate roles in *E. coli* recombination and repair processes (Parsons *et al.*, 1992; Lloyd and Sharples, 1993). The gonococcal *recG* and *ruvA* mutants both showed ~100-fold reduced survival to UV damage, while *E. coli* *ruvA* mutants are more sensitive to UV damage than *recG* mutants (Lloyd, 1991). These data suggest that the gonococcal *recG* and *ruvA* genes may play more similar roles in repair of DNA damage than the *E. coli* enzymes. The Gc *recG* and *ruvA* mutants exhibited similar levels of colony variation (Table 1), but neither mutant completely blocks pilin variation as a *recA* mutant does. It is possible that these Holliday junction-associated proteins play redundant roles in pilin variation and that double mutants will be absolutely deficient. Regardless, the identification of *ruvA* and *recG* in this genetic screen represents the first evidence that Holliday junctions form and that branch migration is required for pilin variation.

In addition to the identification of novel gene products important for pilin variation, two different transposon insertions within the non-coding sequences upstream of *pilE* were identified. As the transposon insertions are not located within a predicted *orf*, there are two main hypotheses to explain the effect of these insertions on pilin variation, but the distinction between the two hypotheses is not clear. The first hypothesis is that the participation of

upstream sequences is altered by the transposon insertions preventing them playing their normal function in pilin variation. Two pilin-associated repeats, RS1 and RS2 (Haas and Meyer, 1986), as well as the *pilE*-associated silent pilin copy, lie directly upstream of these transposon insertions. Our laboratory has previously proposed that sequences external to the *pilE* region may be required for pilin variation (Wainwright *et al.*, 1994; Howell-Adams and Seifert, 2000) and these conserved sequences could be playing a role in this recombination system. Alternatively, the sequences disrupted by the transposon insertions may play a direct role in antigenic variation. Regardless of which of these models is correct, these insertions show that the sequences upstream of *pilE* are important for pilin variation. We are currently determining how and when these *cis*-acting sequences function.

Several of the mutants with strong pilin variation phenotypes contained insertions in genes whose role in this process are not obvious. We have no suggestions as to why inactivation of a membrane-associated protease (*ecfE/yael*), a predicted apolipoprotein acyltransferase (*cutE*), or two of out of the eight ribosomal rRNA encoding genes (16S and 23S rRNA genes), would severely disrupt pilin variation. We have speculated that two genes involved in threonine biosynthesis (*thrB* and *thrC*), and one or more components of a predicted ABC transporter (NGO.2117–2119) act to allow small molecules to influence pilin variation but are unaware of any previously described mechanisms whereby metabolites or transported molecules alter recombination processes. It seems plausible that all of these mutants point to more unappreciated aspects of Gc physiology that directly influence this recombination process, but additional experimentation will be required to reveal how these genes are involved in this pathogenic process.

Experimental procedures

Bacterial strains and growth conditions

Neisseria gonorrhoeae FA1090 variant 1-81-S2 *recA6* is a translucent variant of the volunteer isolate 1-81-S2 containing the IPTG-inducible *recA6* allele (Seifert, 1997). Gc were grown on GCB which consists of 36.25 g l⁻¹ of Gc medium base (Difco) containing 1.25 g l⁻¹ of agar, Kellogg's supplements I [0.4% glucose (Sigma), 0.01% glutamine (Sigma), 0.000002% cocarboxylase (Sigma)], and Kellogg's supplements II [ferric nitrate (0.0005 g l⁻¹; Difco)] at 37°C + 5% CO₂. Gonococcal liquid medium (GCBL) consists of 1.5% Proteose Peptone No. 3 (Difco), 0.4% K₂HPO₄, 0.1% KH₂PO₄, 0.1% NaCl (Difco), pH 7.2. Gonococcal frozen stocks were generated by swabbing (Dacron swab from Puritan) a lawn of Gc into 1 ml of GCBL containing 20% glycerol (GCBG).

When appropriate 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Diagnostic Chemicals, Ltd) was added to the media to allow expression from the regulatable promoter of

the *recA6* construct. Kanamycin was added to media when necessary at a concentration of 40 µg ml⁻¹. Deferoxamine mesylate (Desferal, Sigma) was used at a concentration of 4 and 6 µM in solid medium and 100 µM in liquid medium. Polyacrylamide gel electrophoresis and Western blot analysis of TbpB expression were performed as described previously to monitor iron availability (Serkin and Seifert, 2000).

Generation of gonococcal mTn5 mutants

Gonococcal genomic DNA was isolated as follows. Briefly, a confluent lawn of Gc was collected after 24 h of growth and lysed by the addition of 50 µl of 10% SDS with 3 µl of RNaseA at 10 mg ml⁻¹. DNA was extracted twice with phenol and chloroform:isoamyl alcohol (24:1), and once with chloroform:isoamyl alcohol. The genomic DNA was isopropanol precipitated, washed with 70% ethanol, resuspended in TE, and quantitated using the Low DNA Mass Ladder (Stratagene).

In vitro transposition was performed using the EZ::TN transposition system (Epicentre Technologies). The EZ::TN transposon and genomic DNA were combined in a 100:1 molar ratio with transposition buffer, transposase and water to a final volume of 15 µl and incubated at 37°C for 2 h as instructed. Stop solution (1% SDS) was added to the transposition reaction and incubated at 70°C for 10 min. The DNA was then ethanol precipitated and resuspended in 10 µl H₂O, and the gapped transposon insertion was repaired using T4 DNA polymerase and T4 DNA ligase (Pelicic *et al.*, 2000).

The transposon insertions were then introduced into the Gc genome by transformation. Gonococcal transformation was performed essentially as previously described (Mehr and Seifert, 1998). Transformants were then selected on Gc solid medium containing kanamycin, IPTG and 6 µM Desferal.

After 30 h of growth transformants were screened for PDCMC. Colonies with a PDCMC score of ≤ 1 (see kinetic PDCMC assay) were passaged onto Kan containing GCB. After 24 h of growth a single piliated colony of each transformant was again passaged onto Kan containing GCB. After an additional overnight growth a single piliated colony of each transformant was passaged to obtain a lawn. A frozen stock was then generated the following day.

Pilus-based colony variation assays

Percent phase variation assay. Gonococci were revived from frozen stocks. After 24 h of growth a single P⁺ colony was passaged onto medium containing IPTG to obtain single isolated colonies. After 24 h five representative colonies were picked using a piece of sterile filter paper and disrupted in 1 ml of GCBL and then serially diluted 10-fold. One hundred microlitres of the 10⁻³ and 10⁻⁴ dilutions were plated onto plain GCB. After overnight incubation the number of P⁻ and the total colonies were counted and the frequency of pilin variation was determined by the ratio of the number of P⁻ colonies to the total number of colonies.

Kinetic PDCMC assay. Gonococci were revived from frozen stocks. After 24 h of growth a single P⁺ colony was passaged onto the appropriate medium to obtain single iso-

lated colonies. Seven transformants and the parental control were passaged onto each plate into equal sectors. Colony variation was scored after 22, 24, 26, 28 and 30 h of growth by observing the number of P⁻ regions on the same 5–10 colonies per time point with a stereomicroscope. Colony variation was scored by assessing the number of P⁻ regions exhibited by each colony. A colony that showed no P⁻ regions was given a score of 0. A colony that had one P⁻ region was scored as 1. A colony with two P⁻ regions was scored a 2. Colonies that had three of four P⁻ regions were scored as 3 or 4 respectively. Any colony with more than four regions was given a score of 4. The individual colony scores for each time point were averaged and graphed.

Identification of transposon insertion site

The site of transposon insertion was identified one of two ways: (i) by using an adapted anchored PCR approach and (ii) by RATE (Ducey, 2002). The anchored PCR was performed as follows. Using the Stratagene Taq Plus Long PCR system a linear amplification was performed using 10 ng of genomic DNA and either the KANFOR or KANREV primer (Table 4). The cycling parameters used were: 94C for 30 s, 56C for 30 s and 72C for 3 min for 30 cycles. The products from the first reaction were diluted 1:10 and 1 µl was added to 24 µl of step 2 reaction mix containing 17.7 µl H₂O, 2.5 µl 10× high salt buffer, 3 µl dNTPs at 2 mM, 0.25 µl of either the KANFOR or KANREV primer, 0.25 µl of either the NOTIGCUFOR or NOTIGCUREV primer (Table 4), and 0.3 µl of the Taq Plus Long enzyme. The NOTIGCU primers are designed to anneal to the gonococcal DUS, and contain a NotI linker on the 5' end to increase their annealing temperature. The GCU sequence is randomly distributed throughout the genome and acts as an internal anchor during PCR. The Step 2 cycling parameters were 30 cycles at 94C for 30 s, 58C for 30 s, 72C for 4 min. This strategy successfully identified the site of insertion in 37 of the 46 mutants. The remaining 9 were identified by RATE PCR (Ducey, 2002) using 10 ng

Table 4. Oligonucleotides used for real-time PCR.

Name	Sequence 5'→3'
CYSGFOR	CCGTCCGCATCGAACCG
CYSGREV	TCCAAAACGCTTTCAATTTCC
EZTNFOR	ACCTACAACAAAGCTCTCATCAACC
EZTNREV	GCAATGTAACATCAGAGATTTTGAG
HP1503FOR	GGGAAGTTATATTCGGATGC
HP1503REV	AAACGGCGCGGTCTGTTTG
IS1016FOR	GTAGAAGCAGATGAAAGTTATT
IS1016REV	CTTTCTACAAGTAACAGGGC
KANFOR	TTGATGCTCGATGAGTTTTTCTAA
KANREV	GTTTCCCGTTGCCTATGGCTCATA
MQOFOR	AACGAAGACCACTGCCGT
MQOREV	GGTGCGGAGGGTGAGCT
NOTIGCUFOR	TTGCGGCCGCAAGCCGTCTGAA
NOTIGCUREV	TTGCGGCCGCAATTCAGACGGC
OMP3FOR	AGCAGGCTCCTCAATATGTT
OMP3REV	CTTGAGTCATTTGCGCTTGA
SURAFOR	ATGCAGCTTGCAACCAATG
SURAREV	CAGGATGTGTTGGGGCGC
TBPBFOR	ACCATTGCAAGATTCCAGTC
TBPBREV	ATATAATCCAACCTCCCGGAA

of isolated Gc genomic DNA. The anchored PCR approach was successful at identifying six of the nine sites of insertion because of the lack of a DUS in the region of the insertion. The reasons that the remaining three inserts were not identified using the anchored approach are unclear as all three insertions were located near a DUS.

The products were analysed by gel electrophoresis and quantitated using the Low DNA Mass Ladder (Stratagene). Excess dNTPs and primers were removed from the PCR products using the shrimp alkaline phosphatase and Exonuclease I enzymes (USB) at 37C for 15 min followed by incubation at 80C for 15 min. The sequencing reaction was then performed using EZTNFOR or EZTNREV (Table 3) and the CEQ-DTCS (Beckman-Coulter) reagents as directed. The products were then ethanol precipitated and sequences determined using a Beckman Coulter CEQ 2000XL.

UV sensitivity assays

Iron-dependent. Survival of Gc mutants with iron-dependent PDCMC phenotypes to UV irradiation was performed in liquid medium as described previously (Serkin and Seifert, 2000). Piliated Gc were streaked and grown for 10 h, then collected and resuspended at approximately 5×10^7 cfu ml⁻¹ in GCBL + Kellogg's supplements I +0.0042% Na₂CO₃. After 14 h the culture was diluted 1:10 in fresh medium and grown for an additional 3–4 h. The culture was then diluted 1:5 into iron-replete and iron-limiting medium, each containing 1 mM IPTG. These cultures were grown for 3 h, then serially diluted from 10⁻¹ to 10⁻⁶. Twenty microlitres of spots were plated of each dilution and the spots were exposed to 0, 20, 40, 60, or 80 mJ m⁻² in a Stratagene UV Stratalinker 1800.

Iron-independent. Survival of gonococcal mutants with iron-independent PDCMC phenotypes was assessed by collecting Gc using a Dacron swab to a concentration of ~10⁸ cfu ml⁻¹. These cultures were immediately serially diluted, plated, and exposed to UV radiation as described above. After approximately 20 h of growth, unirradiated and irradiated colonies were counted and the ratio of percent survival was calculated.

DNA transformation efficiency assays

Transformation efficiency assays of Gc mutants with iron-dependent phenotypes were performed as previously described (Serkin and Seifert, 2000). After growth in either iron-replete or iron-limiting liquid medium the transformations were performed as described above using 40 µl of culture and 50 ng of the plasmid pSY6. Transformation efficiency assays of Gc mutants with iron-independent PDCMC phenotypes were performed by collecting Gc from solid medium, resuspending to a concentration of ~10⁸ cfu ml⁻¹, and then performing the transformation as described above using 40 µl of culture and 50 ng of pSY6 plasmid.

For both iron-dependent and iron-independent assays, the transformation culture was serially diluted from 10⁻¹ to 10⁻⁶ and plated on both plain and nalidixic acid containing GCB. After overnight incubation the colonies on both plain and Nal-

containing medium were counted. The transformation efficiency is the mean number of NaI^{R} transformants/cfu.

Generation of cDNA

RNA was isolated from $\sim 10^8$ Gc cfu using the Qiagen RNeasy purification kit with DNase treatment, as per the instructions. cDNA was synthesized as follows. Five micrograms total RNA and 30 μg random hexamer primers (Roche) were combined and denatured at 70C for 10 min, then placed on ice for 10 min. DTT, dNTP mixture, RNasin (Promega), SuperscriptII (Invitrogen), and H_2O was added to a final volume of 30 μl . Reverse transcription was carried out at 42C for 2.5 h, and the Superscript was inactivated by heating at 94C for 10 min. H_2O was added to a final volume of 100 μl , 2 μl of RNaseI (Promega) was added and the reaction was incubated at 37C for 30 min. The cDNA was cleaned and concentrated using the Qiagen PCR Purification Kit.

Analysis of predicted operons

A gene was predicted to reside in an operon if the gene immediately downstream was located within 500 bp and in the same orientation. Predicted operons were examined by PCR amplification of cDNA generated as described previously. The PCR reactions consisted of 1 μl of cDNA, 16.4 μl of H_2O , 3.0 μl of 25 mM MgCl_2 , 2.5 μl 10 \times PCR Buffer, 0.25 μl each primer at 50 pmol μl^{-1} , and 0.1 μl *Taq* (Promega). Reactions were performed using the following parameters: 94C for 2 min, followed by 30 cycles of 94C for 45 s, 62C for 45 s and 72C for 90 s. In each reaction primer 1 targeted the 3' region of the upstream gene and primer 2 targeted the 5' region of the downstream gene in one pair of genes. Each gene downstream of and including the gene containing the mTn5 insertion was examined in this manner until no PCR product was obtained or a gene in the opposite orientation was found. Primer sequences are available upon request.

Analysis of transcript levels using real-time PCR

Relative RNA transcript levels were determined using the Roche LightCycler and SYBR Green technology. Sequences of oligonucleotides used for real-time analysis are listed in Table 4. The real-time PCR reactions consisted of 10.6 μl H_2O , 4 mM final concentration of MgCl_2 , 0.5 pM final concentration of each primer, and 2 μl SYBR Green Master Mix (Roche), added to 1 μl of cDNA for a total volume of 20 μl . Reactions were performed using the following parameters: denature at 94C for 1 s, anneal at 55C for 5 s, followed by extension for 10 s at 72C for 40 cycles.

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References

- Alba, B.M., Leeds, J.A., Onufryk, C., Lu, C.Z., and Gross, C.A. (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev* **16**: 2156–2168.
- Alonso, J.C., Stiege, A.C., and Luder, G. (1993) Genetic recombination in *Bacillus subtilis* 168: effect of *recN*, *recF*, *recH* and *addAB* mutations on DNA repair and recombination. *Mol Gen Genet* **239**: 129–136.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.
- Chaussee, M.S., Wilson, J., and Hill, S.A. (1999) Characterization of the *recD* gene of *Neisseria gonorrhoeae* MS11 and the effect of *recD* inactivation on pilin variation and DNA transformation. *Microbiology* **145**: 389–400.
- Chen, I., and Dubnau, D. (2004) DNA uptake during bacterial transformation. *Nature Reviews* **2**: 241–249.
- Cohen, M.S., and Cannon, J.G. (1999) Human experimentation with *Neisseria gonorrhoeae*: progress and goals. *J Infect Dis* **179**(Suppl.2): S375–S379.
- Daniel, W.W. (1999) *Biostatistics: A Foundation for Analysis in the Health Sciences*. New York, NY: John Wiley and Sons, Inc.
- Dartigalongue, C., Loferer, H., and Raina, S. (2001) EcfE, a new essential inner membrane protease: its role in the regulation of heat shock response in *Escherichia coli*. *EMBO J* **20**: 5908–5918.
- Delany, I., Ieva, R., Alaimo, C., Rappuoli, R., and Scarlato, V. (2003) The iron-responsive regulator *fur* is transcriptionally autoregulated and not essential in *Neisseria meningitidis*. *J Bacteriol* **185**: 6032–6041.
- Ducey, T.F., and Dyer, D.W. (2002) Rapid identification of EZ::TN transposon insertion sites in the genome of *Neisseria gonorrhoeae*. *Epicentre Forum* **9**: 6–7.
- Genco, C.A., and Desai, P.J. (1996) Iron acquisition in the pathogenic *Neisseria*. *Trends Microbiol* **4**: 179–184.
- Genevris, S., Steeghs, L., Roholl, P., Letesson, J.J., and van der Ley, P. (2003) The Omp85 protein of *Neisseria meningitidis* is required for lipid export to the outer membrane. *EMBO J* **22**: 1780–1789.
- Goodman, S.D., and Scocca, J.J. (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. *Proc Natl Acad Sci USA* **85**: 6982–6986.
- Goryshin, I.Y., and Reznikoff, W.S. (1998) Tn5 *in vitro* transposition. *J Biol Chem* **273**: 7367–7374.
- Haas, R., and Meyer, T.F. (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell* **44**: 107–115.
- Hagblom, P., Segal, E., Billyard, E., and So, M. (1985) Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. *Nature* **315**: 156–158.
- Hamrick, T.S., Dempsey, J.A., Cohen, M.S., and Cannon, J.G. (2001) Antigenic variation of gonococcal pilin expression *in vivo*: analysis of the strain FA1090 pilin repertoire and identification of the *pilS* gene copies recombining with *pilE* during experimental human infection. *Microbiology* **147**: 839–849.

- Henrichsen, J. (1975) The occurrence of twitching motility among Gram-negative bacteria. *Acta Pathol Microbiol Scan B* **83**: 171–178.
- Hill, S.A. (2000) *Neisseria gonorrhoeae* *recJ* mutants show defects in recombinational repair of alkylated bases and UV-induced pyrimidine dimers. *Mol Gen Genet* **264**: 268–275.
- Howell-Adams, B., and Seifert, H.S. (2000) Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation. *Mol Microbiol* **37**: 1146–1159.
- Jonsson, A.-B., Ilver, D., Falk, P., Pepose, J., and Normark, S. (1994) Sequence changes in the pilus subunit lead to tropism variation of *Neisseria gonorrhoeae* to human tissue. *Mol Microbiol* **13**: 403–416.
- Kellogg, D.S., Jr, Peacock, W.L., Deacon, W.E., Brown, L., and Pirkle, C.I. (1963) *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J Bacteriol* **85**: 1274–1279.
- Kline, K.A., Sechman, E.V., Skaar, E.P., and Seifert, H.S. (2003) Recombination, repair and replication in the pathogenic *Neisseriae*: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol Microbiol* **50**: 3–13.
- Koomey, J.M., and Falkow, S. (1987) Cloning of the *recA* gene of *Neisseria gonorrhoeae* and construction of gonococcal *recA* mutants. *J Bacteriol* **169**: 790–795.
- Koomey, M., Gotschlich, E.C., Robbins, K., Bergstrom, S., and Swanson, J. (1987) Effects of *recA* mutations on pilus antigenic variation and phase transitions in *Neisseria gonorrhoeae*. *Genetics* **117**: 391–398.
- Kroll, J.S., Loynds, B.M., and Moxon, E.R. (1991) The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. *Mol Microbiol* **5**: 1549–1560.
- Laskos, L., Ryan, C.S., Fyfe, J.A., and Davies, J.K. (2004) The RpoH-mediated stress response in *Neisseria gonorrhoeae* is regulated at the level of activity. *J Bacteriol* **186**: 8443–8452.
- Lloyd, R.G. (1991) Conjugational recombination in resolvase-deficient *ruvC* mutants of *Escherichia coli* K-12 depends on *recG*. *J Bacteriol* **173**: 5414–5418.
- Lloyd, R.G., and Sharples, G.J. (1993) Dissociation of synthetic Holliday junctions by *E. coli* RecG protein. *EMBO J* **12**: 17–22.
- Mehr, I.J., and Seifert, H.S. (1998) Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation, and DNA repair. *Mol Microbiol* **30**: 697–710.
- Meyer, T.F., Mlawer, N., and So, M. (1982) Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell* **30**: 45–52.
- Meyer, T.F., Billyard, E., Haas, R., Storzbach, S., and So, M. (1984) Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proc Natl Acad Sci USA* **81**: 6110–6114.
- Parsons, C.A., Tsaneva, I., Lloyd, R.G., and West, S.C. (1992) Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc Natl Acad Sci USA* **89**: 5452–5456.
- Pellicic, V., Morelle, S., Lampe, D., and Nassif, X. (2000) Mutagenesis of *Neisseria meningitidis* by *in vitro* transposition of Himar1 mariner. *J Bacteriol* **182**: 5391–5398.
- Salvatore, P., Bucci, C., Pagliarulo, C., Tredici, M., Colicchio, R., Cantalupo, G., et al. (2002) Phenotypes of a naturally defective *recB* allele in *Neisseria meningitidis* clinical isolates. *Infect Immun* **70**: 4185–4195.
- Sebastian, S., Agarwal, S., Murphy, J.R., and Genco, C.A. (2002) The gonococcal *fur* regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. *J Bacteriol* **184**: 3965–3974.
- Segal, E., Billyard, E., So, M., Storzbach, S., and Meyer, T.F. (1985) Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. *Cell* **40**: 293–300.
- Segal, E., Hagblom, P., Seifert, H.S., and So, M. (1986) Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proc Natl Acad Sci USA* **83**: 2177–2181.
- Seifert, H.S. (1992) Molecular mechanisms of antigenic variation in *Neisseria gonorrhoeae*. *Mol Cell Bio Hum Dis Series* **1**: 1–22.
- Seifert, H.S. (1997) Insertionally inactivated and inducible *recA* alleles for use in *Neisseria*. *Gene* **188**: 215–220.
- Seifert, H.S., Ajioka, R.S., Paruchuri, D., Heffron, F., and So, M. (1990) Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. *J Bacteriol* **172**: 40–46.
- Serkin, C.D., and Seifert, H.S. (2000) Iron availability regulates DNA recombination in *Neisseria gonorrhoeae*. *Mol Microbiol* **37**: 1075–1086.
- Skaar, E.P., Lazio, M.P., and Seifert, H.S. (2002) Roles of the *recJ* and *recN* genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. *J Bacteriol* **184**: 919–927.
- Sparling, P.F. (1966) Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. *J Bacteriol* **92**: 1364–1371.
- Stein, D.C. (1991) Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. *Can J Microbiol* **37**: 345–349.
- Stohl, E.A., and Seifert, H.S. (2001) The *recX* gene potentiates homologous recombination in *Neisseria gonorrhoeae*. *Mol Microbiol* **40**: 1301–1310.
- 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.
- Swanson, J., Kraus, S.J., and Gotschlich, E.C. (1971) Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J Exp Med* **134**: 886–906.
- Swanson, J., Bergström, S., Barrera, O., Robbins, K., and Corwin, D. (1985) Pilus- gonococcal variants. Evidence for multiple forms of piliation control. *J Exp Med* **162**: 729–744.
- Swanson, J., Robbins, K., Barrera, O., Corwin, D., Boslego, J., Ciak, J., et al. (1987) Gonococcal pilin variants in experimental gonorrhoea. *J Exp Med* **165**: 1344–1357.
- Theze, J., Kleidman, L., and St Giron, I. (1974) Homoserine kinase from *Escherichia coli* K-12: properties, inhibition by 1-threonine, and regulation of biosynthesis. *J Bacteriol* **118**: 577–581.
- Thomas, C.E., and Sparling, P.F. (1994) Identification and

- cloning of a *fur* homologue from *Neisseria meningitidis*. *Mol Microbiol* **11**: 725–737.
- Thomas, C.E., and Sparling, P.F. (1996) Isolation and analysis of a *fur* mutant of *Neisseria gonorrhoeae*. *J Bacteriol* **178**: 4224–4232.
- Thome, B.M., and Muller, M. (1991) Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for export. *Mol Microbiol* **5**: 2815–2821.
- Thompson, S.A., and Sparling, P.F. (1993) The RTX cytotoxin-related FrpA protein of *Neisseria meningitidis* is secreted extracellularly by meningococci and by HlyBD+ *Escherichia coli*. *Infect Immun* **61**: 2906–2911.
- Umezu, K., Chi, N.W., and Kolodner, R.D. (1993) Biochemical interaction of the *Escherichia coli* RecF, RecO, and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proc Natl Acad Sci USA* **90**: 3875–3879.
- Umezu, K., and Kolodner, R.D. (1994) Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. *J Biol Chem* **269**: 30005–30013.
- Virji, M., Everson, J.S., and Lambden, P.R. (1982) Effect of anti-pilus antisera on virulence of variants of *Neisseria gonorrhoeae* for cultured epithelial cells. *J Gen Microbiol* **128**: 1095–1100.
- Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., and Tommassen, J. (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**: 262–265.
- Wainwright, L.A., Pritchard, K.H., and Seifert, H.S. (1994) A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. *Mol Microbiol* **13**: 75–87.
- Wolfgang, M., Lauer, P., Park, H.-S., Brossay, L., He'bert, J., and Koomey, M. (1998) PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol Microbiol* **29**: 312–330.

Supplementary material

The following material is available for this article online:

Fig. S1. Operon structure and mini-transposon insertions.

Table S1. Insertions sites located within operons with unidentified effector genes.