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

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Antigenic variation of gonococcal pilin involves a family of variable genes that undergo homologous recombination, resulting in transfer of variant sequences from the *pilS* silent gene copies into the complete *pilE* expression locus. Little is known about the specific recombination events that are involved in assembling new variant pilin genes *in vivo*. One approach to understanding pilin variation *in vivo* is to carry out experimental human infections with a gonococcal strain having a fully characterized repertoire of pilin genes, so that the specific recombination events occurring *in vivo* can be determined. To this end, the authors cloned, sequenced and mapped the pilin genes of strain FA1090 of *Neisseria gonorrhoeae*. This strain contains one *pilE* locus and 19 silent gene copies that are arranged in five *pilS* loci; the *pilE* locus and four of the *pilS* loci are clustered in a 35 kb region of the chromosome. The general features of the pilin loci in FA1090 are similar to those in strain MS11, in which the mechanism of pilin variation has been extensively studied. However, none of the silent copy sequences are identical in the two strains, which emphasizes the extreme variability in this gene family among gonococci. Three male volunteers were inoculated with the same variant of strain FA1090 and developed urethritis within 2–4 d. The *pilE* gene sequences from a total of 23 colonies cultured from the subjects were analysed, determining which *pilS* silent copy donated each portion of the expressed *pilE* genes. There were 12 different pilin variants, one of which was the original inoculum variant, among the *in vivo*-expressed *pilE* gene sequences. The *pilE* of the inoculum variant was derived entirely from a single silent copy (*pilS6c1*). However, the *pilE* genes in the majority of the colonies cultured from the infected subjects were chimeras of sequence derived from two or three silent copies. Recombination to generate new *pilE* sequences involved exchange of single variable minicassettes, multiple minicassettes, entire silent gene copies, or (rarely) recombination within a minicassette.

Keywords: *Neisseria gonorrhoeae*, gonococcus, recombination, human challenge

INTRODUCTION

The strict human pathogen *Neisseria gonorrhoeae* has complex mechanisms for constantly altering the anti-

genic nature of many of its surface components, including the type 4 pili that are believed to be responsible for the initial attachment of the organisms to the mucosal epithelium of the human host (reviewed by Nassif & So, 1995; Nassif *et al.*, 1999). The antigenic type of the pilus changes at a high frequency via changes in the primary amino acid sequence of the major subunit pilin protein. Pilin antigenic variation may play a role in

Abbreviations: mc, minicassette; PAR, pilin-associated repeat.

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immune evasion during gonococcal infection (Zak *et al.*, 1984; Hagblom *et al.*, 1985; Swanson *et al.*, 1987), and undoubtedly contributed to the failure of a pilin vaccine to provide protection against natural infection (Brinton *et al.*, 1982; Boslego *et al.*, 1991). Pilin subunit sequence changes have also been linked to tissue tropism and to variation in adhesiveness of gonococci in *in vitro* studies, suggesting that expression of different pilins may allow gonococci to colonize different tissues or body sites (reviewed by Nassif & So, 1995; Seifert, 1996; Nassif *et al.*, 1999).

The gonococcal chromosome generally has one expression locus (*pilE*) containing a functional pilin gene and multiple silent loci (*pilS*) containing one or more partial pilin gene copies (silent copies) that are truncated at the 5' end. The *pilE* gene has a 5' constant region, a central semivariable (SV) region and a 3' hypervariable (HV) region. The silent copies contain only the SV and HV regions (reviewed by Nassif & So, 1995; Seifert, 1996; Nassif *et al.*, 1999). In strain MS11, the only strain in which most of the pilin genes have been characterized, there are 17 silent copies clustered into five *pilS* loci (Haas *et al.*, 1992). The silent copies (or the corresponding regions of *pilE*) have also been described as containing six variable regions, or minicassettes (mc), bordered by conserved sequence. The minicassette at the 3' end of a silent copy is designated mc1; mc6 is at the 5' end (Haas & Meyer, 1986; Haas *et al.*, 1992). Pilin antigenic variation involves RecA-, RecO- and RecQ-dependent non-reciprocal recombination between the *pilS* silent copies and the *pilE* locus (Koomey *et al.*, 1987; Mehr & Seifert, 1998). As a result of these recombination events, a newly generated *pilE* gene with previously silent sequences is expressed. The expression locus may receive all or just part of a variant *pilS* silent copy; the final pilin gene may thus be a chimera of sequences originating from multiple *pilS* loci. The minicassette model predicts that recombination will occur at the conserved regions interspersed between the variable minicassettes (Haas & Meyer, 1986; Haas *et al.*, 1992). Seifert and coworkers have proposed alternative models for pilin recombination, suggesting that the minicassettes could be further subdivided and reshuffled, with recombinational end points occurring within them (Seifert, 1996; Howell-Adams & Seifert, 2000).

The potential for variable recombination events leads to an enormous set of possible pilin antigenic types for any given strain. It is not known whether all of those possible types are equally likely to appear in the population of gonococci infecting a human host. There could be mechanistic recombinational constraints operating *in vivo* that affect which stored pilin sequences are assembled into new *pilE* coding sequences. In addition, post-recombinational selection pressures might limit the range of possible pilin types, based on efficiency of assembly, adherence, or some as yet unknown pilus function.

The human challenge model of gonococcal urethritis in male volunteers has provided an opportunity to study

the importance of pilus expression and pilin antigenic variation in early infection. Nonpilated colony variants (Kellogg *et al.*, 1963) and a *pilE* deletion mutant (authors' unpublished results) are reduced in infectivity in the human challenge model. Swanson *et al.* (1987) showed that antigenic variation occurred during infection of volunteers with strain MS11 by determining the DNA sequence of *pilE* in isolates cultured from infected subjects. We carried out a similar study using strain FA1090 (Seifert *et al.*, 1994), and found considerable pilin variation in the early stages of infection with this strain. However, without information about the complete pilin gene repertoire of strain FA1090, we could not determine the precise recombinational events occurring in *pilE* during infection. We have now cloned and sequenced all of the *pil* loci in strain FA1090 and determined their organization on the chromosome. This information has allowed us to identify the *pilS* silent copies recombining with *pilE* to generate new expressed pilins during experimental infection of volunteers with this strain and therefore to localize the end points of the recombination events occurring in the pilin gene during human infection.

METHODS

Bacterial strains. *N. gonorrhoeae* FA1090 is a serum-resistant, proline-requiring strain of serotype P.IB (Black *et al.*, 1984; Jerse *et al.*, 1994). FA1090 variant A21, which was used for experimental infection of volunteers 1, 2 and 62, is a pilated, Opa-negative variant with three lipooligosaccharide species, one of which binds monoclonal antibody 3F11 (Jerse *et al.*, 1994, and data not shown). The variable regions of the *pilE* gene of variant A21 are derived entirely from silent copy 1 in *pilS6* (*pilS6c1*). Gonococci were grown on GCB agar (Difco) with the supplements of Kellogg *et al.* (1963) at 37 °C in a 5% CO₂ atmosphere. *Escherichia coli* strain DH5 α MCR (Gibco-BRL) was grown on LB agar with or without 50–100 μ g ampicillin ml⁻¹.

Cloning and sequencing methods. To clone the *pil* genes, *N. gonorrhoeae* strain FA1090 chromosomal DNA was digested with *Clal* and separated on a 1% agarose gel. The 4.3 kb, 3.4–3.2 kb, 2.4 kb, 1.6 kb and 0.9 kb fractions were eluted from the agarose using a Qiaex DNA extraction kit (Qiagen). The 3.4–3.2 kb, the 2.4 kb and the 0.9 kb fractions were ligated into the *AccI* site of pUC18 (Gibco-BRL). The 4.3 kb and the 1.6 kb fragments were ligated into the *Clal* site of pBluescriptSK+ (Stratagene). Ligated products were transformed into *E. coli* DH5 α MCR (Gibco-BRL). Clones were identified by colony blot hybridization (Sambrook *et al.*, 1989) with the *Sma*/*Cla*-specific oligonucleotide (see below). The 3.4 kb fragment containing the *pilE* locus could not be cloned into these high-copy-number plasmids, presumably due to toxic overexpression of pilin protein, but was successfully cloned into pBR322. The *pilE* clone was identified by hybridization with the *pilE* promoter-specific oligonucleotide pilstart (see below). Southern blot analysis of the clones and of FA1090 chromosomal DNA digested with *Clal* confirmed that no deletions or gross rearrangements had occurred during the cloning (data not shown).

To sequence the *pil* loci, nested deletions of each cloned insert were generated using exonuclease III and S1 nuclease

(Promega Erase-a-Base Kit). DNA was sequenced by the UNC-CH Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The reactions were typically primed with the M13 forward or the M13 reverse oligonucleotide primers. The *pilE* gene and several regions of the other clones that lacked sufficient overlaps with the nested deletions were sequenced using custom primers. To sequence the *pilE* clone, pBR322-specific oligonucleotides pBR#1 (5'-AATAGGCGTATCAC-GAGGCC-3'), pBR#2 (5'-GTTGCGGCCGTTGAGCACC-G-3') and pBR#3 (5'-CGGTGCGTACTGCGTTAGC-3') were used as primers, after generating nested deletions. Sequences were analysed using the GCG Sequence Analysis Software Package. All pilin-gene-containing regions of the clones were sequenced on both strands. The FA1090 pilin gene sequences are deposited in GenBank under accession numbers U58846–U58851.

Oligonucleotides. Oligonucleotides were synthesized using the Applied Biosynthesis System (ABI) and were used for Southern hybridization and PCR without further purification. Oligonucleotide pilstart (5'-GAGATAAACGCATAAAATTTCACC-3') was homologous to the promoter region of the *pilE* gene (Seifert *et al.*, 1994; Wainwright *et al.*, 1994). Oligonucleotides *cys1c* (5'-CGGTAAATGTTCTGCGGAC-3') and *cys2r* (5'-GCAGTTGACGGCAGGTGC-3') hybridized to the conserved regions 5' and 3', respectively, of *mc2* in all pilin genes. Oligonucleotide Sma/Cla (5'-TTGCAAGGCGGGCGGGTTCGTCG-3') was homologous to the Sma/Cla repeat. The TS6 oligonucleotide (5'-CGCCTGTCAGATAAAAACCG-5') hybridized to the region of the PAR (pilin-associated repeat) sequence extending past the Sma/Cla repeat. The RS1' oligonucleotide (5'-CACGCGCCGATTTCAAACACTTCC-3') was a shortened version of the RS1 oligonucleotide of Haas & Meyer (1986), hybridizing to the RS1 repeat sequence.

The chromosome mapping experiments required DNA probes specific for each of the *pil* loci of FA1090. The pilstart oligonucleotide (see above) was specific for the *pilE* locus. Oligonucleotide TS3 (5'-CAGAGAACGGTTCGCGGCAG-3') was specific for the *pilS3* locus and hybridized 3' of the *NheI* site in that clone. Oligonucleotide TS1 (5'-AACGTCGTCGTCGTTGGCGG-3') was specific for the *pilS2* locus, hybridizing to *mc2* in *pilS2c3*. Oligonucleotide TS2 (5'-AAGAAGGAATGCCCGAACCG-3') was specific for *pilS7*, hybridizing to DNA outside of the pilin coding region. Oligonucleotide TS4 (5'-GAATCACGGCACATGGCCGG-3') hybridized to several silent copies in the *pilS1* locus, and also to *pilS7* in *mc6*. Oligonucleotide TS5 (5'-GCGCACCGAAGCCAACGCCG-3') was generated to *mc2* of *pilS3c3*, and also hybridized to silent gene copies in *pilS1* and *pilS2*. Results of Southern blot experiments with TS4 and TS5 were interpreted by comparing them with results obtained with probes with partially overlapping specificities. The probe for the *pilS6* locus was a clone consisting of *pilS6* flanking sequence that was generated when making the nested deletions for sequencing.

Pulsed-field electrophoresis. Gonococcal chromosomal DNA was prepared in agarose blocks (Dempsey *et al.*, 1991) or purified by caesium chloride density-gradient centrifugation (Sambrook *et al.*, 1989). The DNA was digested with restriction endonucleases and resolved by contour-clamped homogeneous electric field electrophoresis, then transferred to BAS membrane (Schleicher and Schuell) and cross-linked as previously described (Dempsey *et al.*, 1991).

DNA hybridization. Southern blots, colony blots and ³²P end-labelling of oligonucleotide probes were as described by Sambrook *et al.* (1989). Hybridization and washing were done at 55 °C for TS5, TS1, TS3 and Sma/Cla, and at 50 °C for TS2, TS4 and pilstart. The hybridization solution contained 4 × SSPE, 2 × Denhardt's solution, 20 mM sodium pyrophosphate and 0.2% SDS. Filters were washed twice for 30 min in a solution of 1 × SSC, 0.1% SDS and 5 mM sodium pyrophosphate, and once for 20 min in 0.5 × SSC, 0.1% SDS and 5 mM sodium pyrophosphate.

DNA fragments were labelled with ³²P by random priming using an oligo-labelling kit (Pharmacia). These probes were hybridized at 60 °C for Southern blots of pulsed-field gels or 65 °C for Southern blots of conventional agarose gels in a solution of 6 × SSC, 0.5% SDS and 5 × Denhardt's solution. Filters were washed once for 5 min in a solution of 2 × SSC and 0.1% SDS at room temperature, once for 15 min in a solution of 2 × SSC and 0.1% SDS at room temperature, and twice for 1 h in a solution of 0.1 × SSC and 0.5% SDS at the hybridization temperature.

Experimental human infection. Procedures for recruiting male subjects, obtaining informed consent, carrying out intra-urethral inoculation of gonococci, and processing of urethral swab and urine samples have been described (Cohen *et al.*, 1994). Briefly, male subjects between the ages of 18 and 35 gave informed consent and were admitted to the General Clinical Research Center of UNC Hospitals, which is an inpatient research unit. Subjects were inoculated intra-urethrally with a suspension of gonococci using a pediatric catheter. They were examined twice daily by a physician and treated with antibiotics immediately upon developing signs or symptoms of infection or at the end of the trial, which lasted 4–6 d. Daily urine specimens were cultured on GCB agar containing VCN inhibitor (Unipath), as were urethral swab specimens obtained when a subject developed a urethral discharge. Colonies growing on the primary isolation plates after 24 h incubation were picked into 30 µl freezer storage medium (FSM) in microtitre wells and stored at –70 °C as previously described (Jerse *et al.*, 1994). All protocols were reviewed and approved by the Institutional Review Board and the General Clinical Research Center Advisory Board of the University of North Carolina at Chapel Hill. Subjects were compensated for their participation.

To determine the DNA sequence of *pilE* from the colonies in FSM, the *pilE* gene was amplified by PCR using aliquots of the stored colony suspension as the DNA template. The primers were oligonucleotides pilstart and SP3A (Wright *et al.*, 1994). The PCR profile was 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 30 cycles. PCR products were sequenced using the oligonucleotide pilAW (5'-CCTACCAAGACTACACCGCCC-3') as the primer.

RESULTS

Pilin genes of strain FA1090

To clone the *pil* loci of strain FA1090, we took advantage of the conservation of portions of *pil* coding sequence and of certain repeated elements near gonococcal *pil* genes. The Sma/Cla repeat is a 65 bp sequence element located at the 3' end of each *ClaI* restriction fragment containing a *pil* locus in strain MS11 (Meyer *et al.*, 1984). Oligonucleotide probes derived from the Sma/Cla repeat and from *pil* coding sequence elements in

Fig. 1. Schematic representation of *N. gonorrhoeae* strain FA1090 pilin loci. White boxes represent pilus gene copies; the arrows show the orientation of the genes. The 5' ends of the silent copies are at different positions in mc6; the numbers above the boxes indicate the first amino acid encoded by the translated silent copy, where amino acid 1 is the first amino acid of complete pilin protein. The silent copy at the 3' end of each *pilS* locus contains a stop codon at its 3' end (indicated by *); most of the other silent copies lack stop codons. Seven silent copies contain a run of 5–9 cytosine residues in mc5; the gene is out of frame in four of those copies (▽ indicates the shift in translational reading frame). Repeated elements: RS2 (49 bp), diagonally striped boxes; RS4 (32 bp), vertically striped boxes; RS1 (39–40 bp), checkered boxes; Sma/Cla (65 bp), black boxes. The silent gene copies that do not contain repeated elements between them overlap or are separated by one nucleotide. The multiple copies of the repeated sequence elements are identical or differ in only a few nucleotides. The *pilE* locus contains the *pilE* gene, with a complete pilin coding sequence (starting with amino acid 1) and promoter (bent arrow at 5' end of the gene). The *pilE* locus also contains a silent copy plus a partial copy consisting of portions of a pilin gene interrupted by 190 bp of sequence that is unrelated to pilin coding sequence but is similar to *pilS* flanking sequences from FA1090 and from other gonococcal and meningococcal strains. The designations of the *pilS* loci correspond to those in strain MS11 (Bihlmaier *et al.*, 1991; Haas *et al.*, 1987), based on the map locations of the genes.

strain MS11 hybridized to six bands in a Southern blot of *Cla*I-digested FA1090 DNA; one of those bands also hybridized to the oligonucleotide pilstart, specific for the promoter region of *pilE* (Seifert *et al.*, 1994) (data not shown). We cloned the six *pil*-containing *Cla*I fragments and sequenced the *pil*-containing regions on both strands.

The *pilE* locus and the five *pilS* loci (*pilS1*, *pilS2*, *pilS3*, *pilS6* and *pilS7*) of strain FA1090 contained a total of 19 silent gene copies (Fig. 1). The general features, conserved domains and organization of the *pil* loci were similar to those in strain MS11, the only other gonococcal strain for which the pilin gene repertoire has been characterized. However, the number of silent copies in each locus differed in the two strains, as did the number and distribution of the *pil*-associated repeat elements RS1, RS2 and RS4 (Haas & Meyer, 1986; Haas *et al.*, 1992). The designations of the FA1090 *pilS* loci correspond to those in strain MS11; we assigned them on the basis of map location of the genes in the two strains (see below).

None of the *pilS* silent copy sequences were completely duplicated in more than one of the silent copies. In most of the six minicassette regions, all of the silent copies had unique sequences or at most two gene copies had the same sequence. Comparing the 19 sequences at each of the variable minicassette domains showed that regions of sequence similarity were often present in some or all of the silent copies, extending through either the entire minicassette or a portion of it. The mc1 and mc3 regions were the least variable, with some versions of these shortest minicassette regions repeated in several of the silent copies. Hypervariable mc2 showed the greatest sequence divergence, although there were a few copies with regions of identical sequence for up to 50 bp.

Gonococcal strain MS11 has 17 silent gene copies, 11 of which have been sequenced (Haas & Meyer, 1986; Haas *et al.*, 1992). None of the sequences from strains FA1090 and MS11 were identical throughout the entire length of a silent gene copy. The two strains shared three versions of mc6, six versions of mc5, three versions of mc4, four versions of mc3 and one version of mc2.

Fig. 2. Diagram of the major pilin gene cluster, including all but one of the FA1090 *pil* loci, based on restriction mapping and PCR analysis. Open boxes designate *pil* gene copies; the different gene copies within each locus are designated C1, C2, etc. Designations correspond to those in Fig. 1. The orientation of the *Cla*I fragments in this figure is reversed from that in Fig. 1, to be consistent with the arrangement of the loci on the macrorestriction map of the FA1090 chromosome (Dempsey *et al.*, 1991; Dempsey & Cannon, 1994). The horizontal arrow above copy 1 in the *pilE* locus indicates that it is the full-length, transcribed pilin gene. All of the other gene copies on the diagram are truncated silent copies. Black boxes at the 3' end of each *pil* locus designate the pilin-associated repeat (PAR) sequence, within which is the previously described *Sma*/*Cla* repeat. Restriction sites used in mapping: N, *Nhe*I; C, *Cla*I; S, *Sma*I; H, *Hind*III; P, *Pme*I; E, *Eco*RI; G, *Bgl*II. The *pilS7* locus, which is the only FA1090 *pil* locus not included on this diagram, is located ~400 kb from this gene cluster on the chromosome.

Map locations of the FA1090 *pil* genes

In previous studies, we constructed a macrorestriction map of the 2200 kb FA1090 chromosome, using rare-cutting restriction enzymes including *Nhe*I (Dempsey *et al.*, 1991; Dempsey & Cannon, 1994). To define the locations of the individual *pil* loci, we carried out Southern blot analysis of *Nhe*I-digested FA1090 chromosomal DNA separated by contour-clamped homogeneous field (CHEF) electrophoresis and probed with oligonucleotides or cloned DNA specific to each *pil* locus. The *pilE*, *pilS2*, *pilS6* and *pilS1* loci mapped to fragment N10; *pilS3*, which contains a *Nhe*I site within it, mapped to the junction of N10 and adjacent fragment N4. The *pilS7* locus mapped to fragment N6, located ~400 kb on the map from the major N10/N4 *pil* gene cluster (data not shown).

To map the relative positions of the loci in the major *pil* gene cluster, we used a modified Smith–Birnstiel mapping technique (Smith & Birnstiel, 1976), taking advantage of the *Nhe*I site contained in *pilS3*. FA1090 chromosomal DNA was digested with *Nhe*I and with a second enzyme (either partial or complete digests), and the resulting fragments were resolved by conventional or CHEF gel electrophoresis. Southern blots were probed with the *pilS3*-specific oligonucleotide TS3, which hybridized to fragment N10 near the *Nhe*I site, and with DNA probes specific for the other *pil* loci, to map the positions of the loci within the 62 kb N10 fragment (data not shown). The mapping experiments showed that the *pilS3*, *pilS6*, *pilE*, *pilS2* and *pilS1* loci were contained in a region of the chromosome less than 35 kb in size, with *pilS3* and *pilS6* close together at one

end and *pilE*, *pilS2* and *pilS1* tightly clustered at the other end (Fig. 2).

The *Cla*I fragments containing *pilS3*, *pilS2* and *pilE*, each of which had the *Sma*/*Cla* repeat at the 3' end, also had 98% identity over 130 bases at their 5' ends. Through PCR and sequence analysis we determined that the *pilS3* and *pilS6* loci were on *Cla*I fragments located next to each other on the FA1090 chromosome and that the 130 bp of repeated sequence at the 5' end of the *pilS3* *Cla*I fragment was contiguous with the *Sma*/*Cla* repeat at the 3' end of *pilS6*. Similarly, Southern blot analysis of DNA digested with different restriction enzymes and probed with oligonucleotides specific for the conserved regions at both ends of the *pil*-containing *Cla*I fragments confirmed that *pilE*, *pilS2* and *pilS1* were contiguous on the FA1090 chromosome, so that the *Sma*/*Cla* repeat associated with each locus would be extended by the 130 bp of repeated sequence from the 5' end of the adjacent *Cla*I fragment. Examining the sequences of the assembled fragments showed that there was a 265 bp repeated sequence element, containing within it the originally described *Sma*/*Cla* and RS4 repeats, 3' of each *pil* locus (Fig. 3). We designated this conserved region as the PAR (pilin-associated repeat).

Recombination events generating new *pilE* sequences during experimental human infection

Experimental gonococcal infection in males results in urethritis that is similar to natural infection in both clinical signs and time course (Cohen *et al.*, 1994). To characterize the recombination events generating new

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1  Stop          RS4
   TGAAGGCAAAT TAGGCCTTAA ATTTTAAATA AATCAAgCGG TAAGTGATTT
51 CCCACGGCCG CCCGGATCAA CCCGGGCGGC TTGTCTTTTA AGGGTTTGCA
   SmaI
101 AGGCGGGCGG GGTCTGTCCT TCCGGTGGAA ATAATATATC GATTGCGCTT
   ClaI
151 CAAGGCCCTG CATGTaCCTC ATTGCCACCC GTTTAAACAC GGTTTTTATC
201 TGACAGGCGC GCAATCCGCC CCCcCATTG CCGAACAAGC GGTCCGGACT
251 CCCGCCCGCG CGGGAATGAC GGCGGA

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Fig. 3. Complete sequence of the PAR. The repeat begins immediately following the stop codon of the 3'-most silent gene copy (copy 1) of each *pilS* locus. The previously described RS4 and Sma/Cla repeats (Haas *et al.*, 1992) are boxed and underlined, respectively. Lower-case letters show where one or more of the sequenced copies of the repeat has a different base than the consensus nucleotide indicated. The copy of PAR associated with *pilS7* stops at position 230, and the copy between the *pilE* and *pilS2* loci stops at position 268.

pilin variants *in vivo*, we inoculated a human volunteer (volunteer 62) intraurethrally with a pilated variant of strain FA1090 (variant A21) in which the predominant expressed pilin sequence was derived entirely from copy 1 in the *pilS6* locus (*pilS6c1*). This variant produces both full-length pilin protein and S-pilin, which is a soluble, truncated form of pilin is that is secreted by some gonococcal variants and is not assembled into pili (Haas *et al.*, 1987). The function of S-pilin, if any, is not known. Variant A21, which was originally isolated *in vitro*, is not as heavily pilated as most variants that have been recently isolated from patients with gonorrhoea (data not shown). The subject was monitored for development of signs or symptoms of infection, and urine specimens were cultured daily. He was treated as soon as he developed a purulent urethral discharge, which occurred 2 d after inoculation. Colonies growing on the primary isolation plates from urine or urethral swab specimens all had pilus⁺ colony morphology. We amplified and sequenced the *pilE* gene from 12 colonies that were cultured from this subject at different times. The *pilE* sequence of one colony that was cultured from a specimen obtained 2 h after inoculation was identical to that of the inoculum variant. A colony analysed from the first day after inoculation had a portion of the original *pilS6c1* sequence replaced with new information. Ten colonies from urine and urethral swab cultures obtained at the time the subject developed a urethral discharge on day 2 had four new *pilE* sequences among them, in which different portions of the inoculum sequence were replaced. We compared each of the newly expressed pilin sequences to the set of silent copy sequences and identified the silent copy that was the source for each part of the *pilE* sequence. The expressed sequences were all identical to the relevant portions of the matching silent copy sequences. The recombination end points involved in generating the expressed sequences were localized by finding the point at which an expressed sequence changed from matching one silent copy to matching a different one (Fig. 4).

We carried out similar analyses of the derivation of the *in vivo*-expressed *pilE* sequences from our earlier study of two subjects (volunteers 1 and 2) who were also experimentally infected with the FA1090 variant expressing *pilE* derived entirely from *pilS6c1*. In that study, we determined the DNA sequence of *pilE* from individual colonies cultured from the subjects, as well as

the *pilE* sequence from organisms in urethral swab specimens or urine sediments that were frozen without *in vitro* passage. PCR amplification and sequencing of *pilE* from the latter specimens had sometimes resulted in multiple bands at some positions in the sequencing gels, although it was usually possible to identify a predominant sequence based on band intensity (Seifert *et al.*, 1994). When we compared the *pilE* sequences of the reisolates from volunteers 1 and 2 to the *pilS* storage copies, we could determine unambiguously which *pilS* gene copies were the sources of the expressed sequences only for the seven sequences that had been obtained from colonies growing on the primary isolation plates (Fig. 4). For the sequences obtained from the frozen urine sediment samples or urethral swab specimens, especially those in which a predominant sequence was determined by analysing band intensity in sequencing gels showing mixed sequences, it was difficult to determine a *pilS* source for all regions of the *pilE* genes. We therefore confined the analysis to the single-colony isolates from volunteers 1 and 2 that gave unambiguous results in terms of identifying the *pilS* sources for the entire expressed sequences. Other sequences from the earlier study (Seifert *et al.*, 1994) may contain false mosaics or incorrect nucleotides that were assigned based on the subtraction analysis, and should be considered less reliable.

There were 12 unique *pilE* sequences among the 23 isolates from the three subjects, one the same as the inoculum variant and 11 new sequences (Fig. 4). Information from at least one silent copy in *pilS1*, *pilS2*, *pilS3* and *pilS7* was used to generate one or more *pilE* sequences in different isolates. Sequence from the silent copy located nearest to *pilE* on the chromosome did not appear in any of the expressed sequences, and the only *pilS6* sequence used was that of the inoculum (*pilS6c1*). In one isolate from volunteer 2, the entire inoculum *pilE* sequence was replaced with sequence derived from *pilS7c1*. In most cases, however, the *pilE* sequences represented chimeras of minicassettes derived from different *pilS* copies, with recombination occurring at the previously described minicassette borders (Haas & Meyer, 1986). Many of the *pilE* sequences retained portions of the *pilS6c1*-derived inoculum sequence. However, these *pilE* sequences must have resulted from independent recombination events, since they retained different portions of the original sequence. In two

Fig. 4. Diagram of *pilE* sequences from individual colonies of FA1090 cultured from experimentally infected human subjects. The DNA sequences are identified by their GenBank accession numbers. The *pilE* sequences were determined for colonies cultured on the primary isolation plates from urine or urethral swab specimens from volunteers 1, 2 and 62. All three subjects were inoculated with a variant of FA1090 expressing *pilE* derived entirely from silent copy 1 of the *pilS6* locus (*pilS6c1*). Volunteer 1 developed urethritis 3 d after inoculation; volunteer 2, 4 d after inoculation; and volunteer 62, 2 d after inoculation. The sequences from volunteers 1 and 2 with accession numbers beginning with the letter L were determined in a previous study (Seifert *et al.*, 1994). The diagrammed sequences do not include the conserved residues encoding amino acids 1–44 at the N-terminus of pilin protein. Regions of sequence identical to that in the inoculum variant (*pilS6c1*) are represented by the diagonally striped boxes. All other *pilS* copies are shown as open boxes. Horizontally split boxes indicate regions of sequence that could have been derived from either of two *pilS* sources. In sequence U58844, the designation *pilS** in part of mc4 indicates that the sequence could correspond to any of the following silent copies: *pilS2c5*, *pilS2c6*, *pilS3c1*, *pilS3c3*, *pilS6c3*, *pilS7c1*. Recombination end points that are located within the conserved regions flanking the variable minicassettes are drawn in the centre of the conserved regions, although it is not possible to identify the exact end points within those regions. The diagrams were constructed using the minimum number of recombination events required to generate a particular pilin sequence from the different storage copies, and could therefore be an underestimate of the actual amount of recombination that occurred.

instances, the only model we could construct to account for the expressed *pilE* sequence involved recombination occurring within a minicassette. In one isolate from volunteer 2 (U61269), the sequence of mc2 matched *pilS1c1* through part of the minicassette and then changed to match *pilS1c5*. In one of the isolates from volunteer 62 (U58844), recombination appeared to have occurred within mc4, between *pilS1c5*-derived sequences and DNA from a different silent copy. The other recombination partner might have been any of six different silent copies that have the same sequence in the 3' portion of mc4.

DISCUSSION

Intrastrain and interstrain variation in gonococcal pilin sequences

Despite the intensive effort that has been devoted to studies on the mechanism of gonococcal pilin variation, information about the actual range of intrastrain and interstrain variation in pilin primary sequences is

surprisingly limited. Our results showed that the genome of strain FA1090 contained one *pilE* locus plus 19 silent gene copies organized into five *pilS* loci. The 19 silent copies contained an extensive set of stored variant sequences: none of the silent copy sequences were completely duplicated and most of the variable minicassettes were unique or were shared by at most two silent gene copies. The variable minicassette domains sometimes contained short regions of relatively conserved sequence present in some or all of the silent gene copies. Even hypervariable mc2, with the greatest amount of sequence diversity, contained regions up to 50 bp in length that several silent copies had in common. The relative conservation of certain residues within the variable minicassettes in the FA1090 sequences is consistent with the analysis by Seifert (1996), who compared the amino acid sequences of 50 published pilin variants and showed that there are positions with little or no variation located within the originally defined minicassettes. These regions of similarity within otherwise distinct silent copies presumably reflect the evolution of

the silent copies through a process of gene duplication and subsequent mutation.

The *pilE* locus and most of the *pilS* loci were clustered in a 35 kb region of the FA1090 chromosome, with only one *pilS* locus (containing a single silent copy) outside the major cluster. The map positions and sequences of the *pil* loci match identically to those in the recently completed genome sequence of strain FA1090 (see <http://www.genome.ou.edu/gono.html>; the GenBank accession number for the completed genome is AE004969). The tight clustering of the *pil* loci and the numerous DNA repeats surrounding them had the potential to confound the use of shotgun sequencing to assemble the sequence in this region of the gonococcal chromosome. Validating the genome sequencing results with information obtained by a different method increases the reliability of the genome sequence in the region of the chromosome containing these extensively studied genes.

The mapping experiments also revealed a 265 bp repeated sequence at the 3' end of each of the *pilS* loci (the PAR sequence); the previously described 65 bp Sma/Cla repeat was actually a portion of this larger repeat, as was a copy of the repeat element RS4. The Sma/Cla repeat, which was proposed by Wainwright *et al.* (1994) to have a key role in promoting recombination leading to pilin antigenic variation, has homology to previously identified recombinase target sequences. We did not detect any additional homology to those target sequences in the regions of the complete PAR sequence that extend the boundaries of the Sma/Cla element.

The locations of most of the FA1090 *pil* loci generally corresponded to the mapped positions of the *pil* loci on the chromosome of strain MS11 (Bihlmaier *et al.*, 1991; Haas *et al.*, 1987). There were, however, some differences in the arrangement and content of the *pil* genes in the two strains. There is no *pil* locus in FA1090 corresponding to *pilS5* of MS11, which is 180° across the circular map from the major *pil* gene cluster (Bihlmaier *et al.*, 1991; Dempsey & Cannon, 1994). In the mapped variant of strain MS11, a second *pilE* locus (*pilE2*) is in the place occupied by *pilS3* in FA1090. The two strains had different total numbers of silent copies (19 in FA1090; 17 in MS11); the numbers of silent copies in corresponding *pilS* loci differed, as did the sequences of the corresponding copies and the identity and arrangement of the repeats flanking them. The differences are consistent with continued rearrangement and mutation of *pil* genes occurring since the evolutionary divergence of these two strains.

Until now, strain MS11 has been the only one for which extensive pilin sequence information was available (11 of the 17 silent copies) (Haas & Meyer, 1986; Haas *et al.*, 1992). Comparing the FA1090 sequences with those from MS11 showed that the repertoire of stored pilin information differs in the two strains. None of the silent copies were completely identical in FA1090 and MS11, although a few of the variable minicassettes were the same. There were also short regions of similar or

identical deduced amino acid sequence within some variable minicassettes, including the hypervariable mc2, without the exact duplication of the entire minicassette in the two strains. The mc2 sequence that FA1090 and MS11 had in common is not present in all strains (Haas *et al.*, 1992). Since identical mc2 sequences are not present in all strains, as would be expected if this region were a critical functional domain, it is likely that the appearance of the same mc2 sequences in multiple strains is a consequence of frequent horizontal genetic exchange of pilin genes. Haas *et al.* (1992) estimated that there are no more than approximately 100 different hypervariable mc2 sequences distributed among gonococci. However, since the comparison of the FA1090 and MS11 sequences shows that mc2 regions may contain stretches of sequence that are shared by other silent copies without conservation of an entire minicassette, oligonucleotide hybridization experiments may well underestimate the total number of different sequence variants that exist in the universe of gonococcal strains.

Pilin variation during experimental human infection

One of the major objectives of this study was to use the information about the *pil* sequence repertoire of FA1090 to begin characterizing pilin variation occurring during experimental gonococcal infection with this strain. Sequencing the *pilE* genes in 23 colonies cultured from three experimentally infected volunteers and comparing them to the sequences of the different silent copies showed that at least one copy from each of the *pilS* loci was used for recombination into *pilE* in the 11 newly generated sequences, including the *pilS7* locus located ~ 400 kb from the major *pil* gene cluster. There did not appear to be a strong bias towards preferential use of silent copy information from particular *pilS* loci *in vivo*, although the sample size is relatively small.

In their original model for pilin antigenic variation and recombination, Meyer and coworkers identified the boundaries of the six minicassettes by comparing the DNA sequences of several silent copies and proposed that the end points of recombination between *pilS* silent copies and *pilE* would be located within the blocks of absolutely conserved sequence flanking the minicassettes (Haas & Meyer, 1986; Haas *et al.*, 1992). More recently, Seifert and coworkers have challenged the notion that the variable minicassettes represent the minimal units of coding sequence that could be transferred from a silent copy to the *pilE* locus by recombination, and have suggested that recombination may occur within the minicassettes (Wainwright *et al.*, 1994, 1997; Mehr & Seifert, 1998). The great majority of the recombination events that occurred in the *in vivo*-generated pilin variants in this study involved crossing-over within the conserved blocks of sequence flanking the minicassettes, with transfer of individual minicassettes, multiple minicassettes, or entire *pilS* gene copies. We did, however, observe two examples of probable intra-minicassette recombination. Comparing the sequences that were involved in the intra-minicassette recombination suggests that 11 base pairs of identity was sufficient for

pil sequence recombination. Seifert and coworkers have hypothesized that efficient recombination between *pil* sequences with such limited homology would require specific protein factors in addition to the homologous recombination machinery (Wainwright *et al.*, 1994, 1997; Mehr & Seifert, 1998).

The pattern of variation in pilin expression in the colonies cultured from volunteer 62 was similar to that in our earlier study of volunteers 1 and 2 (Seifert *et al.*, 1994). Removing from the analysis several sequences that we now realize were less reliable did not alter the conclusions from that earlier study. In all three subjects, new pilin variants appeared early in the infection, with changes occurring throughout the length of the *pilE* gene. Multiple variants were present simultaneously, although the number of colonies analysed was not sufficient to draw conclusions about the relative proportions of different variants in the infecting population. Volunteer 62 developed urethritis less than 48 h after inoculation; none of the colonies cultured at that time contained the same *pilE* gene as the inoculum variant. The 10 colonies analysed from the second day contained four different pilin variants. The culturing of multiple colonies with identical *pilE* sequences from a specimen suggests that the variants were generated *in vivo*, and not by switching occurring after isolation of the bacteria from the infected subject. The fact that some of the variants appeared to be recombinants derived from others that appeared earlier in the infection argues that they arose from recombination occurring *in vivo* (i.e. sequences U58843 and U58844; L28990 and L28991) rather than from clonal expansion of minority variants that were present in the original inoculum. It is not possible to use this type of analysis of pilin variation to determine if the rapid appearance of new pilin variants was a consequence of an elevated rate of variation *in vivo*, since it is complicated by selection for or against different pilin variants that probably occurred after the actual recombination events.

Gonococcal and meningococcal pilin proteins often contain post-translational modifications, including glycosylation, phosphorylation and modification with glycerophosphate or phosphorylcholine (reviewed by Nassif *et al.*, 1999). In general, these modifications do not appear to have major effects on pilus-mediated adherence to eukaryotic cells *in vitro*, but they do influence the structure of the pilus fibre and the tendency of the pili to gather into bundles (Virji *et al.*, 1993; Marceau *et al.*, 1998; Jennings *et al.*, 1998; Forest *et al.*, 1999). The function and importance of these pilin modifications *in vivo* are unknown. The translated sequences of the 19 silent copies in FA1090 differed in some of the amino acid residues that are the potential sites for modification: 11 copies contained serine-63, the site for glycosylation; 18 copies contained serine-68, the site for phosphorylation; 17 copies contained serine-94, the site for modification with α -glycerophosphate. Thus, for each of these three modifications, this strain has the potential to express pilin protein that would not be modified, which might be advantageous under particular

conditions *in vivo*. The deduced amino acid sequence of the pilin produced by the *pilS6c1*-expressing inoculum variant contained an alanine residue at position 63 and serines at positions 68 and 94, and would therefore presumably not be glycosylated but could be phosphorylated and/or modified with α -glycerophosphate. In the pilin variants recovered from the volunteers, whenever a new mc6 (the region containing residue 63) was introduced by antigenic variation, it encoded serine-63. However, some colonies retained the inoculum sequence with alanine-63. It would be necessary to follow infected subjects past the time they develop signs of urethritis to determine if the apparent trend towards expression of potentially glycosylated pilins holds true, but there are both ethical and practical impediments to carrying out such experiments.

The rapid shifts in preponderance of different pilin variants *in vivo* suggest that the infecting population of gonococci was subjected to powerful selective pressures during these early stages of uncomplicated urethral infection in men. Growth of the inoculum variant *in vitro* did not result in similar shifts in the identity of the predominant variants: after growth of variant A21 on GCB agar for 24 h, more than 98% of the organisms expressed the original *pilS6c1*-derived pilin (G. Haskell & J. G. Cannon, unpublished data). Selection of new pilin variants *in vivo* was probably not based on evasion of a specific acquired antibody response, given the short duration of the infection. It is possible, however, that immune selection based on pre-existing antibodies or broad non-specific host defences could occur. Alternatively, there may be a selective advantage for gonococci expressing certain pilin types, based on function or on efficiency of pilus assembly. Differences in the primary amino acid sequence of gonococcal or meningococcal PilE influence attachment of the bacteria to different cell types (Lambden *et al.*, 1980; Nassif *et al.*, 1993; Jonsson *et al.*, 1994; Marceau *et al.*, 1995; Marceau & Nassif, 1999; Long *et al.*, 1998), even though the PilC protein is thought to be the actual adhesin (Rudel *et al.*, 1995; Källström *et al.*, 1997). Sequence differences in PilE may affect adherence indirectly, by changing the structure and properties of the pilus fibre and thus altering the exposure or local density of the PilC adhesin (Nassif *et al.*, 1999). The deduced amino acid sequences of the *in vivo*-expressed pilins do not provide any clues to the structural basis of such potential functional differences, since the sequences and their silent copy sources were different in the colonies cultured from the three different volunteers. The emergence of different pilin variants in different infected individuals could reflect differences in the binding specificity required for attachment to cellular receptors in those individuals. Alternatively, there may be multiple pilin variants with different primary sequences that can promote the same function. To investigate this question further, we plan to infect subjects twice with the same variant of strain FA1090, characterizing pilin expression in reisolates from both episodes of infection. Such experiments, coupled with *in vitro* characterization of

the properties of the pili expressed by *in vivo*-generated variants, may help in resolving some of the persistent questions surrounding structure–function relationships for gonococcal pilin and the contribution that pilin antigenic variation makes to the success of the gonococcus as a pathogen.

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