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Characterization of the *recD* gene of *Neisseria gonorrhoeae* MS11 and the effect of *recD* inactivation on pilin variation and DNA transformation

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Pilin antigenic variation in *Neisseria gonorrhoeae* may result following intrachromosomal recombination between homologous *pil* genes. Despite extensive study, *recA* is the only previously characterized gene known to be involved in this process. In this study, the gonococcal *recD* gene, encoding one subunit of the putative RecBCD holoenzyme, was characterized and its role in pilin variation assessed. The complete *recD* gene of *N. gonorrhoeae* MS11 was cloned and its nucleotide sequence determined. The gonococcal *recD* gene complemented a defined *Escherichia coli recD* mutant, based on plaque formation of bacteriophage λ and the restoration of ATP-dependent nuclease activity. Inactivation of the gonococcal *recD* gene had no measurable effect on cell viability or survival following UV exposure, but did decrease the frequency of DNA transformation approximately threefold. The frequency at which non-parental pilin phenotypes were spawned was 12-fold greater in MS11 *recD* mutants compared with the parental MS11 *rec*$^+$ strain. Similar results were obtained using *recD* mutants that were not competent for DNA transformation. Complementation of the MS11 *recD* mutant with a wild-type *recD* gene copy restored the frequency of pilin phenotypic variation to approximately wild-type levels. The nucleotide changes at *pilE* in the *recD* mutants were confined to the variable regions of the gene and were similar to changes previously attributed to gene conversion.

Keywords: *Neisseria*, *recD*, pilin variation, DNA transformation

INTRODUCTION

Neisseria gonorrhoeae (gonococcus) is particularly adept at modulating the expression and antigenicity of several surface-exposed proteins (reviewed by Swanson *et al.*, 1992). Mechanistically these changes can occur through RecA-independent processes (phase variation of *opa* gene expression and lipooligosaccharide biosynthesis genes) and by RecA-dependent homologous recombination, as exemplified by pilin antigenic variation. Furthermore, because the gonococcus is naturally competent for DNA transformation, additional genetic diversity can occur through the horizontal transfer of rearranged alleles (Smith *et al.*, 1991). The dynamic nature of the gonococcal surface may potentiate the survival of the bacterium within its human host, by contributing to its ability to evade the human immune response.

The pilin polypeptide (encoded by the pilin expression locus *pilE*) can be polymerized to form the major component of the pilus organelle (reviewed by Seifert, 1996; Swanson & Koomey, 1989). Pili are thin hair-like structures on the surface of the bacterium that are required for virulence (Kellogg *et al.*, 1963; Swanson, 1973; Swanson *et al.*, 1971, 1987). Pilin variants arise through the genetic alteration of *pilE*. Rearrangement at *pilE* occurs primarily through an intracellular (Facius & Meyer, 1993; Swanson *et al.*, 1990; Zhang *et al.*, 1992) gene-conversion-like event whereby a variant *pil* gene

Abbreviation: DUS, DNA uptake sequence.

The GenBank accession number for the sequence reported in this paper is AF058330.

sequence (*pilS*) replaces the homologous gene sequence that resides at the *pilE* locus (Haas & Meyer, 1986; Swanson *et al.*, 1986). Pilin variants also occasionally arise through *pilE* deletion events between direct oligonucleotide repeats (Hill *et al.*, 1990) or by an intercellular DNA-transformation-mediated route (Gibbs *et al.*, 1989; Seifert *et al.*, 1988). However, in contrast to intracellular events where discrete segments of *pil* DNA are transferred from *pilS* loci to *pilE*, genetic variation via the transformation route tends to be restricted to the exchange of intact *pilE* alleles (Hill, 1996). Despite extensive study, the molecular mechanism of pilin antigenic variation has not been defined.

In other organisms (e.g. *Escherichia coli*, *Salmonella typhimurium* and *Saccharomyces cerevisiae*) insight into genetic recombination has relied heavily on the use of defined *rec* mutations (reviewed by Kowalczykowski *et al.*, 1994). In contrast, surprisingly few gonococcal *rec* mutants have been constructed. Previous efforts have demonstrated a requirement for RecA in pilin gene rearrangements (Koomey & Falkow, 1987; Koomey *et al.*, 1987). However, RecA is required for homologous strand invasion and participates in essentially all pathways of homologous recombination. As a result, a *recA* null mutation does not provide insight into the roles that other *rec* gene products may play in the initial steps of homologous recombination, including pilin antigenic variation. Therefore, it is anticipated that numerous other *rec* gene products, including RecB, RecC and RecD, may be involved in the initial steps of pilin antigenic variation. Identification of the gene products involved in pilin variation, coupled with the knowledge of their substrate specificities and enzymic activity, should help to define the molecular pathway of pilin antigenic variation.

The *recD* gene encodes one subunit of the RecBCD holoenzyme, an ATP-dependent exonuclease present in many bacterial species (Brcic-Kostic *et al.*, 1991; Kowalczykowski *et al.*, 1994; Kuzminov *et al.*, 1994; Myers & Stahl, 1994). RecBCD, or exonuclease V (Exo V), is involved in the generalized recombination pathway of *E. coli*. Exo V activity is specific for the end of duplex DNA and the enzyme acts by degrading duplex DNA until it encounters an octomeric DNA sequence (a Chi site), at which point the enzyme changes from an ATP-dependent exonuclease into a DNA helicase (reviewed by Myers & Stahl, 1994). Accompanying this transition is either the loss or inactivation of the RecD subunit (Dixon & Kowalczykowski, 1993; Myers *et al.*, 1995a), which allows recombination to be focused within the interval adjacent to the encountered Chi site (Myers *et al.*, 1995b). *E. coli recD* mutants are hyper-recombinogenic for recombination (Thaler *et al.*, 1989), in contrast to *recB* or *recC* mutants which are recombination-deficient in the absence of an alternative pathway for recombination (reviewed by Clark, 1973; Sargentini & Smith, 1986). Moreover, *recD* mutations also enhance recombination following bacterial conjugation in *E. coli* or DNA transduction in *S. typhimurium* (Lovett *et al.*, 1988; Miesel & Roth, 1994), as well as allow chromosomal transformation of *E. coli* with linearized plasmid DNA molecules (Russell *et al.*, 1989).

In this study, the *recD* gene from *N. gonorrhoeae* MS11 was identified and characterized. The effect of inactivation of the *recD* gene in this strain on pilin variation and transformation was analysed.

METHODS

Bacterial strains, plasmids, media and growth conditions. Bacterial strains, plasmids and bacteriophage are described in Table 1. *N. gonorrhoeae* was grown on phosphate-buffered agar medium containing IsoVitaleX (Becton Dickinson; GC agar) at 37 °C in 5% CO_2 (Swanson, 1982). When assessing pilin switching rates, individual colonies were transferred from agar plates on a small piece of sterile Whatman filter paper and resuspended in 1 ml sterile H_2O; appropriately sized aliquots were then plated on fresh agar medium. To select for antibiotic-resistance markers, the following concentrations of antibiotics were added to gonococcal growth medium: 6 μg erythromycin ml^{-1}, 10 μg chloramphenicol ml^{-1}. *E. coli* was grown on/in Luria–Bertani (LB) agar or broth at 37 °C and supplemented with the following concentrations of antibiotics, when appropriate: 100 μg carbenicillin ml^{-1}, 80 μg chloramphenicol ml^{-1}, 200 μg erythromycin ml^{-1}.

Recombinant DNA techniques. Plasmid DNA was isolated using a plasmid purification kit (Qiagen) as described by the manufacturer. Restriction enzymes were purchased from New England Biolabs. The genomic library of strain MS11 was constructed by cloning a partial *Sau*3A digest of chromosomal DNA isolated from *N. gonorrhoeae* MS11 into dephosphorylated, *Bam*HI-digested pBR322 (New England Biolabs). Following ligation, the mixture was used to transform *E. coli* DH10B and transformants were selected on agar plates containing carbenicillin. The nucleotide sequence of the gonococcal *recD* gene and the *pilE* gene of pilin variants was determined by using custom designed oligonucleotides (Table 2) (Genemed Synthesis) and the Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Applied Biosystems). The sequencing reactions were run on an Applied Biosystems 373 DNA sequencer (Applied Biosystems). The sequencing data were analysed using Sequencher version 3.0 software (Gene Codes). PCR was performed using a Perkin Elmer 9600 Thermocycler using either *Taq* DNA polymerase (Perkin Elmer) or the Expand Hi-Fidelity kit (Boehringer Mannheim). Southern blotting was done by transferring DNA to Nytran membranes (Schleicher & Schuell) by capillary action followed by baking at 80 °C (Sambrook *et al.*, 1989). For probes, oligonucleotides were labelled with [γ-^{32}P]dATP (Dupont) using T4 polynucleotide kinase (New England Biolabs); DNA fragments were labelled with [α-^{32}P]dATP (Dupont) by using the Klenow DNA polymerase (Sambrook *et al.*, 1989).

Directed mutagenesis of *recD*. Oligonucleotides RecD1 and RecD4 were used to amplify the *recD* gene from pSH24. The amplicon was cloned into pCRII$^+$ (Invitrogen) and subsequently excised by digestion with *Eco*RI. The ends were filled-in using Klenow DNA polymerase (New England Biolabs), and the blunt-ended *recD* fragment was purified from agarose gels using a Qiaex II Gel Extraction kit (Qiagen) and ligated into an *Eco*RV-linearized pSKII+ vector that carried the gonococcal DNA uptake sequence (DUS). The recombinant plasmid was purified from *E. coli*, digested with *Eco*RV, which cut within the *recD* gene (the vector *Eco*RV site was destroyed following ligation of the *recD* fragment), and

Table 1. Strains, phage and plasmids

Strain/phage/plasmid	Relevant characteristics	Source/reference
Strains		
E. coli		
DH10B	*recA1*	BRL
C600	*rec*$^+$ *Su*$^+$	Appleyard (1954)
FS1576	*recD*: *recD1009 Su*$^+$, derived from C600	F. Stahl, University of Oregon, USA
N. gonorrhoeae		
P9	*rec*$^+$	Perry *et al.* (1987)
MS11	*rec*$^+$	J. Swanson, Rocky Mountain Laboratories, Hamilton, USA
	recD::*ermC*	This study
	dud-1	This study – *dud-1* allele from P. F. Sparling, University of North Carolina, USA
	dud-1 recD::*ermC*	This study
	comA::*cat*	This study – *comA*::*cat* allele from T. F. Meyer, Max-Planck-Institut für Biologie, Tubingen, Germany
	comA::*cat recD*::*ermC*	This study
	recD::*ermC* (*opaC*::*recD*$^+$)	This study
λ phage		
MMS805	b1453	F. Stahl, University of Oregon, USA
STU4	*cI857 P80 S7*	Hill *et al.* (1997)
Plasmids		
pSKII+	Cloning vector	Stratagene
pCR2.1	Cloning vector	Invitrogen
pCRII	Cloning vector	Invitrogen
pSH24	*N. gonorrhoeae recD* genomic clone (Amp^R) in pBR322	This study
pRML170	*N. gonorrhoeae recD*::*ermC* (Amp^R Kan^R Erm^R)	This study
pRML180	*N. gonorrhoeae recD* ORF in pCR2.1 (Amp^R Kan^R)	This study
pRML185	*cat* ORF in pCR2.1 (Amp^R Kan^R Cam^R)	J. Carlson, Rocky Mountain Laboratories, Hamilton, USA
pUC18::*opaC*	*opaC* cloned in pUC18	C. Grant, Rocky Mountain Laboratories, Hamilton, USA
pRML200	*opaC*::*recD*::*cat*::*DUS* (Amp^R Cam^R)	This study

Table 2. Oligonucleotides

Designation	Nucleotide sequence (5′–3′)	Reference/source
RecD1	GCAAACCTGTCCACCGACTGC	This study
RecD4	TACCCGCTCAAGCATACTGC	This study
RecDF2	CAGTAGTAGGTTGAGCCCGTTGAAG	This study
RecDB2	TTATACGACCCCGAATCTGCG	This study
PilEF1	TCCCCTTTCAATTAGGAGT	Hill (1996)
PilER2	CCGATATATTATTTCCACC	Hill (1996)

ligated to a blunt-ended *ermC* gene fragment. Strains containing the *recD*::*ermC* recombinant plasmid (pRML170) were selected on plates containing erythromycin and carbenicillin following transformation of *E. coli* DH10B (Life Technologies). Plasmid pRML170 was used to transform *N. gonorrhoeae* strain P9 and transformants were selected on agar plates containing erythromycin. PCR and Southern blotting indicated that the transformants contained a single

copy of the *ermC* gene in the *recD* locus, which was consistent with allelic replacement of the wild-type *recD* gene with the mutated allele. Genomic DNA isolated from P9 *recD* was used to transform MS11 and transformants were selected on GC agar plates containing erythromycin.

Construction of MS11 *recD* merodiploid strain. The *recD* gene was amplified by PCR from pSH24 using oligonucleotide primers RecDF2 and RecDB2 and cloned into the pCR2.1 (Invitrogen) vector to form the recombinant plasmid designated pRML180. Plasmid pRML180 was cleaved with *Spe*I in the multiple cloning region of the vector, 3′ to the cloned *recD* gene. The DNA was blunt-ended by using Klenow DNA polymerase, and then ligated with a blunt-ended fragment containing a promoterless *cat* gene and the gonococcal DUS. The ligation mixture was used to transform *E. coli* DH10B and transformants were selected on LB agar plates containing chloramphenicol. Following confirmation of the recombinant plasmid construction, the *recD-cat*-DUS region of the plasmid was excised on a *Xba*I and *Bam*HI fragment. The ends were filled-in using Klenow enzyme and ligated into a blunt-ended *Cla*I site of a pUC18::*opaC* recombinant plasmid. *E. coli* transformants were selected on LB agar plates containing chloramphenicol. PCR and restriction enzyme analysis confirmed the construction of the plasmid subsequently designated pRML200. Plasmid pRML200 was used to transform wild-type MS11. Insertion of the *recD* and *cat* genes into the *opaC* locus was confirmed by Southern blotting and PCR. Inactivation of the wild-type *recD* locus was achieved by allelic replacement using genomic DNA isolated from the MS11 *recD*::*ermC* strain. Transformants were selected on agar plates containing erythromycin and construction of the merodiploid was confirmed by Southern blotting and PCR.

Nucleotide sequence of pilin variants. Colonies with non-parental pilin phenotypes were grown for 18 h on agar plates and cell lysates were prepared by boiling cell suspensions in 50 µl H_2O for 10 min. The *pilE* gene was amplified by PCR in a Perkin Elmer 9600 thermocycler with AmpliTaq polymerase (Perkin Elmer) using previously defined primers (Table 2) (Hill, 1996). The PCR product was purified by using Centricon-100 columns (Amicon), and the nucleotide sequence of both strands of the *pilE* gene was determined as described above.

UV sensitivity and cell viability assays. The sensitivity of gonococcal strains to UV radiation was determined essentially as previously described by Koomey & Falkow (1987). Non-piliated, non-opaque gonococci were grown overnight on plates and resuspended and serially diluted in water to a density of approximately 1×10^8 c.f.u. ml^{-1}. Subsequently, 25 µl was exposed to UV radiation (0, 25, 50, 75, 125, 150 and 200 µJ × 100) by using Stratalinker 1800 UV cross-linker (Stratagene). An MS11 *recA* strain was used as a control. Cell viability assays were done as previously described by Sandler (1996). Briefly, non-piliated, non-opaque variants of the strains to be tested were grown in liquid broth. At various stages of growth, samples were taken, the OD_{600} was determined, and viable cell counts were determined by plating defined volumes.

^{3}H-labelling of bacteriophage DNA for exonuclease assay. Labelled λ DNA was prepared as described previously (Hill *et al.*, 1997). Briefly, a 100 ml culture of an *E. coli* λ lysogen (*cI857 P80 S7*) was grown at 34 °C in the presence of 0·25 mCi (9·25 MBq) [^{3}H]thymidine (Dupont). After 6 h incubation, the temperature of the culture was increased to 42 °C for 15 min to induce the phage lytic cycle and the culture was then grown at 37 °C for an additional 3 h. Cells were then disrupted by lysozyme treatment in conjunction with a chloroform extraction. Phage were purified by CsCl isopycnic centrifugation. The ^{3}H-labelled phage DNA was extracted using phenol/chloroform and precipitated with ethanol.

Bacteriophage plaque size assay for RecD function. Phage strain MM805 Gam$^-$ Red$^-$ Chi$^-$ was kindly provided by Frank Stahl (University of Oregon) and was used to assess RecD function as previously described by Thaler *et al.* (1989). Plaque size was measured by using a micrometer and dissecting microscope and the data are reported in the text as the mean diameter (mm) ± SEM.

DNA transformation. DNA transformation of piliated MS11 strains was performed as previously described (Swanson *et al.*, 1990). Bacteria were grown on solid medium for 18–24 h. Typically, 20 colonies were then transferred to 0·5 ml broth medium containing 20 mM $MgCl_2$ using small pieces of sterile Whatman filter paper. Purified chromosomal DNA isolated from *N. gonorrhoeae* that confers resistance to nalidixic acid was added at a concentration of 3·3 µg ml^{-1} and the suspension was incubated at 37 °C for 30 min. After incubation, the cell suspension was diluted 10-fold in pre-warmed broth medium containing 1% IsoVitaleX (Becton Dickinson) and incubated for an additional 5 h at 37 °C, 5% CO_2 prior to plating on agar plates containing 2·0 µg nalidixic acid ml^{-1}. The frequency of transformation was determined by dividing the number of nalidixic-acid-resistant c.f.u. by the total number of c.f.u. and is given in the text as the mean frequency ± SEM.

RESULTS

Cloning and nucleotide sequence analysis of the *recD* gene from *N. gonorrhoeae* MS11

To clone the *recD* gene from *N. gonorrhoeae*, the amino acid sequences of the RecD polypeptides of *E. coli* (Finch *et al.*, 1986a) and *Haemophilus influenzae*

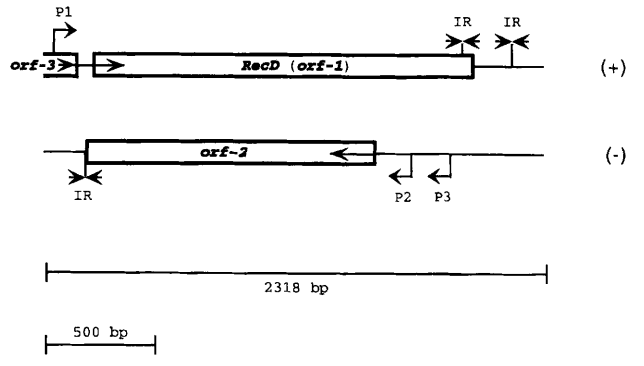

Fig. 1. Physical map of the *recD* (*orf-1*) genomic region of MS11. The nucleotide sequence of 2318 bp DNA which contained the *recD* gene (*orf-1*) was determined. The *recD* (*orf-1*) gene was preceded by a putative promoter labelled P1. Two inverted repeats, designated IR, were present downstream of *recD* (*orf-1*). Upstream of *recD* (*orf-1*) on the same DNA strand (+), a partial ORF (*orf-3*) was encoded that shared similarity to genes encoding ABC transporters. *orf-2* was encoded on the opposite (−) DNA strand, compared to *recD* (*orf-1*), and was preceded by two putative promoters labelled P2 and P3. An inverted repeat (IR) composed of the gonococcal DNA uptake sequence (DUS) was present proximal to the predicted translational stop codon of *orf-2*.

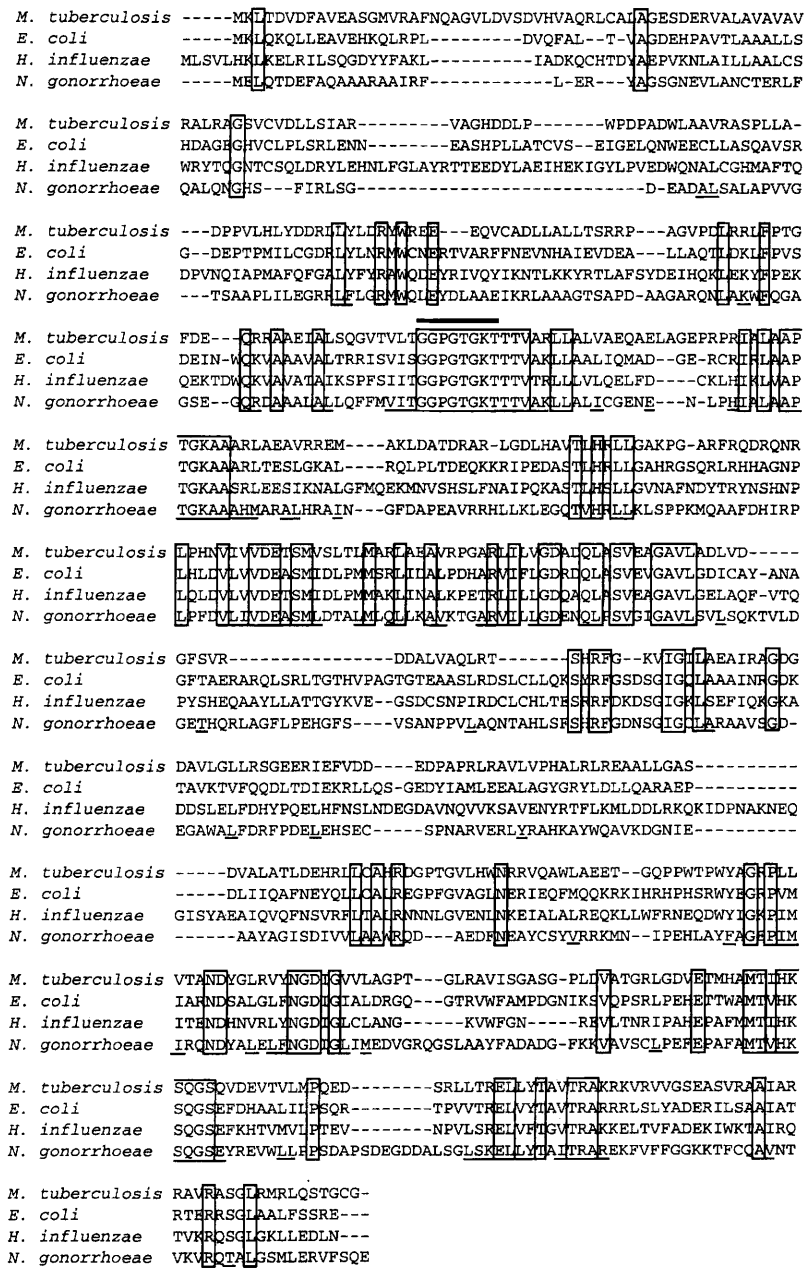

Fig. 2. Alignment of the gonococcal RecD polypeptide (ORF-1) with selected RecD polypeptides. The deduced amino acid sequence of the RecD polypeptide from *N. gonorrhoeae* MS11 (ORF-1) is compared to the amino acid sequences of the RecD polypeptides from *M. tuberculosis* (Cole *et al.*, 1998), *E. coli* (Finch *et al.*, 1986b) and *H. influenzae* (Fleischmann *et al.*, 1995). Boxed and underscored regions indicate regions of identity and similarity, respectively. The conserved 'P-loop' sequence is overscored with a bar.

(Fleischmann *et al.*, 1995) were used to search for a similar polypeptide in the translated *N. gonorrhoeae* FA1090 genome database (University of Oklahoma) prior to its completion and annotation (Roe *et al.*, 1998). A putative *recD* gene was identified and the nucleotide sequence facilitated the design of oligonucleotides complementary to the 5′ and 3′ regions of the gene (RecD1, RecD4). By using PCR, a 1·1 kb portion of the putative *recD* gene was amplified using genomic DNA isolated from strain MS11 as template DNA. The nucleotide sequence of the PCR product was determined, which confirmed that a *recD*-like gene had been amplified. Subsequently, the PCR product was radiolabelled and was used to screen a genomic library of *N. gonorrhoeae* MS11 by colony hybridization. The probe hybridized to several clones in the library; a single clone (pSH24) was selected and the nucleotide sequence of 2318 bp neisserial DNA was determined. A schematic depiction of the sequenced region is shown in Fig. 1. Two complete ORFs were identified. One ORF (ORF-1, consisting of 1746 bp) encoded a 582-amino-acid polypeptide with a predicted molecular mass of 63 kDa. A potential Shine–Dalgarno sequence (AAGG) was present 7 bp upstream of the predicted start codon (AUG). By using MacTargsearch (Mulligan *et al.*, 1984), a potential promoter (P1), centred 169 bp upstream of the translational start site of *orf-1*, was identified that shared 38·5% similarity with the consensus *E. coli* promoter. Using the BLASTX program, a high scoring match was found between ORF-1 and the RecD polypeptide of *E. coli* (Finch *et al.*,

Table 3. Nuclease activity in cellular extracts

Results are expressed as the percentage of TCA-soluble [^{3}H]DNA. Assay conditions are described in detail in Methods.

Strain and relevant genotype	Nuclease activity		Ratio (+/−ATP)
	−ATP	+ATP	
E. coli rec$^+$	8	34	4·3
E. coli recD	52	24	0·5
E. coli recD/pSH24 (*N. gonorrhoeae recD*$^+$)	21	33	1·6

1986a). In addition, high scoring matches were found with the putative RecD polypeptides of *H. influenzae* (Fleischmann *et al.*, 1995) and *Mycobacterium tuberculosis* (Cole *et al.*, 1998). The amino acid sequence of the ORF-1 polypeptide is 58·5 % similar and 34·8 % identical to the RecD protein of *E. coli*. An alignment of selected RecD polypeptides and ORF-1 showed that several regions are conserved among the polypeptides (Fig. 2), including a consensus 'P-loop', a feature typical of several ATP/GTP-binding proteins (reviewed by Saraste *et al.*, 1990). Based on the similarity of the amino acid sequence of ORF-1 to the *E. coli* RecD polypeptide, ORF-1 was designated RecD and the corresponding gene was designated *recD*.

A potential translational start site (AUG) for a second ORF (ORF-2, consisting of 1326 bp) was preceded by 8 bp by a possible Shine–Dalgarno sequence (GAAA). ORF-2 was encoded on the opposite DNA strand compared to RecD. By using MacTargsearch (Mulligan *et al.*, 1984), two potential promoter regions were identified 159 bp and 324 bp upstream of ORF-2 that were 40·8 % and 42·6 % similar, respectively, to the *E. coli* consensus promoter. An inverted repeat composed of the gonococcal DUS overlapped with the translational stop codon. A search of the GenBank databases did not identify a protein similar to ORF-2.

In contrast to *E. coli* and other bacterial species, the putative gonococcal *recD* gene in MS11 was not flanked by the genes encoding either the RecB or RecC subunits of the RecBCD holoenzyme. A portion of a third ORF (ORF-3, consisting of 153 bp) was present upstream of the *recD* gene that was similar to a variety of genes encoding ABC transporters. There was 66 bp between the end of *orf-3* and the predicted start codon of *recD*. A BLASTX search of the GenBank databases using the 354 bp of sequenced DNA that is downstream of the *recD* gene as the query sequence did not identify a similar protein.

Complementation of an *E. coli recD* mutant with the gonococcal *recD* gene

E. coli recD mutants are deficient in Exo V (ATP-dependent exonuclease) activity. To determine if the gonococcal *recD* gene could restore ATP-dependent nuclease activity to an *E. coli recD* mutant, a plasmid that contained the gonococcal *recD* gene (pSH24) was introduced into a defined *E. coli recD* mutant by electroporation. Cell extracts were prepared from *E. coli rec*$^+$, *E. coli recD* and *E. coli recD*/pSH24 (*N. gonorrhoeae recD*$^+$). Subsequently, the nuclease activity present in the extracts was determined, both with and without the addition of ATP, by using ^{3}H-labelled λ DNA as a substrate. Nuclease activity rendered the ^{3}H-label acid-soluble, which was quantified by liquid scintillation counting; the results are summarized in Table 3. As expected, the nuclease activity in extracts prepared from the *E. coli rec*$^+$ strain was 4·3-fold greater when exogenous ATP was added (34 % acid-soluble ^{3}H) compared to that in the absence of exogenously added ATP (8 % acid-soluble ^{3}H). In contrast, the nuclease activity in extracts from the *E. coli recD* strain decreased following the addition of ATP (52 % acid-soluble ^{3}H in the absence of exogenously added ATP compared to 24 % acid-soluble ^{3}H following ATP addition), resulting in a ratio of ATP-dependent to ATP-independent nuclease activity of 0·5. In the absence of exogenously added ATP, the nuclease activity in extracts prepared from the *E. coli recD* strain was greater (52 % acid-soluble ^{3}H) compared to that in extracts from the parental *rec*$^+$ strain (8 % acid-soluble ^{3}H). This result may indicate that either there is a compensatory increase in ATP-independent nuclease activity in the absence of Exo V, or alternatively that Exo V inhibits ATP-independent nuclease activity. Similar to results obtained using *E. coli rec*$^+$ extracts, the nuclease activity in extracts prepared from the *recD* mutant strain that contained the gonococcal *recD* gene [*E. coli recD*/pSH24 (*N. gonorrhoeae recD*$^+$)] was 1·6-fold greater when exogenous ATP was added (33 % acid-soluble ^{3}H) compared to that in the absence of exogenously added ATP (21 % acid-soluble ^{3}H). In addition, in the absence of exogenously added ATP, the nuclease activity detected in extracts from the complemented *E. coli recD* mutant was less compared to the activity in extracts prepared from the *recD* mutant (21 % acid-soluble ^{3}H, 52 % acid-soluble ^{3}H, respectively). These results indicated that the gonococcal *recD* gene could restore ATP-dependent nuclease activity to a defined *E. coli recD* mutant.

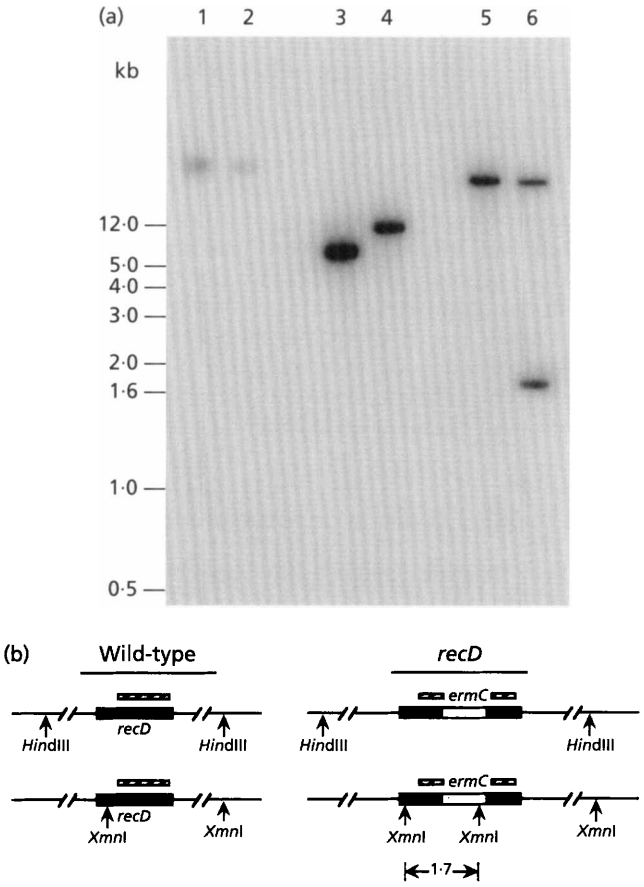

Fig. 3. Southern blot analysis of chromosomal DNA isolated from strains MS11 wild-type and MS11 *recD*. DNA was isolated from strains MS11 wild-type and MS11 *recD* and was examined by blot hybridization (a). Lanes: 1, MS11 wild-type genomic DNA (uncut); 2, MS11 *recD* genomic DNA (uncut); 3, *Hin*dIII digest of MS11 wild-type genomic DNA; 4, *Hin*dIII digest of MS11 *recD* genomic DNA; 5, *Xmn*I digest of MS11 wild-type genomic DNA; 6, *Xmn*I digest of MS11 *recD* genomic DNA. The blot was probed with gonococcal *recD* specific probe. (b) Schematic presentation of the gonococcal *recD* locus showing the relative position of the *Hin*dIII and *Xmn*I sites. The cross-hatched bar shows the approximate hybridization position of the *recD* probe.

The plaque size of Red$^-$ Gam$^-$ bacteriophage λ is significantly influenced by the presence of Exo V activity in the *E. coli* host (McKittrick & Smith, 1989; Myers *et al.*, 1995a; Russell *et al.*, 1989). Specifically, larger plaques are formed when the phage infect *recD* mutants of *E. coli*, due to the lack of host cell Exo V activity (reviewed by Myers & Stahl, 1994; Thaler *et al.*, 1989). Therefore, as an additional test to determine if the gonococcal *recD* gene could complement a *recD* mutation in *E. coli*, the plaque size of Red$^-$ Gam$^-$ phage was used as an indicator of Exo V activity. In addition, since the recombinant plasmid used to assess ATP-dependent nuclease activity (pSH24) contained a large genomic DNA fragment from MS11, the gonococcal *recD* gene was amplified by PCR from pSH24 and cloned into the pCR2.1 vector to form the recombinant plasmid designated pRML180. As a control, a *cat* gene was similarly cloned into pCR2.1 and the plasmid was designated pRML185. These plasmids were then introduced into the *E. coli recD* strain by electroporation. The strains were infected with Red$^-$ Gam$^-$ bacteriophage and the diameter of the resulting plaques was measured to assess the activity of Exo V. The mean diameter of the plaques formed by Red$^-$ Gam$^-$ phage on *E. coli recD*/pRML180 (*N. gonorrhoeae recD*$^+$) was $1{\cdot}5 \pm 0{\cdot}039$ mm; significantly smaller compared to the mean diameter of the plaques that formed on the control strain *E. coli recD*/pRML185 (*cat*) ($2{\cdot}1 \pm 0{\cdot}056$ mm; P value $< 0{\cdot}0001$), but slightly larger than those formed on the parental *E. coli rec*$^+$ strain ($1{\cdot}2 \pm 0{\cdot}025$ mm; $P < 0{\cdot}0001$). These results showed that the gonococcal *recD* gene was necessary and sufficient to restore Exo V activity to an *E. coli recD* mutant.

Inactivation of the *recD* gene in MS11 and complementation of the mutant with an intact gene copy

N. gonorrhoeae MS11 *recD* mutants were obtained by allelic replacement using donor DNA that carried a *recD*::*ermC* insertional mutation. The replacement of the wild-type copy with the insertionally inactivated copy of *recD* was confirmed by PCR analysis (data not shown) and Southern blotting (Fig. 3a). To address potential polar effects due to insertion of the heterologous *ermC* marker into the *recD* locus, an MS11 *recD*::*ermC* (*opaC*::*recD*$^+$) merodiploid strain was constructed that contained an intact copy of the *recD* gene inserted into the *opaC* locus. Piliated *recD* mutants of both MS11 and P9 strains had a longer lag phase and an approximately 10% longer generation time in broth medium compared to piliated MS11 *rec*$^+$ or piliated MS11 *recD*::*ermC* (*opaC*::*recD*$^+$) merodiploid strains (data not shown). Thus the growth defect was associated with the inactivated *recD* allele and was not strain-specific. However, there was no apparent difference in growth among non-piliated variants of MS11 *rec*$^+$, MS11 *recD* and MS11 *recD*::*ermC* (*opaC*::*recD*$^+$) (data not shown). No difference was detected among the three strains [MS11 *rec*$^+$, MS11 *recD* and MS11 *recD*::*ermC* (*opaC*::*recD*$^+$) merodiploid] in viability or their susceptibility to UV irradiation (data not shown). Since *N. gonorrhoeae* is naturally competent for DNA transformation (Sparling, 1966) the frequency of DNA transformation was also evaluated among the strains using purified chromosomal donor DNA that carried a single base pair mutation conferring resistance to nalidixic acid. The frequency of transformation of the MS11 *rec*$^+$ strain was $0{\cdot}018 \pm 0{\cdot}004$ compared to $0{\cdot}006 \pm 0{\cdot}003$ in the MS11 *recD* mutant strain, a threefold difference ($P = 0{\cdot}073$). The *recD* merodiploid strain had a frequency of transformation of $0{\cdot}012 \pm 0{\cdot}005$; this was not significantly different from the *rec*$^+$ strain ($P = 0{\cdot}442$) or the *recD* mutant ($P = 0{\cdot}358$).

Table 4. Effect of *recD* mutation on the frequency of pilin phenotypic variation

The data represent the mean (SEM) from at least 16 individual colony platings.

Strain	Frequency of pilin switching (SEM)
Wild-type 37 °C	0·009 (0·01)
Wild-type 33 °C	0·011 (0·00)
recD::ermC	0·108 (0·02)
recD::ermC (*opaC::recD*$^+$)	0·014 (0·00)
dud-1	0·010 (0·00)
recD dud-1	0·092 (0·06)
comA	0·032 (0·01)
recD comA	0·228 (0·04)

Effect of a gonococcal *recD* mutation on pilin phenotypic variation

Differences in colony morphology correlate with the expression (or lack of expression) of different pilin polypeptides (Swanson *et al.*, 1986) and is therefore a sensitive indicator for genetic rearrangement occurring at the *pilE* locus. To determine if inactivation of the *recD* gene affected pilin variation, the frequency at which progeny displayed a non-parental pilin phenotype was compared among the MS11 *rec*$^+$, MS11 *recD* and MS11 *recD::ermC* (*opaC::recD*$^+$) merodiploid strains. For each experiment, a single colony was plated at low density on agar plates and the progeny were assessed for the presentation of either a parental or non-parental (which includes both pilus$^+$ and pilus$^-$ variants) pilin phenotype. As shown in Table 4, the frequency of progeny with a non-parental pilin phenotype was approximately 12-fold greater in strain MS11 *recD* compared with the parental MS11 *rec*$^+$ strain. The presence of an intact *recD* gene copy in the *opaC* locus of the *recD* mutant restored the frequency of pilin switching to that of the MS11 *rec*$^+$ strain (Table 4) and confirmed that the effect on colony morphology was associated with the mutated *recD* allele. Several control experiments were designed to determine if the increase in variant pilin phenotypes in the *recD* mutant strain was due to either the growth defect or the decrease in the frequency of DNA transformation; both properties associated with piliated *recD* mutants. To determine if the reduced growth rate stimulated changes in the pilin phenotype in wild-type bacteria, the frequency of pilin phenotype variation in MS11 *rec*$^+$ was determined following growth at 33 °C to mimic the growth rate of the *recD* mutant strain. No significant effect on the frequency of pilin variation was observed under this condition (cf. wild-type *rec*$^+$ bacteria following growth at 33 and 37 °C; Table 4). Although a dramatic difference in the frequency of DNA transformation was not evident following inactivation of the *recD* gene, it remained unclear if the minor difference in transformation (threefold decrease) could significantly influence the frequency of pilin variation. Several gonococcal mutants are available that are not competent for transformation, including *dud-1* and a *comA* mutant (Biswas *et al.*, 1989; Facius & Meyer, 1993), both of which are deficient in the ability to transport donor DNA to the cytoplasm. To determine if the effect of a *recD* mutation on the frequency of pilin variation was related to the minor effect on transformation, the frequency of pilin variation was also determined for the following non-competent strains of MS11: *dud-1*, *dud-1 recD*, *comA*, *comA recD*. As shown in Table 4, an increased frequency of progeny with a non-parental pilin phenotype in non-competent strains of MS11 again correlated with the inactivated *recD* allele.

Genetic changes at *pilE* in gonococcal *recD* mutants

To confirm that the variant pilin phenotypes spawned by MS11 *recD* mutants reflected gene-conversion-like events at *pilE*, as seen in *rec*$^+$ bacteria, and to confirm that the changes in colony morphology used to estimate the frequency of pilin variation correlated with changes in the amino acid sequence of the pilin polypeptide, the *pilE* nucleotide sequences from 15 piliated colonies displaying a non-parental pilin phenotype were determined and compared to the parental MS11 *recD pilE* gene sequence. At the amino acid level (Fig. 4), the pilin polypeptide sequences from all 15 non-parental progeny differed compared with the parental polypeptide sequence, with changes occurring exclusively within the variable portions of the polypeptide. Although not all the variable segments in the non-parental *pilE* genes could be ascribed to a specific published *pilS* gene copy, several variable segments were traced back to specific *pilS* gene copies. For example, the gene segment that incorporated the amino acid sequence EMASTGVNKEIKD (residues 98–110) in non-parental pilin variants numbers 6 and 13 originated from *pilS1* copy 3; likewise, the amino acid sequence GAGNAGKADDVTKAGNDNEKIN (residues 135–156) of variant number 13 was introduced following recombination of *pilS1* copy 2 with *pilE* (Haas & Meyer, 1986). Moreover, similar types of pilin variants, with characteristic *pilE* gene-conversion-like motifs, were also found in those non-parental variants that were obtained by plating MS11 *comA recD* bacteria (data not shown). These results confirmed that the characteristic colony morphology of the *recD* bacteria that were scored as presenting a non-parental pilin phenotype correlated, without exception, to amino acid changes encoded at *pilE*. In addition, this analysis showed that the variation at the *pilE* locus in MS11 *recD* mutants was reminiscent of previously ascribed gene-conversion-like events.

DISCUSSION

The purpose of this study was to characterize the *recD* gene of *N. gonorrhoeae*, which encodes one subunit of

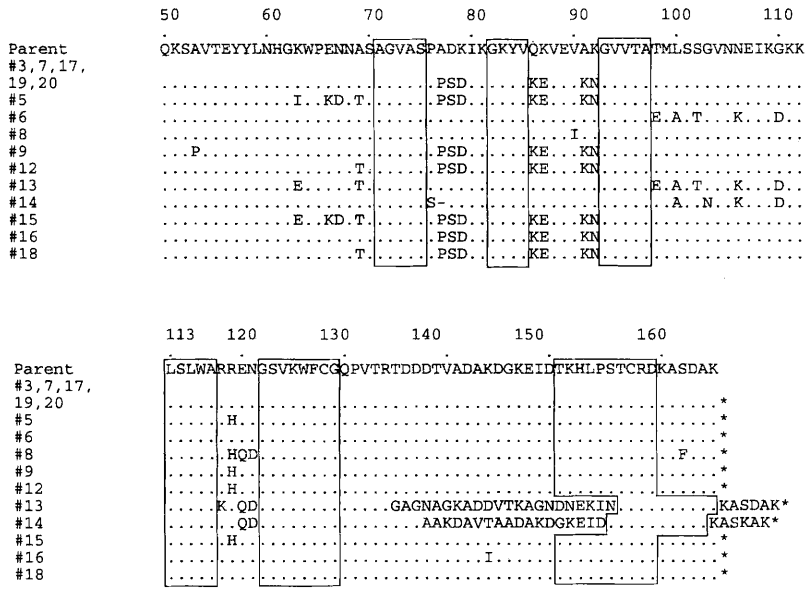

Fig. 4. Amino acid sequence comparison of the MS11 *recD* parental and non-parental pilin polypeptides. The nucleotide sequence of the *pilE* gene was determined for a parental MS11 *recD* colony and for 15 progeny colonies that displayed a non-parental pilin phenotype. The deduced amino acid sequences of the variable regions of the pilin polypeptide are compared and are aligned with respect to the position of the constant regions (boxed segments). Amino acids are noted by the single-letter code and their relative numerical positions are designated. Amino acids that differ from the parental sequence are indicated; stops indicate identical amino acids.

the putative gonococcal RecBCD holoenzyme, and assess its contribution, if any, to genetic variation at the *pilE* locus. The *recD* gene was selected for study based on the prediction that if the RecBCD holoenzyme is involved in recombination at *pilE*, mutations in either of the genes encoding the RecB or RecC subunits would be likely to abolish pilin variation, similar to a *recA* mutation. However, because a mutation in the *recD* gene of *E. coli* has been previously shown to abrogate Exo V activity, while maintaining the recombinogenic helicase activity of RecBC, such a mutation in *N. gonorrhoeae* would be predicted to increase the frequency at which pilin recombinants are formed. Towards this end, we cloned the *recD* gene from strain MS11, determined its nucleotide sequence, and showed that the gene could complement a defined *E. coli recD* mutant. In addition, inactivation of the gene in MS11 increased the frequency at which non-parental pilin phenotypes were spawned. Complementation of the mutant with an intact *recD* gene copy restored the frequency of pilin phenotypic variation to essentially wild-type levels. Several control experiments showed that the increased frequency of pilin variation in the gonococcal *recD* mutant was not due to either the decreased growth rate of piliated *recD* mutants or the decreased frequency of DNA transformation which was also associated with the inactivated *recD* allele.

Analysis of the gonococcal *recD* gene revealed both similarities to homologous genes from other bacterial species and a novel feature that may have important implications with regard to the function of the putative RecBCD holoenzyme of *N. gonorrhoeae*. Several regions of amino acid identity were shared between the gonococcal RecD homologue and the RecD polypeptides of *E. coli*, *M. tuberculosis* and *H. influenzae*, including a conserved 'P-loop' motif (Fig. 2). The 'P-loop' motif is defined as a series of glycine residues followed by a conserved lysine and either serine or threonine (reviewed by Saraste *et al.*, 1990). The gonococcal 'P-loop', and that of other RecD polypeptides, consisted of the amino acid residues GGPGTGKT (Fig. 2). The three-dimensional structure of the glycine-rich region is predicted to form a loop which interacts with a phosphate group of the nucleotide ATP or GTP. The conservation of the 'P-loop' in the gonococcal RecD polypeptide is consistent with the previous finding that the RecD protein of *E. coli* binds ATP (Julin & Lehman, 1987). In contrast to *E. coli*, *H. influenzae* and *M. tuberculosis*, the gonococcal *recD* gene was not linked to either the *recB* or *recC* genes. In *E. coli*, the *recD* gene is directly downstream of the *recB* gene and the genes may be expressed as part of an operon (Amundsen *et al.*, 1986; Finch *et al.*, 1986b). The lack of genetic linkage of the *recD* and the *recB* and *recC* genes in the gonococcus may significantly affect their coordinate expression. Moreover, the small intergenic region between the gene encoding a putative ABC transporter and the downstream *recD* gene in MS11 suggests that expression of *recD* may be linked to the expression of the putative ABC transporter gene. In general, ABC transporters are expressed in response to environmental conditions such as the presence of a specific substrate (reviewed by Higgins, 1992). As a result, it seems reasonable to speculate that under certain physiological conditions, the gonococcus may exist as a $RecD^-$ phenocopy, similar in phenotype to a *recD* mutant. Thus the enhanced frequency of pilin phenotypic variation observed in this study, following inactivation of the *recD* gene, may occur *in vivo* under certain physiological conditions in which the expression of *recD* is not strictly coordinated with that of *recB* or *recC*. Such a strategy could represent yet another means used by the gonococcus to alter its genetic composition.

The gonococcal *recD* gene restored Exo V activity to an *E. coli recD* mutant; however, the activity was not

restored to the levels observed in the parental *rec*$^+$ strain. Several possibilities could account for this observation, including inefficient expression of the gonococcal *recD* gene in *E. coli*. Although the gene was introduced into *E. coli* on a multi-copy plasmid there are no experimental data regarding the relative expression of the gonococcal *recD* gene in *E. coli* compared to expression in *N. gonorrhoeae*. In addition, the association of the gonococcal RecD subunit with the heterologous RecB and RecC subunits of *E. coli* may be inefficient or result in an enzyme with decreased enzymic activity.

Surprisingly, the frequency of DNA transformation in the MS11 *recD* mutant was approximately threefold lower compared to both the wild-type MS11 strain and the *recD* merodiploid, in contrast to the apparent increase in the frequency of intrachromosomal recombination at *pilE* associated with this mutation. Although the decrease was not quite statistically significant, the results were consistent upon repeated experiments. Several possible explanations could account for these disparate *recD* mutant phenotypes. One possibility is that the pathway used for transformation-mediated recombination is not identical to that used for intrachromosomal recombination. We recently showed that during DNA transformation of competent gonococci, donor DNA is converted, at least partially, to a single-stranded form (Chaussee & Hill, 1998). It is conceivable then that only single-stranded donor DNA is transported to the cytoplasm of competent gonococci, which would make it unlikely that the putative RecBCD pathway of recombination would be involved in recombination since the holoenzyme acts primarily on double-stranded DNA substrates. However, this rationale does not explain the decrease in the frequency of transformation-mediated recombination associated with an inactivated *recD* allele. One possible explanation is that there is a compensatory enhancement of ATP-independent nuclease activity in an Exo V mutant (RecD$^-$), similar to that observed in an *E. coli recD* mutant (Table 3). The nuclease activity in an *E. coli recD* mutant is dependent on RecBC and involves the *recJ* and *xonA* genes which both encode single-stranded nucleases (Rinken *et al.*, 1992). Thus the decreased frequency of DNA transformation in a *recD* mutant of gonococci may be due to enhanced degradation of single-stranded transforming DNA and an associated decrease in the formation of recombinants. Alternatively, if transforming DNA is predominantly double-stranded following transport to the cytoplasm, the helicase activity of RecBC may create single-stranded DNA which would then be susceptible to RecJ or other single-stranded exonucleases which may have increased activity in an Exo V mutant, also resulting in a decrease in the frequency of DNA transformation.

In this study, differences in colony morphology were used to estimate the frequency of pilin variation among isogenic strains of MS11. As additional evidence that the variations in colony morphology correlated with changes at the *pilE* locus, the nucleotide sequences of the *pilE* genes from a total of 20 colonies which exhibited a non-parental pilin phenotype were determined. In each case, a non-parental pilin polypeptide was encoded at *pilE*. Although the majority of changes in colony morphology scored in this study were among piliated colonies, non-piliated variants were also scored as presenting a non-parental pilin phenotype. Since phase variation of pilin expression can result from both antigenic variation related recombination and unrelated events [including *pilC* phase variation and deletion of the *pilE* gene (Meyer *et al.*, 1984; reviewed by Swanson & Koomey, 1989)], the frequency of pilin phenotype variation reported here is not necessarily specific to antigenic variation. However, no difference was detected in the frequency of colony opacity phase variation among the isogenic strains, which suggests that phase variation, in general, is not affected by inactivation of the *recD* gene (S. A. Hill, unpublished results).

The increased frequency at which colonies with non-parental pilin colony morphology were spawned in *recD* mutants of MS11 could be either directly or indirectly associated with the *recD* mutation, since essentially nothing is known about the various pathways of homologous recombination that may exist in gonococci, the manner in which these hypothetical pathways interact, or the potential role of Chi. Genes encoding polypeptides with similarity to both the RecB and RecC polypeptides of *E. coli* are present in the genome database of *N. gonorrhoeae* FA1090 (Roe *et al.*, 1998). Thus while the presence of the gonococcal RecBCD holoenzyme has not been experimentally established, the phenotype of the MS11 *recD* mutant suggests that the RecBCD holoenzyme is functional in *N. gonorrhoeae*. Nonetheless, we can only speculate on the molecular mechanism that may be responsible for the increase in pilin variation in *recD* mutants. Clearly, the generation of additional defined gonococcal *rec* mutants, in conjunction with *in vivo* physical analysis of recombination intermediates, is needed to define the molecular events involved in pilin antigenic variation.

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