A Homologue of the Recombination-Dependent Growth Gene, *rdgC*, Is Involved in Gonococcal Pilin Antigenic Variation

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ABSTRACT

Neisseria gonorrhoeae pilin undergoes high-frequency changes in primary amino acid sequence that aid in the avoidance of the host immune response and alter pilus expression. The pilin amino acid changes reflect nucleotide changes in the expressed gene, *pilE*, which result from nonreciprocal recombination reactions with numerous silent loci, *pilS*. A series of mini-transposon insertions affecting pilin antigenic variation were localized to three genes in one region of the Gc chromosome. Mutational analysis with complementation showed that a Gc gene with sequence similarity to the *Escherichia coli rdgC* gene is involved in pilus-dependent colony phase variation and in pilin antigenic variation. Furthermore, we show that the Gc *rdgC* homologue is transcriptionally linked in an operon with a gene encoding a predicted GTPase. The inability to disrupt expression of this gene suggests it is an essential gene (*engA*, *e*ssential *n*eisserial *G*TPase). While some of the transposon mutations in *rdgC* and insertions in the 5′-untranslated portion of *engA* showed a growth defect, all transposon insertions investigated conferred an aberrant cellular morphology. Complementation analysis showed that the growth deficiencies are due to the interruption of RdgC expression and not that of EngA. The requirement of RdgC for efficient pilin variation suggests a role for this protein in specialized DNA recombination reactions.

THE obligate human pathogen *Neisseria gonorrhoeae* (the gonococcus, Gc) expresses type IV pili, which are filamentous, surface-exposed organelles essential for full infectivity (Kellogg *et al.* 1963; Swanson *et al.* 1987). Gc pili are required for both adherence to the urogenital epithelium (Swanson 1973) and for full DNA transformation competence (Sparling 1966; Seifert *et al.* 1990). The pilus is composed primarily of the *pilE* gene product, pilin (Swanson *et al.* 1971). Gc pilus antigenic variation occurs when DNA sequences from one of several silent pilin gene copies (*pilS*) are transferred unidirectionally to replace variable sequences within the *pilE* gene (Hagblöm *et al.* 1985; Haas and Meyer 1986; Segal *et al.* 1986). The Sma/Cla DNA repeat, located at the 3′ end of all pilin loci, is necessary for efficient pilin antigenic variation (Wainwright *et al.* 1994). The *pilE* sequence changes alter the amino acid sequence of the pilin protein and the antigenicity of the pilus (Virji *et al.* 1983; Virji and Heckels 1984). Presumably, these antigenic changes aid in the avoidance of the host immune system (Brinton *et al.* 1978; Boslego *et al.* 1991) and alter pilus-mediated epithelial cell adherence (Virji *et al.* 1982; Rudel *et al.* 1992; Jonsson *et al.* 1994; Long *et al.* 1998). Antigenic variation of pilin can confer changes in pilus-dependent colony morphology (Hagblöm *et al.* 1985; Haas and Meyer 1986; Swanson *et al.* 1986; Long *et al.* 1998), which reflect a phase switch between a number of states of pilus expression (Swanson and Barrera 1983; Haas and Meyer 1986; Long *et al.* 1998), although colony morphology phase switches can also occur by means independent of antigenic variation (Meyer *et al.* 1984; Segal *et al.* 1985; Haas *et al.* 1987; Jonsson *et al.* 1991).

In Gc, DNA recombination is required for natural DNA transformation, DNA repair, and pilus antigenic variation, and all the processes are mediated by RecA (Koomey *et al.* 1987; Seifert 1996; Mehr and Seifert 1997). In *Escherichia coli*, RecA promotes DNA renaturation and strand exchange during homologous recombination, and mediates recombinational repair and SOS induction (Radding 1989; West *et al.* 1992; Cox 1993; Kowalczykowski *et al.* 1994). Gc and *E. coli* RecA share 65% identity and 81% similarity (Fyfe and Davies 1990) and can cross-complement for some recombination functions (Koomey and Falkow 1987; Fyfe and Davies 1990). Since *E. coli* RecA acts in concert with other proteins, the necessity for Gc RecA in pilin antigenic variation suggests that other factors involved in homologous recombination also have a role in this process.

Although Gc pilin antigenic variation is mediated by RecA-dependent homologous recombination, several attributes of this system suggest that both general and *pil*-specific recombination factors are involved. First, Gc pilin antigenic variation is usually unidirectional,

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whereas homologous recombination often results in the reciprocal exchange of DNA sequences. Second, pilin antigenic variation occurs at a high frequency of $>10^{-2}$ *pilE* variants per total *pilE* (Serkin and Seifert 1998). Third, 30–450 bp of *pilS* DNA sequence is transferred into the *pilE* gene during each recombination, which is postulated to be mediated by recombination events at limited regions of homology of 8–30 bp (Howell-Adams *et al.* 1996; B. S. Howell-Adams and H. S. Seifert, unpublished results). Lastly, the pilin-associated Sma/Cla repeat, which is required for efficient pilin antigenic variation (Wainwright *et al.* 1994), is the target for multiple site-specific binding proteins (Wainwright *et al.* 1994, 1997). Thus, it is likely that both homologous recombination activities and pilin-specific factors combine to accomplish pilin antigenic variation.

Previously, we screened random Gc transposon-generated mutants to identify genes essential for pilin antigenic variation (Mehr and Seifert 1997). We identified 22 unique transposon mutations that conferred an *a*ntigenic *v*ariation *d*eficient (Avd) phenotype through multiple backcrosses (Mehr and Seifert 1997). An Avd phenotype was defined as the inability to produce a pilin antigenic variation-specific 460-bp PCR product in a colony-based assay. All Avd mutants also showed a reduced frequency of colony morphology phase variation. Of the transposon mutations, 11 were located in two regions of the Gc chromosome-encoding homologues of the *E. coli* RecF-pathway genes *recQ* and *recO*, suggesting that a Gc RecF-like recombination pathway is involved in pilin antigenic variation (Mehr and Seifert 1998). Furthermore, mutation of genes in the Gc RecBCD pathway showed that they are not involved in pilin antigenic variation, but are involved in DNA transformation (Mehr and Seifert 1998). Mutations in either Gc recombination pathway altered the repair of DNA damage (Mehr and Seifert 1998). Some of these findings are in contrast to the results of Chaussee *et al.* (1999), who reported that mutations in the Gc *recD* gene did not alter DNA repair capabilities, showed a minor decrease in DNA transformation competence, and increased pilus-dependent colony variations. The basis for the descrepancy between these two reports is not presently known.

In this article, we show that the other 11 transposon insertions that produce an Avd phenotype are grouped in a region of the Gc chromosome that encodes three genes; *orf2*, *engA*, and *rdgC*. Some of the transposon-generated mutations produced a growth deficiency in addition to interfering with pilin antigenic variation, and selected transposon insertions resulted in abnormal cellular morphologies that were independent of the growth phenotype. *engA* was shown to be an essential gene that is transcriptionally linked to *rdgC*. Complementation analysis proves that interruption of RdgC expression is responsible for both pilin variation and growth-deficient phenotypes of the transposon mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *N. gonorrhoeae* human volunteer isolate FA1090 1-81-S2 (Seifert *et al.* 1994) was grown on Gc medium base (GCB; Difco, Detroit) plus Kellogg supplements (Kellogg *et al.* 1963) at 37° in 5% CO_2. Antibiotic resistance of Gc strain FA1090 was selected at 2 μg/ml erythromycin (Erm), 50 μg/ml spectinomycin (Spc), 2 μg/ml chloramphenicol (Cam), and 0.2 μg/ml tetracycline (Tet). *E. coli* strain DH10B (GIBCO BRL, Gaithersburg, MD) was grown on Luria-Bertani medium (Difco) at 37°. Antibiotic resistance of *E. coli* was selected at 40 μg/ml kanamycin (Kan), 250 μg/ml Erm, 25 μg/ml Cam, and 150 μg/ml ampicillin (Amp).

Molecular methods: Unless otherwise noted, all enzymes used in this study were supplied and used under the conditions recommended by New England Biolabs (NEB, Beverly, MA). Isolation of DNA from agarose gels was accomplished using GeneClean (Bio101, Vista, CA). DNA ends with 5′ or 3′ overhangs were blunted using T4 DNA polymerase. Blunted vector DNA ends were treated with shrimp alkaline phosphatase (SAP) (United States Biochemical, Cleveland) prior to ligation with blunted insert DNA. Ligations were performed and prepared for transformation as previously described (Mehr and Seifert 1997). *E. coli* were transformed by electroporation using the Gene Pulser II electroporation system (Bio-Rad, Richmond, CA) according to the manufacturer's specifications. Southern blots were performed as described (Sambrook *et al.* 1989). Filters were probed with [α-^{32}P]dCTP-labeled PCR products as described previously (Mehr and Seifert 1997). PCR was carried out essentially as described previously (Mehr and Seifert 1997), except for increasing extension times from 1.5 min/kb to 2 min/kb for *Pfu* thermostable polymerase (Stratagene, La Jolla, CA). RNA was prepared for Northern blotting by the TRIzol method (GIBCO BRL). Northern blots were performed as described (Sambrook *et al.* 1989). cDNA for RT-PCR analysis was synthesized according to the method described by Wainwright *et al.* (1994) using the primer RDGC1 and total RNA isolated from FA1090 variant 1-81-S2 at the late exponential stage of growth. PCR reactions were done in duplicate on 3 μl of the resultant cDNA with primer pairs GTP7/GTP8 or GTP5/RDGC2, to show linkage between *engA* and *rdgC*.

Cloning, sequencing and mutation of *orf2*, *engA*, and *rdgC*: The transposon mutations conferring the Avd phenotype were cloned from the Gc chromosome into pHSS6 (Seifert *et al.* 1986). The clones were named according to the Avd strain from which they were created. For example, clone pAvd-J7 contains the *Cla*I chromosomal fragment harboring the mTn*EGNS* insertion from Avd strain J7. DNA sequencing of these mutations was accomplished using an Applied Biosystems International (Foster City, CA) ABI373 sequencer, and Perkin-Elmer (Norwalk, CT) AmpliTaq DNA sequencing kit with primers listed in Table 1. Sequence similarities to known genes or proteins were found by searching GenBank at the National Center for Biotechnology Information. The Gapped-BLAST search function (Altschul *et al.* 1997) was used to determine percent similarity and identity to known proteins. Double-strand DNA sequences of *orf2*, *engA*, and *rdgC* were deposited in the DDBJ/EMBL/GenBank databases under accession no. AF058711.

To mutate *engA*, the 3-kb region of the chromosome containing *orf2*, *engA*, and the 5′ half of *rdgC* was PCR amplified using HFLU1 and RDGC1 (Table 1). The PCR product was cloned into pCR-Blunt (Invitrogen) to create pENGA. There are three *Cla*I sites within *engA*, at ~160, 650, and 690 bp downstream of the start codon. pENGA was digested completely with *Cla*I, and the large vector-containing fragment

TABLE 1

Oligonucleotides used in PCR and sequencing

Name	5′-Sequence-3′	Reference
ENGA-1	GCCTCAAGATTCTGTCGGTCGC	This work
ENGA-2	TCTCTTCCTTTCGGCCTTCGC	This work
ENGA-3	TGACACAGAAAGACCCAATCATG	This work
ENGA-4	CCTTAATTAAGCCTCAAGATTCTGTCGGTCGC	This work
ENGA-5	TGCATGCATTCTCTTCCTTTCGGCCTTCGC	This work
ENGA-6	CCTTAATTAATGACACAGAAAGACCCAATCATG	This work
GTP1	CTGCCGACTTTGCCGTGTCC	This work
GTP2	GTGGAACTTGGCAAAATCGAGG	This work
GTP3	CCCCGCGACCGCCATTAC	This work
GTP4	ATCGGCTTCTTCGGCTTCAG	This work
GTP5	CCTCGATTTTGCCAAGTTCCAC	This work
GTP7	GCGACGGCGTGTATTACCTG	This work
GTP8	TCAAACAAACCGTCTATACCGC	This work
HFLU1	CCGCCACCCATTTGACAGATAC	This work
HFLU2	CAGTTCGGCATTGATTTCGCTT	This work
HV-1	TCGTCGGCGCCGGTTTTGG	Seifert *et al.* (1994)
ORF1-1	CATTTGAAGCAATTCGCGACC	This work
PHSS6L-24	TTACTGAACGGTGATCCCCACCGG	This work
PHSS6R-24	ATGCTGGAGTTCTTCGCCCACCCC	This work
PILRBS	GGCTTTCCCCTTTCAATTAGGAG	Wright *et al.* (1994)
RDGC1	CGGCGGTTAAGTCTTGTTTGG	This work
RDGC2	CGCAAGGTAAAGTCGGCAGG	This work
RDGC3	TTCAGCAAATAATTCCCGAC	This work
RDGC4	AGGGCGAACATCATGGCG	This work
RDGC9	CCTTAATTAATTCAGCAAATAATTCCCGAC	This work
RDGC10	TGCATGCATAGGGCGAACATCATGGCG	This work
TN3L-24	TGATAATCTCATGACCAAAATCCC	This work
TN3R-GNS	GACTAGTGCTAGCGGATCCCC	This work

was isolated, blunted, and ligated to the blunted *Not*I fragment from pJD1145 (J. P. Dillard, unpublished results) that contains the nonpolar *ermC** gene. pENGA/Erm clones contained an ~530-bp deletion of *engA* replaced by *ermC** fragment. The *engA::ermC** mutation was transferred to Gc by transformation. Southern analysis showed that all Gc *engA::ermC** insertions were merodiploid maintaining both the mutant and wild-type (WT) copy of *engA* (data not shown).

To complement the mutations in *engA* and *rdgC*, the wild-type versions of the genes were amplified from the genome using *Pfu* polymerase without their promoters. The PCR products were cloned into pGCC6 (I. J. Mehr and H. S. Seifert, unpublished results), which contains a fragment of the gonococcal chromosome where a mini-transposon insertion is in an intragenic region of the chromosome with no detectable transcription (I. J. Mehr and H. S. Seifert, unpublished results). Within the mini-transposon is a multi-cloning site adjacent to the *lac* regulatory sites and the *lac* repressor. The construct allows controlled expression of genes *in trans* on the gonococcal chromosome. Oligonucleotides RDGC9 and RDGC10 were used to amplify the wild-type *rdgC* (Table 1). The PCR product was digested with *Pac*I and *Nsi*I, specific for the 5′ ends of RDGC9 and RDGC10, respectively, and ligated to *Pac*I, *Nsi*I double-digested pGCC6, creating clone pGCC6/*rdgC*. Similarly, oligonucleotides ENGA-6 and ENGA-5 were used to amplify and clone *engA* (Table 1). *engA* was also PCR amplified with its own promoter using oligonucleotides ENGA-4 and ENGA-5. This PCR product was cloned into pGCC5, which has the mini-transposon insertion without the *lac* regulatory cassette (I. J. Mehr and H. S. Seifert, unpublished results). Presence and orientation of the PCR products within the GCC vectors were determined by restriction enzyme digestion. Transposon mutants *engA3*::mTn*EGNS*, *rdgC1*::mTn*EGNS*, and *rdgC4*::mTn*EGNS* were each transformed with either the GCC6 allele alone, or the complementation alleles in clones pGCC6/*rdgC* and pGCC6/*engA*. Presence of the GCC constructs within the transformants was determined by PCR and Southern analysis (data not shown). Complemented mutants were grown in the presence of 1 mm IPTG to provide transcription from the *lac* promoters in the mTn*GCC6* construct. The GCC5/*engA* was not grown with IPTG, as *engA* was under the control of its own promoter.

Phenotypic analyses of mutations: Phenotypic analyses of the mutations were performed as described previously (Mehr and Seifert 1997). Briefly, the colony-based PCR assay for pilin antigenic variation was performed using an oligonucleotide specific for the *pilE* ribosomal binding site, PILRBS, and an oligonucleotide specific for two different *pilS* copies, HV-1 (Table 1). Individual Gc colonies expressing Erm^R were collected with sterile filter paper, placed in a lysis solution, and boiled. A portion of each colony lysate was used as template in the PCR reaction. Avd mutants did not produce a 460-bp PCR product. Growth on solid media was assessed by picking and suspending Gc colonies in GCBL media, plating serial dilutions, and determining the mean colony-forming units (CFUs) per colony. DNA transformation efficiency was determined in liquid medium by standard techniques (Mehr and Seifert 1998). A total of 1 μg/ml of chromosomal DNA encoding Spc^R was incubated with ~10^7 cfu/ml of Gc at 37°, 5% CO_2 for 15 min, followed by a 1:10 dilution and another 37°,

5% CO_2 incubation of 4–6 hr. Efficiency was expressed as the mean number of Spc^R transformants per colony-forming unit. UV resistance was assessed by exposing different amounts of Gc plated on solid media to 0, 2, 4, 6, or 8 J/m^2, and comparing the surviving colony-forming units at each dose to the unexposed colony-forming units.

Colony phase variation was assessed by suspending 5–10 Gc colonies exhibiting a highly piliated colony morphology in GCBL, and plating on solid media (Wainwright *et al.* 1994). Colony phase variation was expressed as the percent colonies exhibiting a nonpiliated colony morphology (Swanson *et al.* 1971).

Resistance to ionizing radiation was determined by exposing Gc suspended in GCBL to 0, 9, 18, or 27 krad of γ-rays in a Nordion Gammacell40 irradiator (Kanata, Ontario, Canada). The Gc suspension was diluted and plated on GCB. Relative resistance at each dose was calculated as percent survival compared to the number of colony-forming units in the unirradiated sample.

Transmission electron microscopy: Bacterial cell morphology was examined using transmission electron microscopy of thin sections and whole mounts of gonococci. For thin sections, Gc were grown overnight in GC liquid medium (1.5% proteose peptone no. 3 [Difco], 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl) with Kellogg supplements and 0.042% sodium bicarbonate (Morse and Bartenstein 1974) with or without 1 mm IPTG at 37° with rotation. After 16–18 hr of growth, cultures were diluted back into log phase, and grown in identical conditions for an additional 3–4 hr. Gc from 1.5 ml of each culture were pelleted, washed in ice-cold 100 mm $NaPO_4$ buffer (pH 5.5), and pelleted again. Gc were fixed with 4% glutaraldehyde in 50 mm $NaPO_4$ buffer for 3 hr at room temperature. Fixed samples were washed four times at room temperature with 50 mm $NaPO_4$ buffer, and were postfixed for 1 hr at room temperature with 1% osmium tetroxide in 100 mm $NaPO_4$ buffer. After washing the samples four times for 15 min each with 50 mm $NaPO_4$ buffer, samples were dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100%) and finally in propylene oxide. The samples were infiltrated with EM bed-812 resin (Electron Microscopy Sciences, Fort Washington, PA), embedded in Beem capsules, and sectioned (80 nm, silver) on a Reichert-Jung Ultracut E microtome. The sections were poststained with 4% uranyl acetate for 15 min and with Reynolds lead for 2 min.

For preparation of whole mounts, Gc were grown on GCB plates with or without 1 mm IPTG for 18–20 hr. Poly-l-lysine (1 μg ml^{-1})-treated, carbon-coated nickel grids (Ladd Research Industries, Inc., Williston, VT) were used to lift cells from colonies. Grids were then incubated in drops of the following solutions: 1% glutaraldehyde in 100 mm cacodylate buffer for 2 min, twice in sterile water for 3 sec, and 1% uranyl acetate for 30 sec, and then were air dried. All samples were examined with a Jeol (Peabody, MA) JEM-100 CX II transmission electron microscope at 60 or 80 kV.

RESULTS

Cloning and sequencing of targets of the Avd mutations: Gc chromosomal *Cla*I fragments containing each of the 22 transposon-generated mutations that conferred an Avd phenotype (Mehr and Seifert 1997) were cloned into *E. coli*. The DNA sequences flanking the transposon insertions were determined using oligonucleotide probes specific for the mini-transposon ends and were analyzed by comparison with sequences from GenBank and the Gonococcal Genome Sequencing Project database (GGSP; B. A. Roe, S. P. Lin, L. Song, X. Yuan, S. Clifton, and D. W. Dyer, University of Oklahoma Advance Center for Genome Technology [http://dna1.chem.ou.edu/gono.html]). Of the transposon insertions, 11 were shown to affect expression of the Gc homologues of *recQ* or *recO* (Mehr and Seifert 1998). The other 11 transposon insertions were located in a single region of the Gc chromosome containing three putative *orf*s. A total of 3 transposon insertions (from mutants E1, M1, and E2) was located in the noncoding region in between the first and middle *orf*s, 2 transposon insertions (from mutants J6 and B1) were located in the 3′ coding region of the middle *orf*, and 6 transposon insertions (from mutants J7, C1, L1, L2, L3, and M4) were located in the last *orf* (Figure 1).

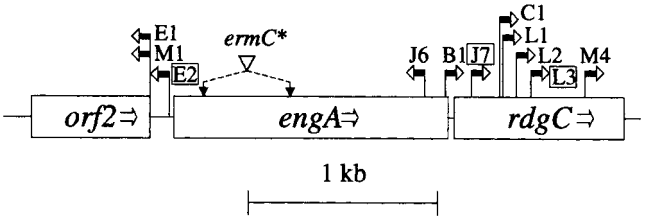

Figure 1.—Map of the *orf2*, *engA*, and *rdgC* chromosome region. Orf2 is similar to a hypothetical protein from *H. influenzae*. EngA is similar to bacterial GTPases. RdgC is similar to a protein in *E. coli* involved in recombination-dependent growth (Ryder *et al.* 1996). Open arrows within the genes indicate the predicted direction of transcription. Sites of mTn*EGNS* insertions are indicated by thin lines connecting the open-head arrows to the genes. The open head of the arrow indicates the direction of transcription of the *ermC* gene in the mini-transposon. The names of the Avd mutants are indicated above the arrows. The boxed insertions were chosen for further study. The small shaded triangle above *engA* indicates the region of *engA* deleted between the *Cla*I sites that was replaced by the transcriptionally nonpolar *ermC** fragment.

The most 5′ *orf*, *orf2*, encodes a predicted product that exhibits 16% identity, 45% similarity and 23% identity, 53% similarity to putative *trans*-membrane proteins from *E. coli* (GenBank accession no. AE000337–gi1788860) and *Haemophilus influenzae* (SwissProt accession no. P43989), respectively (data not shown). The second *orf*, *engA* (see below), encodes a predicted protein that exhibits consensus GTP binding domains (Figure 2; Bourne *et al.* 1991; March 1992; Pillutla *et al.* 1995). Gc EngA is highly similar to hypothetical proteins found in every sequenced and most of the partially sequenced prokaryotic genomes (data not shown). Comparisons to the *E. coli* (GenBank accession no. AE000337-gi1788858) and *H. influenzae* (SwissProt accession no. P44536) homologues show 48% identity, 72% similarity and 43% identity, 66% similarity, respectively, to Gc EngA (Figure 2). The gene immediately downstream of *engA* encodes a predicted protein exhibiting 35% identity, 64% similarity to *E. coli* RdgC and 16% identity, 41% similarity to an *H. influenzae* ORF that is also likely to be an RdgC homologue (Figures 1

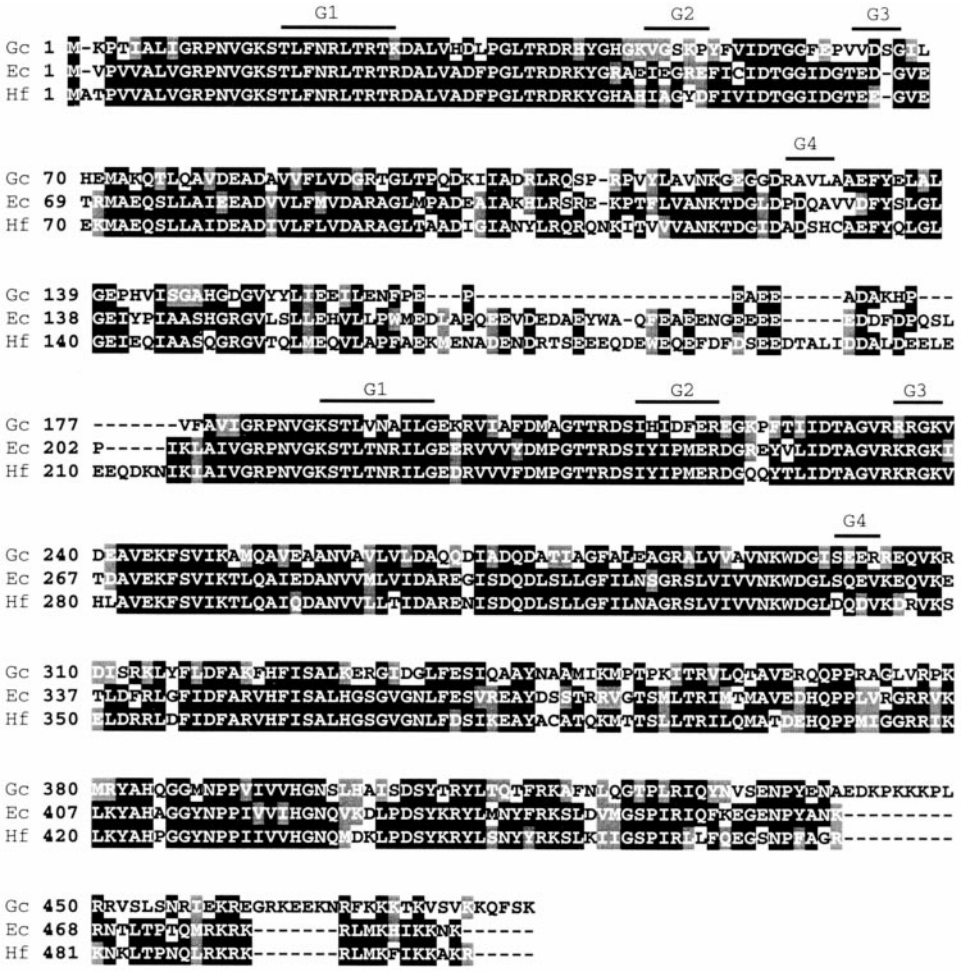

Figure 2.—Sequence alignment of *N. gonorrhoeae* EngA with *E. coli* and *H. influenzae* homologues. Gc, *N. gonorrhoeae*; Ec, *E. coli*; and Hf, *H. influenzae*. Black shading indicates conserved residue, gray shading represents similar residues, and no shading shows no conservation. Consensus G1, G2, G3, and G4 GTPase domains are indicated with an overline (Bourne *et al.*, 1991). The G2 domain is the GTPase domain with the least sequence conservation.

and 3; Ryder *et al.* 1996). The *E. coli rdgC* gene was identified as a gene that conferred a growth defect when mutated in a recombination-deficient genetic background (Ryder *et al.* 1996). While RdgC was proposed to be involved in the reinitiation of chromosomal replication after a collapse of a replication fork (Ryder *et al.* 1996), the exact role of this gene product in replication, recombination, or growth is unknown.

Many, but not all, of the transposon mutations affected both growth and antigenic variation (Mehr and Seifert 1997). To determine whether these phenotypes were due to the disrupted expression of *engA* or *rdgC*,

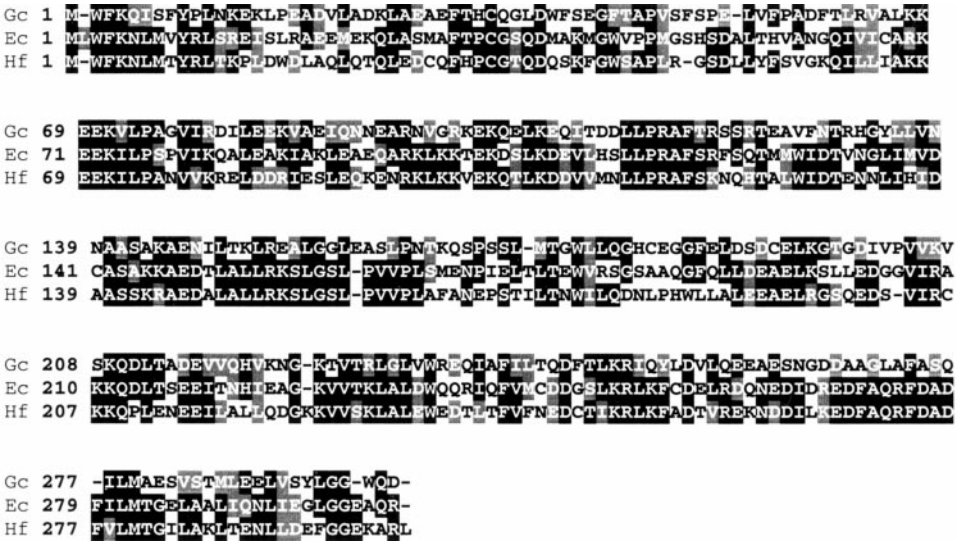

Figure 3.—Sequence alignment of *N. gonorrhoeae*, *E. coli*, and *H. influenzae* RdgC homologues. Gc, Ec, and Hf and shading are the same as in Figure 2.

three transposon mutations where chosen for further study. E2 (*engA3*::mTn*EGNS*, the mutation in mutant Avd-E2) was located in the 5′-noncoding region of *engA* (Figure 1), and exhibited both an Avd phenotype and a growth defect (Mehr and Seifert 1997). J7 (*rdgC1*::mTn*EGNS*, the mutation in Avd-J7) was located in the 5′ portion of *rdgC* (Figure 1), and exhibited an Avd phenotype, but wild-type colony growth (Mehr and Seifert 1997). L3 (*rdgC4*::mTn*EGNS*, the mutation in Avd-L3) was located in the 3′ portion of *rdgC* (Figure 1), and exhibited both an Avd phenotype and a growth defect (Mehr and Seifert 1997).

Expression of *rdgC in trans* complements Avd mutations for pilin phase and antigenic variation: Only a few plasmid complementation systems have been developed for *Neisseria*, and for single-copy stable complementation, chromosomal insertions are preferred. Previously, we had identified a random mini-transposon insertion mutant (Cg8; Mehr and Seifert 1997) that did not alter any recombination-associated phenotypes, and was not in an expressed region of the chromosome. This mini-transposon insertion has been used to create a Gc chromosomal complementation system (NICS; I. J. Mehr and H. S. Seifert, unpublished results). The Gc *engA* or *rdgC* gene was introduced into the unexpressed site (carrying the Erm^R, mini-transposon insertion of mutant Cg8) in the chromosome of Gc strain FA1090, variant 1-81-S2 (Seifert *et al.* 1994) under the control of the *lac* regulatory system (Seifert 1997). Transformants were confirmed by Southern blot analysis, and were named GCC6/*engA* and GCC6/*rdgC*. The E2 (*engA3*), J7 (*rdgC1*), and L3 (*rdgC4*) transposon mutations were then moved by DNA transformation from a *recA6* background to the wild-type *recA* background of the *engA* and *rdgC* complementation strains. Six phenotypes were tested: pilin antigenic variation as measured by the colony-based PCR assay, pilus-based colony morphology phase variation, growth, DNA transformation efficiency, UV resistance, and γ-ray resistance. None of the three transposon mutants carrying a wild-type *recA* gene exhibited a significant decrease in their resistance to DNA damage by UV light or γ-ray radiation, and all three transposon mutants were wild type for DNA transformation (data not shown).

Interestingly, none of the *engA3* (E2), *rdgC1* (J7), or *rdgC4* (L3) transposon mutations in the wild-type *recA* background carrying an empty complementation locus (GCC6) produced the consistent Avd phenotype found by the colony-based PCR assay of the parental *recA6* strains. Approximately one-fifth of all PCR reactions done on colony lysates of these mutants with a wild-type *recA* gene exhibited the 460-bp recombination-dependent products. This is in contrast to these same mutations in an induced *recA6* background ($RecA^+$), where none of the PCR reactions generated product (data not shown; Mehr and Seifert 1997). This suggested that the L3, E2, and J7 Avd mutations in a wild-type *recA* background were disabled only partially for pilin antigenic variation. Analysis of pilus-dependent colony morphology phase variation supported this conclusion since the E2 (*engA3*), J7 (*rdgC1*), and L3 (*rdgC4*) in the wild-type *recA* background decreased phase variation 2- to 5-fold (Figure 4), while these same mutations decreased colony phase variation >20-fold in the induced *recA6* background (data not shown; Mehr and Seifert 1997). Since a fully induced *recA6* gene does not produce wild-type levels of RecA (Seifert 1997), the differences in phase and antigenic variation phenotypes are likely to be due to different RecA levels. However, a role for the *recA* promoter (which is not used in the *recA6* gene) cannot be ruled out. Expression of *rdgC*, but not *engA*, *in trans* complemented the phase variation deficiencies observed for all three transposon insertion mutations, showing that it was the interruption of RdgC activity that was responsible for the Avd phenotypes of all three mutants (Figure 4). The fact that expression of RdgC from the *lac* promoter resulted in a level of colony phase variation higher than the wild-type strain in mutant L3 (L3-6/*rdgC*, Figure 4) suggests that a truncated form of RdgC produced in mutant L3 can complement the wild-type proteins for increased frequencies of antigenic variation.

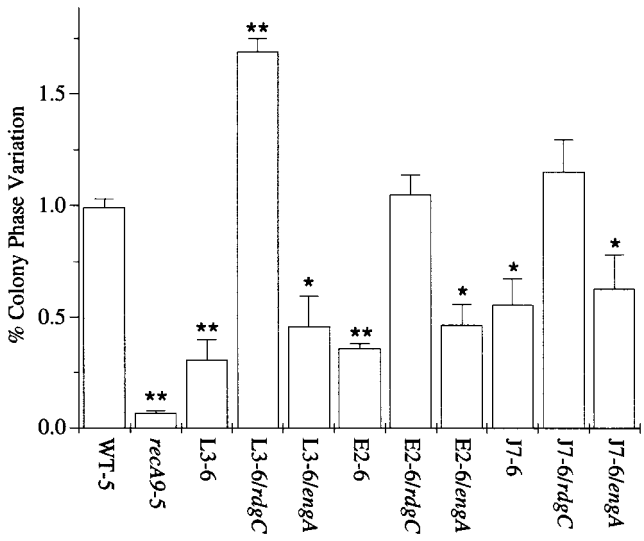

Figure 4.—Colony morphology phase variation of complemented *rdgC* and *engA* mutants. All strains are derived from FA1090 1-81-S2 (Seifert *et al.* 1994). L3 contains the *rdgC4*::mTn*EGNS* mutation. E2 contains the *engA3*::mTn*EGNS* mutation. J7 contains the *rdgC1*::mTn*EGNS* mutation. Complemented strains and controls are indicated by a −5 for the GCC5 construct, which is the same as GCC6 without the *lac* sequences, a −6 for the GCC6 construct, a −6/*rdgC* for the GCC6/*rdgC* construct, and a −6/*engA* for the GCC6/*engA*. All strains are $recA^+$, except for *recA9*, which has *recA* disrupted by the *ermC* gene. The mean and standard error of six experiments are shown. $*P < 0.05$, $**P < 0.001$ relative to WT-5 by the Student's *t*-test.

Expression of *rdgC in trans* complements Avd muta-

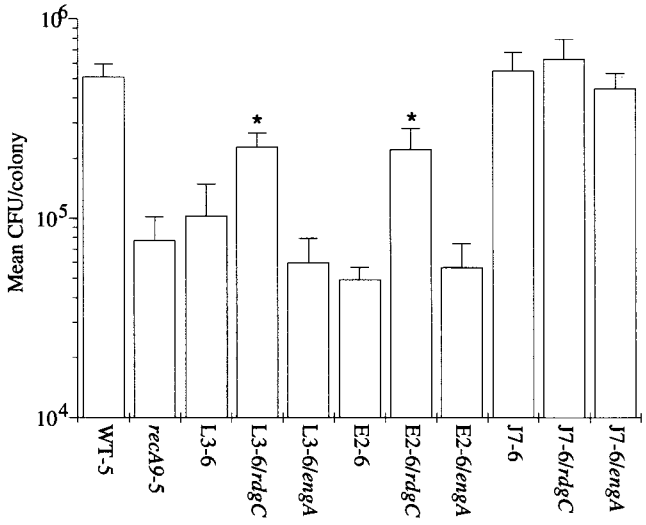

Figure 5.—Growth of complemented *rdgC* and *engA* mutants. Strains are the same as previously shown. The mean and standard error of six experiments are shown. $*P < 0.05$ compared to WT-5, *recA9*-5, and uncomplemented mutants by the Student's *t*-test.

tions for growth: The E2 (*engA3*) and L3 (*rdgC4*) mutations in the GCC6 background produced growth defects (Figure 5) similar to those observed in the *recA6* background (Mehr and Seifert 1997). As expected, the J7 (*rdgC1*) transposon mutation in the GCC6 background did not confer a growth defect (Figure 5), consistent with its phenotype in the *recA6* background (Mehr and Seifert 1997). The *engA3* and *rdgC4* growth defects were partially complemented by *trans* expression of *rdgC*, but not *engA* (Figure 5).

***engA* and *rdgC* are in an operon:** The isolation of several Avd mutations in the 5′-noncoding region of *engA* (Figure 1) and the lack of promoter sequences directly upstream of the *rdgC* coding region (data not shown) suggested that these two genes were arranged in an operon. RT-PCR analysis confirmed the genetic evidence that *engA* and *rdgC* are transcriptionally linked (data not shown).

***rdgC* and *engA* mutations confer altered cellular morphology:** Because the E2 (*engA3*) and L3 (*rdgC4*) mutants exhibited a growth defect, we investigated whether these and the J7 (*rdgC1*) mutant showed changes in cellular morphology. Transmission electron microscopy of both thin sections and whole mounts of the mutant strains and of wild-type FA1090 demonstrated that all three mutant strains exhibited abnormal cellular morphologies (Figure 6). While FA1090 showed the normal diplococcal shape with one septum-like structure in the center of diplococci, the three mutants had a more rounded cell shape, and often contained more than one septum-like structure per cell (Figure 6, bottom). The thin sections confirmed the presence of multiple septal-like structures in some of the mutant cells (as indicated by arrowheads in Figure 6, top). Furthermore, the septum-like structures of some of the mutant cells were off center (as indicated by arrows in Figure 6, top). Finally, the mutant strains expressed an electron-dense layer surrounding each cell, whereas FA1090 only exhibited this electron-dense material between neighboring cells (Figure 6, bottom). These alterations in cellular morphology in mutants that are impaired in pilin antigenic variation suggest a link between these processes, but show that the growth phenotypes are not explained solely by abnormal cell division.

***engA* is an essential gene:** The *E. coli* Era GTPase is essential, shown by a conditionally lethal mutation (March *et al.* 1988). The *engA* gene product shows sequence similarity to the consensus GTPase domains of Era (Figure 2), although EngA is not a close homologue to Era. Since no transposon mutations were found in the 5′ portion of the coding region of *engA* (Figure 1) despite the isolation of several independent insertions upstream and downstream of the gene, it was possible that *engA* is an essential gene in Gc. To test this hypothesis, *engA* was cloned and disrupted with the *ermC** gene (Figure 1). When this mutation was transformed into Gc, Erm^R transformants were acquired infrequently, at $\sim 1 \times 10^{-7}$ Erm^R transformants/cfu, and all Erm^R transformants were merodiploid for *engA* in that each contained both the wild-type *engA* gene and an *ermC** disrupted *engA* (data not shown). This result is consistent with the *engA* gene product being essential.

engA was also cloned into the GCC5 expression vector. GCC5 is in the same locus as GCC6 but does not carry *lac* regulatory sequences. This construct allowed expression of *engA* under the control of its own promoter (I. J. Mehr and H. S. Seifert, unpublished results; see materials and methods). The GCC5/*engA* construct was introduced into the chromosome of wild-type Gc (Figure 4). Gc GCC5/*engA* positive clones were then transformed with mutated versions of *engA* that was disrupted by *ermC** (Figure 1). Transformants that contained an *engA* disruption at the wild-type chromosomal locus were acquired only in the presence of the GCC5/*engA* construct (data not shown), confirming that disruption of *engA* is lethal and confirming that a promoter sequence is present upstream of *engA*.

DISCUSSION

Cloning and sequence analysis identified that 11 of 22 transposon mutations conferring an Avd phenotype (Mehr and Seifert 1997) were located in a region of the gonococcal chromosome containing three putative *orf*s: *orf2*, *engA*, and *rdgC* (Figure 1). Database searches suggested that Orf2 might be a *trans*-membrane protein of unknown function, that EngA was likely to be a GTPase, and that RdgC was likely to be involved in growth linked to recombination. Analysis of three mutants, E2 (*engA3*::mTn*EGNS*, located in the 5′-noncoding region of *engA*), J7 (*rdgC1*::mTn*EGNS*, located in

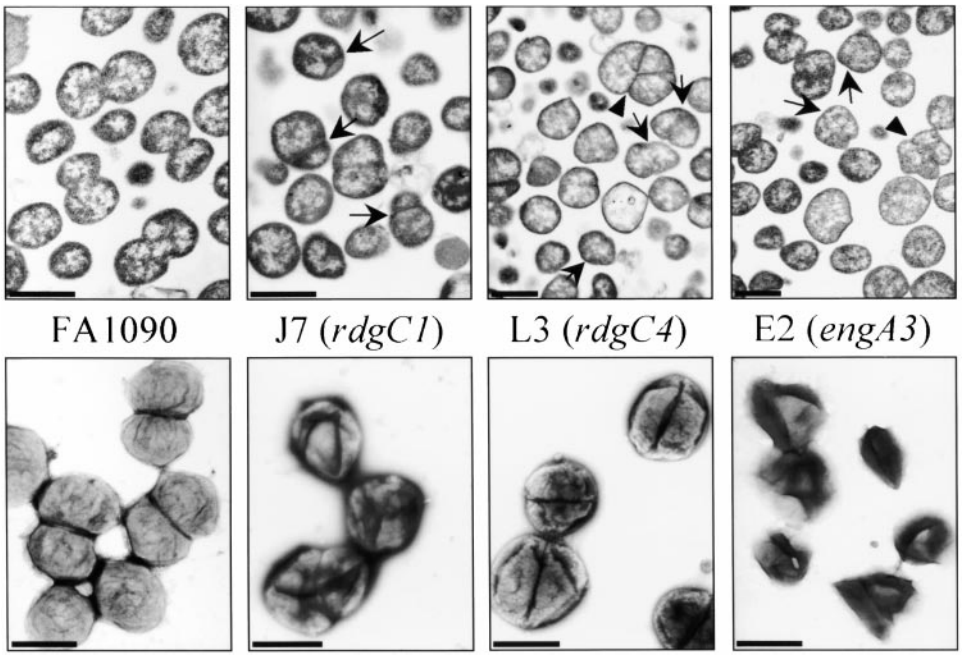

Figure 6.—Transmission electron microscopy of *rdgC* and *engA* mutants. FA1090 denotes wild-type 1-81-S2, and mutant strains are the same as previously shown. The top shows uranyl-acetate- and Reynolds-lead-stained thin sections of gonococci. Arrows indicate aberrantly placed septa. Arrowheads indicate cells with multiple septa. The bottom shows uranyl-acetate-stained whole mounts of gonococci. The bar in each panel represents 1 μm.

the 5′ portion of *rdgC*), and L3 (*rdgC4*::mTn*EGNS*, located in the 3′ portion of *rdgC*), revealed that both the growth and pilin antigenic variation phenotypes conferred by these mutations were complemented by the expression of *rdgC in trans*. Transmission electron microscopy demonstrated that an abnormal cellular morphology was also associated with each of these three transposon mutations, but that the abnormal cellular morphology did not correlate with the different growth phenotypes.

EngA is somewhat similar to the Era/ThdF family of essential GTPases, although closer homologues are found in most sequenced bacterial genomes. All of these EngA homologues are hypothetical proteins with no known function, but the conserved GTPase domains and ubiquitous presence in bacteria suggest a primary conserved function. Era has been studied extensively in *E. coli*, and is thought to be a membrane-localized (March 1992; Lin *et al.* 1994), RNA binding protein (Chen *et al.* 1999; Meier *et al.* 1999) involved in cell division (March *et al.* 1988). *E. coli* ThdF is involved in thiophene and furan oxidation (Alam and Clark 1991). Both proteins bind and hydrolyze GTP by similar motifs, although their roles in bacterial physiology are apparently quite divergent. There is significant sequence divergence between EngA, Era, and ThdF, except for the four highly conserved GTP binding domains, of which EngA has two sets (Figure 2). We show that EngA is essential to Gc growth, and assume that EngA is a GTPase, based on sequence similarity to *E. coli* Era and ThdF (Bourne *et al.* 1991; March 1992; Pillutla *et al.* 1995). It will be interesting to determine whether the close homologues in other bacterial species are also essential, and whether they might be a target for novel antimicrobials.

It is puzzling that growth defects were caused by transposon insertions in both the 5′-noncoding region of *engA* and the 3′-coding region of *rdgC*, but not the 5′-coding region of *rdgC*. Furthermore, growth defects caused by transposon insertions in either region can be complemented by *rdgC* expression *in trans*, suggesting that disruption of *rdgC* expression alone was responsible for the growth defects. One hypothesis for the variable growth phenotypes is that intermediate expression of *rdgC* is detrimental to the cell, but full or no expression of *rdgC* has no growth phenotype. This hypothesis assumes that the insertions in the 5′-noncoding region of *engA* reduce transcription of the operon where enough EngA is produced for cell survival, but not enough RdgC is produced to avoid a growth defect. The 3′-coding region insertions in *rdgC* would be predicted to produce a truncated version of RdgC, which could affect the growth phenotype by binding and inactivating a putative interacting protein. In support of this hypothesis is the observation that the growth phenotype became more severe as the mini-transposon insertions left more of the RdgC sequence intact (Mehr and Seifert 1997). Therefore, mutations in the 5′-coding region of RdgC would effectively block expression of the interfering domains of RdgC, providing a null phenotype and allowing for normal growth on solid media.

The behaviors of some *rdgC* mutations in Gc are in contrast to *rdgC* mutations in *E. coli* (Ryder *et al.* 1996). In *E. coli*, *rdgC* mutations only showed a growth phenotype when combined with other mutations in recombination genes, *recBC*, *sbcBC*, and *recA* (Ryder *et al.* 1996). Our analysis of the repair phenotypes of gonococcal *recB recC* and *recD* mutants suggested that gonococci are naturally *sbcB* deficient and resemble *E. coli recBC sbcBC* mutants (Mehr and Seifert 1998). It is clear from the

present study that in the gonococcus RdgC contributes to pilin antigenic variation and is necessary for wild-type growth and normal cellular morphology. The phenotypes in the gonococcus are reminiscent of the *E. coli* phenotypes, but show some differences. First, only some of the gonococcal *rdgC* mutations show a growth phenotype, and the expression of these is independent of other mutations. Second, since gonococcal *rdgC* mutations are deficient in pilin antigenic variation, the RdgC protein plays a role in this specialized recombination system. Finally, no recombination phenotype was observed for *E. coli rdgC* mutants (Ryder *et al.* 1996), although *E. coli* does not carry a complex recombination system analogous to the pilin system.

Two other gonococcal genes have been isolated that link DNA recombination and growth or cell division: *comL* (Fussenegger *et al.* 1996a) and *tpc* (Fussenegger *et al.* 1996b). Both genes were identified by screening for reduced transformation competence. A *comL* mutant shows smaller bacterial cell size (Fussenegger *et al.* 1996a), while *tpc* mutants exhibit abnormal septation by growing in clusters of four instead of two (Fussenegger *et al.* 1996b). The occurrence of three genes that link cell division to DNA recombination may indicate a close relationship between recombination machinery and the cell envelope in this gram-negative, diplococcal organism. The presence of *engA* and *rdgC* in an operon, the possible role for RdgC in cell division, and the essential nature of EngA raise the possibility that these proteins cooperate during cell division. The inability to mutate *engA* will make determining whether a relationship exists between these proteins more difficult.

The data presented here demonstrate that in the gonococcus RdgC plays a role in pilin antigenic variation, but that *rdgC* mutants are only partially disabled in a wild-type *recA* background. The role that RdgC plays in pilin antigenic variation is less obvious than the role of RecA, RecO, or RecQ, which are likely involved in DNA strand exchange and homologous pairing (Mehr and Seifert 1998). All of our molecular models for the gene conversion events leading to pilin antigenic variation require two gonococcal chromosomes (Howell-Adams *et al.* 1996; B. S. Howell-Adams and H. S. Seifert, unpublished results), and are therefore compatible with a function that links pilin variation to replication. Clearly, further biochemical characterization of RdgC is required to elucidate RdgC's role in pilin antigenic variation, and possibly chromosome replication. It is interesting to note that RdgC homologues are found only in 2 of the 12 completely sequenced bacterial genomes, *E. coli* and *H. influenza*, and only a small subset of partially sequenced organisms besides Neisseria species, while *engA* homologues are found in most of the fully and partially sequenced organisms. The identification of a role for RdgC in the recombinations leading to pilin variation provides the first direct role for this protein in a recombination process.

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