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Inversion of *Moraxella lacunata* Type 4 Pilin Gene Sequences by a *Neisseria gonorrhoeae* Site-Specific Recombinase

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A plasmid library of *Neisseria gonorrhoeae* sequences was screened for the ability to mediate recombinations on a sequence containing the *Moraxella lacunata* type 4 pilin gene invertible region in *Escherichia coli*. A plasmid containing the *N. gonorrhoeae* sequence encoding the putative recombinase (*gcr*) was identified and sequenced. Plasmids containing *gcr* were able to mediate site-specific recombinations despite a weak amino acid homology to Piv, the native *M. lacunata* pilin gene invertase. The *gcr* gene is present only in pathogenic strains of *Neisseria* tested; however, in our assays gene knockouts of *gcr* did not alter the variation of surface features that play a role in the pathogenesis of *N. gonorrhoeae*.

The Piv recombinase is the only *Moraxella* gene product required to mediate inversion of the cloned *Moraxella lacunata* and *Moraxella bovis* type 4 pilin gene regions in *Escherichia coli* (25, 28). The stop codons for two partial 3′ pilin domains (*tfpQ* and *tfpI*) lie within a 2.1-kb invertible region, while the short, conserved 5′ domain and pilin promoter lie just outside this segment (27, 28). Site-specific recombinations between the boundaries of the invertible region result in transcriptional fusions of the *tfpQ* or *tfpI* domain to the conserved 5′ pilin domain and promoter (27, 31). These inversions are clearly demonstrated by use of restriction sites located asymmetrically in the invertible region and by the different mobilities of pilin subunits produced in *E. coli* by immunoblot analysis (31). Further highlighting the essential role for Piv in site-specific recombination, insertions into or deletions in the *M. lacunata piv* gene eliminate inversion of the pilin gene segment (28). Inversions of these phase-locked constructs can be restored by a plasmid carrying the *piv* gene (28).

The type 4 pili produced by *M. lacunata* and *M. bovis* are also produced by a variety of gram-negative mammalian pathogens, including *Neisseria gonorrhoeae* (19, 42). Pili are generally thought to play an important role in the establishment of infection by mediating the attachment to host epithelial cells and are also associated with competence for DNA transformation (4, 40), twitching motility (18), and autoagglutination (44). The gonococcal genome is well known for its ability to alter the pilin structural gene by recombination of silent pilin sequences (*pilS*) into the expression locus (*pilE*) (6, 16, 17, 26, 45). Deletions in the *recA* allele decrease antigenic variation of the pilin gene 100- to 1,000-fold (23), yet homologies between recombining sequences are as low as 30 bp (29), leaving open the possibility that a site-specific recombinase or other locus-specific factor facilitates the recombination process. Interestingly, there is no evidence of site-specific recombinases, phage, transposons, or insertion sequences characterized in the gonococcal genome despite this well-documented genomic plasticity. We hypothesized that if a site-specific recombinase exists in gonococci, it may be able to mediate inversions on the cloned *M. lacunata* type 4 pilin gene region in *E. coli*. We constructed a suitable tester plasmid to screen a plasmid library of *N. gonorrhoeae* genomic DNA segments and report our findings below.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* JM109 *recA* (50) cells were grown aerobically at 37°C in Luria broth (LB) or on Luria agar (LA) plates (containing 1.5% [wt/vol] agar) supplemented with the appropriate antibiotic. Antibiotics for selection of plasmids in *E. coli* were used at the following concentrations for both plates and liquid media: ampicillin (AP), 100 μg/ml; kanamycin (KM), 30 μg/ml; chloramphenicol (CM), 25 μg/ml. *Neisseria* strains used in this study are from the laboratory collection of Thomas Meyer and include the pathogenic strains *N. gonorrhoeae* MS11-A and VP1, *N. meningitidis* B1940 and C1701, and the commensal *Neisseria* strains *N. lactamica*, *N. mucosa*, *N. flavescens*, *N. subflava*, *N. sicca*, *N. elongata*, and *N. flava*. *Neisseria* were grown on GC agar base with vitamin supplements (Becton Dickinson) at 37°C in 5% CO_2. *Neisseria* epithelial cell invasion assays, DNA transformations, and tests for pilin variation were performed as previously described (10, 22). For selection of *Neisseria* transformants, antibiotics were used at the following concentrations: rifampicin, 50 μg/ml; erythromycin, 7 μg/ml; CM, 10 μg/ml; KM, 200 μg/ml. Plasmids used in this study are described in Table 1.

DNA procedures. Standard procedures were used for plasmid DNA isolation, restriction enzyme digests, gel electrophoresis, isolation of DNA fragments, ligations, and transformations into *E. coli*, as described by Sambrook et al. (35). Genomic *Neisseria* DNA was isolated by using a modified protocol for *N. meningitidis* (34).

Plasmid inversion assay. Plasmid pMIT1 was constructed to serve as a substrate for putative Piv-like recombinases contained on a gonococcal plasmid library. Plasmid pMIT1 has a deletion in the *piv* gene and contains a promoterless chloramphenicol acetyltransferase (Cat) cassette within the invertible pilin gene region from *M. lacunata* (31) (see Table 1 for details on cloning). Inversion to the pMIT2 configuration (Fig. 1) results in a transcriptional fusion of Cat to the pilin promoter, conferring resistance to CM. The orientation can also be determined by restriction sites located asymmetrically within the invertible region (Fig. 2). Competent JM109(pMIT1) was transformed with a plasmid library containing *N. gonorrhoeae* MS11 sequences (22, 41) and plated at low cell density onto LA-AP-KM plates to maintain both pMIT1 and the *Neisseria* library plasmids. Transformants resistant to AP-KM were then replica plated onto LA-KM-CM plates in order to score for inversions and onto LA-AP plates in order to later rescue the *piv*-complementing plasmid. Plasmid DNA from Km-Cm-resistant colonies was digested with *Eco*RI-*Sal*I or *Sca*I to determine the orientation of Cat on pMIT1. An inversion to the pMIT2 configuration was identified by both restriction digest profile (Fig. 2) and resistance to CM. The plasmid containing the *N. gonorrhoeae* sequence encoding the putative recombinase was rescued from the LA-AP plate and purified by transformation into fresh competent cells. The isolated plasmid, pGCR9, could reproducibly invert pMIT1 to pMIT2. Large-scale preps were made of plasmid pGCR9 for restriction mapping and DNA sequence analysis.

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference
pMxL1	*M. lacunata* pilin gene invertible region; piv^+ Ap^r	28
pMxL5	*piv* knockout of pMxL1dR13; Km^r Ap^r	28
pMxL20	*piv*-containing plasmid lacking invertible pilin gene region; Ap^r	This work
pMIR1	Religation of 6.5-kb *Xba*I-*Hin*dIII (filled) fragment from pMxL5; Ap^r	This work
pMIR2	1.7-kb *Spe*I (filled) fragment from pMIR1 replaced with 700-bp *Bam*HI (filled) promoterless Cat cassette from pCM4; Ap^r	This work; 5
pMIR3	2.1-kb *Nsi*I-*Kpn*I insert region from pMIR2 cloned into the *Pst*I-*Kpn*I sites of vector pOK12; Km^r	This work; 47
pMIT1	2.1-kb *Nsi*I-*Xba*I insert region from pMIR3 cloned into the *Pst*I-*Xba*I sites of vector pWSK130; *piv* knockout; invertible region contains promoterless Cat cassette; Km^r Cm^s; pSC101 origin of replication	This work; 49
pMIT2	pMIT1 containing the Cat cassette in the inverted configuration; Km^r Cm^r	This work
pGCR9	3-kb genomic region from *N. gonorrhoeae* cloned into a pBR322 derivative contains *gcr*; Ap^r	This work
pGCR91	pBluescriptII (KS+)::2.94-kb *Pst*I pGCR9[a]; Ap^r	This work
pGCR92	pBluescriptII (KS+)::2.94-kb *Pst*I pGCR9[b]; Ap^r	This work
pGCR93	1.65-kb *gcr* PCR product from *N. gonorrhoeae* MS11 (3orf2 and 5orf2 primers) cloned into *Eco*RI and *Sal*I sites of pUC19; Ap^r	This work; 50
pGCR94	pBluescriptII (KS+)::1.6-kb *Hin*dIII pGCR93[a]; Ap^r	This work
pGCR95	pBluescriptII (KS+)::1.6-kb *Hin*dIII pGCR93[b]; Ap^r	This work
pGCR96	1.57-kb *gcr* PCR product from *N. gonorrhoeae* MS11 (3orf2 and 5rbf2 primers) cloned into *Eco*RI site of pBluescriptII (KS+)[a]; Ap^r	This work
pGCR97	pBluescriptII (KS+)::1.57-kb *Eco*RI pGCR96[b]; Ap^r	This work
pGCR100	pK19::3.0-kb *Eco*RI-*Bam*HI pGCR9; Km^r	This work; 30
pGCR101	pBluescriptII (KS+)::3.0-kb *Eco*RI-*Bam*HI pGCR100[a]; Ap^r	This work
pGCR102	pBluescriptII (KS+)::3.0-kb *Eco*RI-*Sal*I pGCR100[b]; Ap^r	This work
pGCR110	Antiparallel insertion of *Bam*HI-flanked kan_{GC} (pMF52) cassette replacing a 750-bp *Bcl*I fragment on *gcr* in pGCR102; Km^r Ap^r	This work; 13
pDFT7	pDFG4 containing a transposon insertion (TnMax1) in *comA*	7
pGP1-2	Source for heat-inducible T7 RNA polymerase	43
pHTR72	Contains stable in-frame *pilC* on conjugative plasmid p*tet*M	32

[a] *gcr* under the control of the *lacZ* promoter.
[b] *gcr* under the control of the T7 promoter.

Exonuclease III digestions. To facilitate DNA sequencing and protein expression studies, a 3-kb *Pst*I fragment containing all but 60 bp of the *Neisseria* insert of pGCR9 was cloned in both orientations into the *Pst*I site of pBluescriptII (KS+) (Stratagene) to yield pGCR91 and pGCR92 (Table 1). Exonuclease III deletions of plasmid pGCR91 were constructed by using a nested double-stranded deletion kit (Pharmacia) according to the manufacturer's instructions.

DNA sequencing. Gonococcal sequences contained on pGCR9 were sequenced from exonuclease III derivatives of pGCR91 and subcloned fragments of pGCR9 on pBluescriptII (KS+) or were sequenced directly from pGCR9 by using synthetic oligonucleotide primers. Sequencing was performed by the dideoxy chain termination method (36) with double-stranded plasmid DNA templates, α-^{35}S-dATP as the radiolabel, and a Sequenase version 2.0 kit (United States Biochemicals) according to the manufacturer's protocol. Regions with high GC content were resolved on 40% formamide–6% polyacrylamide gels (46a). The BLAST (1) program was used to search the EMBL and GenBank nucleotide sequence databases (release 31) for homology to identified candidate reading frames. The nucleotide sequences across the Piv- and Gcr-mediated recombination sites of pMIT2 were determined by using oligonucleotide primers (Microsynth AG) 5′-GGAGTTCATTATGAACG-3′ (mirR) and 5′-CACCATCAGCTATGCC-3′ (mirL) based on the nucleotide sequence of pMxL1 (28); these primers are positioned outside both junctions of the invertible region according to Fig. 1 and 4.

PCR amplification. For amplification of *gcr* from *N. gonorrhoeae* MS11 chromosomal DNA, primers 5′-CTGAATTCAAGCTTGAACAATTTTTATACGG-3′ (3orf2) and 5′-CGGGATCCGTCGACCGATTCTTCCTTCGTCGC-3′ (5orf2) were designed with restriction sites (underlined) *Eco*RI and *Hin*dIII in 3orf2 and *Bam*HI and *Sal*I in 5orf2 for subsequent subcloning; these primers are based on the DNA sequence from the insert region of pGCR9. The possibility of altering the expression of *gcr* in *E. coli* was examined by incorporating the ribosome binding site (RBS) (double underlined) from the *N. gonorrhoeae opa* gene (10, 41) and an *Eco*RI site (underlined) into the 5′ primer 5rbf2: 5′-GGAATTCGAATAAGGAGCCGAAAATGTTCGACCAATGTTTCAG-3′. Amplification of *gcr* was carried out in an A Protocol thermocycler (AMS Biotechnology). Thermostable *Pwo* DNA polymerase (Boehringer Mannheim) was used under the manufacturer's buffer conditions. The first denaturation step was carried out for 5 min at 95°C, after which the polymerase was added. The sample was subjected to 30 cycles of PCR (94°C for 60 s, 50°C for 120 s, and 72°C for 120 s), with a final extension step of 72°C for 5 min. The *gcr* PCR product obtained by using primers 3orf2 and 5orf2 was then digested with *Eco*RI and *Sal*I and run on a 1% low-melting-point agarose gel, and a 1,650-bp band was excised for cloning into the *Eco*RI and *Sal*I sites on pUC19 (50) to yield pGCR93 (Table 1). This allowed a 1,600-bp *Hin*dIII fragment to be subcloned in both orientations with respect to the T7 promoter into the pBluescriptII (KS+) *Hin*dIII site to yield pGCR94 and pGCR95 for use in the T7 expression assay. Similarly, the 1,570-bp *gcr* PCR product from primers 3orf2 and 5rbf2 was digested with *Eco*RI and cloned directly into the pBluescriptII (KS+) *Eco*RI site, giving pGCR96 and pGCR97.

T7 promoter expression. *E. coli* K38 containing plasmid pGP1-2 carrying a thermoinducible T7 RNA polymerase (43, 46) was transformed with pGCR91, pGCR92, pGCR94, and pGCR95 (without heat shock). Cells were grown in M9 medium with AP-KM at 30°C to mid-log phase. Five hundred microliters was pelleted and washed three times with M9 medium (35). Cells were resuspended in 1 ml of M9 medium and grown for 1 h at 30°C, followed by a heat pulse of 42°C for 15 min. A 1.2-μl aliquot of rifampicin (50 μg/ml) was added, and the cells were incubated for an additional 10 min at 42°C. Cells were then cooled to 30°C for 20 min, after which 1 μl of [^{35}S]methionine was added for 15 min at 30°C. Cells were pelleted and taken up in 40 μl of Laemmli buffer (24). Ten microliters was run on a 12.5% polyacrylamide gel, which was then dried and autoradiographed.

Southern hybridizations. Southern blots were performed with *Cla*I-digested genomic DNA from several *Neisseria* species as well as *M. lacunata* ATCC 17956 and *M. bovis* Epp63 (kindly provided by Carl Marrs). Restriction fragments were separated by electrophoresis on 0.8% (wt/vol) agarose gels in 1× TAE buffer (35), denatured, and transferred by capillary blotting to BioDyne A membranes (Pall). Membranes were vacuum baked at 80°C for 2 h, and DNA hybridizations were performed at 68°C under high-stringency conditions (39). Membranes were probed with an internal 750-bp *Bcl*I fragment of *gcr* isolated from pGCR9. Probe DNA was prepared using an [α-^{32}P]dATP random-primed labeling kit (Bio-Rad) according to the manufacturer's instructions.

Nucleotide sequence accession number. The sequence for *gcr* has been deposited in the GenBank database and assigned the accession number U82253.

RESULTS

Cloning of a putative *N. gonorrhoeae* site-specific recombinase. An assay was devised to detect a *Neisseria* site-specific recombinase by its ability to mediate inversions at the Piv recombination sites on plasmid pMIT1. A positive-control plasmid containing *piv* (pMxL20) was first used to determine if Piv could invert a modified inversion region carrying the Cat

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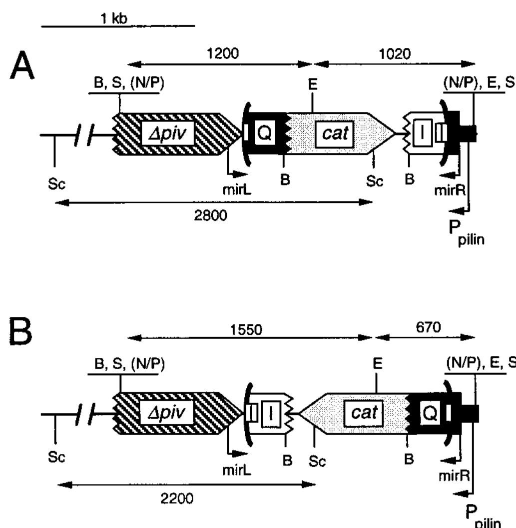

FIG. 1. Insert region of the *Moraxella* inversion tester plasmid in the Cm^s pMIT1 (A) and Cm^r pMIT2 (B) orientations. See Table 1 for cloning details. Recombination boundaries of the invertible region are shown by heavy curved lines. Small white boxes represent the 19-bp repeat sequences (see Fig. 4). A promoterless Cat cassette between the two 3′ truncated pilin genes, *tfpI* (I) and *tfpQ* (Q), is expressed from the pilin promoter (P_{pilin}) only in the pMIT2 configuration. Deletion of the 5′ region in the *piv* gene abolishes recombination of the Cat on pMIT1 (see Results). The direction and location for pilin expression and sequencing primers (mirL and mirR) are shown by bent arrows outside the invertible region. Neither plasmid contains *N. gonorrhoeae*-derived sequence. Interrupted vector segments containing the *Sca*I site are shown on the left. Relevant restriction sites are shown along with fragment sizes used to test for the orientation of the invertible region. E, *Eco*RI; B, *Bam*HI; N/P, *Nsi*I-*Pst*I junction; S, *Sal*I; Sc, *Sca*I.

cassette on pMIT1. JM109 cells containing both pMIT1 and pMxL20 were first grown on LA-AP-KM plates and then replica plated to LA-KM-CM plates. Most (>95%) colonies tested grew within 24 h of incubation, and after 36 h, all colonies expressed resistance to CM. Plasmid DNA prepared from Km-Cm-resistant colonies produced *Eco*RI-*Sal*I fragments of 6.8, 1.55, and 0.67 kb, compared to the original pMIT1 plasmid, which produced *Eco*RI-*Sal*I fragments of 6.8, 1.2, and 1.02 kb (Fig. 1 and 2). Likewise, digestion of pMIT1 with *Sca*I produces fragments of 6.2 and 2.8 kb, compared with 6.8- and 2.2-kb fragments from digestion of the plasmid in the inverted pMIT2 configuration (Fig. 1 and 2). The sizes of these bands and the expression of Cm resistance are clear markers for inversion on this plasmid substrate. It is unlikely that some undefined recombination between the tester plasmid and the host chromosome resulted in the pMIT2 configuration, since we were able to cure JM109(pMIT2) using an incompatible plasmid replicon, pWSK29 (49). To ensure that pMIT1 is homogenous and that the Cat gene does not invert from uncharacterized *E. coli* recombinases, pMIT1 was purified by transformation and grown in LB-KM. High-density inoculations of JM109(pMIT1) into LB-KM-CM or onto LA-KM-CM plates failed to result in growth, indicating that *E. coli*-mediated inversions of pMIT1 do not occur.

To identify the *N. gonorrhoeae* recombinase, *E. coli* JM109 (pMIT1) was transformed with the gonococcal plasmid library and plated onto LA-AP-KM plates at low density. Approximately 300 Ap-Km transformants were patched onto LA-KM-CM plates, yielding 13 Km-Cm-resistant colonies. Twelve colonies that initially expressed resistance to KM-CM failed to grow on a second passage either on LA-KM-CM plates or in LB-KM-CM. The small amount of plasmid DNA prepared from the original Km-Cm-resistant colonies showed no indications of inversions to pMIT2 and were considered false positives. One colony maintained resistance to KM-CM after multiple passages, presumably by a gonococcal sequence promoting a recombination so that the Cat gene is expressed in the pMIT2 configuration. The *Eco*RI-*Sal*I and *Sca*I restriction digest profiles of pMIT2 arising from the native *piv* recombinase and the putative gonococcal recombinase were indistinguishable (Fig. 2). The plasmid expressing the putative gonococcal recombinase (pGCR9) was isolated by transformation. The ability of pGCR9 to mediate recombinations on pMIT1 was confirmed by transforming pGCR9 into JM109(pMIT1). Three hundred Km-Ap-resistant colonies were replica plated to LA-CM plates in two independent experiments, yielding 15 and 32 Km-Cm-resistant colonies (5 to 10%). Plasmid DNA prepared from LB-KM-CM cultures of these colonies all contained inversions to the pMIT2 configuration, as determined by restriction digest analysis (data not shown).

Nucleotide sequence of the *Neisseria* insert region. The *N. gonorrhoeae* insert on pGCR9 contains two large, nonoverlapping open reading frames with a convergent transcriptional direction; they are separated by an apparent stem-loop structure which may represent the transcriptional terminators for both genes, as proposed for the *N. gonorrhoeae comA* and *comL* genes (9). This stem-loop structure contains a perfect match to the DNA uptake sequence used to confer genus-specific recognition in the transformation process (15) (Fig. 3). *orf1* failed to mediate DNA recombinations by our inversion assay (data not shown) and failed to produce signals in the T7 promoter expression assay (see below). Subclones containing *orf1* under the control of the *lacZ* promoter grew poorly and produced a low plasmid yield, suggesting that high expression of the *orf1* gene product is toxic to the *E. coli* host. *orf2* or *gcr* (gonococcal recombinase) proved to be solely responsible for mediating inversions on pMIT1 in plasmid inversion assays using subclones (see below). The *gcr* reading frame is 1,316 bp in

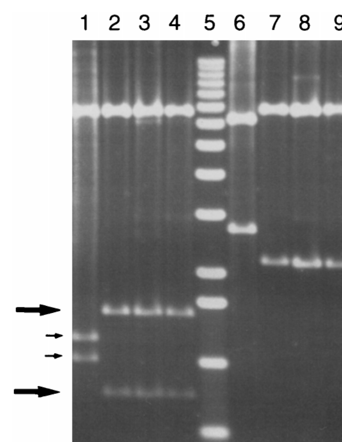

FIG. 2. Piv- and Gcr-mediated inversions of pMIT1 to the pMIT2 configuration. *E. coli* JM109 containing pMIT1 was transformed with plasmids containing either *piv* or *gcr* and plated onto LA-AP-KM plates; colonies were then tested for inversion to pMIT2 by growth on LA-KM-CM plates. Plasmid DNA was isolated from these Km-Cm-resistant colonies and retransformed by using KM-CM to select for plasmid pMIT2 alone. Plasmid DNA was isolated and digested with *Eco*RI-*Sal*I (lanes 1 to 4) or *Sca*I (lanes 6 to 9) and electrophoresed on 0.9% Tris-acetate-EDTA agarose gels. Small arrows indicate characteristic *Eco*RI-*Sal*I fragments of 1,200 and 1,020 bp from pMIT1, while large arrows indicate 1,550- and 670-bp fragments from pMIT2. Lanes: 1, pMIT1 control; 2 through 4, pMIT1-to-pMIT2 transition mediated by pMxL20, pGCR9, and pGCR96, respectively; 5, 1-kb ladder; 6, pMIT1; 7 through 9, pMIT1-to-pMIT2 transition mediated by pMxL20, pGCR9, and pGCR96, respectively.

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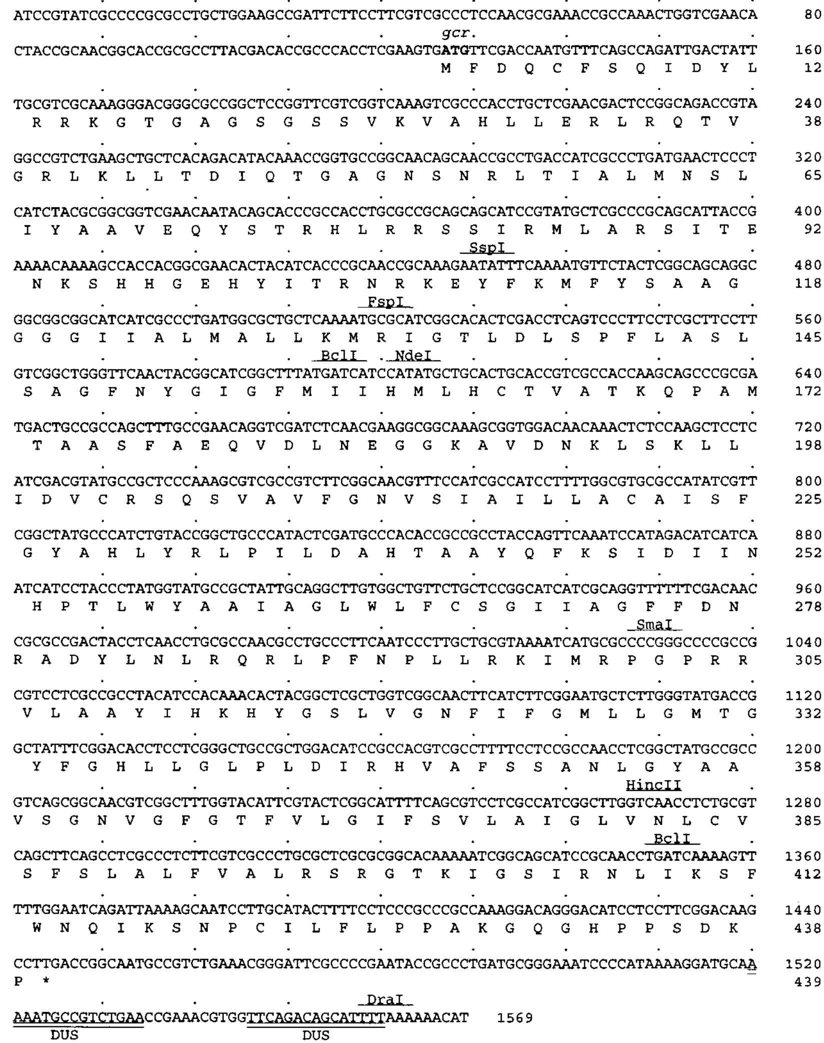

FIG. 3. Nucleotide sequence for *gcr* and deduced amino acid sequence. Relevant restriction sites are overlined. The ATG start codon for *gcr* is in boldface. Inverted repeats forming a putative transcriptional terminator and/or DNA uptake sequence (DUS) are double underlined.

length and codes for a predicted protein of 439 amino acids with a predicted molecular mass of 48 kDa (Fig. 3). A BESTFIT (Genetics Computer Group) comparison between the amino acid sequences of Gcr and Piv revealed only 18% identity and 34% similarity, which is lower than expected for two functionally interchangeable proteins. As expected, a BLAST search failed to identify significant amino acid homology to Piv or any other protein in the database. We were unable to find homology to *Neisseria* or *E. coli* RBSs and promoters for *gcr.*

pGCR9 subclones mediate inversions on pMIT1. *E. coli* JM109 (pMIT1) was transformed with the pGCR9 insert cloned in either orientation in pBluescriptII (KS+) (pGCR91 and pGCR92) and with pBluescriptII (KS+) carrying *gcr* cloned from amplified genomic *N. gonorrhoeae* MS11 DNA (pGCR94, pGCR95, pGCR96, and pGCR97) (Table 1). These transformants were then used in the plasmid inversion assay described above. One hundred Ap-Km-resistant transformants for each plasmid were replica plated to LA-KM-CM, yielding inversion frequencies up to 10%, similar to that of the pGCR9 control. Only subclones that contained *gcr* (Fig. 2) were able to invert the region containing the Cat gene. Gcr-mediated inversions of this region were confirmed to be identical to Piv-mediated inversions by *Eco*RI-*Sal*I and *Sca*I restriction digests (Fig. 2) and by sequencing across the recombination junction sites of the resulting pMIT2 (see below). The ability of *gcr* to mediate inversions of pMIT1 irrespective of its orientation to the *lacZ* promoter of pBluescriptII (KS+) indicates that the original gonococcal promoter and RBS are recognized by the *E. coli* translation apparatus despite the lack of homology to the *E. coli* consensus RBS. Subclones containing 5′ deletions in *gcr* failed to produce Cm-resistant colonies (data not shown).

The Gcr and Piv recombination sites are identical. The putative Gcr recombinase mediates recombination of the *M. lacunata* type 4 pilin inversion region on pMIT1. The junction sites of various Gcr-mediated recombinations of the tester plasmid were sequenced with the mirL and mirR primers. In each case the nucleotide sequences across both recombination sites were found to be identical to the sequences for *M. lacunata* Piv-mediated recombinations (Fig. 4). Inversions were confirmed by using sequencing primers mirL and mirR to determine the presence or absence, respectively, of a 19-bp repeat within the *M. lacunata* pilin genes. Nucleotide differences between the *tfpI* and *tfpQ* pilin genes located farther 3′ confirmed these results (31) (Fig. 1 and 4).

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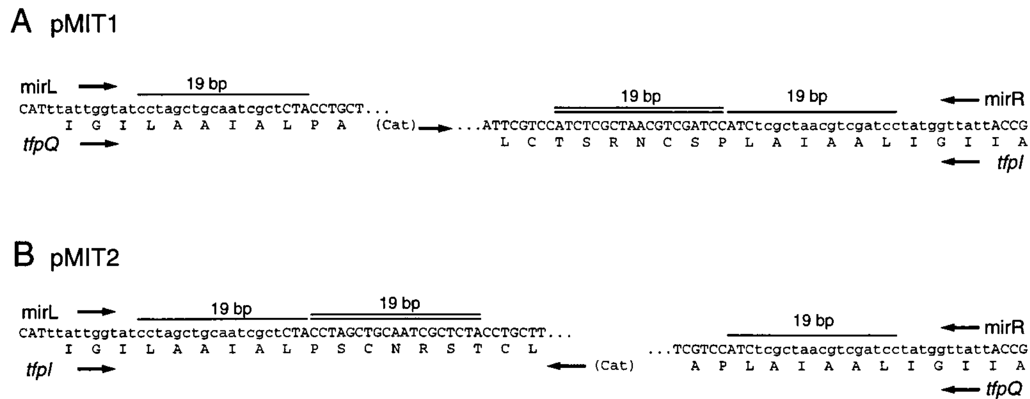

FIG. 4. Recombination junction sites of pMIT1 (A) and pMIT2 (B) before and after Piv- or Gcr-mediated inversions. The orientation is the same as that shown in Fig. 1. The 26-bp Piv or Gcr recombination sites are shown in lowercase letters; the 19-bp sequence is marked with a single overline in *tfpQ* and *tfpI* and with a double overline where it is duplicated in *tfpI*. The transcriptional directions of Cat, *tfpQ*, and *tfpI* are shown by arrows. The directions of the oligonucleotide primers mirL and mirR, used for sequencing across the junction sites, are shown above the sequences; however, the actual primer sequences begin 65 and 61 bp outside each recombination site for mirL and mirR, respectively.

T7 promoter expression of the open reading frames contained on pGCR9. In order to monitor the proteins encoded by the insert of the selected library clone pGCR9, we cloned the corresponding *Pst*I fragment in both orientations into pBluescriptII (KS+), thus placing either *orf1* (pGCR91) or *gcr* (pGCR92) under the control of the phage T7 RNA polymerase promoter. Although the sequencing data indicate two convergent open reading frames, we could express protein only from the T7 promoter of pGCR92. Expression of *orf1* under the control of the *lacZ* promoter was shown in DNA inversion assays to decrease cell viability. Since pGCR92 carries *orf1* under the control of the *lacZ* promoter, the resulting decrease in cell viability required longer exposure to autoradiography relative to pGCR95 (Fig. 5). The expressed protein in *E. coli* was approximately 46 kDa, which is slightly less than the predicted size of 48 kDa for Gcr (Fig. 5). To alleviate stability problems associated with *orf1* expression and to provide further evidence for *gcr* encoding the 48-kDa protein, clones containing only the amplified *gcr* from genomic *N. gonorrhoeae* were tested in this system. Plasmid pGCR95, which has *gcr* under T7 promoter control, produced the same 46-kDa protein as pGCR91 with a 25% shorter exposure time on autoradiography than pGCR92. Similar results were obtained by using pGCR97, which contains the RBS of the *N. gonorrhoeae* opacity gene (41) with the correct spacing engineered in front of *gcr* (data not shown).

Distribution of *gcr* among *Neisseria* species. Under high-stringency conditions only pathogenic *Neisseria* species appear to contain sequences that hybridize to *gcr*. The most closely related commensal strain, *N. lactamica*, also hybridized to *gcr*. These samples hybridized to a single 3-kb *Cla*I band, indicating that *gcr* is highly conserved among pathogenic *Neisseria* species (Fig. 6). *Cla*I-digested genomic DNA from *M. lacunata* ATCC 17956 and *M. bovis* Epp63, both of which carry the *piv* gene, failed to hybridize to *gcr* (data not shown). Likewise, *Neisseria* genomic DNA digested with *Cla*I failed to hybridize to a *piv* probe (data not shown).

Gonococcal *gcr* mutants show no alteration in pathogenicity, transformation competence, or pilin variation. Gonococcal *gcr* mutants were constructed in order to investigate the role of *gcr* in the pathogenesis and pilin variation of *N. gonorrhoeae*. To disrupt *gcr*, the *Bam*HI-flanked kan_{GC} cassette from pMF52 (10) was used to replace a 750-bp *Bcl*I fragment of pGCR102 to yield pGCR110 (Table 1). Wild-type *N. gonorrhoeae* MS11 was transformed with pGCR110 to allow for allelic exchange between the chromosomal *gcr* and the kan_{GC}-containing *gcr*. Knockouts for *gcr* were selected by resistance to high concentrations (200 μg/ml) of KM. The presence of the kan_{GC} insert into *gcr* was confirmed by DNA hybridization (data not shown). The *N. gonorrhoeae gcr* mutant showed a wild-type colony morphology, grew normally, and was affected neither in transformation competence nor in the ability to invade human epithelial cells. The frequency of pilin variation, measured via piliated to nonpiliated transitions and vice versa, did not change significantly compared to that of corresponding isogenic control strains. Since alterations in pilin variation were not detected in a *gcr* mutant, we eliminated two other sources of pilin variation. The gonococcal *gcr* mutants was transformed with pDFT7 (7), rendering the cells transformation deficient and unable to take up silent pilin loci from neighboring cells due to the knockout in the transformation competence gene *comA* (7, 33). The variable expression of PilC, implicated in pilin variation (21), was stabilized by conjugating the *pilC*-containing plasmid pHTR72 (32) into the *gcr comA* double mutant. However, inhibition of these pathways did not reveal any differences in pilin variation solely due to *gcr*.

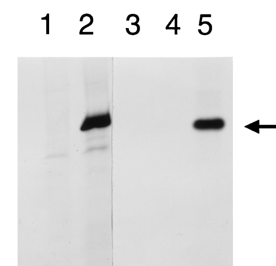

FIG. 5. T7 promoter expression of Gcr. See Materials and Methods for details. The arrow points to the 46-kDa band. The vector for all lanes is pBluescriptII (KS+). Lanes: 1, pGCR91 (*gcr* under the control of the *lacZ* promoter; *orf1* under the control of the T7 promoter); 2, pGCR92 (*gcr* under the control of the T7 promoter; *orf1* under the control of the *lacZ* promoter); 3, pBluescriptII control; 4, pGCR94 (amplified *gcr* from *N. gonorrhoeae* MS11 under the control of the *lacZ* promoter); 5, pGCR95 (amplified *gcr* from *N. gonorrhoeae* MS11 under the control of the T7 promoter). Gels were exposed for 8 days (lanes 1 and 2) or for 2 days (lanes 3 to 5).

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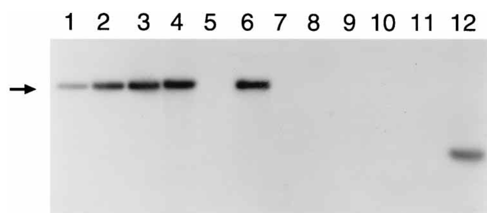

FIG. 6. Conservation of *gcr* across different *Neisseria* species. *Cla*I-digested genomic DNA from various *Neisseria* species hybridized to a [^{32}P]dATP-labeled *gcr* probe. The arrow corresponds to 3 kb. Lanes: 1, *N. gonorrhoeae* VP1; 2, *N. gonorrhoeae* MS11; 3, *N. meningitidis* (C1701); 4, *N. meningitidis* (B1940); 5, *N. mucosa*; 6, *N. lactamica*; 7, *N. flavescens*; 8, *N. subflava*; 9, *N. flava*; 10, *N. sicca*; 11, *N. elongata*; 12, 1-kb marker.

DISCUSSION

Although the requirement for RecA in the antigenic and phase variation of gonococcal pilin is firmly established, the possibility that a site-specific recombinase might also play a role has been suggested previously (23, 37, 38, 48). The fact that Piv is involved in type 4 pilin variation of *M. lacunata* and *M. bovis* led to our hypothesis that a Piv-like recombinase may exist in *N. gonorrhoeae*, although it would not necessarily be involved in pilin variation. The conservation and location of the 62-bp *Sma-Cla* repeat at the untranslated 3′ regions of all *N. gonorrhoeae* and *N. meningitidis pilS* and *pilE* loci (16) have implicated this repeat as a potential binding site for a DNA site-specific recombinase (48). The *Sma-Cla* repeat also contains a perfect match (5′-GTTTGCAAGGCGGGC-3′) to the consensus Fis binding site (20). Two other Fis sites are located just outside this region where the homology between *pilS* and *pilE* extends an additional 100 bp (11). Fis binds and induces bends in DNA, which enhances the frequency of site-specific recombination (8). However, it remains unclear whether a Fis homolog is present in *N. gonorrhoeae* or if this similarity is fortuitous. We tested our hypothesis by using a tester plasmid containing the *Moraxella* invertible region to isolate a putative gonococcal recombinase by direct complementation in *E. coli.*

Our findings show that *N. gonorrhoeae* and perhaps other pathogenic *Neisseria* species encode a protein that is functionally able to replace the *M. lacunata piv* gene in DNA inversion assays. The low frequency of Gcr-mediated compared with Piv-mediated inversions on the pMIT1 substrate may be explained by rather poor amino acid homology between the two proteins. This is made even more apparent at the nucleotide level by the lack of hybridization between *piv* and several *Neisseria* DNAs and between *gcr* and *Moraxella* DNA (data not shown). Another explanation for the low frequency of Gcr-mediated recombination on pMIT1 is steric blockage of the recombinase. Although the two *M. lacunata* Piv recombination sites (contained on pMIT1) are identical, the *tfpI* pilin gene contains a 19-bp tandem repeat that overlaps the recombination site by 16 bp (Fig. 1 and 4). The presence of this tandem repeat not only shifts the coding sequence for *tfpI* out of frame but probably hinders Piv binding at the normal recombination site by creating a partial secondary recombination site overlapping the primary site (31). The duplication of this sequence may reflect the transposase-like properties of Piv (25) or an evolutionary outcome of maintaining "variety generators" on genome structures (2, 3). Previous attempts to clone the *M. bovis piv* gene and invertible region intact from genomic libraries and from existing subclones have failed (31). One possibility is that *M. bovis* lacks the tandem repeat or secondary Piv recombination site, resulting in destabilizing inversions that render such constructs nonviable on high-copy-number plasmids (31). Gcr may better mediate recombinations on an *M. bovis* rather than an *M. lacunata* inversion substrate. Finally, Gcr may have greater efficiency at mediating recombination between sites in direct repeat orientation instead of the inverted repeat configuration present on pMIT1. Clearly, more work is required to elucidate the optimum recombinogenic substrate for Gcr.

Our preliminary experiments indicate no major effect of Gcr on the frequency of pilus phase variation in gonococci. The *gcr* knockouts were mutated to abolish any transformation-mediated changes and were transformed with a constitutive *pilC* gene to prevent PilC-dependent phase variation, therefore increasing the sensitivity of the assay. The assay was not designed to detect sequence changes within the *pilE* gene that would not affect the piliation status. Therefore, we cannot exclude an effect of GCR on pilin variation. One could speculate that Gcr is a locus-specific factor that promotes RecA-mediated recombination of pilin sequences. The conservation and location of the Sma-Cla repeats make them an attractive candidate as a substrate for recombinase binding. Binding of gonococcal Fis-like protein could facilitate recombination by Gcr and RecA by inducing bends in the DNA, similar to the Hin family of DNA invertases (8, 14).

An intriguing hypothesis is the possible involvement of Gcr in the formation of large genome inversions, as recently identified in strain MS11 (11). In this study, mapping experiments indicated that the recombination site for the inversion was located in the intergenic region between the *pilE1* and *opaE* loci. This region also harbors the Sma-Cla repeat and the predicted recombination of Gcr. Unfortunately, the observed genome inversion seems to be a rare event and therefore complicates the assessment of the possible role of Gcr.

Alternatively, *gcr* may be a cryptic gene or an evolutionary remnant from gonococcal strains that could mediate recombinations only in a simple binary fashion that was later supplanted by high-frequency, RecA-dependent recombinations, which generate much greater antigenic diversity in pilin. The role, if any, of Gcr in *N. gonorrhoeae* pilin gene recombination requires further analysis.

We did consider that inversions of pMIT1 might be due to some mechanism besides Gcr. However, due to the facts that the inversion assays were performed in a RecA-deficient strain and the sequences across the inversion junction sites of both Piv- and Gcr-mediated recombinations were identical, we consider other mechanisms unlikely. We are currently investigating whether Gcr mediates deletions using recombination sites in the direct repeat configuration and whether Gcr binds to the *Sma-Cla* repeat in order to further substantiate the role of Gcr in gonococcal pilin antigenic variation.

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