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Neisserial Correia Repeat-Enclosed Elements Do Not Influence the Transcription of *pil* Genes in *Neisseria gonorrhoeae* and *Neisseria meningitidis*▽†

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Two human-specific neisserial pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, require the expression of type IV pili (tfp) for initial attachment to the host during infection. However, the mechanisms controlling the assembly and functionality of tfp are poorly understood. It is known that the gonococcal *pilE* gene, encoding the major subunit, is positively regulated by IHF, a multifunctional DNA binding protein. A neisserial specific repetitive DNA sequence, termed the Correia repeat-enclosed element (CREE) is situated upstream of three *pil* loci: *pilHIJKX* (*pilH-X*), *pilGD*, and *pilF*. CREEs have been shown to contain strong promoters, and some CREE variants contain a functional IHF binding site. CREEs might therefore be involved in the regulation of tfp biogenesis in pathogenic *Neisseria*. Site-directed and deletion mutagenesis on promoter::*cat* reporter constructs demonstrated that transcription of *pilH-X* and *pilGD* is from a σ^{70} promoter and is independent of the CREE. The insertion of a CREE in the *pilF* promoter region in *N. meningitidis* generated a functional σ^{70} promoter. However, there is also a functional promoter at this position in *N. gonorrhoeae*, where there is no CREE. These results suggest CREE insertion in these three *pil* loci does not influence transcription and that IHF does not coordinately regulate tfp biogenesis.

Both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are obligate human pathogens. *N. gonorrhoeae* colonizes nonciliated columnar epithelial cells of the urogenital tract and causes the sexually transmitted disease gonorrhoea (55). *N. meningitidis* colonizes the human upper respiratory tract and exists in a carriage state in 10 to 20% of the human population (10, 11). *N. meningitidis* is also a major bacterial agent of cerebrospinal meningitis and septicemia. Both *N. gonorrhoeae* and *N. meningitidis* initiate infection via type IV pili (tfp) (56), an inner membrane-anchored organelle that participates in a diverse range of cellular functions, such as formation of bacterial aggregates (28, 30, 58), DNA uptake during natural transformation (1, 53, 54), and twitching motility (41, 62, 63). Nonpiliated *N. gonorrhoeae* is known to be avirulent (34).

The main component of a tfp filament is pilin protein encoded by the *pilE* gene (43). Although the overall structure of the tfp filament is relatively simple, the tfp biogenesis apparatus consists of as many as 23 ancillary Pil proteins involved in the assembly and functionality of the organelle (5, 8, 33). The tfp biogenesis process, in brief, involves the maturation of prepilin through the cleavage of the signal peptide by a prepilin peptidase encoded by *pilD* (22, 57). The assembled pilus filament is translocated through a membrane secretin, PilQ (20), which itself is stabilized by PilW (9). The mechanical energy required for pilus elongation and retraction is generated by two ATPases, known as PilF (22) and PilT (4). The products of *pilG*, *pilH*, *pilI*, *pilJ*, *pilK*, and *pilW* are known to be important for tfp homeostasis by counteracting PilT-mediated pilus retraction (8, 60). Furthermore, PilX (28, 29) and PilC1 and PilC2 (35, 44, 49) are important for the fine tuning of tfp adhesiveness and functionality. Many of these Pil proteins show homology to components of type II protein secretion systems (36).

Despite extensive research into the roles of these Pil proteins, little is known about the transcriptional control of the genes encoding the proteins, except for the transcription of the gonococcal *pilE* gene. The *pilE* gene is transcribed from a σ^{70} promoter during growth *in vitro* (24). A DNA binding protein, known as the integration host factor (IHF) (23), binds to the *pilE* promoter region and enhances *pilE* transcription (31). Deletion of this IHF binding site (BS) resulted in a 10-fold reduction of *pilE* transcription (31). IHF, a small heterodimeric protein encoded by *ihfA* and *ihfB* binds to the target DNA in a sequence-specific manner and causes the DNA molecule to bend (21, 23). Fyfe and Davies demonstrated that the AT-rich tract positioned between the −35 box of the *pilE* promoter and the IHF BS is required for maximal *pilE* transcription (25). It appears that the binding of IHF to the *pilE* promoter bends the DNA and allows the DNA to wrap around RNA polymerase bound to the promoter, and a second AT-rich region upstream of the IHF BS seems to facilitate this event (C. S. Ryan and J. K. Davies, unpublished data).

The genomes of pathogenic *Neisseria* isolates contain large amounts of repetitive DNA (2, 15, 46, 59). Some of these repetitive sequences have been shown to affect gene expression (39, 51). One of the common repetitive sequences is the Correia repeat-enclosed element (CREE), which occupies 1 to 2% of the genome (17, 37). The CREE consists of two terminal inverted repeats, termed the Correia repeats (CR), which are 25 to 27 bp long and of which there are two types, α and β. The CR flank a central core sequence (6, 14, 37, 40). The two types of CR can be further categorized depending on whether they

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TABLE 1. NG MS11-A derived reporter strains used in this study

Reporter strain	Description
JKD5222	Contains an empty vector[a]
JKD5243	Contains P*pilG*::*cat* cassette with CREE and IHF BS
JKD5244	Contains P*pilH*::*cat* cassette with CREE and IHF BS
JKD5245	Contains P*pilF*::*cat* cassette without CREE
JKD5250	Contains P*pilH*::*cat* cassette with CREE and IHF BS; sequence upstream of DAP1751 deleted (Fig. 1b)
JKD5253	Contains P*pilG*::*cat* cassette with sequence upstream of DAP1752 deleted (Fig. 3b)
JKD5256	Contains P*pilH*::*cat* cassette with sequence downstream of DAP1763 deleted (Fig. 1b)
JKD5259	Contains P*pilG*::*cat* cassette with sequence upstream of DAP1753 deleted (Fig. 3b)
JKD5261	Contains P*pilH*::*cat* cassette with P1 −10 box mutated to CCCCCC (Fig. 1b)
JKD5262	Contains P*pilF*::*cat* cassette with P1 −10 box mutated to CCCCCC (Fig. 2b)
JKD5264	Contains P*pilH*::*cat* cassette with P2 −10 box mutated to GGGGGG (Fig. 1b)
JKD5265	Contains P*pilF*::*cat* cassette with P2 −10 box mutated to GGGGGG (Fig. 2b)
JKD5269	Contains P*pilH*::*cat* cassette without CREE (amplified from *N. gonorrhoeae* strain MS11-A)
JKD5271	Contains P*pilF*::*cat* cassette with CREE and IHF BS (amplified from *N. meningitidis* strain FAM18)
JKD5275	Contains P*pilG*::*cat* cassette with P1 −10 box mutated to ATCTTG (Fig. 3b)

[a] C. S. Ryan, unpublished data.

are present on the left- or right-hand side of the core sequence. This is of particular importance, as both types of terminal CR can generate functional promoters, but the conferred promoter strengths can be different, as demonstrated by Siddique et al. (51). In general, the first 6 nucleotides on the left-end CR resemble a −10 box of a σ^{70} promoter, and the −35 box is contributed by the native sequence upstream of the CREE. This type of promoter was first described by Snyder et al. for the transcription of *dcw* in *Neisseria lactamica* (52). The right-end CR can contain a −35 box, and the last 4 nucleotides are usually TATA, which forms part of a −10 motif and can be completed by the native sequence to generate a fully functional promoter. The functionality of this promoter was first demonstrated by Black et al. upstream of the *uvrB* gene (3). Later studies have shown that the transcription of *drg* (7), *lst* (45), and *mtrCDE* (48) are also dependent on this type of promoter.

The CREE can also regulate gene expression posttranscriptionally. When the CREE is transcribed as part of the mRNA, the two inverted CR can form a stem-loop, which may be targeted by RNase III, potentially altering the level of translation (16, 18, 40). Moreover, some CREE variants contain an IHF BS (6). It was shown by Rouquette-Loughlin et al. that IHF can bind to the IHF BS contained within the CREE to negatively regulate the expression of the *mtrCDE* operon (48).

In this study, we found the CREE upstream of 3 *pil* loci in *N. gonorrhoeae* and *N. meningitidis*, namely, *pilHIJKX* (*pilH-X*), encoding minor pilins that are known to be essential for wild-type (WT) level tfp expression and tfp dynamics (28, 61); *pilF*, encoding an ATPase belonging to the GspE family, which provides mechanical energy to power pilus assembly (8, 22); and *pilGD*, encoding a tfp biogenesis protein that can bind DNA (13, 60) and a prepilin peptidase (22, 57), respectively.

Given that the CREE may generate promoters and that some of these CREE variants contain an IHF BS, the aim of this study was to investigate the effect of the CREE on the transcription of these *pil* loci. Here, we provide evidence suggesting the CREE does not affect the transcription of *pil* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* DH5α (24) was used for propagating plasmids carrying *pil* promoter::*cat* reporter constructs (P*pil*::*cat*) constructed in this study. Standard *E. coli* competent cell preparation and transformation methods were used (27). Transformed *E. coli* cells were selected on LB agar containing 100 μg ml^{-1} ampicillin and 10 μg ml^{-1} chloramphenicol. Plasmids containing P*pil*::*cat* were extracted from 50 to 100 ml of overnight *E. coli* broth cultures using the High Pure Plasmid Isolation Kit (Roche) according to the manufacturer's instructions. Genomic DNA needed for subsequent recombinant DNA manipulation was extracted from *N. gonorrhoeae* strains FA1090 (accession number AE004969; http://www.genome.ou.edu/gono.html) and MS11-A (50) and *N. meningitidis* strain FAM18 (2) using the GenElute Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's instructions. All P*pil*::*cat* reporter plasmids were transformed into *N. gonorrhoeae* strain MS11-A (50) to allow recombination into the recipient chromosome. Neisserial transformations were performed using the spot transformation method on GC agar plates (19). Ten microliters of uncut plasmid DNA (approximately 1 μg to 10 μg) was used, and selection was on GC agar containing spectinomycin (60 μg ml^{-1}). All P*pil*::*cat* reporter plasmids and neisserial reporter strains used in this study are listed in Table S1 in the supplemental material and Table 1, respectively. The broth and plate growth conditions used for *E. coli*, *N. gonorrhoeae*, and *N. meningitidis* have been described previously (24).

Total RNA extraction. *N. gonorrhoeae* cells were grown to mid-exponential phase (optical density at 600 nm [OD_{600}], 0.6 after approximately 3.5 h) in GC broth (24). For RNA protection, 0.5 volume of RNA*later* RNA stabilization reagent (Qiagen) was added immediately to the liquid culture and allowed to incubate with the bacterial suspension for 15 min at room temperature. The cells were pelleted at 8,500 × *g* for 5 min, followed by the TRIzol-chloroform RNA extraction method described by Rio et al. (47). After the addition of TRIzol (Invitrogen), the samples were heated to 65°C for 15 min and allowed to cool to room temperature for 5 min before the addition of chloroform. RNA was treated with DNase twice using the Turbo DNA-*free* kit (Ambion) according to the manufacturer's instructions. RNA was immediately purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNA-free RNA was confirmed by the absence of signals in PCR. For RNA storage at −80°C, 2 volumes of 100% ethanol and 0.1 volume of 1 M NaCl (prepared in diethyl pyrocarbonate [DEPC]-treated water) were added. For use, the appropriate volume was removed, and the RNA was pelleted by centrifugation at 13,000 × *g* for 30 min at 4°C. The RNA pellet was washed with 75% ethanol, air dried, and suspended in an appropriate volume of DEPC-treated water.

Fluorescent primer extension analysis. Ten micrograms of total RNA was used in the fluorescent primer extension protocol described by Lloyd et al. (38), except for the following modifications: 100 units of SuperScript II reverse transcriptase (RT) (Invitrogen) was used in both the initial primer extension reaction and the enrichment step, and the final concentration of high-performance liquid

chromatography (HPLC)-purified 5′-6-carboxyfluorescein (FAM)-labeled oligonucleotides (see Table S2 in the supplemental material) was 10 μM. Gel electrophoresis and data analysis of the dried-down FAM-labeled cDNA was performed by the Australian Genome Research Facility Ltd. (Melbourne, Australia).

cDNA synthesis, RT-PCR, and qRT-PCR. RNA was extracted from *N. gonorrhoeae* strains FA1090 and MS11-A and *N. meningitidis* strain FAM18. Five micrograms of total RNA was used for the first-strand cDNA synthesis using SuperScript II reverse transcriptase as instructed by the manufacturer (Invitrogen). For quantitative real-time PCR (qRT-PCR), the reverse transcription step was extended to 2.5 h at 42°C.

The standard RT-PCRs were performed with *Taq* DNA polymerase (Roche) using the annealing and extension conditions recommended by the supplier.

In qRT-PCR, a 20-μl reaction mixture contained 10 μl Power SYBR green (Applied Biosystems), 1.6 μl of both the forward and reverse primers (see Table S2 in the supplemental material) at 625 nM, 2.5 μl of cDNA, and 4.3 μl of water. All qRT-PCRs were performed in triplicate. The standards for the qRT-PCR were genomic DNAs of *N. gonorrhoeae* strains FA1090 and MS11-A and *N. meningitidis* strain FAM18. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 35 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting-curve analysis. qRT-PCR was performed on 3 biological replicates. Quantification of transcripts was done using the Realplex real-time PCR system (Eppendorf).

Construction of promoter::*cat* reporter constructs. The oligonucleotide primers used to generate WT, deletant, and mutant promoter-reporter constructs are listed in Table S2 in the supplemental material. The WT promoter sequence was PCR amplified from the genomic DNA of *N. gonorrhoeae* strain FA1090, unless otherwise specified. The promoterless *cat* gene was amplified from pJKD699 (26) using the appropriate promoter-specific forward P*pil*::*cat* primers and oligonucleotide primer M13RP (42). The PCR products were gel purified using the QIAquick Gel Purification Kit (Qiagen) according to the manufacturer's instructions. Approximately equal amounts of the promoter fragment and *cat* fragment were fused using site-overlapping extension (SOE) PCR (25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by 94°C for 1 min and 72°C for 10 min) (32). The fusion PCR product was gel purified (QIAquick Gel Purification Kit; Qiagen) into 40 μl water. The DNA in this 40 μl was blunt ended (24) in a 50-μl reaction mixture containing a final concentration of 1× T4 polynucleotide kinase buffer (NEB), 20 units T4 polynucleotide kinase (NEB), 30 units T4 DNA polymerase (NEB), 0.2 mM ATP (NEB), and 0.04 mM PCR deoxynucleoside triphosphates (dNTPs) (Promega). The blunt-ending reaction mixture was incubated at 37°C for 1 h and cleaned with a QIAquick PCR Purification Kit (Qiagen).

All P*pil*::*cat* constructs were cloned into pJKD3172, which contains a spectinomycin resistance cassette inserted into a copy of the gonococcal *iga* gene (Ryan and Davies, unpublished; see Table S1 in the supplemental material). pJKD3172 was linearized by digestion with BglII, blunt ended as described above, and alkaline phosphatase (Boehringer Mannheim) treated. The blunt-ended P*pil*::*cat* SOE products were cloned into the BglII site in the *iga* gene in pJKD3172 (in opposite orientation to the *iga* gene). Transformation of *N. gonorrhoeae* strain MS11-A resulted in the integration of the P*pil*::*cat* constructs into the chromosomal *iga* gene via homologous recombination (25). The potential transformants carrying P*pil*::*cat* constructs were selected by spectinomycin resistance and screened by PCR and sequencing to ensure the promoter and reporter sequences were correct. Each of the respective WT P*pil*::*cat* constructs was used as a template for the construction of deletant and mutant P*pil*::*cat* constructs.

Determination of CAT production. Cell extracts were prepared from liquid cultures of *N. gonorrhoeae* and *N. meningitidis* P*pil*::*cat* reporter strains grown to mid-exponential phase as described previously (25). The total protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce) according to the manufacturer's instructions. At least three cell extracts were prepared on separate days for each reporter strain. A minimum of one chloramphenicol acetyltransferase (CAT) assay was performed for each cell extract.

RESULTS

The complete genome sequences of *N. gonorrhoeae* strains FA1090 (accession number AE004969; http://www.genome.ou.edu/gono.html) and NCCP (12) and *N. meningitidis* strains Z2491 (46), MC58 (59), and FAM18 (2) are available. As many as 23 genes have been annotated as being involved in pilus biogenesis in *N. gonorrhoeae* and *N. meningitidis*. All sequenced strains of *N. gonorrhoeae* and *N. meningitidis* share similar *pil* gene arrangements. An alignment of DNA sequences of each *N. gonorrhoeae* and *N. meningitidis pil* gene revealed that these *pil* loci are very similar at the nucleotide level (data not shown).

Sequences upstream of each of the *pil* genes were also aligned. CREEs were found to be present upstream of 3 *pil* genes, *pilH*, *pilG*, and *pilF*, in some but not all backgrounds. For example, the CREE is found upstream of *pilH* in all the *N. gonorrhoeae* and *N. meningitidis* strains examined, except in *N. gonorrhoeae* strain MS11-A (Fig. 1b), whereas the CREE is found upstream of *pilF* only in *N. meningitidis* strains, but not in *N. gonorrhoeae* strains (Fig. 2b). Furthermore, the CREE is found upstream of *pilG* in all *N. gonorrhoeae* strains but in only one *N. meningitidis* strain, FAM18 (Fig. 3b). It was therefore tempting to speculate that the transcriptional control of these *pil* genes may be different because of the presence or absence of the CREE and IHF BS.

***pilHIJKX* transcription does not appear to be affected by the CREE.** *pilH-X* are closely spaced and oriented in the same direction, suggesting they may be transcribed as an operon in both *N. gonorrhoeae* and *N. meningitidis* (Fig. 1a). RT-PCR was able to demonstrate the cotranscription of *pilH-X* (data not shown), indicating there is a promoter upstream of *pilH*. In *N. gonorrhoeae* strain NCCP, the CREE was found to be inserted 143 bp upstream of the ATG start codon (Fig. 1b). The inserted CREE does not have an IHF BS and is only 108 bp in length. Both the left and right ends of the CREE belong to the α type. Given that this CREE was inserted away from the start of the coding sequence, it is unlikely that it is involved in the transcription of *pilH-X* in *N. gonorrhoeae* strain NCCP. In *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains Z2491, MC58, and FAM18, the CREE was found to be much closer to the ATG start codon (22 bp upstream) and is the longer 155- to 156-bp variant, which contains an IHF BS (Fig. 1b). In all 4 strains, the left end of the CREE belongs to the α type and contains a −10 box sequence (-10^{L}) that has 5 out of 6 bases identical to the σ^{70} promoter sequence consensus and a corresponding −35 box sequence (-35^{L}) located 17 bp further upstream that is contributed by the native sequence (Fig. 1b). The right end of the CREE belongs to the β class, with the first 4 bases at the terminus (-10^{R}) being GATA in all 3 *N. meningitidis* strains and TATA in *N. gonorrhoeae* strain FA1090. The CREE insertion has not generated a −10 box in *N. meningitidis*, and the −10 box in *N. gonorrhoeae* has only 4 out of 6 bases identical to the consensus (Fig. 1b). The corresponding −35 box (-35^{R}) located 17 bp further upstream is found in all 4 strains. Lastly, the IHF BS contained within the CREE in *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains Z2491 and FAM18 is identical to the IHF BS contained within the CREE found upstream of the *mtrCDE* genes (48).

Fluorescence-based primer extension (FPE) analysis was performed on total RNA extracted from *N. gonorrhoeae* strain MS11-A, the strain that does not have the CREE in the *pilH-X* promoter region, and two FPE products were detected (Fig. 1b). Based on the two transcriptional start point (tsps) suggested by the FPE results, two putative −10 motifs, termed P1 and P2, could be deduced. In order to determine which, if any, of these putative promoters was functional, each −10 motif was separately mutated in strains JKD5261 (P1 mutated) and

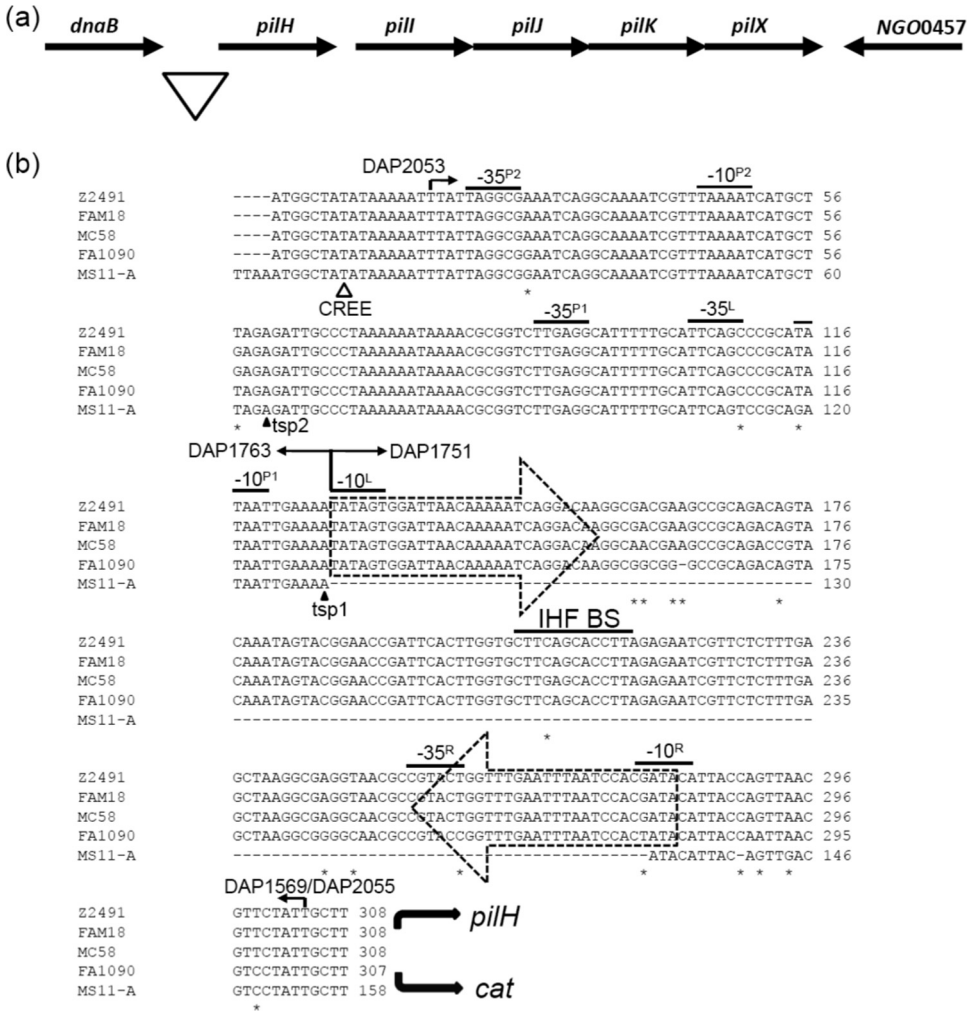

FIG. 1. The *pilHIJKX* locus. (a) Schematic representation of *pilHIJKX* in *N. gonorrhoeae* strain FA1090. The triangle marks the region of DNA sequence examined. (b) Sequence alignment of the promoter region for *pilH-X* in *N. gonorrhoeae* strains FA1090 and MS11-A and *N. meningitidis* strains Z2491, MC58, and FAM18. The triangle indicates the position where the CREE was inserted into *N. gonorrhoeae* strain NCCP. The dashed arrows enclose the two terminal CR. The −10 and −35 boxes of the four putative σ^{70} promoters, designated P1, P2, L, and R, are overlined. The transcriptional start points (tsps 1 and 2) are marked by solid arrowheads. The IHF BS contained within the CREE is overlined. Base differences among the five sequences are marked with asterisks. The curved arrows indicate the directions of *pilH* and *cat* transcription. The numbered arrows indicate the oligonucleotide primers used (see Table S2 in the supplemental material). DAP1569 is specific for *N. gonorrhoeae* strain FA1090, and DAP2055 is specific for *N. gonorrhoeae* strain MS11-A.

JKD5264 (P2 mutated) (Table 1), and the amount of CAT produced by each strain was measured and compared to the WT strain, JKD5244 (Table 1).

The two negative-control strains, MS11-A and JKD5222, which contain the vector alone, did not produce CAT, as shown by CAT enzyme-linked immunosorbent assay (ELISA) (Fig. 4). The CAT ELISA data suggested P2 is the functional promoter, because altering this sequence resulted in substantially reduced CAT levels in JKD5264, whereas when the P1 sequence was mutated, the CAT levels in JKD5261 remained the same as in the WT strain, JKD5244 (Fig. 4). A −35 box (-35^{P2}) was found to be located 18 bp upstream of P2 and had 3 out 6 bases matching the consensus (Fig. 1b).

Deletional analysis of the P*pilH*::*cat* strain JKD5244 was performed to assess the role of the CREE in the transcription of *pilH-X*. Two deletion mutants, JKD5250 and JKD5256 (Table 1), were made. Deleting the sequence upstream of the CREE (primer DAP1751) (Fig. 1b) significantly affected transcription, as CAT produced by JKD5250 is negligible compared to that of the WT strain JKD5244 (Fig. 4), despite the fact that it still contains an intact −10 box (-10^{L}) at the left-end CR (Fig. 1b). This result implies that either -10^{L} is nonfunctional or the activity of the promoter is abolished because the corresponding −35 box (-35^{L}) (Fig. 1b) is absent. A significant amount of CAT was detected for JKD5256, which contains the sequence upstream of the CREE but not the CREE itself (the sequence between primers DAP1751 and DAP1569 is deleted) (Fig. 1b). However, compared to the WT strain, JKD5256 exhibited a 75% reduction in the CAT level (Fig. 4). In addition, CAT produced by strain JKD5269 carrying the MS11-A version of the P*pilH*::*cat* construct, that is, the *pilH* promoter sequence without the CREE, also showed more

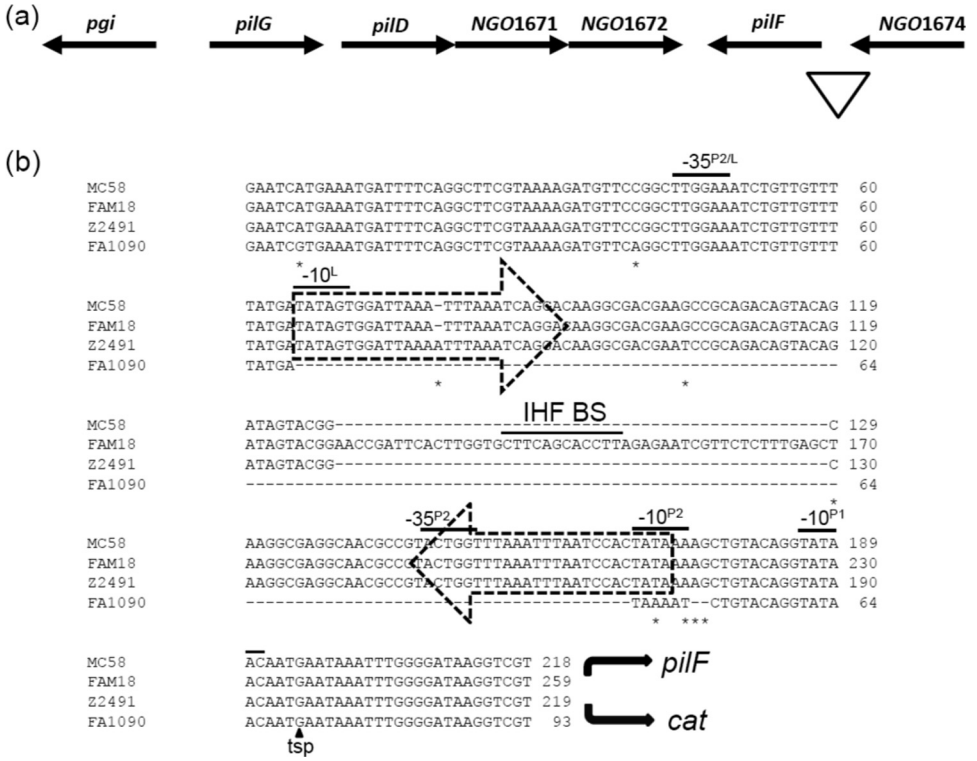

FIG. 2. The *pilF* locus. (a) Schematic representation of *pilF* in *N. gonorrhoeae* strain FA1090. The triangle marks the region of DNA sequence examined. (b) Sequence alignment of the promoter regions for *pilF* in *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strains Z2491, MC58, and FAM18. The dashed arrows enclose the two terminal CR. The −10 and −35 boxes of the three putative σ^{70} promoters, designated P1, P2, and L, are overlined. The −35 box for P2 in *N. gonorrhoeae* strain FA1090 is shown further upstream, due to the absence of the CREE in this strain. The mapped tsp is marked by a solid arrowhead. The IHF BS contained within the CREE in strain FAM18 is overlined. Base differences between the four sequences are marked with asterisks. The curved solid arrows indicate the directions of *pilF* and *cat* transcription.

than 50% reduction compared to the WT strain, JKD5244 (Fig. 4). In summary, it seems that P2 is the only functional promoter present upstream of *pilH-X*.

As the CAT ELISA data implied different levels of *pilH* transcription in different reporter strains, we directly compared the relative expression levels, using qRT-PCR, of *pilH* in *N. gonorrhoeae* strains FA1090 (P*pilH-X* containing the CREE) and MS11-A (P*pilH-X* without the CREE) (Fig. 1b). To ascertain the reliability of the data, two housekeeping genes, *recA* and *rpoD*, were included as internal controls. It was shown that the average ratios of *pilH* to *recA* transcript levels in FA1090 and MS11-A were 0.41 and 0.39, respectively (Table 2). The average ratios of *pilH* to *rpoD* transcript levels in FA1090 and MS11-A were 0.87 and 0.83, respectively (Table 2). This indicates that the level of *pilH* transcripts present in strains FA1090 and MS11-A are very similar and that the CREE found in the *pilH* promoter region in *N. gonorrhoeae* strain FA1090 and the 3 *N. meningitidis* strains have no effect on transcription.

A CREE insertion in *N. meningitidis* has generated a functional promoter for the transcription of *pilF*. In *Neisseria*, *pilF* is located downstream of and in the opposite orientation to the *pilGD* locus (Fig. 2a). Detailed analysis of the sequence upstream of *pilF* revealed the insertion of a CREE, with a β left and β right CR, 47 bp upstream of the ATG start codon in the *N. meningitidis* strains examined in this study (Fig. 2b). The CREE insertion generated two putative promoters at either end of the CREE (L and P2) (Fig. 2b). In *N. meningitidis* strains Z2491 and MC58, the inserted CREE is 108 bp in length and does not contain an IHF BS. The CREE inserted in the same location in *N. meningitidis* strain FAM18 is 157 bp in length and contains an IHF BS at its center. The DNA sequence of the *pilF* promoter regions in *N. gonorrhoeae* strains FA1090, NCCP, and MS11-A is the same as in *N. meningitidis*, except that it does not contain the CREE. FPE analysis on total RNA extracted from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain FAM18 gave rise to the same extension product (data not shown). The mapped tsp and a putative −10 box, designated P1, are indicated in Fig. 2b.

In order to determine which of the putative promoter sequences was functional, mutational analysis of the *N. gonorrhoeae* strain FA1090 *pilF* promoter region was performed. JKD5245 (WT P*pilF*::*cat*) and mutant strains JKD5262 (P1 mutated) and JKD5265 (P2 mutated) were assayed by CAT ELISA. The CAT ELISA results suggested P2 is the functional promoter for *pilF* transcription, because JKD5265 produced negligible amounts of CAT (Fig. 5). This was not surprising, because P2 has a corresponding −35 box (-35^{P2}) (Fig. 2b) while P1 does not. Given that the CREE insertion generated a −10 sequence slightly different from that in *N. gonorrhoeae* and the CREE upstream of P*pilF* in *N. meningitidis* strain FAM18 contained an IHF BS, we constructed a reporter strain,

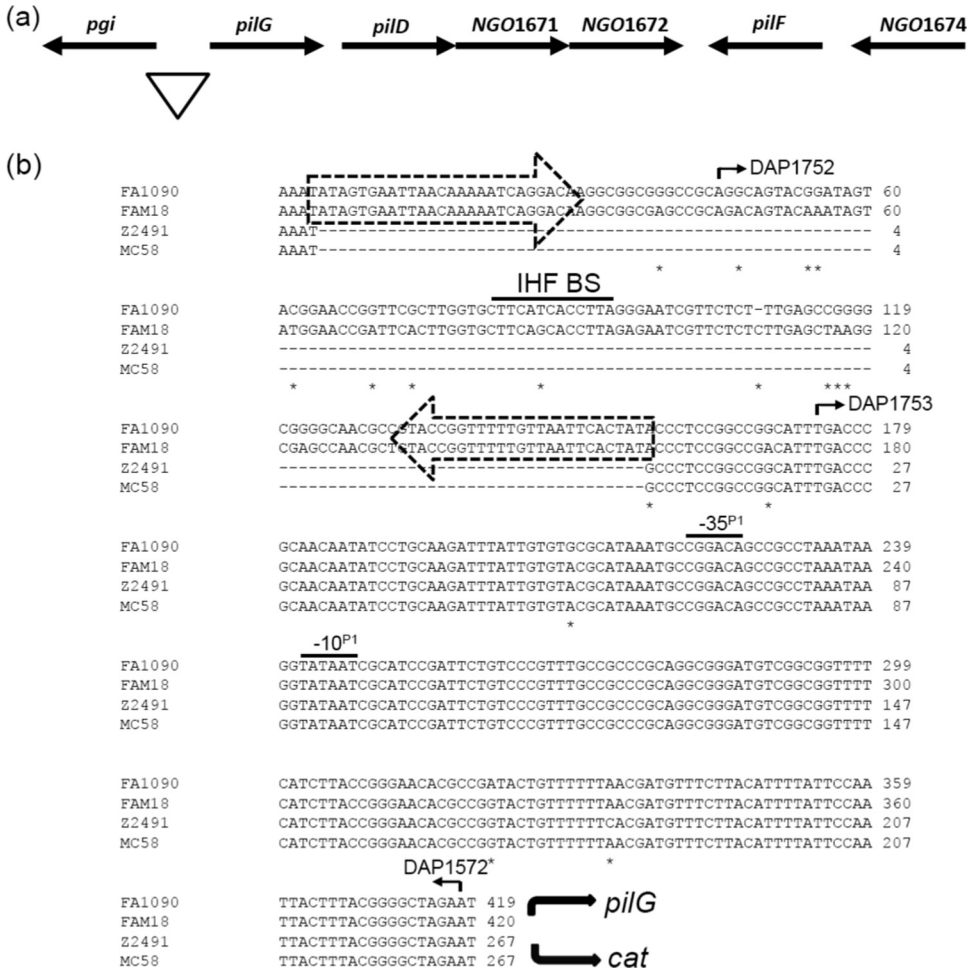

FIG. 3. The *pilGD* locus. (a) Schematic representation of *pilGD* in *N. gonorrhoeae* strain FA1090. The triangle marks the region of DNA sequence examined. (b) Sequence alignment of the promoter regions for *pilG* in *N. gonorrhoeae* strains FA1090 and *N. meningitidis* strains Z2491, MC58, and FAM18. The dashed arrows enclose the two terminal CR. A −10 box and the corresponding −35 box of a σ^{70} promoter, termed P1, are shared by all strains and are overlined. The IHF BS contained within the CREE is overlined. Base differences among the four sequences are marked with asterisks. The curved solid arrows indicate the directions of *pilG* and *cat* transcription. The numbered arrows indicate the oligonucleotide primers used (see Table S2 in the supplemental material).

JKD5271 (FAM18 P*pilF*::*cat* with the CREE plus IHF BS), to determine if the CREE and the IHF BS have an effect on *pilF* transcription. There was no significant difference in promoter strength conferred by JKD5245 and JKD5271, as indicated by the CAT ELISA results (Fig. 5). This suggests that *pilF* transcription does not seem to be affected by the presence or absence of the CREE.

To provide direct evidence that the CREE has no effect on *pilF* transcription, qRT-PCR was performed on total RNA extracted from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain FAM18 to quantify the levels of *pilF* transcripts (Table 3). The average ratios of *pilF* to *recA* transcripts were 0.99 and 0.28 in *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain FAM18, respectively. The average ratios of *pilF* to *rpoD* transcripts were 2.38 and 0.32, respectively. This result suggests *N. meningitidis* strain FAM18 produced significantly lower levels of *pilF* transcripts than *N. gonorrhoeae* strain FA1090.

The upstream CREE does not influence transcription of the *pilG* gene. RT-PCR showed that *pilG* is cotranscribed with *pilD* and the two genes (*NGO1671* and *NGO1672*) (Fig. 3a) downstream of *pilD* encoding hypothetical proteins (data not shown). A CREE, with α right and α left CR types and a putative IHF BS, was found to be inserted 220 bp upstream of the start of the *pilG* coding sequence in *N. gonorrhoeae* strains FA1090, MS11-A, and NCCP and *N. meningitidis* strain FAM18 (Fig. 3b). *N. meningitidis* strains Z2491 and MC58 have identical upstream sequences, except that they do not have CREE insertions (Fig. 3b).

To assess if the CREE is needed for *pilG* transcription, the promoter region was PCR amplified from *N. gonorrhoeae* strain FA1090 and used as the WT template in strain JKD5243. From this, two deletion mutant reporter strains were made by deleting part of the CREE (the sequence upstream of primer DAP1752 was deleted) (Fig. 3b) and the entire CREE (the sequence upstream of primer DAP1753 was deleted) (Fig. 3b)

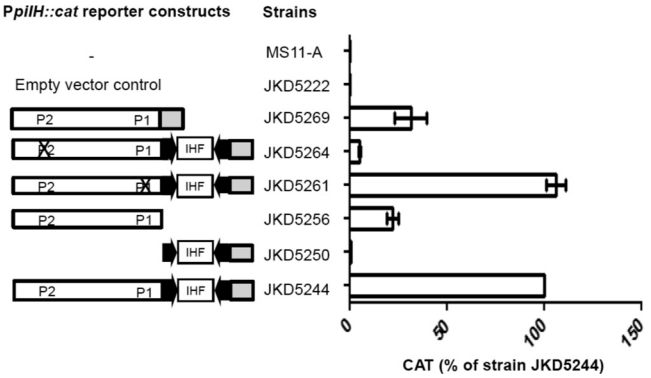

FIG. 4. Transcriptional analysis of the *pilH* promoter region in an *N. gonorrhoeae* strain MS11-A background. P*pilH*::*cat* reporter constructs are shown schematically. The white boxes indicate sequence upstream of the CREE, and the gray boxes indicate sequence downstream of the CREE. The arrows are the terminal CR. The Xs indicate promoter mutations. The amount of CAT protein produced by each *N. gonorrhoeae* reporter strain was quantified by CAT ELISAs, and at least 3 independent biological samples were prepared. The CAT level is expressed as the percentage of the wild-type (*N. gonorrhoeae* strain JKD5244) level. Strains MS11-A and JKD5222 were included as negative controls. The error bars represent standard deviations of the mean.

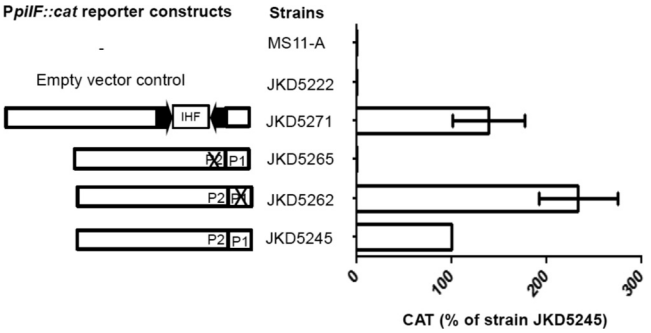

FIG. 5. Transcriptional analysis of the *pilF* promoter (P*pilF*) region in an *N. gonorrhoeae* MS11-A background. The arrows are the terminal CR. An X indicates a particular promoter that was mutated. The amount of CAT protein produced by each *N. gonorrhoeae* reporter strain was quantified by CAT ELISAs, and at least 3 independent biological samples were prepared. The CAT level is expressed as the percentage of the wild-type level produced by JKD5245. The error bars represent standard deviations of the mean.

in strains JKD5253 and JKD5259, respectively. The CAT levels produced by the two *N. gonorrhoeae* deletant strains were similar to those of the WT strain, JKD5243, confirming that the CREE is not involved in *pilG* transcription (Fig. 6).

FPE was performed on total RNA extracted from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain MC58, but it was unable to show a tsp (data not shown). A potential promoter, based on its sequence similarity to the promoter consensus sequence, was found to be downstream of the CREE in all strains examined (Fig. 3b). It consists of a perfect −10 box (-10^{P1}) and a corresponding −35 box (-35^{P1}) located 15 bp further upstream (Fig. 3b). The −10 box was mutated in strain JKD5275, and CAT activity was completely abolished (Fig. 6).

DISCUSSION

CREEs have been proposed to be mobile elements, although the associated mobilization mechanism(s) remains unknown (6). The insertion of the CREE upstream of genes can generate promoters (51), further modulate gene expression by IHF binding (48), and affect transcript stability by forming secondary structures that can either be targeted by or protected from RNase III (16, 18). In contrast to these reports, we have evidence suggesting the CREEs present upstream of *pilH-X*, *pilGD*, and *pilF* in some pathogenic *Neisseria* strains are not involved in the regulation of *pil* gene expression. To support this, we have mapped the σ^{70}-dependent promoters for the relevant *pil* loci and have shown that these promoters are not part of the CREE sequence for P*pilH-X* and P*pilGD*.

It remains unclear to us why both P*pilH*::*cat* reporter strains JKD5244 (FA1090 P*pilH* with the CREE) and JKD5261 (P1 mutant) produced substantially more CAT protein than strains JKD5269 (MS11-A P*pilH* without the CREE) and JKD5256 (FA1090 P*pilH* without the CREE) (Fig. 4). It should be noted that the IHF BS present within the CREE is positioned downstream of the functional P2 sequence, which is unlike the situation in the *N. gonorrhoeae pilE* promoter region, where it is located 55 bp upstream of the promoter motif (31). The presence of an IHF BS downstream of a promoter has been shown to have a negative impact on gene expression, and this is exemplified by the transcription of the *mtrCDE* operon in *N.*

TABLE 2. qRT-PCR performed on total RNA extracted from *N. gonorrhoeae* strains FA1090 and MS11-A

Strain	Sample[a]	RNA fold change		
		pilH-recA	*pilH-rpoD*	*recA-rpoD*
MS11-A	1	0.45	0.88	1.98
	2	0.44	0.87	1.99
	3	0.29	0.73	2.53
	Avg	0.39	0.83	2.17
FA1090	1	0.61	1.0	1.64
	2	0.34	0.75	2.18
	3	0.28	0.87	3.10
	Avg	0.41	0.87	2.31

[a] Three biological RNA samples were obtained from each strain for qRT-PCR.

TABLE 3. qRT-PCR performed on total RNA extracted from *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain FAM18

Strain	Sample[a]	RNA fold change		
		pilF-recA	*pilF-rpoD*	*recA-rpoD*
FAM18	1	0.24	0.37	1.57
	2	0.20	0.20	1.00
	3	0.39	0.40	1.02
	Avg	0.28	0.32	1.20
FA1090	1	1.02	2.10	2.06
	2	1.16	2.82	2.43
	3	0.78	2.23	2.85
	Avg	0.99	2.38	2.45

[a] Three biological RNA samples were obtained from each strain for qRT-PCR.

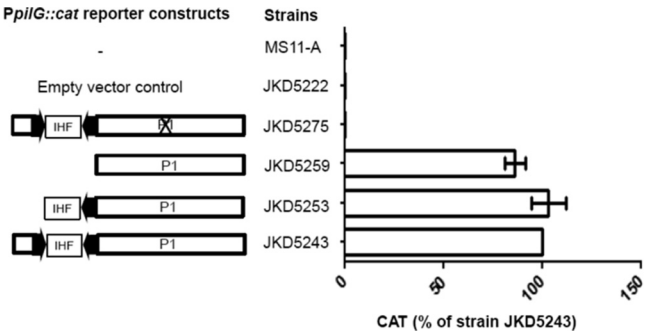

FIG. 6. Transcriptional analysis of the *pilG* promoter (P*pilG*) region in an *N. gonorrhoeae* MS11-A background. The arrows are the terminal CR. The X indicates the mutation of a putative −10 box termed P1. The amount of CAT protein produced by each *N. gonorrhoeae* reporter strain was quantified by CAT ELISAs, and at least 3 independent biological samples were prepared. The CAT level is expressed as the percentage of the wild-type level produced by strain JKD5243. The error bars represent standard deviations of the mean.

meningitidis (48). Therefore, the larger amount of CAT protein produced by the WT strain, JKD5244, does not appear to be due to the presence of the IHF BS.

It was also possible that the higher levels of CAT observed with strains JKD5244 and JKD5261 was due to the presence of the CREE, which might help stabilize the transcripts when the CREE is transcribed as part of the message (18). However, the qRT-PCR results showed that the *pilH* transcript levels are similar in *N. gonorrhoeae* strains FA1090 and MS11-A, suggesting the CREE has no effect on transcription. We therefore suggest that in this specific instance the presence of the CREE may somehow positively influence translation of the mRNA.

Although the P*pilF* promoter is partly contained within the CREE in *N. meningitidis*, the strength of the promoter is similar to that of the *N. gonorrhoeae* version, which does not have the CREE (Fig. 5). The difference in the *pilF* transcript levels in *N. gonorrhoeae* strain FA1090 and *N. meningitidis* strain FAM18 may be attributable to the difference in the mRNA turnover rates in these strains. Additionally, it was noted that the level of CAT produced by JKD5262 (P1 mutant) was 2-fold higher than that of the WT strain, JKD5245 (Fig. 5). As P2 was shown to be the functional promoter, the actual tsp is therefore likely to be located upstream of P1 (Fig. 2b), and as a result, the P1 sequence would be transcribed. It may be that the P1 mutation has resulted in relaxation of a secondary structure in the mRNA, which would allow more efficient translation. We have searched for DNA sequences that might contribute to the formation of a secondary mRNA structure, but none were identified. An alternative explanation could be that the P1 −10 motif is part of an IHF BS, as it has 10 out of 13 bases matching the IHF consensus. The binding of IHF to this site may partially repress *pilF* transcription, and the mutation introduced into the P1 −10 motif could relieve that repression.

Since the CREE insertions upstream of the three *pil* loci are neither strain nor species specific, it seems that they are the result of random transposition. The data generated in this study suggest that in these specific cases, CREEs have no effect on gene expression. Nonetheless, given its abundance and the differential distribution across the neisserial genomes, there is no doubt that the CREE has a significant role in controlling neisserial gene expression on a global scale.

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