

# Characterization of a Novel Antisense RNA in the Major Pilin Locus of *Neisseria meningitidis* Influencing Antigenic Variation

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## ABSTRACT

Expression of type four pili (Tfp) is essential for virulence in *Neisseria meningitidis*. Pili mediate adhesion, bacterial aggregation, and DNA uptake. In *N. meningitidis*, the major pilin subunit is encoded by the *pilE* gene. In some strains, PilE is subject to phase and antigenic variation, which can alter Tfp properties and together offer a possible mechanism of immune escape. Pilin expression and antigenic variation can be modulated in response to environmental cues; however, the precise mechanisms of such regulation remain unclear. We identified a promoter in the *pilE* locus, 3' of the *pilE* coding sequence, on the antisense (AS) strand which is conserved in meningococci. We show that this promoter directs transcription of an AS RNA that is expressed during specific growth phases and in response to salt stress. Furthermore, we demonstrate that the transcript encompasses sequences complementary to the entire *pilE* coding sequence and 5' untranslated region. AS RNAs can regulate the gene on the sense strand by altering transcript stability or translation. However, by using Northern blotting, quantitative reverse transcription-PCR (RT-PCR), and Western blotting, we found no significant AS RNA-dependent changes in *pilE* transcript or protein level. Instead, our data indicate that the AS RNA influences pilin antigenic variation. This work provides further insights into the complex regulation of pilin expression and variation in pathogenic *Neisseria*.

## IMPORTANCE

Pathogenic *Neisseria* spp. express type four pili (Tfp) which are important for adhesion, aggregation and transformation. Some strains of *N. meningitidis* are able to vary the sequence of the major subunit (PilE) of the Tfp. The mechanisms underlying this variation are not fully defined, but the process requires several noncoding elements that are found adjacent to the *pilE* gene. In this work, we identified a *cis*-encoded RNA antisense to *pilE* in *N. meningitidis*. By using Northern blotting and RT-PCR analysis, we found that the RNA is expressed in stationary phase or following salt stress. Our work also indicates that this RNA does not significantly affect *pilE* or pilin expression levels but instead appears to modulate pilin variation.

*Neisseria meningitidis* (meningococcus) is a Gram-negative bacterium that is frequently carried asymptotically in the human upper respiratory tract. Occasionally, the meningococcus causes meningitis and septicemia, which can result in death or devastating sequelae. *N. meningitidis* has evolved a number of strategies to colonize the upper respiratory tract, including adhesion to epithelial cells and immune evasion. The expression of type four pili (Tfp) by this important human pathogen contributes to both colonization and disease (1).

In the meningococcus, Tfp are filamentous structures composed of thousands of subunits of the major pilin PilE, along with accessory pilins arranged in a helical configuration to form the pilus fiber (2). Pili are important for interactions with host cells (3). *In vitro*, strains lacking *pilE* have a reduced capacity to adhere to epithelial cells or explanted human airway organ culture (4–6). In addition, Tfp are essential for colonization of the vascular endothelium, where they promote bacterial survival by enabling microcolony formation and resistance to blood flow (7), as well as pathogenesis through signaling events that lead to inflammation and migration across the blood-brain barrier (8, 9). Finally, Tfp are also required for transformation in *Neisseria* spp. (10).

*N. meningitidis* expresses one of two distinct classes of Tfp which are distinguished by the sequence of PilE and the *pilE* expression locus (11, 12). Among disease-causing isolates, class II *pilE* genes are highly conserved, while class I pilins are subject to high-frequency phase and antigenic variation (Av), enabling the bacterium to modulate its pilin sequence (13). Pilin variation has

consequences on host cell interactions (14, 15) and signaling (16) and may also influence the formation of bacterial aggregates and confer resistance to host immune responses (17). Meningococci expressing class I Tfp harbor a single pilin locus containing multiple copies of silent *pilS* sequences upstream of the *pilE* gene. The *pilS* cassettes lack predicted promoters, ribosome binding sites, and sequences encoding the N-terminal region of PilE necessary for pilus assembly. Pilin Av has been well studied in the related species *Neisseria gonorrhoeae* and arises through homologous re-

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TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Description and/or relevant property(ies)	Reference or source
<i>E. coli</i> DH5 $\alpha$	<i>fhuA2 lac<math>\Delta</math>U169 phoA glnV44 <math>\phi</math>80<math>\Delta</math> lacZ<math>\Delta</math>M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	
<i>N. meningitidis</i> strains		
8013	Serogroup C, serotype 18, class I <i>pilE</i>	40
8013 $\Delta$ G4	Kanamycin cassette inserted into G4 sequence	This study
WT_ery	Erythromycin cassette upstream of AS promoter	This study
Mut_ery	Erythromycin cassette upstream of AS promoter, AS promoter mutation	This study
Plasmids		
pGEM-T	Cloning and expression vector, carbenicillin resistance	Promega
pCR.2.ITOPOery	Cloning and expression vector, erythromycin resistance	R. Exley, unpublished data
pEGFP-N2	Cloning and expression vector, kanamycin resistance	Clontech
pEGFP-N2(Insert1)	<i>N. meningitidis</i> 8013 AS promoter region cloned into pEGFP-N2	This study
pEGFP-N2(Insert1_M1)	<i>N. meningitidis</i> 8013 AS promoter region cloned into pEGFP-N2; mutation of –35 site	This study
pEGFP-N2(Insert1_M2)	<i>N. meningitidis</i> 8013 AS promoter region cloned into pEGFP-N2; mutation of –10 site	This study
pEGFP-N2(Insert1_M3)	<i>N. meningitidis</i> 8013 AS promoter region cloned into pEGFP-N2; mutation of –35 and –10 sites	This study
pEGFP-N2 (AS_lpx_ery)	Vector for construction of WT_ery	This study
pEGFP-N2 (AS_lpx_ery_M3)	Vector for construction of Mut_ery	This study

combination between *pilS* cassettes and the *pilE* coding sequence, an event known as gene conversion (18, 19). This process requires RecA (20) and RecQ and RecO (21) and is governed by additional genetic elements within the *pilE* locus. For example, a sequence that is predicted to form a guanine quartet (G4) structure is located 5' to *pilE* and is essential for gene conversion (22), while a Sma/Cla repeat situated 3' of *pilE* enhances the efficiency of this process (23). Furthermore, transcription of a G4-associated *cis*-acting small noncoding RNA is thought to be required for G4 formation and subsequent pilin antigenic variation (24). Thus, noncoding DNA and RNA sequences have important roles in pilin variation.

Despite its key role in virulence, regulation of pilin expression and variation in *N. meningitidis* is not well understood. The majority of pilin regulation studies have been performed in *N. gonorrhoeae*, but the two organisms differ in their host niches and may thus exhibit distinct expression profiles or responses to environmental changes. There is variation in *pilE* promoter sequences both between *Neisseria* species and also among different *N. meningitidis* strains (25). In *N. meningitidis*, a number of proteins participate in pilin regulation upon bacterial contact with host cells. CrgA, a transcriptional regulator induced following initial contact of bacteria with epithelial cells, downregulates the expression of *pilE* (26). NafA, a protein which is upregulated and surface expressed after adhesion of *N. meningitidis* to host cells, is proposed to control piliation at a posttranscriptional level by preventing pilus bundling (27). In addition, the RNA binding protein Hfq influences *pilE* expression in *N. meningitidis* in a strain-specific manner. For example, in strain H44/76, loss of Hfq leads to a reduction in PilE levels (28), while in MC58 loss of Hfq results in PilE upregulation (29). Hfq functions by facilitating the interaction of small noncoding RNAs (sRNAs) with target mRNAs (30), or it can facilitate antisense RNA pairing (31); therefore, this finding indicates that expression of *pilE* may involve regulatory RNAs.

Through genome analysis, we identified a putative promoter for a *cis*-encoded antisense (AS) RNA within the region 3' of the *pilE* gene of *N. meningitidis*. The aim of this work was to investi-

gate the function of this RNA. AS RNAs have been identified in numerous bacterial species, and their roles are largely to regulate the sense transcript at the level of transcription, mRNA stability, or translation (for reviews, see references 32 and 33). We therefore examined the role of RNA in modulating pilin expression in *N. meningitidis*. Moreover, given the importance of noncoding *pilE*-proximal elements in modulating pilin Av, we also sought to establish whether this RNA has any influence on this important process and found that expression of the RNA modulates pilin variation. Our work provides further insights into the complexity of the *pilE* locus.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** Strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown on Luria-Bertani media at 37°C containing carbenicillin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), or erythromycin (200  $\mu$ g/ml) when required. *N. meningitidis* was grown overnight at 37°C, 5% CO<sub>2</sub>, either on brain heart infusion (BHI; Oxoid) agar supplemented with 5% heat-denatured horse serum and 0.1% starch or in liquid BHI. Erythromycin (2  $\mu$ g/ml) was added for selection.

For stress experiments, liquid cultures of *E. coli* or *N. meningitidis* were grown for approximately 3 h in LB or BHI medium, respectively, to an optical density at 600 nm (OD<sub>600</sub>) of 0.5 to 0.6 and subjected to acid stress (HCl, pH 2.5), envelope stress (5% Triton X-100 or 5% ethanol), oxidative stress (0.15% H<sub>2</sub>O<sub>2</sub>), salt stress (0.5 M NaCl or 0.5 M KCl), osmotic stress (6% sucrose), or temperature stress (10°C) for 10 min. For analysis of transcript and protein expression during growth, *N. meningitidis* strains were grown overnight on BHI agar. Bacteria were recovered from the plate, resuspended in phosphate-buffered saline (PBS), and quantified using the A<sub>260</sub> to measure DNA in a lysed sample. A volume equivalent to 1  $\times$  10<sup>9</sup> CFU was inoculated into 25 ml of BHI medium in 125-ml conical flasks (Corning), and cultures were shaken at 180 rpm at 37°C. OD<sub>600</sub> readings were taken at periodic intervals to determine growth phase, and samples were taken at various time points for RNA and protein analyses.

**Strain construction.** A 595-bp fragment, including the AS promoter (downstream), and a 752-bp adjacent fragment (upstream) were amplified from *N. meningitidis* 8013 using primers *pilE*\_AS-F/*pilE*\_AS-1R and *lpxC*\_frag\_F/R (Table 2), respectively. The PCR products were cloned

TABLE 2 Oligonucleotides used in this study

Oligonucleotide purpose and name	Sequence	Purpose
<b>Strain construction</b>		
<i>pilE</i> _AS-F	GGGGGAATTCCGCGCTGTCAGATAAAACC	Amplification of <i>pilE</i> antisense; EcoRI site is underlined
<i>pilE</i> _AS-1R	GGGGCCCCGGGCGGAAGCCATCCTTTTGGC	Amplification of <i>pilE</i> antisense; XmaI site is underlined
Ery_HindIII_F	GGGGAAGCTTCCGATACCCCGATGACG	Amplification of Ery; HindIII site is underlined
Ery_SacI_R	GGGGGAGCTCGAATTCGCCCTTCCCGGGG	Amplification of Ery; SacI site is underlined
<i>lpxC</i> _fragment_F	GGGGGAGCTCCCTGTCGCGTCATTCC	Amplification of <i>lpxC</i> fragment; SacI site is underlined
<i>lpxC</i> _fragment_R	GGGGGCTAGCGCTTCAGTCAATCAGCG	Amplification of <i>lpxC</i> fragment; NheI site is underlined
SDM_mut3_F	CACTTACCGCTCCCTTTATTTAAAAATTTATGGT <b>CCAC</b> <b>ATT</b> TACCTTAGC	Site-directed mutagenesis of –35 and –10 sites of putative AS promoter; mutations are in bold
SDM_mut3_R	GCTAAGGTAAAT <b>GTG</b> ACCATAAAATTTAAATA <b>AAAGGG</b> AGCGGTAAGTG	Site-directed mutagenesis of –35 and –10 sites of putative AS promoter; mutations are in bold
pEGFP_seq_F	GGTGGGAGGTCTATATAAGC	Sequencing of AS fragment in pEGFP-N2
insert1_seq_R	GGTTTATCTGACAGGCGCG	Sequencing upstream of AS fragment
GFP-seq-R	CGTCGCGTCCAGTCCGACCCAG	Sequencing of AS fragment in pEGFP-N2
<i>lpxC</i> _frag_R2	GGAAAAATAGAAAGCGTTATCC	Amplification and sequencing of <i>lpxC</i> in <i>N. meningitidis</i> 8013
Ery_F_R	GCACGAGCTCAAGCTTCCG	Sequencing of WT_ery and Mut_ery
<b>Strand-specific qRT-PCR</b>		
ssQRTPCR_tag_F	CCGTCTAGCTCTCTCTAATCG	Strand-specific RT-PCR (ssRT-PCR) tag
ssRTPCR_AS_tag	<u>CCGTCTAGCTCTCTCTAATCCGTAAGCTTGAGGCATTTC</u>	cDNA synthesis for ssRT-PCR with AS; tag sequence is underlined
ssQRTPCR_AS_F	CGCCAAAATGCCGACGATG	ssRT-PCR with AS
ssRTPCR_ <i>pilE</i> _tag	<u>CCGTCTAGCTCTCTCTAATCCGATGGCTTCGAAACTTGTG</u>	cDNA synthesis for ssRT-PCR with <i>pilE</i> ; tag sequence is underlined
ssQRTPCR_ <i>pilE</i> _F	CGAGCTGATGATTGTGATTGC	ssRT-PCR with <i>pilE</i>
ssRTPCR_tmRNA_tag	<u>CCGTCTAGCTCTCTCTAATCCATTGACCGACTGCTGC</u>	cDNA synthesis for ssRT-PCR with tmRNA; tag sequence is underlined
<b>Northern blotting</b>		
Insert1_probe	GCCGCCGCCAACGGCAAGACCGACGACAAAAATCAACA CCAAGCACCTGCC	Probe for AS transcript from pEGFP-N2(Insert1)
(AS) <i>pilE</i> -1	ACCGATGGTCAAATACATTGCATAATGCCGATGGCGT AAGCTTGAGGCAT	AS probe
<i>pilE</i> _probe	GGCTGATTTTTGACCTTCAGCCAAAAGGATGGCTTCGG AAACTTGTGCGC	<i>pilE</i> probe
8013_tmRNA_F	GGTTGCGAAGCAGATGCC	Amplification of <i>N. meningitidis</i> 8013 tmRNA PCR probe
8013_tmRNA_R	CCAGTCAATGTAAGATGACG	Amplification of <i>N. meningitidis</i> 8013 tmRNA PCR probe
ssrA_EC_F	CGAAACCCAAGGTGCATGC	Amplification of <i>E. coli</i> tmRNA probe
ssrA_EC_R	CAGGGCTTCCACGCG	Amplification of <i>E. coli</i> tmRNA probe
Antigenic variation assay primer <i>pilE</i> _F	CGATGGCGTAAGCTTGAGG	Amplification of <i>pilE</i>
<b>Walking RT-PCR</b>		
RT_F	AGCTGGCAGATGAATCATCG	Amplification of cDNA for mapping of 3' end
RT_R1	CCCTTCAAAAAGGTTTTACCC	Amplification of cDNA for mapping of 3' end
RT_R2	CAAACCTTGATACCAATCTTGCT	Amplification of cDNA for mapping of 3' end
RT_R3	CCATGCCAATAGAGATACCC	Amplification of cDNA for mapping of 3' end
RT_3.5	TATGCTACCGCGCAAATTCAAA	Amplification of cDNA for mapping of 3' end
RT_R4	CCAACCCACCTTATGCTAC	Amplification of cDNA for mapping of 3' end
RT_4.5	CCAAGAAAACGGAATTTTTAAAA	Amplification of cDNA for mapping of 3' end
T7_iv_temp_F	GGGGGAATTCTAATACGACTCACTATAGGAGCCTTG AAGCGCAGTCG	Amplification of template for <i>in vitro</i> transcription; T7 promoter is underlined
iv_temp_R	GCGGAGCGGTTTCTGTTGC	Amplification of template for <i>in vitro</i> transcription

flanking an erythromycin resistance cassette to obtain plasmid pEGFP-N2(AS\_ *lpx\_ery*). Site-directed mutagenesis (QuikChange kit; Agilent) was carried out using primers SDM\_mut3\_F/R to obtain plasmid pEGFP-N2(AS\_ *lpx\_ery*\_M3). Plasmids were digested with HindIII and SacI, and the purified inserts were used to transform *N. meningitidis* 8013 to obtain the strains WT\_ery and Mut\_ery, respectively. Strain 8013ΔG4 was constructed by using fusion PCR to insert a kanamycin resistance cassette, including two *rrnB* terminators (34), into the guanine quartet region (C CCACCCAA^CCCACCC) so that the transcriptional orientation of the cassette was divergent to *pilE* (see Fig. S1 in the supplemental material). For the construction of strains WT\_ery(RecA6) and Mut\_ery(RecA6), genomic DNA from strain 8013 containing the *recA6* allele (gift from Hank Seifert) was used to transform the strains WT\_ery and Mut\_ery,

respectively. Single colonies of the transformants obtained were confirmed by Western blotting of whole-cell extracts for RecA expression in the presence or absence of isopropyl-β-D-thiogalactopyranoside (IPTG).

**SDS-PAGE and Western blotting.** Whole-cell extracts were prepared from cultures at different time points by boiling bacterial suspensions in SDS-PAGE lysis buffer (35) at a dilution of 1 ml per OD<sub>600</sub> unit. Proteins were separated by SDS-PAGE alongside molecular weight markers (All Blue; Bio-Rad) and transferred to nitrocellulose membranes (Hybond-C Extra; Amersham) for Western blotting. PilE was detected using SM1 (1:10,000) (36) and IRDye 800CW-goat anti-mouse IgG (1:10,000; Li-Cor). A GroEL loading control was detected using anti-GroEL antibody (1:8,000; gift from Jörgen Johansson) and IRDye 680-goat anti-rabbit IgG (1:10,000; Li-Cor). RecA was detected using an anti-RecA antibody

(1:6,000; Abcam) and IRDye 680–goat anti-rabbit IgG (1:10,000; Li-Cor). Bands were visualized and quantified using the Odyssey Sa infrared imaging system. Experiments were carried out in triplicate using strains from independent transformations. Pile band intensities were normalized to the respective GroEL band intensities and expressed as the ratio to the normalized Pile value of the first lane.

**RNA isolation and Northern blotting.** Total RNA was isolated from *E. coli* by using the Fast RNA Blue kit (MP Biomedicals) followed by DNase treatment (Roche) or from *N. meningitidis* by using the RNeasy midikit and on-column DNase treatment (Qiagen). RNA (20 µg) was separated by electrophoresis in 1.5% agarose formaldehyde gels in HEPES buffer, transferred onto Hybond-N membranes (GE Healthcare), and cross-linked by UV light. Probes were generated by end-labeling primers [*pilE*\_probe, (AS)*pilE*-1, or Insert1 probe], or a PCR product generated using primers 8013\_tmRNA\_F/R with [ $\gamma$ -<sup>32</sup>P]ATP (PerkinElmer). Membranes were incubated in hybridization buffer (GE Healthcare) and hybridized with labeled probes overnight at 64°C. Signals were detected using a fluorescent image analyzer (Fuji FLA-5000) and quantified using AIDA image analyzer software. Experiments were carried out in triplicate using strains from independent transformations.

**Strand-specific quantitative reverse transcription-PCR (qRT-PCR).** First-strand cDNA was synthesized from 5 µg total RNA by using AS- or *pilE*-specific primers carrying a 5' tag sequence not found in the *N. meningitidis* genome (Table 2). RNAs were reverse transcribed in the presence of actinomycin to avoid second-strand cDNA synthesis. After RNase H treatment for 20 min at 37°C, cDNA was purified, and quantitative real-time PCR was performed with Power SYBR green PCR master mix (Applied Biosystems) using a unique tag primer, to ensure strand-specific cDNA amplification. The reaction was monitored using a StepOnePlus real time PCR system. Results are reported as the average *R* value (calculated as  $2^{-\Delta\Delta C_T}$ , where  $C_T$  is the threshold cycle) of triplicate experiments performed on WT\_ery and Mut\_ery strains obtained from three independent transformations. The amount of transcript was expressed as the *R* value standardized to the transfer-messenger RNA (tmRNA) control. The efficiencies of each primer pair were evaluated by performing a 10-fold dilution series experiment and determining the slope of the standard curves obtained. The primer pairs were found to have efficiencies ranging from 85 to 100%.

**Primer extension and walking RT-PCR.** RNA (10 µg) extracted from *N. meningitidis* strains following NaCl stress was mixed with labeled probe and denatured at 80°C for 5 min, and the primer was extended by using avian myeloblastosis virus reverse transcriptase in extension buffer (25 mM Tris-HCl [pH 8.3], 30 mM NaCl, 15 mM MgCl<sub>2</sub>, 1.25 mM dithiothreitol, 1 mM deoxynucleoside triphosphates) at 42°C for 1 h. Products were denatured at 80°C for 3 min in formamide sample buffer. A PCR product of the AS promoter region was generated using primers *pilE*\_AS-F and *pilE*\_AS-1R, and sequencing was performed using the labeled *pilE*\_AS-1R primer according to standard methods (35). Products were run on a 5% denaturing polyacrylamide gel. Signals were detected as described previously.

Mapping (walking) PCR was performed according to published protocols (37). Total RNA was used as a template for the synthesis of separate cDNAs using primers R1 to R4.5 (Table 2). Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's directions. The respective cDNA primers and an upstream primer (AS\_RT\_F) were used for PCR amplification, and the PCR products were analyzed by agarose gel electrophoresis with a 1-kb DNA ladder (Biolone) for molecular size marker. Identical RT-PCR analysis was carried out on an *in vitro*-transcribed AS RNA synthesized with primers T7\_iv\_temp\_F and iv\_temp\_R using the RiboMAX large-scale RNA production system (T7; Promega).

**Antigenic variation assays.** Antigenic variation assays in liquid medium were performed with strains 8013 and 8013ΔG4 or with WT\_ery and Mut\_ery strains containing the *recA6* allele. Each Av assay was carried out using bacteria inoculated from frozen stocks made from single colo-

nies of the respective strains, in order to ensure that each experiment started with a homogenous population of cells containing the same *pilE* sequence. Strains grown overnight on solid BHI medium without IPTG were used to inoculate BHI broth and grown to mid-log phase. Where relevant, IPTG was then added to a final concentration of 1 mM and the cultures were incubated with shaking at 37°C for 10 min. For NaCl stress, the cultures were incubated with NaCl (0.5 M) together with IPTG for 10 min. The cultures were then serially diluted and plated onto BHI agar containing 1 mM IPTG and grown overnight for 22 h (see Fig. S2 in the supplemental material). Ninety-six colonies of each strain under each condition were passaged onto BHI agar and incubated overnight. Lysates were prepared by resuspending single colonies in 100 µl PBS and boiling for 10 min. *pilE* was amplified from single-colony lysates using primers AS\_F and *pilE*\_F, which are specific for amplification of *pilE*, and the Herculase II fusion DNA polymerase (Agilent). Products were sequenced and compared to *pilE* progenitor sequence, and changes were mapped to *pilS* donors. Each variant *pilE* was considered to have resulted from a single Av event. The assays with 8013 and 8013ΔG4 were performed once. Av assays with WT\_ery(RecA6) and Mut\_ery(RecA6) were performed three times.

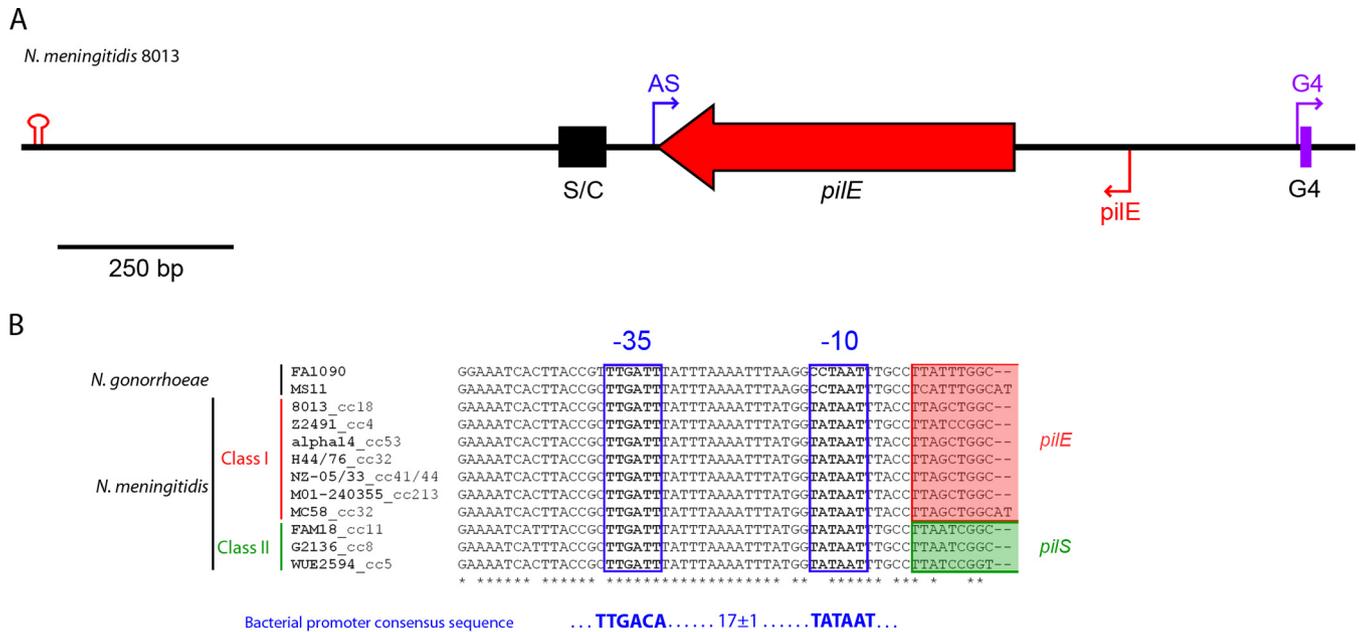
**Statistical analysis.** The Student *t* test was used for the quantification of transcript level by strand-specific qRT-PCR. Fisher's exact test was used for the antigenic variation assay with strains 8013 and 8013ΔG4. Results of the antigenic variation assay with WT\_ery(RecA6) and Mut\_ery(RecA6) were analyzed by fitting generalized linear models (GLMs) (38). Binomial GLMs were fitted, with the response variable defined as a sequence of ordered pairs (number of the variant, number of the same) from *pilE* sequences, and the predictor variables were the following: strain (WT\_ery or Mut\_ery), NaCl induction (yes or no), and assay (replicate 1, 2, or 3). R statistical software was used, specifically, the function GLM (39).

## RESULTS

**Identification of a putative promoter in the class I *pilE* locus in *N. meningitidis*.** Inspection of the available genome sequence of *N. meningitidis* strain 8013 (40) (accession number FM999788) revealed the presence of a putative promoter on the AS strand, 5 nucleotides (nt) downstream of the *pilE* stop codon (Fig. 1A). No other putative promoters were identified on the AS strand within *pilE* in this sequence. The promoter has strong resemblance to the bacterial RpoD  $\sigma^{70}$  promoter consensus sequence and is conserved in meningococcal genomes among strains belonging to different lineages (Fig. 1B). In two *N. gonorrhoeae* genomes, the sequence is conserved apart from three substitutions, two of which occur in the putative –10 TATA box sequence (CCTAAT instead of TATAAT) (Fig. 1B). Interestingly, in meningococci that express class II pilin, the promoter is found in the *pilS* region but not adjacent to the *pilE* gene (Fig. 1B).

We did not identify any sequences resembling a Shine-Dalgarno ribosome binding site within proximity to an ATG start codon within 350 nucleotides of this promoter. We therefore hypothesized that this promoter drives transcription of a noncoding, AS RNA.

**Expression of the AS transcript in *E. coli* and *N. meningitidis*.** To determine whether the AS promoter is functional, we first examined the ability of this sequence to mediate transcription in *E. coli*. A 595-bp region from *N. meningitidis*, including the putative AS promoter and 386 bp of the 3' end of the *pilE* coding sequence (but excluding the *pilE* promoter sequence) was introduced into pEGFP-N2 to make pEGFP-N2(insert1); this plasmid only harbors a cytomegalovirus early-immEDIATE promoter for transcription in eukaryotic but not prokaryotic cells. To examine promoter activity, *E. coli* strain DH5 $\alpha$  was transformed with pEGFP-N2(insert1), grown for 3 h to an OD<sub>600</sub> of 0.5 to 0.6 in liquid



**FIG 1** Location of a promoter sequence in the class I *pilE* locus in *N. meningitidis* 8013. (A) Schematic diagram of the *pilE* locus in *N. meningitidis* 8013. Sma/Cla (S/C) and guanine quartet (G4) are DNA elements in the *pilE* locus that are important for pilin antigenic variation in *N. meningitidis*. The promoters of the *pilE* AS RNA, the G4-associated sRNA, and *pilE* are denoted by the blue, purple, and red arrows, respectively. The predicted *pilE* terminator is denoted by the red stem-loop. (B) Multiple-sequence alignment of the region corresponding to the AS promoter in *N. meningitidis* strains from different clonal complexes (cc) expressing class I or class II pilin and that in *N. gonorrhoeae*. The putative  $-10$  and  $-35$  sequences are boxed in blue, *pilE* sequences are shaded in red, and *pilS* sequences are shaded in green. The promoter sequence is conserved in *N. meningitidis* strains. In the two strains of *N. gonorrhoeae*, the sequence is conserved, apart from three substitutions.

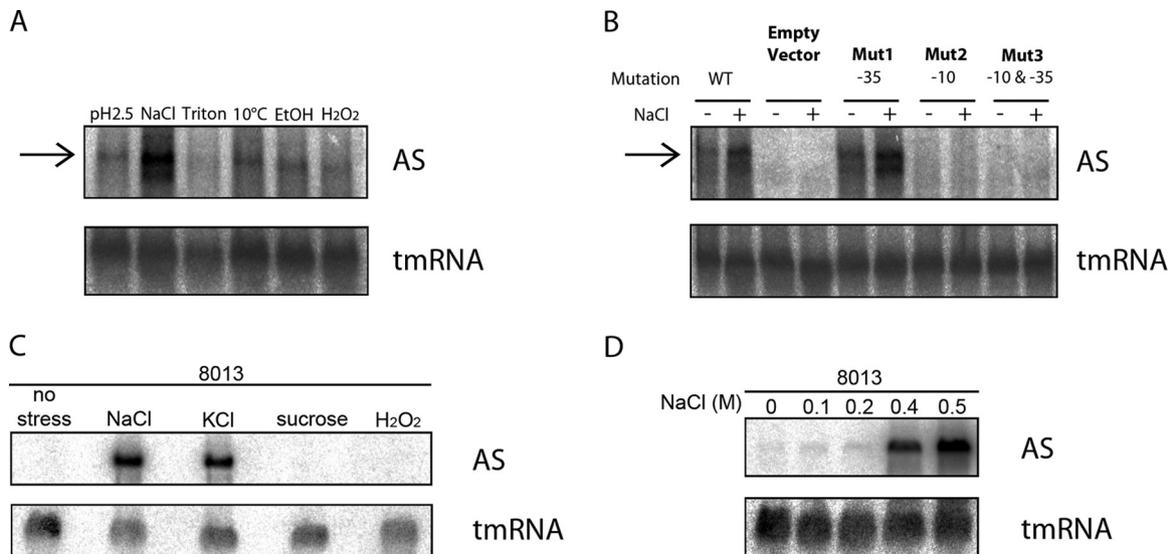
medium, and then subjected to acid, NaCl, temperature, envelope, or oxidative stress for 10 min. The presence of the AS transcript was detected by Northern blotting by using a 50-nt probe that hybridized 39 nt downstream of the predicted  $-10$  sequence. The AS transcript was significantly more abundant when the *E. coli* cells were exposed to high NaCl concentrations (0.5 M for 10 min) but not after other stresses (Fig. 2A). In addition, we generated plasmids in which the predicted  $-35$  sequence (TTGATT  $\rightarrow$  TCCCTT; Mut1), the  $-10$  sequence (TATAAT  $\rightarrow$  TCACAT; Mut2), or both (Mut3) had been modified in an attempt to abolish promoter activity (Fig. 2B). No transcript was detected in *E. coli* harboring pEGFP-N2 alone (empty vector) with or without salt stress. A transcript of the expected size was detected in *E. coli* containing the plasmid with the unmodified promoter sequence (wild type [WT]) or with an alteration in the putative  $-35$  sequence (Mut1), indicating that the promoter is functional and that the  $-35$  sequence is dispensable for expression of the RNA. In contrast, mutation of the  $-10$  sequence (in Mut 2 and Mut 3) was sufficient to abolish transcription.

We also examined the expression of the AS RNA in response to different stresses in wild-type *N. meningitidis*. Strain 8013 was grown overnight on solid medium and used to inoculate liquid BHI medium. After 3 h growth at 37°C with agitation, bacteria were subject to stress conditions by addition of NaCl or KCl to 0.5 M, sucrose to 6%, or H<sub>2</sub>O<sub>2</sub> to 0.15% for 10 min. Using a probe specific for the AS RNA, a single transcript was detected in bacteria treated with NaCl or KCl, indicating that the AS RNA is also expressed in response to salt stress in wild-type *N. meningitidis* (Fig. 2C). We subsequently determined the concentration of salt necessary to induce abundant expression of the AS RNA. Strain 8013

was grown in liquid medium as described above, and NaCl was added to a range of final concentrations from 0 to 0.5 M. As shown in Fig. 2D, AS RNA levels were increased at salt concentrations of 0.4 M or more.

To investigate the role(s) of the AS RNA, we next constructed isogenic strains with either an intact or a mutated AS promoter. The control strain of 8013, designated WT\_ery, carries the erythromycin antibiotic resistance marker but an intact AS promoter sequence (see Fig. S3 in the supplemental material). We confirmed that AS expression in WT\_ery was not affected by the presence of the erythromycin resistance cassette, based on Northern blotting (see Fig. S3). We also introduced the  $-10$  and  $-35$  AS promoter mutations into *N. meningitidis* strain 8013 by homologous recombination to generate the isogenic strain Mut\_ery (see Fig. S3). There was no difference in growth of WT\_ery and Mut\_ery (Fig. 3A), and Northern blot analysis of total RNA isolated at different time points demonstrated that the AS RNA was detected in the WT\_ery strain but not in Mut\_ery after overnight growth (21 h, OD<sub>600</sub> of 1.9) (Fig. 3B). In agreement with results using wild-type strain 8013, exposure of WT\_ery to high salt (0.5 M NaCl) significantly increased the abundance of the AS transcript (by approximately 280-fold;  $P = 0.0003$ ) (Fig. 3D; see also Table S1 in the supplemental material). Therefore, we have identified a *cis*-encoded AS RNA in the class I *pilE* locus of *N. meningitidis* strain 8013 which is present in stationary phase and can be up-regulated by NaCl. In subsequent experiments, we chose to use 0.5 M NaCl stress as a method of inducing overexpression of the AS RNA to allow us to investigate its function.

**The AS RNA encompasses sequences complementary to *pilE* and its 5' UTR.** AS RNA transcription can affect levels of the



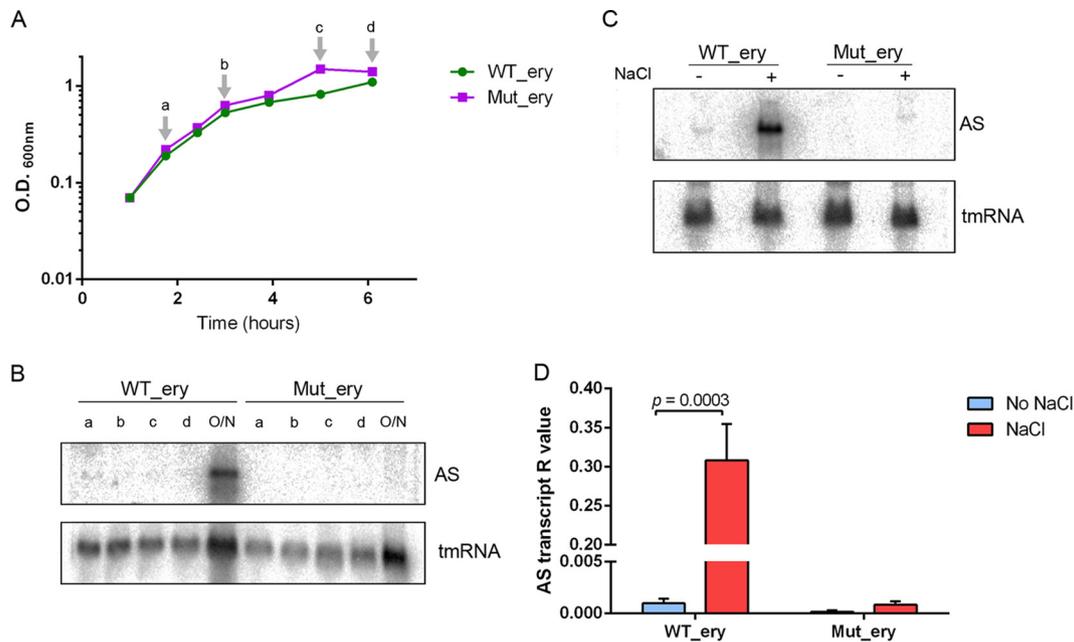
**FIG 2** The AS transcript is induced by salt stress in *E. coli* and *N. meningitidis*. (A) A 595-bp region, including the AS promoter from *N. meningitidis* 8013, was cloned into pEGFP-N2 and transformed into *E. coli*; transformants were subjected to different stresses to identify conditions under which the AS promoter is functional. Northern blot analysis of total RNA was performed using a probe complementary to the cloned insert (AS) or transfer mRNA (tmRNA) as a loading control. The AS transcript was upregulated following NaCl stress. (B) Northern blot analysis of total RNA from *E. coli* strains containing plasmids with mutations to the  $-35$  site (Mut1), the  $-10$  site (Mut2), or both (Mut3) of the *N. meningitidis pilE* AS promoter with (+) and without (-) NaCl stress. No transcript was detected upon mutation of the  $-10$  sequence. (C) Northern blot analysis of total RNA from wild-type *N. meningitidis* strain 8013 subjected to different stresses. The AS transcript was detected using a 50-nt probe [(AS)*pilE*-1] that hybridizes specifically to the AS RNA 533 nt downstream of the AS promoter. tmRNA was used as a loading control. A transcript corresponding to the AS RNA was detected following exposure to NaCl and KCl. (D) Analysis of AS RNA expression in *N. meningitidis* following a 10-min incubation with different concentrations of NaCl. AS RNA levels were increased at higher salt concentrations (0.4 and 0.5 M NaCl).

corresponding sense transcript through a variety of mechanisms, including altered RNA stability or binding to the 5' untranslated regions (UTRs) of the sense mRNA to alter ribosome binding and thus affect protein expression. To determine whether the AS RNA encompasses the region complementary to the full *pilE* coding sequence and/or 5' UTR, we mapped the 5' and 3' ends of the transcript. The 5' end of the AS transcript was mapped by primer extension and found to be to 8 nt downstream of the  $-10$  sequence of the predicted promoter. No extension product was obtained from the strain Mut\_ery (Fig. 4A). RT-PCR was employed to map the 3' end of the transcript using a set of reverse primers which hybridize at various intervals within the region corresponding to the *pilE* promoter and G4 sequence (Fig. 4B and C). As a control, the same PCR was performed using an AS RNA synthesized *in vitro* with T7 RNA polymerase. Results indicated that using RNA isolated from *N. meningitidis* as the template, a PCR product of expected size was obtained with primers F+R3.5 (877 nt) but not with F+R4 (888 nt) or F+R4.5 (933 nt). Products of 888 nt and 933 nt were amplified from *in vitro*-synthesized RNA with primers F+R4 and F+R4.5, respectively (Fig. 4B, red asterisks), demonstrating that the absence of product was not due to primer inefficacy. Smaller-sized products obtained from reactions using primers F+R4 were sequenced. The products obtained from *N. meningitidis* RNA corresponded to nonspecific amplification from rRNA (Fig. 4B, green asterisks) and a truncated PCR product of 727 nt and 733 nt (Fig. 4B, orange asterisk and blue asterisk, respectively). This result indicated that the 3' end of the AS transcript in *N. meningitidis* lies between the site of hybridization of primers R3.5 and R4, which corresponds to the promoter of the G4-associated sRNA (Fig. 4C). Thus, the AS transcript encompasses sequence antisense to the entire *pilE* coding sequence and 5'

UTR and terminates within the promoter of the G4-associated sRNA (Fig. 4C, shaded region).

**AS RNA expression does not impact *pilE* transcript and pilin levels.** Given that the AS RNA is complementary to the entire *pilE* coding sequence and 5' UTR, we next determined whether *pilE* transcript or pilin protein levels are affected by the presence of the AS transcript. Northern blot analysis was performed using a *pilE*-specific oligonucleotide probe. Analysis of total RNA isolated from the strains at different growth phases revealed that *pilE* mRNA was expressed at similar levels throughout growth in both WT\_ery and Mut\_ery (Fig. 5A) and was detected even after overnight growth, when AS levels were highest (Fig. 3B). We also used NaCl stress to examine the levels of *pilE* transcript when expression of the AS RNA was induced. Total RNA was prepared from WT\_ery and Mut\_ery with and without brief incubation in high salt concentrations (0.5 M). We found that in the absence of NaCl stress, both Northern blot analysis and strand-specific qRT-PCR indicated a trend toward elevated *pilE* mRNA levels in Mut\_ery compared to WT\_ery (Fig. 5B and C; see also Table S1 in the supplemental material), but this was not significant ( $P = 0.1223$ ; Student's *t* test). Following NaCl stress which induced AS RNA levels up to 280-fold (Fig. 3D), an approximately 5-fold reduction of *pilE* transcript levels was observed, but this was not dependent upon the AS transcript, as it was observed in both WT\_ery and Mut\_ery ( $P = 0.0184$  and  $P = 0.0224$ , respectively; Student's *t* test).

Although there was no significant alteration in *pilE* transcript levels upon deletion or induction of the AS RNA, we investigated whether there were any changes in pilin protein levels, which might result from posttranscriptional regulation of PilE. We performed Western blot analysis of whole-cell lysates using anti-pilin



**FIG 3** Expression of the AS transcript in *N. meningitidis*. (A) Growth curves of WT\_ery and Mut\_ery cultured at 37°C in BHI broth. Arrows indicate times at which RNA was extracted for Northern blot analysis of AS RNA expression. (B) Northern blot analysis results for total RNA isolated at different time points during growth (h 1.75 [a], h 3 [b], h 5 [c], h 6 [d], and after growth overnight [O/N] for 21.5 h). The AS transcript was detected using a 50-nt probe [(AS)*pilE*-1] that hybridizes specifically to the AS RNA 533 nt downstream of the AS promoter. tmRNA was used as a loading control. (C) Representative Northern blot analysis of total RNA from *N. meningitidis* strains WT\_ery and Mut\_ery, cultured with and without NaCl stress. (D) Strand-specific qRT-PCR analysis of AS transcript levels in WT\_ery and Mut\_ery strains grown with and without NaCl stress. Amount of transcript is expressed as the *R* value standardized to that of the tmRNA control.  $P = 0.0003$ , Student's *t* test. The AS transcript was upregulated under NaCl stress in WT\_ery; no transcript was detected in Mut\_ery.

antibody and fluorescence analysis to quantify relative amounts of pilin in whole-cell lysates from WT\_ery and Mut\_ery either during growth (Fig. 5D) or following salt stress (Fig. 5E). Mutation of the AS promoter or NaCl stress did not lead to a detectable change in PilE protein levels (for quantification, see Tables S3 and S4 in the supplemental material). Therefore, we found no evidence that the AS RNA affects *pilE* transcript or pilin protein levels in meningococcus.

**Effect of AS RNA on pilin antigenic variation.** A small G4-associated RNA is essential for pilin Av in *N. gonorrhoeae* (24). This small RNA is proposed to act in *cis*, forming an RNA:DNA hybrid with the C-rich DNA strand and promoting formation of the G4 structure to initiate gene conversion. Given that the AS RNA transcript overlaps with the promoter region of the G4-associated sRNA, we investigated whether the AS RNA had any impact on pilin Av.

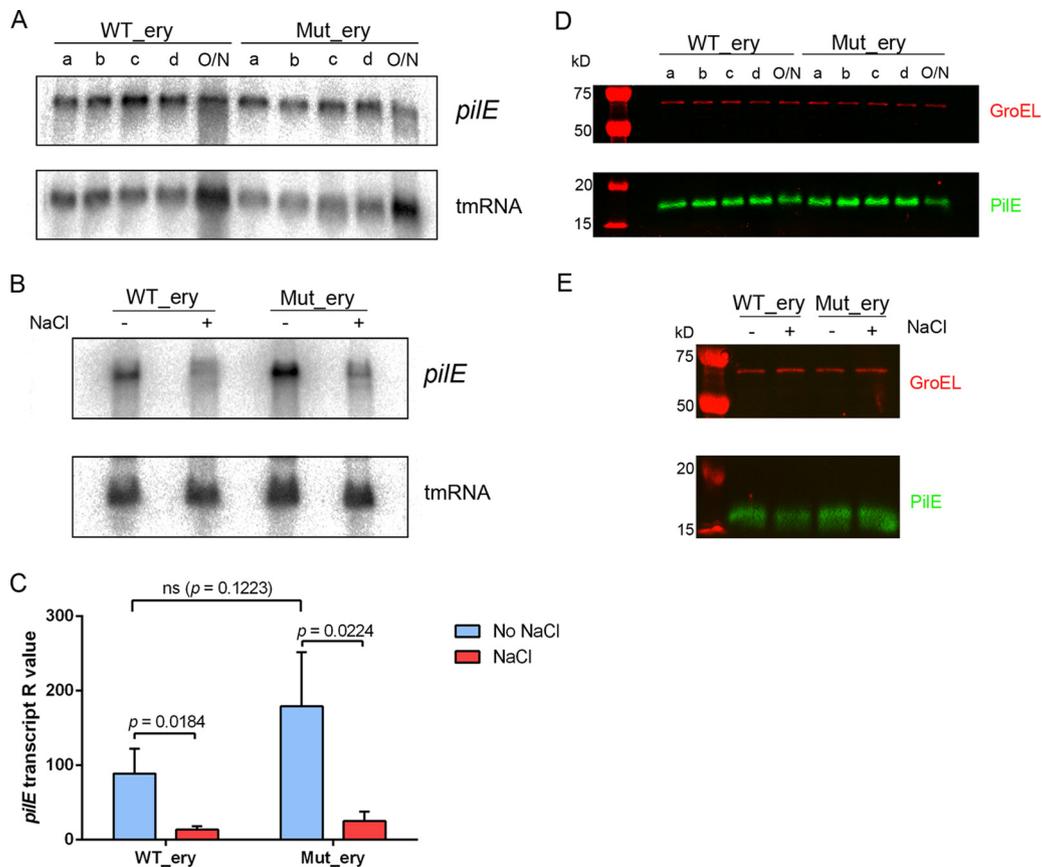
The G4 sequence and position relative to *pilE* is conserved in *N. meningitidis* strain 8013, and so we first performed Av assays using 8013 and 8013ΔG4, which has a kanamycin resistance marker with transcriptional terminators inserted into the G4 sequence (see Fig. S1 in the supplemental material). *pilE* variation was detected in 8013, while no Av events occurred in 8013ΔG4 (Fig. 6A), indicating that the G4 is required for Av in meningococcus and that Av is detectable by our methods. We therefore examined Av in WT\_ery(RecA6) and Mut\_ery(RecA6) in the presence of IPTG to induce RecA expression, with or without NaCl to induce the expression of the AS RNA. The sequence of *pilE* in the resulting colonies was compared to the potential *pilS* donor sequences in the *N. meningitidis* 8013 genome, and the number of variation events was determined. Table 3 and Fig. 6B show results from

three independent assays (a total of 1,150 *pilE* sequences). We fitted a binomial GLM which proved to be satisfactory (goodness of fit statistic = 5.8392 on 6 degrees of freedom;  $P = 0.4414$ ). On their own, neither the presence of NaCl nor the mutation resulted in a statistically significant change in levels of antigenic variation. However, we found that there was a significantly reduced level of antigenic variation when WT\_ery was induced with NaCl ( $z = 2.434$ ,  $P = 0.0149$ ) but this was not observed for Mut\_ery, indicating that the AS RNA transcript or transcription may influence pilin Av.

## DISCUSSION

Here we have described the identification of a novel RNA antisense to *pilE* in *N. meningitidis*. The sequence and position of the promoter of this RNA are conserved in different meningococcal isolates expressing class I *pilE* but is absent from the class II *pilE* locus. Instead, the AS promoter is located in the *pilS* locus in class II-expressing strains, consistent with this region of the class II *pilS* locus having homology to the region 3' of class I *pilE* (12). Analysis of the sequence downstream of *pilE* in two genomes of the related pathogen *N. gonorrhoeae* revealed conservation of the putative -35 sequence but substitutions in the putative -10 sequence. These base changes occur in the TATA box and thus may result in a nonfunctional promoter. Consistent with this, a recent transcriptomic study in *N. gonorrhoeae* did not detect antisense transcription in *pilE* (41); other reports have described the presence of functional antisense promoters in the 5', 3', and midgene regions of *pilE* in this species (S. Hill, T. Le, and S. Wachter, presented at the XIXth International Pathogenic Neisseria Conference, Asheville, NC, 12 to 17 October 2014). These diverse findings may





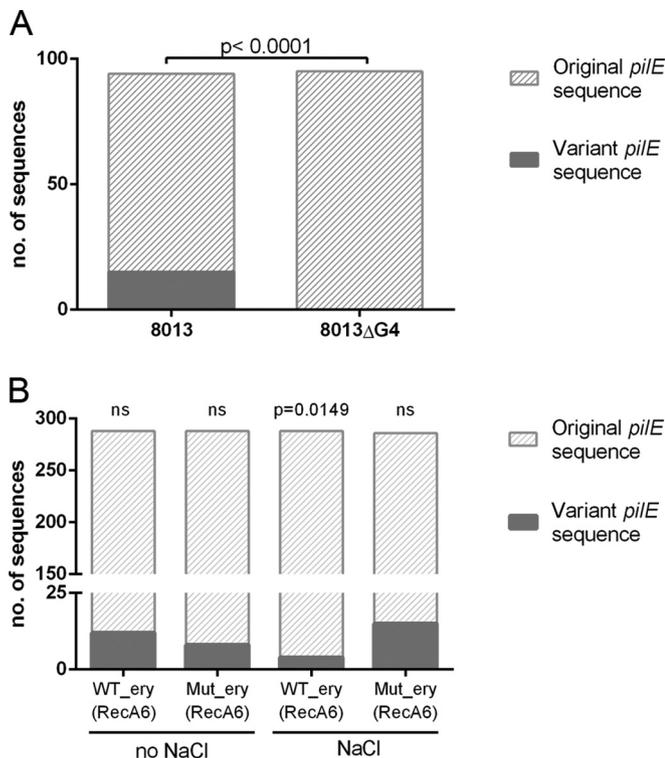
**FIG 5** Effect of the AS RNA on *pilE* mRNA and PilE protein levels in *N. meningitidis*. (A) Northern blot analysis of RNA from WT\_ery and Mut\_ery taken at different time points during growth (shown in Fig. 3A). *pilE* transcript was detected using the *pilE*\_probe. tmRNA was used as a loading control. (B) Detection of *pilE* transcript in *N. meningitidis* with and without NaCl stress. Average *R* values were calculated and analyzed as described in the text. The *pilE* transcript was downregulated under NaCl stress in both WT\_ery and Mut\_ery. (C) Strand-specific qRT-PCR analysis of *pilE* mRNA levels in WT\_ery and Mut\_ery with and without NaCl stress. Average *R* values were calculated and analyzed as described in the text. The *pilE* transcript was downregulated under NaCl stress in both WT\_ery and Mut\_ery. (D) Western blot analysis of whole-cell lysates of WT\_ery and Mut\_ery taken at different time points during growth. GroEL was used as a loading control. (E) Western blot analysis results for WT\_ery and Mut\_ery with and without NaCl stress. GroEL was used as a loading control. Mutation of the AS promoter or NaCl stress did not result in a detectable difference in PilE protein levels.

(<http://pubmlst.org/neisseria/>), the position and sequence of the *pilE* AS promoter identified in this work are conserved, despite variation in the *pilE* coding sequence (data not shown).

*cis*-encoded AS RNAs are abundant in bacteria (33), and 260 such RNAs were recently identified in a transcriptomic study of RNAs that are differentially regulated upon exposure of *N. meningitidis* to human blood (42). Interestingly, the *pilE* AS RNA was not identified in that study, consistent with our observation that the expression is regulated by specific conditions. We detected the AS transcript in late stationary phase or following a period of salt stress. Upregulation by salt was specific to NaCl and KCl, and not sucrose, implying the AS RNA is not induced by osmotic stress. The observation that salt also increases the promoter activity in *E. coli* indicates that the mechanisms may be common to both species, although the molecular basis of salt regulation of the AS RNA is not yet known. Bacterial sRNAs can be regulated in response to different environmental stresses. For example, the *trans*-encoded sRNAs DsrA and RprA, which are upregulated in *E. coli* by low-temperature and cell surface stress, respectively (43). In *N. meningitidis* the small RNA AniS is anaerobically induced (44), while NrrF is expressed by meningococcus under iron-depleted conditions (45).

*cis*-encoded AS RNAs have exact complementarity to the sense transcript and therefore often regulate the expression of the gene encoded on the opposite DNA strand. In most cases, this requires an RNA-RNA interaction and can result from transcription inhibition, alteration of transcript stability, or inhibition of translation (33). To date there has only been a single report of sRNA-mediated regulation of pili in bacteria. The sRNA FasX downregulates pilus expression in group A streptococci by targeting and destabilizing mRNA from the pilus biogenesis operon (46). Our work revealed that in *N. meningitidis*, *pilE* mRNA is detected throughout growth *in vitro* in both the wild type and the strain lacking the AS RNA, suggesting that the AS RNA is not required for stabilization of the *pilE* message. Furthermore, our results indicated that the *pilE* transcript is not destabilized by the AS RNA, as there was no significant AS RNA-dependent change in *pilE* transcript levels either in the mutant or when the AS was induced.

We considered that the AS RNA might alter pilin at the translational level. Our mapping experiments showed that the AS RNA includes the 5' UTR of the *pilE* transcript. Noncoding RNAs can prevent translation by pairing with this region of mRNAs and affecting ribosome binding (47). Based on Western blotting, there was no difference in the amount of pilin in whole-cell lysates in the



**FIG 6** Effects on antigenic variation of AS mutation and AS induction under salt stress. (A) Av assays were performed with wild-type *N. meningitidis* strain 8013 and an isogenic mutant with a kanamycin resistance cassette inserted into the guanine quartet (G4) sequence. *pilE* sequences obtained were compared to the potential *pilS* donor sequences, and the number of variation events was calculated. No *pilE* variation events were detected in the strain with the G4 mutation. (B) Av assays were performed on WT\_ery(RecA6) and Mut\_ery(RecA6) with and without salt stress. Results were analyzed by fitting generalized linear models. NaCl stress resulted in a statistically significant reduction in antigenic variation in WT\_ery(RecA6) ( $P = 0.0149$ ) but not in Mut\_ery(RecA6).

presence or absence of the AS RNA. Of note, however, we did observe that even when a decrease in the amount of *pilE* transcript was detected by Northern blotting following salt stress, there was no corresponding decrease in pilin protein levels detected in cell lysates. Upon expression, pilins are inserted into the inner membrane before cleavage by a prepilin peptidase and assembly into Tfp (48), which may constitute a stable pool of pilin. As such, further work is required to rule out any influence of AS RNA on pilin expression. For example, a sustained high level of AS RNA may impact the abundance of pilin. Consistent with this, piliation and pilin levels in *N. gonorrhoeae* are reduced during growth in 300 mM KCl or NaCl (49).

While our results do not provide any evidence for the AS RNA affecting *pilE* or pilin, this could be related to relative levels of the sense and AS transcripts. We estimate that the *pilE* transcript is over 200 times more abundant than the AS RNA, even when AS is induced by salt stress. Therefore, it is possible that the expression of *pilE* mRNA does in fact dictate AS RNA levels. Our data show that *pilE* mRNA is detected throughout growth, but when *pilE* transcript levels decrease (such as following salt stress or after overnight growth), the AS RNA levels increase. This increase may result from both increased AS promoter activity and the concomitant reduction of *pilE* transcription. Changes in the ratio of

sense/AS transcripts, either in response to environmental cues or as governed by *pilE* transcript levels, could serve as a mechanism of regulation of AS functions.

The promoter of the G4-associated sRNA in *N. gonorrhoeae* is required for Av of *pilE* and has been proposed to result in transcription of an RNA that promotes G4 formation (24). In our assays, under AS-inducing conditions, we observed a significant decrease in *pilE* variation in the wild-type strain, which was not observed in the mutant. This suggested that, in contrast to the loss of the G4-associated sRNA which abolishes Av, AS RNA transcript or transcription modulates *pilE* variation. The mechanism by which this occurs is not yet known. Our mapping experiments revealed that the AS transcript 3' end overlaps with the promoter of the G4-associated RNA. Overlapping transcription units can influence the expression of the downstream transcript, through promoter occlusion (50). Therefore, enhanced transcription of the AS RNA could interfere with the transcription of the G4-associated sRNA and consequently reduce the efficacy or frequency of G4 formation, and thus affect *pilE* variation frequency. Further work is under way to examine this possibility.

Like many noncoding RNAs, the AS RNA appears to be specifically expressed under certain conditions. As discussed, high concentrations of NaCl were used to induce AS RNA expression in this study. This level of salt is nonphysiological, although certain chronic inflammatory airway diseases, such as cystic fibrosis or rhinitis, have been reported to increase Na and Cl ion concentrations up to almost 300 mM in airway surface liquid (51). More importantly, we found that the AS RNA is expressed when bacteria are in stationary phase. This may resemble the environments that meningococci would encounter *in vivo*, for example competitive conditions of nutrient depletion, high cell density, and oxygen limitation (52). Little is known currently about the impact of the microenvironment on pilin expression and variation in the meningococcus. In *N. gonorrhoeae*, Av rates are not affected by temperature, carbon source, or oxygen availability, but an increase in Av frequency was observed when bacteria were grown under iron-starved conditions (53). Pilin variation is thought to be a key mechanism of immune escape, as well as impacting Tfp functions (5, 6, 14, 16, 17). Understanding the conditions which govern and modulate this sophisticated molecular process is therefore important. As is true for many noncoding RNAs, the precise role and

**TABLE 3** *pilE* sequence variants detected in Av assays in liquid media

Strain/presence of NaCl	Assay replicate no.	Total no. of variants sequenced	No. of different <i>pilE</i> sequences	
			Per assay/strain	Per strain
WT_ery (RecA6)/–	1	96	5	12
	2	96	3	
	3	96	4	
Mut_ery (RecA6)/–	1	96	2	8
	2	96	3	
	3	96	3	
WT_ery (RecA6)/+	1	96	0	4
	2	96	1	
	3	96	3	
Mut_ery (RecA6)/+	1	96	7	15
	2	94	4	
	3	96	4	

mechanism of action of the AS RNA in Av remains elusive, but our work has identified a novel noncoding RNA in the *pilE* locus in *N. meningitidis* which influences Av in this important human pathogen. This study provides further insight into the complexity of pilin regulation and variation in pathogenic *Neisseria* spp.

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## REFERENCES

- Trivedi K, Tang CM, Exley RM. 2011. Mechanisms of meningococcal colonisation. *Trends Microbiol* 19:456–463. <http://dx.doi.org/10.1016/j.tim.2011.06.006>.
- Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, Tainer JA. 2006. Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol Cell* 23:651–662. <http://dx.doi.org/10.1016/j.molcel.2006.07.004>.
- Carbannelle E, Hill DJ, Morand P, Griffiths NJ, Bourdoulous S, Murillo I, Nassif X, Virji M. 2009. Meningococcal interactions with the host. *Vaccine* 27(Suppl 2):B78–B89. <http://dx.doi.org/10.1016/j.vaccine.2009.04.069>.
- Nassif X, Beretti JL, Lowy J, Stenberg P, O'Gaora P, Pfeifer J, Normark S, So M. 1994. Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. *Proc Natl Acad Sci U S A* 91:3769–3773. <http://dx.doi.org/10.1073/pnas.91.9.3769>.
- Rayner CF, Dewar A, Moxon ER, Virji M, Wilson R. 1995. The effect of variations in the expression of pili on the interaction of *Neisseria meningitidis* with human nasopharyngeal epithelium. *J Infect Dis* 171:113–121. <http://dx.doi.org/10.1093/infdis/171.1.113>.
- Virji M, Alexandrescu C, Ferguson DJ, Saunders JR, Moxon ER. 1992. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. *Mol Microbiol* 6:1271–1279. <http://dx.doi.org/10.1111/j.1365-2958.1992.tb00848.x>.
- Mikaty G, Soyer M, Mairey E, Henry N, Dyer D, Forest KT, Morand P, Guadagnini S, Prevost MC, Nassif X, Dumenil G. 2009. Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. *PLoS Pathog* 5:e1000314. <http://dx.doi.org/10.1371/journal.ppat.1000314>.
- Bernard SC, Simpson N, Join-Lambert O, Federici C, Laran-Chich MP, Maissa N, Bouzinba-Segard H, Morand PC, Chretien F, Taouji S, Chevet E, Janel S, Lafont F, Coureuil M, Segura A, Niedergang F, Marullo S, Couraud PO, Nassif X, Bourdoulous S. 2014. Pathogenic *Neisseria meningitidis* utilizes CD147 for vascular colonization. *Nat Med* 20:725–731. <http://dx.doi.org/10.1038/nm.3563>.
- Coureuil M, Mikaty G, Miller F, Lecuyer H, Bernard C, Bourdoulous S, Dumenil G, Mege RM, Weksler BB, Romero IA, Couraud PO, Nassif X. 2009. Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science* 325:83–87. <http://dx.doi.org/10.1126/science.1173196>.
- Seifert HS, Ajioka RS, Paruchuri D, Heffron F, So M. 1990. Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. *J Bacteriol* 172:40–46.
- Diaz JL, Virji M, Heckels JE. 1984. Structural and antigenic differences between 2 types of meningococcal pili. *FEMS Microbiol Lett* 21:181–184. <http://dx.doi.org/10.1111/j.1574-6968.1984.tb00207.x>.
- Wormann ME, Horien CL, Bennett JS, Jolley KA, Maiden MC, Tang CM, Aho EL, Exley RM. 2014. Sequence, distribution and chromosomal context of class I and class II pilin genes of *Neisseria meningitidis* identified in whole genome sequences. *BMC Genomics* 15:253. <http://dx.doi.org/10.1186/1471-2164-15-253>.
- Helm RA, Seifert HS. 2010. Frequency and rate of pilin antigenic variation of *Neisseria meningitidis*. *J Bacteriol* 192:3822–3823. <http://dx.doi.org/10.1128/JB.00280-10>.
- Nassif X, Lowy J, Stenberg P, O'Gaora P, Ganji A, So M. 1993. Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol Microbiol* 8:719–725. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb01615.x>.
- Virji M, Saunders JR, Sims G, Makepeace K, Maskell D, Ferguson DJ. 1993. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Mol Microbiol* 10:1013–1028. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb00972.x>.
- Miller F, Phan G, Brissac T, Bouchiat C, Lioux G, Nassif X, Coureuil M. 2014. The hypervariable region of meningococcal major pilin PilE controls the host cell response via antigenic variation. *mBio* 5(1):e01024–13. <http://dx.doi.org/10.1128/mBio.01024-13>.
- Hubert K, Pawlik MC, Claus H, Jarva H, Meri S, Vogel U. 2012. Opc expression, LPS immunotype switch and pilin conversion contribute to serum resistance of unencapsulated meningococci. *PLoS One* 7:e45132. <http://dx.doi.org/10.1371/journal.pone.0045132>.
- Haas R, Meyer TF. 1986. The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell* 44:107–115. [http://dx.doi.org/10.1016/0092-8674\(86\)90489-7](http://dx.doi.org/10.1016/0092-8674(86)90489-7).
- Hagblom P, Segal E, Billyard E, So M. 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. *Nature* 315:156–158. <http://dx.doi.org/10.1038/315156a0>.
- Koomey M, Gotschlich EC, Robbins K, Bergström S, Swanson J. 1987. Effects of *recA* mutations on pilus antigenic variation and phase transitions in *Neisseria gonorrhoeae*. *Genetics* 117:391–398.
- Mehr IJ, Seifert HS. 1997. Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol Microbiol* 23:1121–1131. <http://dx.doi.org/10.1046/j.1365-2958.1997.2971660.x>.
- Cahoon LA, Seifert HS. 2009. An alternative DNA structure is necessary for pilin antigenic variation in *Neisseria gonorrhoeae*. *Science* 325:764–767. <http://dx.doi.org/10.1126/science.1175653>.
- Wainwright LA, Pritchard KH, Seifert HS. 1994. A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. *Mol Microbiol* 13:75–87. <http://dx.doi.org/10.1111/j.1365-2958.1994.tb00403.x>.
- Cahoon LA, Seifert HS. 2013. Transcription of a cis-acting, noncoding, small RNA is required for pilin antigenic variation in *Neisseria gonorrhoeae*. *PLoS Pathog* 9:e1003074. <http://dx.doi.org/10.1371/journal.ppat.1003074>.
- Rendon MA, Hockenberry AM, McManus SA, So M. 2013. Sigma factor RpoN ( $\sigma^{54}$ ) regulates *pilE* transcription in commensal *Neisseria elongata*. *Mol Microbiol* 90:103–113. <http://dx.doi.org/10.1111/mmi.12350>.
- Deghmane AE, Giorgini D, Larribe M, Alonso JM, Taha MK. 2002. Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. *Mol Microbiol* 43:1555–1564. <http://dx.doi.org/10.1046/j.1365-2958.2002.02838.x>.
- Kuwaie A, Sjolinder H, Eriksson J, Eriksson S, Chen Y, Jonsson AB. 2011. NafA negatively controls *Neisseria meningitidis* piliation. *PLoS One* 6:e21749. <http://dx.doi.org/10.1371/journal.pone.0021749>.
- Pannekoek Y, Huis in 't Veld R, Hopman CT, Langerak AA, Speijer D, van der Ende A. 2009. Molecular characterization and identification of proteins regulated by Hfq in *Neisseria meningitidis*. *FEMS Microbiol Lett* 294:216–224. <http://dx.doi.org/10.1111/j.1574-6968.2009.01568.x>.
- Fantappie L, Metruccio MM, Seib KL, Oriente F, Cartocci E, Ferlicca F, Giuliani MM, Scarlato V, Delany I. 2009. The RNA chaperone Hfq is involved in stress response and virulence in *Neisseria meningitidis* and is a pleiotropic regulator of protein expression. *Infect Immun* 77:1842–1853. <http://dx.doi.org/10.1128/IAI.01216-08>.
- De Lay N, Schu DJ, Gottesman S. 2013. Bacterial small RNA-based negative regulation: Hfq and its accomplices. *J Biol Chem* 288:7996–8003. <http://dx.doi.org/10.1074/jbc.R112.441386>.
- Ross JA, Ellis MJ, Hossain S, Haniford DB. 2013. Hfq restructures RNA-IN and RNA-OUT and facilitates antisense pairing in the Tn10/IS10 system. *RNA* 19:670–684. <http://dx.doi.org/10.1261/rna.037747.112>.
- Georg J, Hess WR. 2011. *cis*-antisense RNA, another level of gene regulation in bacteria. *Microbiol Mol Biol Rev* 75:286–300. <http://dx.doi.org/10.1128/MMBR.00032-10>.
- Sesto N, Wurtzel O, Archambaud C, Sorek R, Cossart P. 2013. The excludon: a new concept in bacterial antisense RNA-mediated gene

- regulation. *Nat Rev Microbiol* 11:75–82. <http://dx.doi.org/10.1038/nmicro2934>.
34. Orosz A, Boros I, Venetianer P. 1991. Analysis of the complex transcription termination region of the *Escherichia coli* *rrnB* gene. *Eur J Biochem* 201:653–659. <http://dx.doi.org/10.1111/j.1432-1033.1991.tb16326.x>.
  35. Sambrook J, Russell DW. 2001. *Molecular cloning: A laboratory manual*, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.
  36. Virji M, Heckels JE, Potts WJ, Hart CA, Saunders JR. 1989. Identification of epitopes recognized by monoclonal antibodies SM1 and SM2 which react with all pili of *Neisseria gonorrhoeae* but which differentiate between two structural classes of pili expressed by *Neisseria meningitidis* and the distribution of their encoding sequences in the genomes of *Neisseria* spp. *J Gen Microbiol* 135:3239–3251.
  37. West S, Zaret K, Proudfoot NJ. 2006. Transcriptional termination sequences in the mouse serum albumin gene. *RNA* 12:655–665. <http://dx.doi.org/10.1261/rna.2232406>.
  38. McCullagh P, Nelder JA. 1989. *Generalized linear models*, 2nd ed. Chapman & Hall, Boca Raton, FL.
  39. Venables WN, Ripley BD. 2002. *Modern applied statistics with S*. Springer-Verlag, New York, NY.
  40. Rusniok C, Vallenet D, Floquet S, Ewles H, Mouze-Soulama C, Brown D, Lajus A, Buchrieser C, Medigue C, Glaser P, Pelicic V. 2009. NeMeSys: a biological resource for narrowing the gap between sequence and function in the human pathogen *Neisseria meningitidis*. *Genome Biol* 10:R110. <http://dx.doi.org/10.1186/gb-2009-10-10-r110>.
  41. Remmele CW, Xian Y, Albrecht M, Faulstich M, Fraunholz M, Heinrichs E, Dittrich MT, Muller T, Reinhardt R, Rudel T. 2014. Transcriptional landscape and essential genes of *Neisseria gonorrhoeae*. *Nucleic Acids Res* 42:10579–10595. <http://dx.doi.org/10.1093/nar/gku762>.
  42. Del Tordello E, Bottini S, Muzzi A, Serruto D. 2012. Analysis of the regulated transcriptome of *Neisseria meningitidis* in human blood using a tiling array. *J Bacteriol* 194:6217–6232. <http://dx.doi.org/10.1128/JB.01055-12>.
  43. McCullen CA, Benhammou JN, Majdalani N, Gottesman S. 2010. Mechanism of positive regulation by DsrA and RprA small noncoding RNAs: pairing increases translation and protects *rpoS* mRNA from degradation. *J Bacteriol* 192:5559–5571. <http://dx.doi.org/10.1128/JB.00464-10>.
  44. Fantappiè L, Oriente F, Muzzi A, Serruto D, Scarlato V, Delany I. 2011. A novel Hfq-dependent sRNA that is under FNR control and is synthesized in oxygen limitation in *Neisseria meningitidis*. *Mol Microbiol* 80:507–523. <http://dx.doi.org/10.1111/j.1365-2958.2011.07592.x>.
  45. Mellin JR, Goswami S, Grogan S, Tjaden B, Genco CA. 2007. A novel Fur- and iron-regulated small RNA, NrrF, is required for indirect Fur-mediated regulation of the *sdhA* and *sdhC* genes in *Neisseria meningitidis*. *J Bacteriol* 189:3686–3694. <http://dx.doi.org/10.1128/JB.01890-06>.
  46. Liu Z, Trevino J, Ramirez-Pena E, Sumbly P. 2012. The small regulatory RNA FasX controls pilus expression and adherence in the human bacterial pathogen group A streptococcus. *Mol Microbiol* 86:140–154. <http://dx.doi.org/10.1111/j.1365-2958.2012.08178.x>.
  47. Mustachio LM, Aksit S, Mistry RH, Scheffler R, Yamada A, Liu JM. 2012. The *Vibrio cholerae* mannitol transporter is regulated posttranscriptionally by the MtlS small regulatory RNA. *J Bacteriol* 194:598–606. <http://dx.doi.org/10.1128/JB.06153-11>.
  48. Craig L, Pique ME, Tainer JA. 2004. Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol* 2:363–378. <http://dx.doi.org/10.1038/nrmicro885>.
  49. Larribe M, Taha MK, Topilko A, Marchal C. 1997. Control of *Neisseria gonorrhoeae* pilin gene expression by environmental factors: involvement of the *pilA/pilB* regulatory genes. *Microbiology* 143:1757–1764. <http://dx.doi.org/10.1099/00221287-143-5-1757>.
  50. Palmer AC, Egan JB, Shearwin KE. 2011. Transcriptional interference by RNA polymerase pausing and dislodgement of transcription factors. *Transcription* 2:9–14. <http://dx.doi.org/10.4161/trns.2.1.13511>.
  51. Vanthanouvong V, Kozlova I, Johannesson M, Nääs E, Nordvall SL, Dragomir A, Roomans GM. 2006. Composition of nasal airway surface liquid in cystic fibrosis and other airway diseases determined by X-ray microanalysis. *Microsc Res Tech* 69:271–276. <http://dx.doi.org/10.1002/jemt.20310>.
  52. Navarro Llorens JM, Tormo A, Martínez-García E. 2010. Stationary phase in gram-negative bacteria. *FEMS Microbiol Rev* 34:476–495. <http://dx.doi.org/10.1111/j.1574-6976.2010.00213.x>.
  53. Serkin CD, Seifert HS. 2000. Iron availability regulates DNA recombination in *Neisseria gonorrhoeae*. *Mol Microbiol* 37:1075–1086. <http://dx.doi.org/10.1046/j.1365-2958.2000.02058.x>.
  54. Jolley KA, Maiden MCJ. 2010. BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. <http://dx.doi.org/10.1186/1471-2105-11-595>.