

# H-NS suppresses *pilE* intragenic transcription and antigenic variation in *Neisseria gonorrhoeae*

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Initially, *pilE* transcription in *Neisseria gonorrhoeae* appeared to be complicated, yet it was eventually simplified into a model where integration host factor activates a single  $-35/-10$  promoter. However, with the advent of high-throughput RNA sequencing, numerous small *pil*-specific RNAs (sense as well as antisense) have been identified at the *pilE* locus as well as at various *pilS* loci. Using a combination of *in vitro* transcription, site-directed mutagenesis, Northern analysis and quantitative reverse transcriptase PCR (qRT-PCR) analysis, we have identified three additional non-canonical promoter elements within the *pilE* gene; two are located within the midgene region (one sense and one antisense), with the third, an antisense promoter, located immediately downstream of the *pilE* ORF. Using strand-specific qRT-PCR analysis, an inverse correlation exists between the level of antisense expression and the amount of sense message. By their nature, promoter sequences tend to be AT-rich. In *Escherichia coli*, the small DNA-binding protein H-NS binds to AT-rich sequences and inhibits intragenic transcription. In *N. gonorrhoeae* *hns* mutants, *pilE* antisense transcription was increased twofold, with a concomitant decrease in sense transcript levels. However, most noticeably in these mutants, the absence of H-NS protein caused *pilE/pilS* recombination to increase dramatically when compared with WT values. Consequently, H-NS protein suppresses *pilE* intragenic transcription as well as antigenic variation through the *pilE/pilS* recombination system.

## INTRODUCTION

*Neisseria gonorrhoeae* (gonococcus) is a Gram-negative diplococcus and the aetiological agent of the human sexually transmitted disease gonorrhoea. Gonorrhoea is generally characterized by a pustular discharge due to neutrophil infiltration. However, if left untreated, the infection can lead to more severe sequelae such as bacteraemia, sterility and pelvic inflammatory disease. Pathogenesis associated with a gonococcal infection is best characterized by the sophistication of its major virulence factors that are employed to survive and reproduce within the human host. The elaboration of type IV pili on the cell surface appears to be particularly important as the infectivity of pilus-minus bacteria is severely reduced (Hill & Davies, 2009).

The major protein component of the pilus organelle is PilE polypeptide, which is encoded by the *pilE* gene. Initially, regulation of *pilE* gene expression appeared complex, as

there are three functional promoters (P1, P2 and P3), with the P1 and P2 promoters utilizing the  $\sigma^{70}$  sigma factor, in contrast to P3, which is recognized by the  $\sigma^{54}$  sigma factor (Fyfe *et al.*, 1995). However, in the gonococcus, only the P1 promoter is actively transcribed. In addition, there appeared to be an absence of transcription factors that provided either positive or negative regulation. However, recently CrgA (contact-regulated gene A) has been implicated in maintaining *pilE* transcript levels in the gonococcus in both a contact-dependent and -independent manner (Matthias & Rest, 2014). However, these observations are at odds with a study involving *Neisseria meningitidis*, where CrgA protein did not appear to regulate pilus expression, despite the fact that it had been shown to be an active transcriptional regulator in this organism (Ieva *et al.*, 2005).

What is firmly established in the gonococcus is the role played by integration host factor (IHF) on *pilE* expression. Upstream of the *pilE* gene resides an IHF-binding site, and, in gonococcal mutants where the IHF-binding site has been deleted, *pilE* transcription is reduced approximately 10-fold (Hill *et al.*, 1997). These observations were further embellished through the identification of two AT-rich segments of DNA (UP-like elements) that flank the IHF-binding site and are both required for optimum transcription

Abbreviations: H-NS, histone-like nucleoid structuring protein; IHF, integration host factor; qRT-PCR, quantitative reverse transcriptase PCR; sRNA, small RNA.

Six supplementary figures are available with the online Supplementary Material.

(Fyfe & Davies, 1998). IHF binds to a consensus DNA sequence, and, when bound, causes a bend in the surrounding DNA, which is believed to facilitate contact of the UP-like elements with RNA polymerase, thus potentiating transcription. Recently, small RNA (sRNA) regulation has also been considered as a means by which *pilE* expression is modulated. At the time, no sRNA had been identified that theoretically could hybridize with the *pilE* transcript, yet PilE polypeptide was found to be absent in *N. gonorrhoeae* *hfq* mutants, suggesting that the Hfq RNA chaperone is required for efficient PilE translation, and, by implication, such regulation involves a potential sRNA molecule (Dietrich *et al.*, 2009). Subsequently, *pilE*-specific antisense RNA has been identified in the gonococcus (Wachter & Hill, 2015). Consequently, this antisense RNA may mediate translation efficiency; however, this has not yet been formally tested.

With the advent of high-throughput RNA sequencing, transcriptome analysis, principally using Artemis and RNA-Seq technologies, has allowed the identification of many previously unknown sRNAs within a cell. Recently, gonococcal small transcriptome analysis revealed that the majority of genes within the chromosome were associated with sense and/or antisense intragenic sRNAs (Wachter & Hill, 2015). Moreover, many other sRNAs were also found within the chromosomal intergenic regions (Wachter & Hill, 2015). Consequently, gonococci exhibit the recently described phenomenon known as pervasive transcription (Sorek & Cossart, 2010; Wade & Grainger, 2014; Wachter & Hill, 2015; Wachter *et al.*, 2015). An unusual aspect of this type of transcription is the use of non-canonical promoters that deviate from the accepted rules governing transcription. An in-depth analysis also showed that the majority of the *pil* genes in the chromosome also produced sense and antisense sRNAs, with sRNAs also observed at the *pilE* locus (Wachter *et al.*, 2015). Indeed, antisense sRNA appears to be the predominant sRNA species that is observed at *pilE*. Therefore, given these observations, the purpose of this study was to further examine transcription at *pilE* with the view of identifying these new putative *pilE* promoter elements and determining the biological consequence(s), if any, for sense and antisense *pilE* transcription. In this report we have identified three new promoter elements within *pilE* (one yielding a sense sRNA and two yielding antisense sRNAs) and have shown through mutational analysis in the gonococcus that transcription from these novel promoters appears to

modulate the level of the *pilE* sense transcript. Furthermore, we also observed that the small DNA-binding protein histone-like nucleoid structuring protein (H-NS) changed the level of *pilE* intragenic transcription within the cell, and, more dramatically, influenced the level of *pilE/pilS* recombination.

## METHODS

*N. gonorrhoeae* strain MS11 *pilE*<sub>7:30:2</sub> (Bergström *et al.*, 1986) was grown at 37 °C in a 5 % CO<sub>2</sub> atmosphere on a previously described growth medium (Swanson, 1982). When appropriate, antibiotics were added to the growth medium: erythromycin at a final concentration of 5 µg ml<sup>-1</sup>, chloramphenicol at a final concentration of 10 µg ml<sup>-1</sup> and kanamycin at a final concentration of 80 µg ml<sup>-1</sup>.

All recombinant work utilized *Escherichia coli* DH5α, which was grown in LB medium at 37 °C. Antibiotics were added to the medium when appropriate: carbenicillin at a final concentration of 100 µg ml<sup>-1</sup> and kanamycin at a final concentration of 50 µg ml<sup>-1</sup>.

**In vitro transcription.** *In vitro* transcription was performed as follows: 3 µg purified template DNA was resuspended in 8 µl 5 × RNA polymerase transcription buffer (0.2 M Tris/HCl, 0.75 M KCl, 50 mM MgCl<sub>2</sub> and 0.05 % Triton X-100; Epicentre), 2 µl 100 mM DTT, 4 µl rNTPs (1 µl each 100 mM rATP, rUTP, rCTP and rGTP), 1 µl RNase inhibitor (20 U µl<sup>-1</sup>; Promega), 4 µl *E. coli* RNA polymerase holoenzyme (1 U µl<sup>-1</sup>; Epicentre) and sufficient diethyl pyrocarbonate (DEPC)-treated water to bring the total volume of the reaction to 40 µl. The reaction was then incubated at 37 °C for 2 h. Template DNA was removed by an overnight *Hae*III enzymic digestion at 37 °C. The restriction-digested *in vitro* transcription reaction was then treated with 6 µl 10 × RQ1 DNase buffer (Promega) and 4 µl RQ1 DNase (1 U µl<sup>-1</sup>; Promega) and subsequently incubated for 3 h at 37 °C. This was followed by the addition of 4 µl RQ1 DNase Stop Solution (Promega) and incubation for 10 min in a 65 °C water bath, followed by addition of 36 µl DEPC-treated water. The RNA was then purified as described below. Quantification of *in vitro*-transcribed RNA utilized a NanoDrop ND-2000 1-Position Spectrophotometer (Fisher Scientific).

**RNA analysis.** RNA isolation, size fractionation, standard quantitative reverse transcriptase PCR (qRT-PCR) analysis, and RNA circularization experiments have been described (Wachter & Hill, 2015; Wachter *et al.*, 2015). Oligonucleotide sequences for primers that were used in the qRT-PCR analysis are presented in Tables 1 and 2. For strand-specific qRT-PCR analysis, the initial cDNA reaction utilized a single specific oligonucleotide primer that was complementary to either sense or antisense RNA (the various oligonucleotide sequences are in Table 3). The cDNA sample was then denatured followed by the addition of RNaseA to remove the RNA from the DNA/RNA hybrid. The remaining single-stranded cDNA product was then amplified with *pilE*-specific primer pairs. In control experiments

**Table 1.** Oligonucleotides used in reverse transcription of *pilE* RNAs

Oligonucleotide	Description	Sequence
14728	Random decamer	5'-NNNNNNNNNN-3'
07053	RNA3 reverse primer	5'-GATGTAGCCCATGAGAGCCAAGCAGATACGTGTG-3'
09085	<i>pilE</i> 3' end primer	5'-CGGCAGGTTGACGGCAGGTGCTTGGTG-3'
RC3	<i>pilE</i> 5' end primer	5'-GTCGGCATTGCGGCAGTCG-3'

**Table 2.** Control primers used in qRT-PCR

Primer	Description	Nucleotide sequence
07052	RNA3 forward primer	5'-GGCAAGCAAGCGGGAGGTACTAGCGATGAGAAGC-3'
07053	RNA3 reverse primer	5'-TGGATGTAGCCCATGAGAGCCAAGCAGATACGTGTG-3'
06971	<i>E. coli rrsA</i> (16S rRNA) forward	5'-CGGTGGAGCATGTGGTTTAA-3'
06972	<i>E. coli rrsA</i> (16S rRNA) reverse	5'-GAAAACCTTCCGTGGATGTCAAGA-3'
09484	GC 16S rRNA forward	5'-GTTATCCCAGGAGGCTGCCCTCGCCATCGGTATTCC-3'
09485	GC 16S rRNA reverse	5'-GTAATACGTAGGGTGGCAGCGTTAATCGGAATTACTGGG-3'
09170	GC <i>recA</i> forward	5'-GCCATCATGAAAATGGACGGCAGCCAGCAGGAAG-3'
09171	GC <i>recA</i> reverse	5'-CTGGCATTGGGCGACGGCTTCGAGGCAGAGTGTGG-3'

with gonococcal RNA extracts, a potential RNA secondary structure inhibitor, betaine, was tested. However, with these studies self-priming became evident in the strand-specific scores (data not shown). Therefore, an important consideration in strand-specific qRT-PCR was assessing self-priming of the RNA transcripts at the 5' or 3' end, as further control experiments indicated that reverse transcriptase utilized the looped-back segment of RNA as a primer in cDNA synthesis to a significant degree. Consequently, for the final cycle threshold ( $C_t$ ) values from the strand-specific reactions, no primer controls (which reflect self-priming) were subtracted from the actual strand-specific  $C_t$  score. The viability of this approach was initially tested on *in vitro* transcription reactions, where it was hypothesized that subtraction of the no-primer expression value from the overall strand-specific cDNA values obtained utilizing a specific primer should yield approximate expression levels comparable with cDNA synthesized using a random oligonucleotide. The results (data not shown) of such a comparison from randomly primed, specifically primed and non-primed cDNAs from an *in vitro*-transcribed RNA template confirmed that removing the expression value from a no-primer control allows strand-specific cDNA synthesis to be a viable method of quantifying RNA from either strand at any given location along *pilE*. The Northern blotting protocol that was used has been published previously (Hill *et al.*, 1990, 1997). The sequence of the oligonucleotide primer designed to target *pilE*-specific antisense RNA is 5'-GUCGGCAUUUUGGCGGCAGUCG-3'.

**Site-directed mutagenesis protocol.** Site-directed mutagenesis protocols have been described previously (Wachter *et al.*, 2015). The oligonucleotide sequences that were used are presented in Table 4. The mutant sequence was designed so that it corresponded to a restriction endonuclease cleavage site for ease of analysis.

**Construction of an *N. gonorrhoeae hns* deletion strain.** In order to construct an *N. gonorrhoeae hns* insertional mutation, segments of the *hns* gene were PCR-amplified using the following primer pairs:

**Table 3.** Oligonucleotides used in *pilE* strand-specific qRT-PCR

Nucleotide	Sequence
09085	5'-CGGCAGGTTGACGGCAGGTGCTTGGTG-3'
RC3	5'-GTCGGCATTGCGCGGCAGTCG-3'
09173	5'-TTGACCTTCGGCCAAAAGGATGGCTT CGGAAAC-3'
09263	5'-CATTGTGGCGGTAACGACGCCGTT-3'
10685-tsp4	5'-CGCCGGCAGAAGTGTGTTTTC-3'

5'-GTGAGGAATACAGTGTTTAATGGTTCCGG-3' and 5'-AAGGT ACCATACATTTAAAAATACCTCGGGGCATCC-3'; and 5'-AATCT AGAGCCTATGCGCTCAAATCCTGC-3' and 5'-AAGATATCTTTT GCCGCCTTTTCAATATTCCGCC-3'. The DNA amplicons were then sequentially ligated to a kanamycin antibiotic resistance marker. The final construct was then used to transform gonococci to kanamycin drug resistance using standard protocols to generate an *N. gonorrhoeae hns* : : *kan* strain.

**Construction of gonococcal *hns* complementation strain.**

Previously, no correlation has been demonstrated between *pilE/pilS* recombination and *opa* expression (Swanson & Barrera, 1983). Therefore, a WT copy of the *hns* gene was placed into the *opaE* locus of the *hns*<sup>-</sup> strain, which resides immediately downstream of the *pilE* gene. The entire *hns* segment was PCR-amplified using the appropriate primers described above and was then ligated to a chloramphenicol drug-resistance marker that carried a transformation uptake sequence in plasmid pCR2.1. The resulting *hns* : *cat* construct was then amplified utilizing the M13 forward and reverse primers, with the resulting amplicon being phosphorylated by T4 polynucleotide kinase (New England Biolabs), which allowed for blunt-end ligation into a unique *SalI* site (blunt-ended treatment with Klenow; New England Biolabs). The resulting *hns* : *cat* : *opaE* construct was then used to transform the *N. gonorrhoeae hns*<sup>-</sup> strain to chloramphenicol/kanamycin drug resistance.

***pilE/pilS* recombination assay.** *pilE/pilS* recombination was monitored at both the DNA and RNA levels in various gonococcal strains that carried the *pilE* promoter mutations, in conjunction with either a WT or *hns* mutant genetic background. For the RNA-based recombination assay, total RNA was purified from the various gonococcal strains and quantified, followed by DNase treatment and cDNA synthesis utilizing a random decamer. The newly synthesized cDNAs were then used as templates for either end-point PCRs or qRT-PCR using a conserved *pilE* primer (RC3) and a *pilS*-specific opposing primer (either *pilS5c1* or *pilS6c1*). Recombination efficiency between *pilE* and *pilS* was determined by comparing the expression level of the *pilE/pilS* recombinant cDNAs with the expression of a *recA* internal control. For the recombination assay at the DNA level, recombination PCRs and qRT-PCRs were performed on isolated chromosomal DNAs. Each reaction utilized 120 ng DNA template and the above *pilE/pilS* primer pairs. Amplified recombinant *pilE* products were again quantified against a *recA* control signal. In addition, reactions using conserved 5' and 3' *pilE* primers (RC3 and 09085, respectively) were also included as a positive control in the assays.

**Bioinformatics analysis.** All algorithms that were used for promoter predictions, RNA-Seq analysis and Artemis GC genome analysis have been described previously (Wachter & Hill, 2015; Wachter *et al.*, 2015).

**Table 4.** Oligonucleotides used to generate mutations in *pilE* promoter regions

Oligonucleotide	Description	Sequence
12561-MutC2F	Midgene mutation	5'-CCCCCTCCGACATCAAAGGCAAATATGTGATCAAGGTTGAAGTTAAAAAC-3'
12562-MutC2R	Midgene mutation	5'-GTTTTTAACCTCAACCTTGATCACATATTTGCCTTTGATGTCGGAGGGGGG-3'
12490-P2F	3' end mutation	5'-AAGGCATCTGATGCCAAATGAGGCAAATTAGCGGATCCTTTTAAATAAAT- CAAGCGGTAAGTGATTTTCCA-3'
12491-P2R	3' end mutation	5'-TGGAAAATCACTTACCGCTTGATTTATTTAAAAGGATCCGCCTAATTTGCCT- CATTTGGCATCAGATGCCTT-3'

The NCBI GenBank accession number for the *N. gonorrhoeae* strain MS11 genome sequence that was used is CP003909.1.

## RESULTS

### Antisense RNA identified at the *pilE* locus

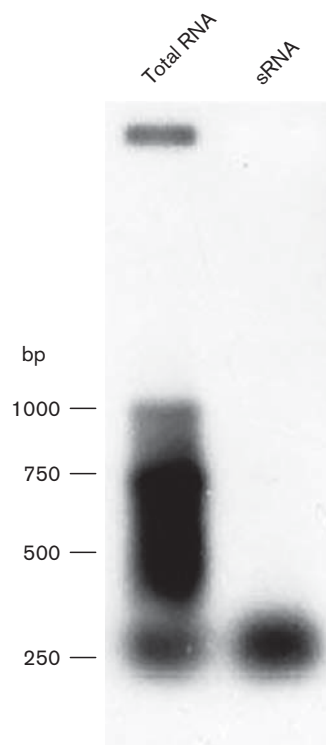
In a previous study, RNA-Seq analysis revealed the presence of *cis*-encoded sense and antisense sRNAs at the majority of loci within the gonococcal chromosome (Wachter & Hill, 2015). When similar analysis focused on the *pil* genes within the chromosome, sense and antisense sRNAs were detected at the *pilE* locus (Wachter *et al.*, 2015), with antisense RNA being observed throughout the entire *pilE* gene (Fig. S1). The presence of *pilE*-specific antisense RNA was confirmed by Northern blotting of total RNA, as well as size-fractionated sRNA, using an oligonucleotide probe designed to hybridize with *pilE*-derived antisense RNA (Fig. 1; the probe targets a unique 5' *pilE* gene sequence). As can be seen in the Northern blot, *pilE* antisense RNA varied in size from approximately 250 to 1000 bp in length. The presence of a 250 bp signal in the size-fractionated sRNA suggests a promoter yielding antisense RNA may be present internally within the *pilE* gene. As qualitatively similar results were obtained with RNA extracted from an *N. gonorrhoeae* *rppH* mutant, this suggests that the smaller RNA species observed in Fig. 1 represent true transcripts and are not a consequence of RNA degradation (S. A. Hill & J. Wachter unpublished observations; Wachter & Hill, 2015). RNA circularization experiments then identified the 3' end points of *pilE*-specific RNAs. The *pilE* sense transcript from the cognate *pilE* promoter ends within the downstream *Sma*/*Cla* repeat at a rho-independent transcription terminator (data not shown). A potential *pilE* antisense transcript was also identified with the 5' end point located within the *pilE* midgene region, with the 3' end point located 20 bp downstream from the AUG start codon at the base of a predicted stem-loop structure (Fig. S2).

### Identification of intragenic antisense promoters within *pilE*

In order to identify the *pilE* antisense promoters, we combined observations from *in vitro* transcription experiments, qRT-PCR analysis, RNA circularization and Northern blotting. From these initial studies, an antisense promoter was

believed to be located within the *pilE* midgene region, with an additional antisense promoter being located towards the 3' end of the *pilE* gene.

Several potential non-canonical midgene promoters were identified using BPROM and Neural Network online prediction tools. In order to further determine whether any of these putative promoters could be transcribed, *in vitro* transcription profiling was utilized, with a sequential series of DNA fragments as transcription templates (Fig. 2a). Each putative promoter was then changed through site-directed mutagenesis (Fig. 2b) and was reassessed for the ability to engage in transcription (Fig. 2c). The analysis was further refined by utilizing a strand-specific qRT-PCR protocol. From such analysis, it was determined that the mutated



**Fig. 1.** Northern analysis of *pilE* antisense RNA. Total RNA and size-fractionated sRNA were isolated from *N. gonorrhoeae* strain MS11. The Northern blot was then probed with an oligonucleotide designed to hybridize with *pilE*-specific antisense RNA.

promoter designated mut2 lost the ability to transcribe both sense and antisense RNA, whereas the mutated promoter designated mut5 lost the ability to transcribe sense RNA (Fig. 2c;  $P < 0.01$ ,  $n = 4$ ). These observations were then confirmed through Northern blotting with strand-specific oligonucleotides in conjunction with deletion analysis (data not shown). Therefore, the *pilE* gene appears to possess two non-canonical midgene promoters that yield both sense (5'-GTTAAAAA-3') and antisense (5'-TAACATAT-3') *pil*-specific RNA, with the midgene antisense promoter being appropriately located to account for the approximate 250 bp antisense RNA species in the Northern blot (Fig. 1).

The two *pilE* midgene promoters are located within the mc5 variable segment of the *pil* genes (Hagblom *et al.*, 1985). Consequently, as *pilE* engages in extensive recombination with the various *pilS* genes, it is possible that the midgene promoter elements could be lost as the *pilE* gene varies. The CLUSTAL analysis presented in Fig. 3 indicates that there is strong conservation of the midgene antisense promoter (promoter 2) across the various *Neisseria pil* gene copies within the database, whereas the sense promoter (promoter 5) appears to be less conserved and may be lost following *pilE/pilS* recombination (Fig. 3).

A similar strategy as outlined above was also employed to identify the 3' antisense promoter that was predicted by RNA-Seq analysis to reside between the end of the *pilE* gene and the downstream Sma/Cla repeat. Several putative promoters were predicted by BPROM analysis, with a single non-canonical promoter (-10 motif, 5'-TTTAAAG-3'; Fig. 4a) found to be transcriptionally active (Fig. 4b, c). When mutated via site-directed mutagenesis, a 90 % reduction in transcription from the mutated construct was observed compared with the non-mutated promoter (Fig. 4c;  $P < 0.001$ ,  $n = 10$ ).

Collectively, these observations indicate that the *pilE* gene contains two antisense promoters, one with the potential to yield full-length antisense RNA, and a second antisense promoter capable of producing an approximately 250 bp antisense RNA, with both antisense RNAs being theoretically able to pair with *pilE* sense RNA arising from the cognate sense promoter.

### ***pilE* antisense transcription in gonococci**

The mutated midgene and 3' antisense promoters were crossed individually, or together, into the MS11 *pilE*<sub>7/30:2</sub> gene utilizing an antibiotic resistance marker located downstream of *pilE* for the initial selection of transformants. Owing to the presence of -35/-10 elements, promoter motifs deviated from the mean chromosomal nucleotide content, which was especially evident for both the midgene and 3' antisense promoters (AT content: midgene promoter 72.5 %, 3' promoter 75 % versus cognate 65 %; Fig. 5a). The small DNA-binding protein H-NS binds to AT-rich segments of DNA and in doing so suppresses intragenic transcription (Navarre *et al.*, 2006; Singh & Grainger, 2013). In light of this, the various promoter mutations

were crossed into both a WT genetic background and an *N. gonorrhoeae hns* mutant background.

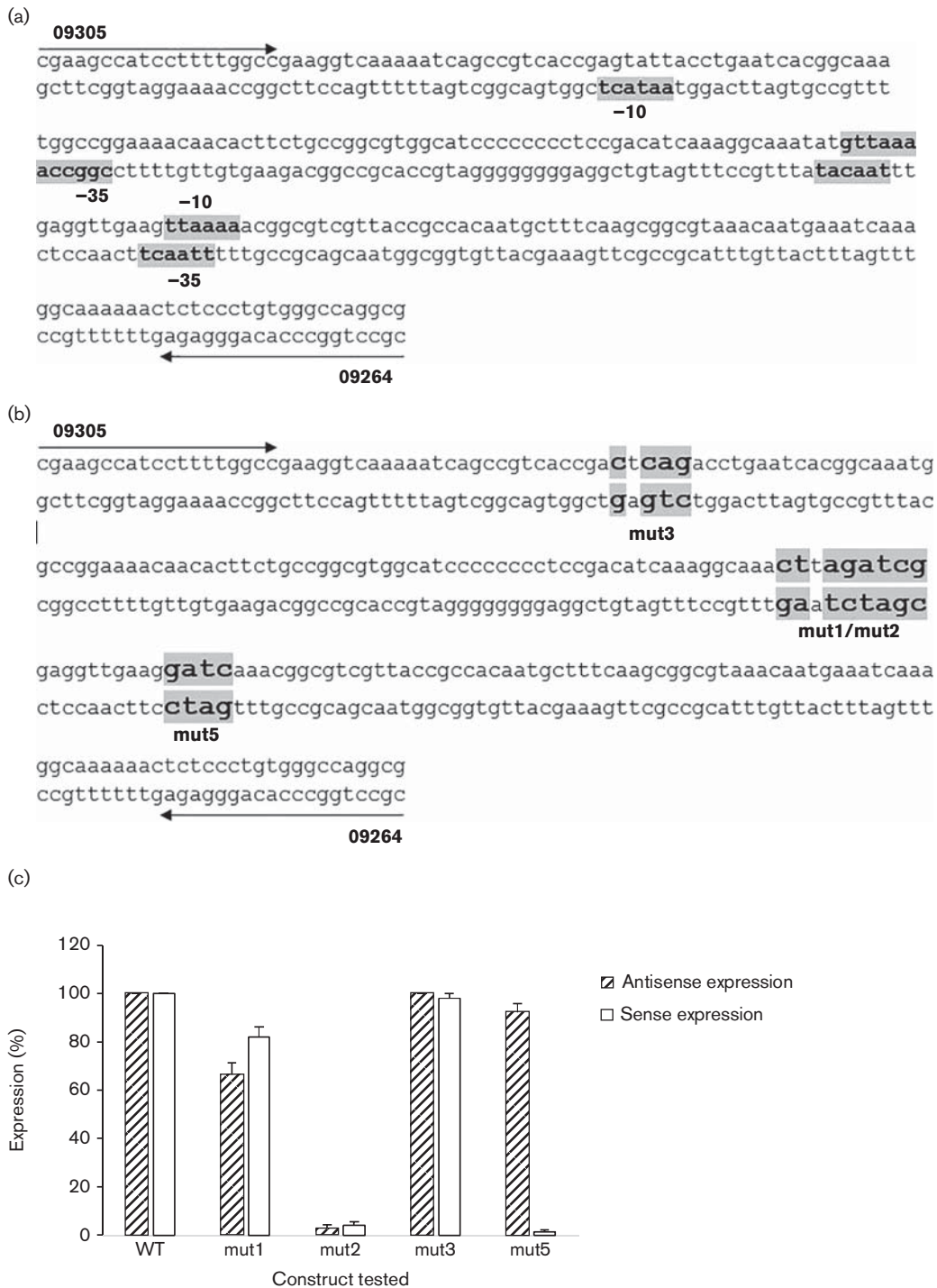
The data in Fig. 5(b, c) present strand-specific qRT-PCR analyses. In a WT genetic background, mutation of the midgene antisense promoter and the 3' antisense promoter, as well as both the midgene antisense and the 3' antisense promoters together, resulted in a reduction of *pilE* antisense RNA relative to the non-mutated promoter (Fig. 5b, left panel; midgene mutated promoter,  $P = 0.001$ ; 3' mutated promoter,  $P = 0.001$ ; both promoters mutated,  $P < 0.001$ ,  $n = 10$ ). In accordance with this reduction of antisense RNA, *pilE* sense RNA inversely increased with respect to the non-mutated promoter (Fig. 5c, left panel; midgene mutated promoter,  $P < 0.001$ ; 3' mutated promoter,  $P < 0.001$ ; both promoters mutated,  $P < 0.001$ ,  $n = 10$ ). When both antisense promoters were mutated, approximately 40 % of *pilE* antisense RNA remained compared with transcript levels from the non-mutated gene, which may reflect antisense transcription that originates from the downstream *opaE* locus (Figs 5b & S1).

In the absence of H-NS protein, a more dramatic reduction in antisense RNA is observed between the non-mutated *pilE* locus and the various *pilE* promoter mutations (Fig. 5b, right panel; midgene mutated promoter,  $P < 0.001$ ; 3' mutated promoter,  $P < 0.001$ ; both promoters mutated,  $P < 0.001$ ,  $n = 10$ ). Again, approximately 40 % *pilE* antisense RNA remains when both antisense promoters are mutated. Regarding sense expression, a perfect inverse correlation is observed with a non-mutated *pilE* gene, with a reduced inverse correlation for the various *pilE* promoter mutation combinations (Fig. 5c, right panel; midgene mutated promoter,  $P = 0.001$ ; 3' mutated promoter,  $P = 0.02$ ; both promoters mutated,  $P < 0.001$ ,  $n = 10$ ). Therefore, these data indicate an imperfect inverse correlation between *pilE* sense and antisense RNAs that manifests most evidently in a WT genetic background. The data also indicate that H-NS protein suppresses transcription from the *pilE* intragenic antisense promoters.

### **Temporal changes of *pilE* sense and antisense RNA**

Northern blotting analysis has previously shown that the *pilE* transcript is not present following growth for 24 h in liquid culture (Wachter *et al.*, 2015). A similar experiment is presented in Fig. 6(a), where *pilE* transcription was monitored over several time points, and the *pilE* transcript appeared to accumulate after approximately 12 h of growth with a subsequent decline. In Fig. 6(b), we determined the temporal ratio of antisense : sense RNA levels using strand-specific qRT-PCR analysis. As  $C_t$  values inversely reflect the amount of RNA that is present within a sample, then an antisense : sense RNA ratio that increases reflects more sense RNA than antisense RNA within the sample. The results indicate that the antisense : sense RNA ratio varies over time, with sense RNA

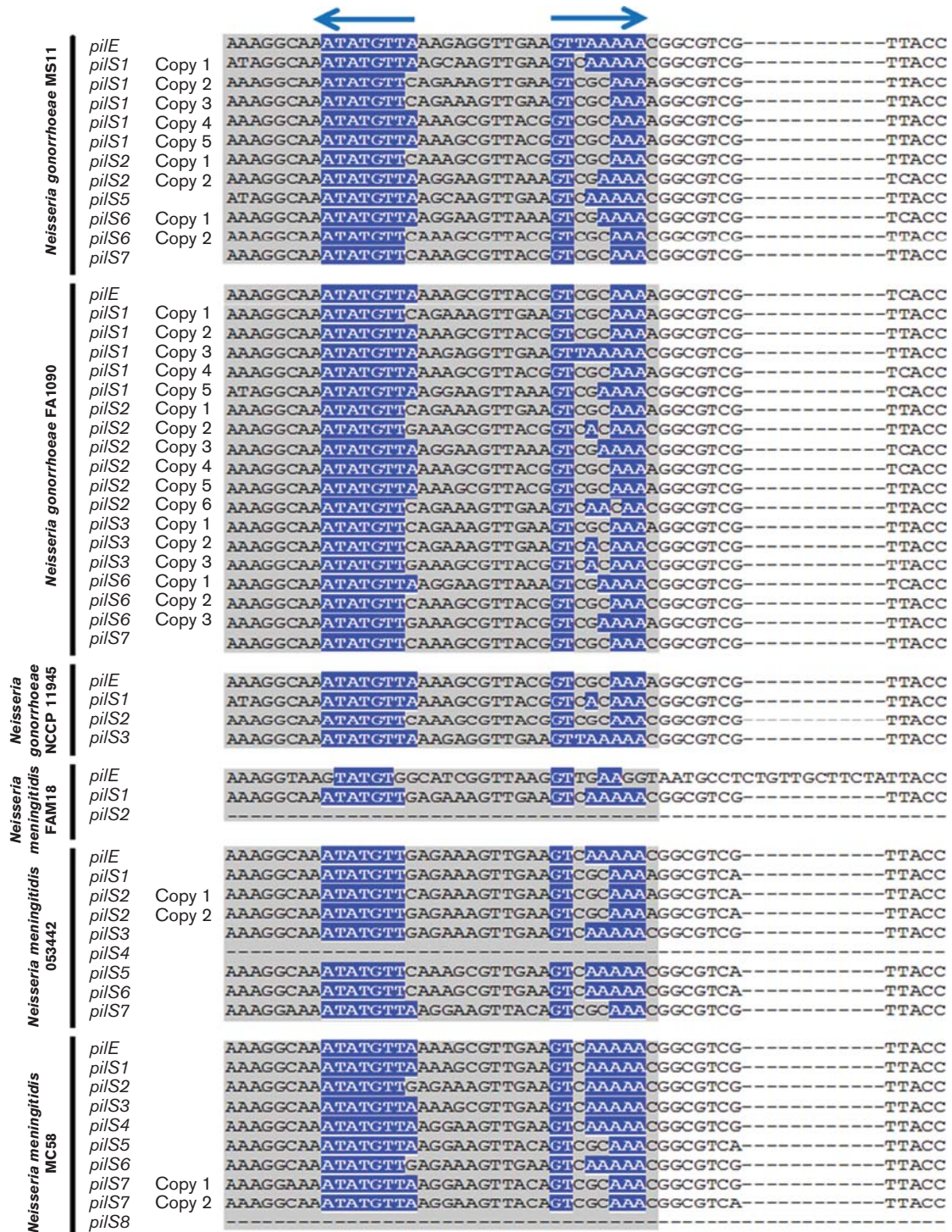




**Fig. 2.** Identification of the *pilE* midgene sense and antisense promoters. (a) Putative promoter elements were initially identified by the BPROM and Neural Network online prediction tools. (b) Mutated promoter sequences. (c) Expression levels of WT and mutated constructs through strand-specific qRT-PCR analysis using *in vitro*-transcribed RNA. Strand-specific cDNA was made utilizing primers 09305 and 09264 for antisense and sense, respectively. WT expression for both antisense and sense expression was normalized to 100 %. Data represent mean  $\pm$  SD;  $n=4$ .

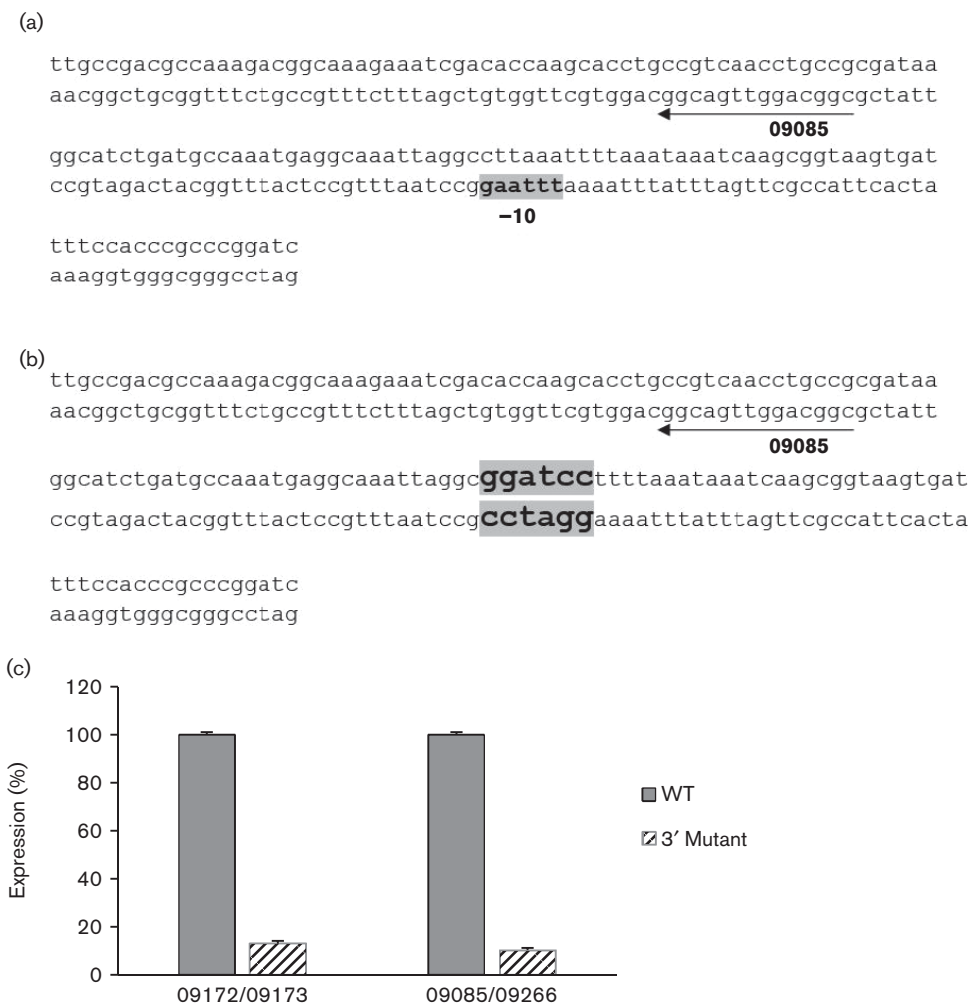
accumulating within the 6 and 12 h cultures, at which point antisense RNA begins to increase, causing the anti-sense : sense ratio to drop thereafter. However, a final

1 : 1 ratio is not observed, which may reflect the fact that antisense transcription from the midgene promoter may also be occurring; the design of the experiment



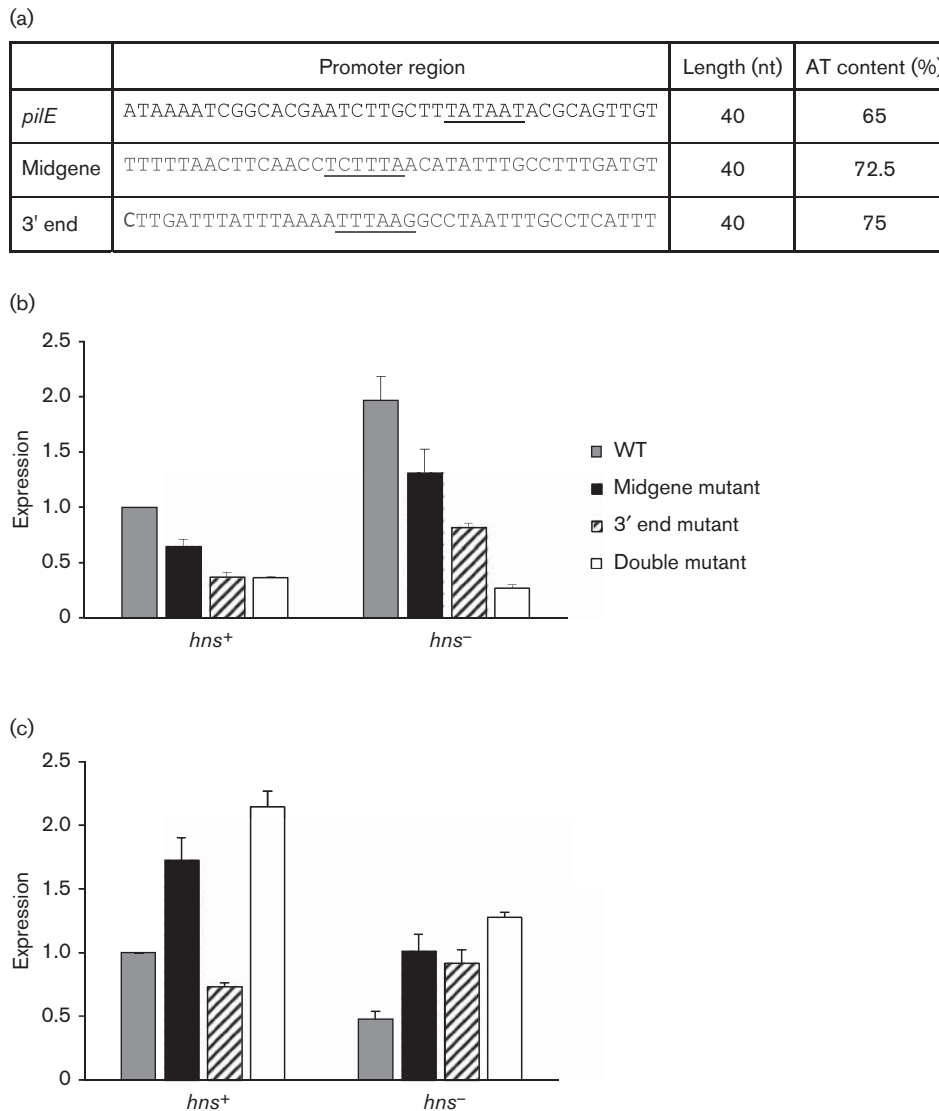
**Fig. 3.** CLUSTAL analysis of *Neisseria pil* gene sequences. The accession numbers for each strain are as follows: *Neisseria gonorrhoeae* MS11 GenBank accession CP003909.1 (*pilE*=NGFG\_01821; *pilS1* copy 1=NGFG\_02253 (2122534–2123035), copy 2=NGFG\_02253 (2123072–2123479), copy 3=NGFG\_02487, copy 4=NGFG\_00014 (2124309–2124659), copy 5=NGFG\_00014 (2124696–2125043); *pilS2* copy 1=NGFG\_02484, copy 2=NGFG\_02485; *pilS5*=NGFG\_02405; *pilS6* copy 1=NGFG\_02481, copy 3=NGFG\_02482; *pilS7*=NGFG\_02431); *Neisseria gonorrhoeae* FA1090 GenBank accession NC\_002946.2 (*pilE*=2038021–2037383\*; *pilS1* copy 1=2043862–2044354\*, copy 2=2044392–2044736\*, copy 3=2044773–2045127\*, copy 4=2045506–2045842\*, copy 5=2045843–2046256\*; *pilS2* copy 1=2040723–2041203\*, copy 2=2041256–2041603\*, copy 3=2041646–2042026\*, copy 4=2042470–2042800\*, copy 5=2042801–2043147\*, copy 6=2043148–2043522\*; *pilS3* copy 1=2013715–2014201\*, copy2=2014238–

2014567\*, copy 3=2014626–2015018\*; *pilS6* NGO2041a copy 1=2015343–2015868, copy 2=2016302–2016669, copy 3=2016707–2017060; *pilS7*=1480693–1481248\*); *Neisseria gonorrhoeae* NCCP 11945 GenBank accession NC\_011035.1 [*pilE*=NGK\_RS08820, *pilS1*=NGK\_RS09875 (1818155–1818627), *pilS2*=NGK\_RS09875 (1818628–1819032), *pilS3*=NGK\_RS09875 (1819033–1819327)]; *Neisseria meningitidis* FAM18 GenBank accession NC\_008767.1 (*pilE*=NMC\_RS01135, *pilS1*=NMC\_RS00015, *pilS2*=NMC\_RS00020); *Neisseria meningitidis* 053442 GenBank accession NC\_010120.1 (*pilE*=NMCC\_RS00970; *pilS1*=NMCC\_RS00130; *pilS2*=NMCC\_RS00125 copy 1=19974–20401, copy 2=19532–19973 ; *pilS3*=NMCC\_RS03380; *pilS4*=NMCC\_RS07650; *pilS5*=NMCC\_RS07655; *pilS6*=NMCC\_RS08075; *pilS7*=NMCC\_RS08080); *Neisseria meningitidis* MC58 GenBank accession NC\_003112.2 (*pilE*=NMB0018; *pilS1*=NMB0019; *pilS2*=NMB0020; *pilS3*=NMB0021; *pilS4*=NMB0022; *pilS5*=NMB0023; *pilS6*=NMB0024; *pilS7*=NMB0025 copy 1=23169–23502, copy 2=22825–23168; *pilS8*=NMB0026); \*Not annotated in final assembly. The midgene antisense promoter is indicated by the left arrow; the midgene sense promoter is indicated by the right arrow. The colour-shaded regions highlight the locations of the antisense and sense promoters within the sequences.



**Fig. 4.** Identification of the antisense promoter at the 3' end of *pilE*. (a) Location of the predicted antisense promoter at the 3' end of *pilE*. (b) 3' Mutated promoter. (c) Expression of *pilE* antisense RNAs from recombinant WT and the 3' mutated constructs in *E. coli* using strand-specific qRT-PCRs. WT expression is normalized to 100 %. Error bars represent mean  $\pm$  SD;  $n=10$ .





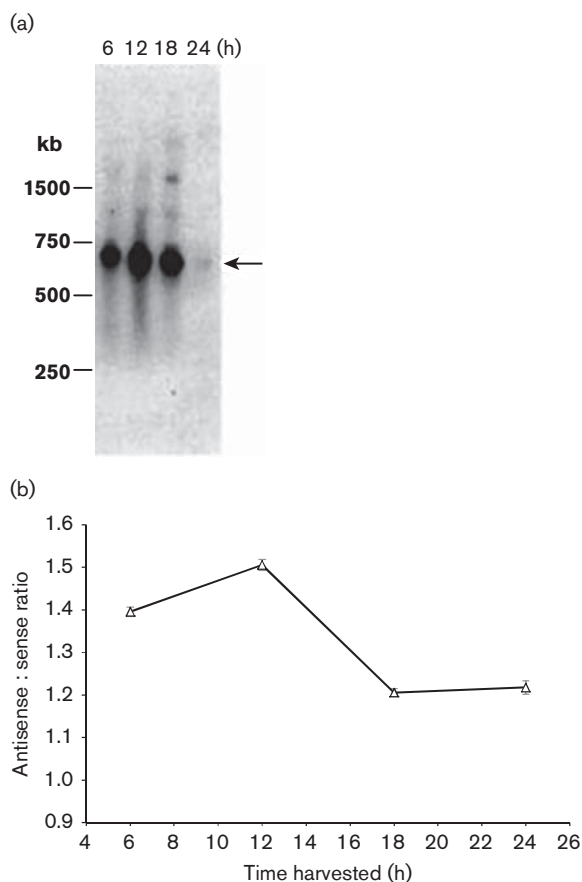
**Fig. 5.** Strand-specific qRT-PCR analyses of *in vivo*-transcribed gonococcal RNAs. Total RNA was isolated from gonococci carrying either a non-mutated *pilE* gene, a *pilE* midgene antisense promoter mutation, a *pilE* 3' antisense mutation, or a *pilE* midgene antisense promoter mutation as well as a *pilE* 3' antisense mutation. Strand-specific cDNAs were synthesized using primer 09172 for antisense expression and 09085 for sense expression. (a) AT-richness of the various promoter sequences and surrounding DNA. The promoter is underlined. (b, c) Expression of *pilE* antisense (b) and sense (c) RNAs in all four GC constructs. The left side of each panel reflects RNAs extracted from WT bacteria; the right side of each panel reflects RNAs extracted from an isogenic *hns* mutant. Expression is normalized relative to the WT value, which is set to 1. Error bars represent mean  $\pm$  SD;  $n=10$ .

would not identify the contribution of this *pilE* antisense RNA to the sense levels.

### H-NS protein suppresses *pilE/pilS* recombination

In a WT genetic background, recombinational repair of *pilE* genes carrying the antisense promoter mutations (which yielded pilus-minus phenotype colonies) appeared

to be impeded when examined microscopically on plates, as they exhibited a lower frequency of reversion to the pilus-plus colony phenotype (data not shown), with the apparent phase variation defect being exacerbated in an *hns*<sup>-</sup> background (Fig. S3). To further examine this apparent H-NS effect on *pilE* recombination, a previously used *pilE/pilS* recombination assay was modified so that it



**Fig. 6.** *pilE* RNA expression over time. (a) Northern blot of total RNA samples isolated from liquid-grown cells at the indicated time points. The total RNAs from each time point were quantified by spectrophotometric analysis and equal amounts of RNA were applied to the gel. The blot was probed with oligonucleotide probe 245, which recognizes *pilE* sense RNA at the 5' end (Wachter *et al.*, 2015). The arrow indicates the full-length *pilE* message. (b) Antisense : sense ratio of *pilE* RNA. The strand-specific  $C_t$  values were determined through qRT-PCR. The initial strand-specific cDNA was made using primer 09172 for antisense expression and primer 09085 for sense expression. As a  $C_t$  value is inversely related to the extent of expression, the higher the ratio that is observed, the more sense expression is apparent when compared with antisense expression. Error bars represent mean  $\pm$  SD;  $n=4$ .

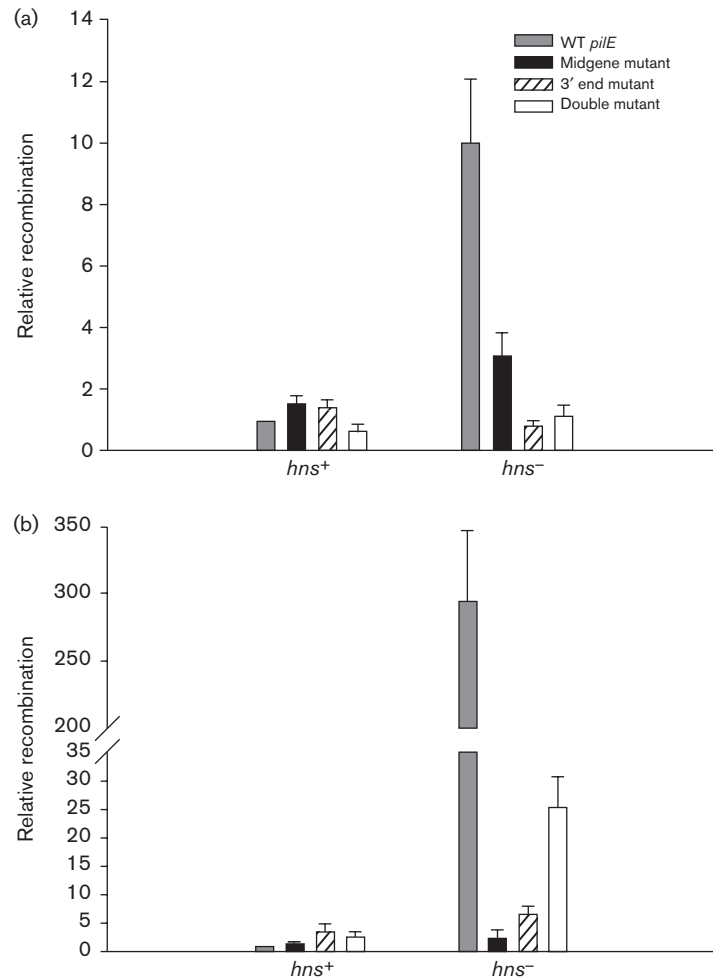
could be quantified by qRT-PCR analysis (Hill *et al.*, 2007; Hill & Davies, 2009). For this analysis, one qRT-PCR primer is specific for *pilE*, and the other, opposing primer, is specific for a *pilS* locus. Therefore, in order to obtain a signal, a *pilE/pilS* recombination event needs to occur. The starting templates that were assessed were either chromosomal DNA extracts (Fig. 7a) or, alternatively, total RNA extracts (Fig. 7b), which were derived from either WT cells or *N. gonorrhoeae* *hns* mutant cells. When nucleic acid was isolated from WT cells, little difference was observed for

recombination between a non-mutated *pilE* gene and one that carried the promoter mutations with *pilS5* copy 1 (Fig. 7a, b, left panels). In contrast, when using a different *pilS*-specific primer that targeted *pilS6* copy 1, a slight decrease in recombination was observed when the *pilE* genes carried the antisense promoter mutations (Fig. S4a, b, left panels). For all *pilE/pilS* recombination experiments, the *pilE* G4 quartet sRNA (Cahoon & Seifert, 2013) was monitored and was found to be equivalent in each sample (data not shown).

In contrast to these observations in a WT genetic background, when nucleic acid was prepared from *N. gonorrhoeae* *hns* mutant cells, a dramatic increase in recombination between an intact *pilE* gene and *pilS5* copy 1 (Fig. 7a, b, right panels) as well as recombination with *pilS6* copy 1 (Fig. 4Sa, b, right panels) was observed. For recombination with *pilS5* copy 1, a 10-fold increase in recombination was observed using DNA templates; a 200–300-fold increase was observed in recombination using total RNA extracts (qualitatively similar increases can be seen for recombination with *pilS6* copy 1; Fig. S4a, b). When the *hns* mutation was complemented with a WT *hns* gene, recombination decreased to levels approaching those observed with WT cells (Fig. S5). In the *hns* mutants, when recombination was monitored between *pilE* genes carrying the various antisense promoter mutations and *pilS*, recombination declined significantly, in contrast to the observations with WT cells [Figs 7a, b (compare the right and left panels) and S4a, b (compare the right and left panels)]. From these results, we conclude; (i) H-NS protein suppresses recombination between *pilE* and *pilS*; (ii) in the absence of *pilE* antisense transcription, *pilE/pilS* recombination is significantly reduced in cells lacking H-NS protein; and (iii), in a WT background, the absence of *pilE* antisense transcription may slightly influence *pilE/pilS* recombination but only in a *pilS*-dependent fashion.

## DISCUSSION

In this study, three additional non-canonical promoters were identified in the gonococcal *pilE* gene; one yielding sense sRNA, with the other two yielding antisense sRNA. Thus, the *pilE* transcription profile conforms to the recently described phenomenon known as pervasive transcription, which was also observed at the multiple *pilS* loci in the gonococcus (Sorek & Cossart, 2010; Wade & Grainger, 2014; Wachter & Hill, 2015; Wachter *et al.*, 2015). Moreover, through mutational analysis of *pilE* in the gonococcus, an inverse correlation is evident between *pilE* sense and antisense RNA levels, most noticeably in a WT genetic background. Therefore, *pilE* antisense RNA may be pairing with the *pilE* sense transcript, thus allowing double-stranded RNA templates to form, which then may serve as substrates for RNase III degradation. Alternatively, transcriptional interference may also be occurring when the respective RNA polymerase enzymes arising from the converging promoters either collide or pause, depending upon

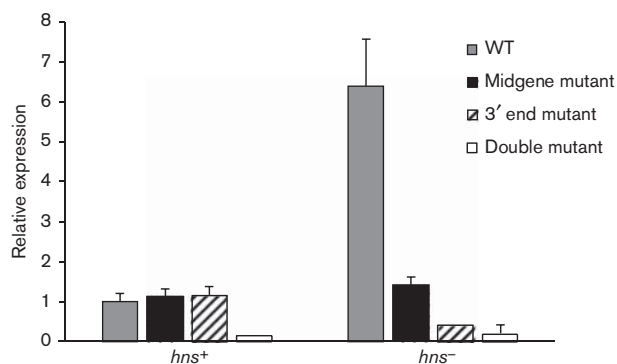


**Fig. 7.** *pilE/pilS* recombination assay. (a) *pilE/pilS* recombination assessed at the DNA level. Identical quantities of chromosomal DNA were used to assess *pilE/pilS* recombinants using qRT-PCR. (b) *pilE/pilS* recombination assessed at the RNA level. Identical amounts of total RNA were used to assess *pilE/pilS* recombinant cDNAs levels using qRT-PCR. The left grouping reflects a WT genetic background (*hns*<sup>+</sup>); the right grouping reflects an *hns*<sup>-</sup> genetic background. The primers that were used for the recombination assay were the *pilE*-specific primer (RC3) and the *pilS5*-specific primer (*pilS5c1*). The relative level of recombinant RNAs/DNAs was initially standardized against a *recA* control. Each group was then standardized against WT *pilE* (*hns*<sup>+</sup>), which was set to 1. The data reflect mean  $\pm$  SD; *n*=3.

the relative strength of the converging promoters (Callen *et al.*, 2004; Shearwin *et al.*, 2005). Consequently, an added layer of complexity in *pilE* transcriptional control may have been revealed, where the levels of *pilE* sense RNA are moderated by concurrent *pilE* antisense transcription. However, in the absence of a perfect inverse correlation between the two RNA species, other unidentified factors/features may also be involved in modulating *pilE* transcript levels. A possibility could be that protective stem-loop structures may form at the 5' ends of both the *pilE* sense and antisense RNAs, as such structures have been shown to stabilize mRNAs in other organisms (Chen *et al.*, 1991). Indeed, RNA secondary structural algorithms do predict the formation of such loop structures, and, if these 5' stem-loops form *in vivo*, then this

may also provide additional protection from degradation (T. L. Masters, J. Wachter, J. Mason and S. A. Hill, in preparation).

In a recent study with *N. meningitidis*, *pilE* antisense RNA was also detected during the stationary growth phase (as well as under salt stress conditions) from a promoter that is located at the 3' end of the *pilE* gene (Tan *et al.*, 2015). However, in contrast to the observations reported herein, this antisense RNA species did not appear to play a role in modulating *pilE* expression, yet antisense expression was shown to negatively impact PilE antigenic variation. The identified meningococcal antisense promoter was located 6 nt downstream of the *pilE* stop codon (which corresponds to promoter P1; Fig. S6). When the equivalent nucleotide



**Fig. 8.** Preferential inhibition of transcription of recombinant genes by H-NS protein. The overall extent of *pilE* transcription was determined by qRT-PCR analysis using primers RC3/173, which specifically amplify all *pilE* transcripts in a total RNA sample. The *pilE* recombinant scores were determined using the primers that were used for the recombination assay, which were the *pilE*-specific primer (RC3) and the *pilS5*-specific primer (*pilS5c1*). The data represent the ratios of the recombinant *pilE*  $C_t$  scores to the overall *pilE*  $C_t$  score. The left grouping reflects a WT genetic background (*hns*<sup>+</sup>); the right grouping reflects an *hns*<sup>-</sup> genetic background. Each group was also standardized against WT *pilE* (*hns*<sup>+</sup>), which was set to 1. The data reflect mean  $\pm$  SD;  $n=3$ .

sequence was mutated in the MS11 *pilE*<sub>7:30:2</sub> gene as well as another potential promoter sequence (designated P3; located further downstream), no significant changes in the amount of *pilE* antisense transcripts were observed (data not shown). Instead, it was only by mutating the 3' P2 promoter (Fig. S6), which is located 12 nt downstream from the stop codon, that significant changes in 3' antisense transcription were observed compared with WT. Consequently, the 3' P2 promoter appears to function in the gonococcus and is responsible for transcription of the antisense RNA originating at the 3' end of *pilE*, which perhaps indicates either a species-specific difference in promoter usage, or possibly reflects other effects due to variations in the respective *pilE* DNA sequences.

In the meningococcal study, no correlation was observed between antisense transcription and *pilE* sense transcript levels (Tan *et al.*, 2015), which contrasts with the analysis with the gonococcal *pilE* RNAs, as an inverse correlation is evident. The observed difference between the two studies may reflect the contribution that self-priming causes in the initial strand-specific reverse transcription reaction for the qRT-PCR analysis as this was factored out in the current study. Reverse transcription due to self-priming has been extensively reported in studies involving RNA viruses, where it significantly impacts the analysis of strand-specific RNA templates (Haddad *et al.*, 2007; Tuiskunen *et al.*, 2010). Whether reverse transcription as a consequence of

self-priming of *pilE* sense and antisense RNAs impacts gonococcal *pilE* transcript levels *in vivo* remains unknown. However, the possibility exists as *N. gonorrhoeae* strain MS11 has been shown to engage in reverse transcription, even though a reverse transcriptase enzyme has not been identified within the gonococcal genome (Barten & Meyer, 1997). In the absence of such an enzyme, it was postulated that reverse transcription may have arisen through DNA polymerase I operating on RNA templates (Barten & Meyer, 1997).

As H-NS protein has been shown to suppress intragenic transcription in *E. coli* (Singh *et al.*, 2014), we next explored whether gonococcal H-NS protein also played a similar role. H-NS protein is widely found in many bacterial species (Dillon & Dorman, 2010), where it has been suggested that the presence of the protein impacts genome evolution through its ability to silence transcription from horizontally acquired DNAs (Navarre *et al.*, 2006; Singh *et al.*, 2014). H-NS appears to be a universal transcriptional repressor and has been shown to regulate many genes that encode virulence factors (Navarre *et al.*, 2006). In the current study, gonococcal H-NS protein modulated the *pilE* antisense transcriptional profiles, with more *pilE* antisense RNA being apparent in *hns* mutants than in WT. Also, with the *hns* mutant, an ordered decrease in the amount of antisense RNA was observed when each of the *pilE* antisense promoters was mutated. Therefore, these observations indicate that H-NS protein may bind to the *pilE* intragenic promoters (presumably owing to the AT-richness of these sequences; Fig. 5a) and suppress *pilE* intragenic antisense transcription.

In WT gonococci, *pilE* antisense transcription did not appear to significantly impact *pilE/pilS* recombination, with negative effects not being universally apparent and seemingly dependent on which *pilS* locus *pilE* was engaged with during recombination (cf. Figs 7 and S4, WT, left panels). H-NS protein has also been shown to influence homologous recombination (Shiraishi *et al.*, 2007; Sharadamma *et al.*, 2010). For example, in *Mycobacterium tuberculosis*, H-NS protein was shown to bind with high affinity to Holliday junctions (an important recombination intermediate), and, in doing so, restrained RecA-mediated strand exchange (Sharadamma *et al.*, 2010). The data presented herein show that the *hns* mutation dramatically influenced the extent of *pilE/pilS* recombination (Figs 7 and S4), indicating that H-NS protein also influences intragenic recombination in the gonococcus. Therefore, gonococcal H-NS protein can be regarded as a suppressor of *pilE/pilS* recombination, which complements the positive effect of the G4-associated sRNA regulation of *pilE/pilS* recombination (Cahoon & Seifert, 2013).

The extent of *pilE/pilS* recombination was monitored using an assay that utilized either total RNA extracts or isolated chromosomal DNA. When *pilE/pilS* recombination was assessed using chromosomal DNA, there was an approximate 10-fold increase in recombination when the *hns*



gene was mutated, with the extent of *pilE/pilS* recombination decreasing when the *pilE* antisense promoters were individually mutated (Figs 7a and S4a). However, when total RNA extracts of *hns* mutants were used for the recombination assay, a 200–300-fold increase in recombination was observed (Figs 7b and S4b), suggesting a differential suppressive effect by H-NS protein on transcripts derived from newly recombined *pilE* genes. A possible explanation for this effect could be that Holliday junctions will naturally form in *pilE* genes engaged in *pilE/pilS* recombination, which would allow targeting of recombining *pilE*s by H-NS protein; if H-NS protein remains bound to the rearranged *pilE* gene, then transcription of those recombinant genes could be preferentially suppressed, which could account for the dramatic recombination effect that was observed when total RNAs were used for the recombination assay (Figs 7 and S4). Indeed, when the extent of transcription from the *pilS5/pilE* recombinant genes was measured against overall *pilE* transcription, H-NS clearly suppressed transcription from the recombinant *pilE* genes in contrast to WT (Fig. 8;  $P < 0.001$ ,  $n = 3$ ). Therefore, besides silencing transcription from horizontally acquired DNA, H-NS protein may preferentially silence transcription from newly rearranged genes that arise by intracellular recombination.

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