INTRODUCTION

*Neisseria gonorrhoeae* causes the sexually transmitted disease gonorrhea where the expression of pili on the cell surface has been shown to be crucial for infectivity (Kellogg et al., 1968; Swanson, 1973; Swanson et al., 1987). The pilus organelle consists of several proteins, with *PilE* polypeptide (encoded by the *pilE* gene) being the major component. Despite the importance of pili to the disease process, very little is known with regard to the regulation of expression of PilE polypeptide. The *pilE* promoter structure is complicated with three fully functional sense promoters being present (designated P1, P2 and P3), yet only the P1 promoter (σ70 dependent) is used in the gonococcus (Fyfe et al., 1995; Carrick et al., 1997). In addition, two *pilE* antisense promoters have been identified: one located within a midgene region and the second one located at the 3′ end of the gene (Masters et al., 2016). Despite considerable effort having been expended on trying to identify regulatory proteins, only one transcriptional cofactor has been found in the form of the small RNA-binding protein, integration host factor (IHF). When IHF binds upstream of the *pilE* promoter, IHF binding facilitates the interaction of two specialized AT-rich promoter sequences (UP elements) with RNA polymerase that increases transcription approximately 10-fold (Hill et al., 1997; Fyfe & Davies, 1998).

mRNA turnover is believed to initiate in regions that are relatively free of bound ribosomes as their constant occupation on transcripts could enhance mRNA stabilization (Bechhofer & Dubnau, 1987). Consequently, 5′ and 3′ untranslated regions (UTRs) are considered to be good candidates as initial cleavage sites for RNases, with the formation of RNA secondary structures within these regions possibly influencing stability and/or translational efficiency (Régnier & Arraiano, 2000; Marzi et al., 2008). Loop structures are predicted in the *Escherichia coli* rpsT P1 mRNA and analysis has shown that the presence of a hairpin at the 5′ end of the *rpsT* P1 transcript hinders both the pyrophosphohydrolase activity of RppH and the single-stranded-dependent cleavage of RNase E, thus prolonging mRNA half-life (Deana et al., 2008). Consequently, the presence of

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**Abbreviations:** DUS, DNA uptake sequence; IHF, integration host factor; qRT-PCR, quantitative real-time PCR; rbs, ribosomal binding site; sRNA, small antisense RNA; UTR, untranslated region.

One supplementary figure is available with the online Supplementary Material.

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Loop structures in the 5′ untranslated region and antisense RNA mediate *pilE* gene expression in *Neisseria gonorrhoeae*

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Regulation of the *Neisseria gonorrhoeae pilE* gene is ill-defined. In this study, post-transcriptional effects on expression were assessed. *In silico* analysis predicts the formation of three putative stable stem–loop structures with favourable free energies within the 5′ untranslated region of the *pilE* message. Using quantitative reverse transcriptase PCR analyses, we show that each loop structure forms, with introduced destabilizing stem–loop mutations diminishing loop stability. Utilizing a series of *pilE* translational fusions, deletion of either loop 1 or loop 2 caused a significant reduction of *pilE* mRNA resulting in reduced expression of the reporter gene. Consequently, the formation of the loops apparently protects the *pilE* transcript from degradation. Putative loop 3 contains the *pilE* ribosomal binding site. Consequently, its formation may influence translation. Analysis of a small RNA transcriptome revealed an antisense RNA being produced upstream of the *pilE* promoter that is predicted to hybridize across the 5′ untranslated region loops. Insertional mutants were created where the antisense RNA is not transcribed. In these mutants, *pilE* transcript levels are greatly diminished, with any residual message apparently not being translated. Complementation of these insertion mutants *in trans* with the antisense RNA gene facilitates *pilE* translation yielding a pilus + phenotype. Overall, this study demonstrates a complex relationship between loop-dependent transcript protection and antisense RNA in modulating *pilE* expression levels.
any pilE mRNA secondary structural features may also moderate transcript turnover.

The presence of naturally occurring small antisense RNAs (sRNAs) has been widely reported (Georg & Hess, 2011). However, the regulatory functions of many of these RNAs are still yet to be determined (Waters & Storz, 2009). Most of the known sRNAs do not encode protein, with their pervasive transcription being initiated from intergenic or intragenic promoters (Wade & Grainger, 2014). A recent analysis of a gonococcal small RNA transcriptome revealed many such sRNAs (Wachter & Hill, 2015). Such trans-encoded sRNAs appear to act primarily by regulating mRNA translation/degradation via complementary binding in an antisense manner that often requires the aid of Hfq protein, which is an RNA chaperone. The absence of Hfq protein causes pleiotropic effects that occasionally involve bacterial pathogenicity (Hoe et al., 2013). Hfq also serves as a major post-transcriptional regulator of numerous stress-responsive genes (Sittka et al., 2007). In a gonococcal hfq mutant, the absence of Hfq protein has been shown to decrease pilE transcript levels, as well as to influence several pilus-associated phenotypes (Dietrich et al., 2009). In a Neisseria meningitidis hfq mutant, PilE polypeptide is absent (Pannekoek et al., 2009). Consequently, these observations suggest that a small RNA may be involved in modulating pilE expression.

In this study, post-transcriptional effects on pilE expression were investigated and a stabilizing role for stem–loop structures in the 5′ UTR of the pilE message was indicated. Furthermore, as one loop structure is predicted to occlude the pilE ribosomal binding site, evidence is presented whereby an sRNA is required for translation of the pilE message. Overall, the data indicate that pilE expression is regulated at the post-transcriptional level adding a further layer of complexity to the regulation of this important virulence determinant.

METHODS

Strains and growth conditions. N. gonorrhoeae strain MS11 (Rocky Mountain Laboratories, Hamilton, MT, USA) was used in this study. Gonococci were passaged daily on a gonococcal typing medium (Swanson, 1982) at 37°C in a 5% CO2 atmosphere. When grown in the presence of antibiotics, the final concentrations were as follows: chloramphenicol, 10 µg ml−1; kanamycin, 80 µg ml−1; erythromycin, 5 µg ml−1.

E. coli cells were grown using LB medium at 37°C. When E. coli carried recombinants, the medium was supplemented with antibiotics at the following concentrations: carbenicillin, 100 µg ml−1; erythromycin, 200 µg ml−1; chloramphenicol, 20 µg ml−1; tetracycline, 15 µg ml−1; and kanamycin, 40 µg ml−1.

Construction of translational fusions. pilE translational fusions were constructed by fusing the pilE 5′ UTR (which included DNA comprising the first 19 codons) in-frame to a reporter gene, either beta-galactosidase (lacZ) or chloramphenicol acetyltransferase (cat), that lacked their cognate ribosomal binding site (rbs). This procedure entailed amplifying pilE with the appropriate set of primers (Table 1) and initially cloning the fragment into the pCR11 (InVitrogen) vector. For construction of the WTpilE-lacZ clone, the appropriate fragment was cut by EcoRI and BamHI enzymes and ligated in the vector pRS414 that carries a truncated lacZ gene preceded by a strong transcriptional terminator, resulting from the loop 2: lacZ deletion fusions, sequential PCR was employed that amplified the flanking regions to each loop followed by ligation of the fragments. The fragment containing the loop deletion was then cloned into vector pRS414 at the unique sites EcoRI and BamHI to produce pRS414-L1del: lacZ (pT16) and pRS414-L2del: lacZ (pT18) constructs.

A similar approach was used to make pilE::cat translational fusions. The promoter-less cat gene was obtained from pCR2.1-cat::DUS (S. A. Hill, unpublished). Various pilE fragments containing intact or the 5′ UTR loop deletions were amplified using the appropriate primers (Table 1), followed by cloning into the pCR1I vector; a double SacI and SallAI digest released the fragment that was then inserted in the pCRI1-cat vector. This protocol allowed us to generate pCRII-WTpilE::cat (pT14), pCRII-L1del: cat (pT15) and pCRII-L2del::cat (pT16) constructs.

In order to introduce the pilE::cat translational fusions into the gonococcal chromosome, the various pilE::cat fusions were cloned into a pBlueScript-opalCerm vector that carries the opaE gene with an ermC gene inserted in the unique SalI site. The fusions were then PCR-amplified and inserted in pBlueScript-opalCerm at the unique Xbal site to create pBlueScript-opaE::erm::pilE::cat constructs. These DNAs were then used to cross the fusions into the gonococcal opaE locus with transformants being selected through erythromycin resistance.

To investigate the effect of pilE antisense transcription on regulation of pilE across the 5′ loops, a kanamycin gene insertion was introduced into the pilE gene such that transcription of antisense RNAs across the loop structures would be disrupted. The pilE::kan construct was generated by blunt-end ligation of the kan gene into the pilE BshHI site. The appropriate orientation of the kan insert was confirmed by PCR to ensure interruption of antisense transcription. The pUC8-pilE::kan plasmid DNA was then used to transform the N. gonorrhoeae MS11 carrying a pilE::cat translational fusion to kanamycin resistance to create a GC pilE::kan opaE::erm::pilE::cat strain.

Site-directed mutagenesis was performed as previously described (Wachter et al., 2015; Masters et al., 2016).

Construction and complementation of the asRNA7 ermC insertion knockout mutants. Regions upstream of the pilE 5′ UTR in N. gonorrhoeae MS11 were disrupted with anermC insertions that were made by sequential ligation of PCR-generated DNA fragments and anermC gene cassette. DNA transformation was then used to make the gonococcal mutants. The resulting constructs (ΔasRNA7::ermC1-6) were then tested for transcription of full-length pilE message through Northern blot and endpoint reverse transcriptase PCR analysis. To determine the translational efficiency of these insertion mutants, a cat gene was transcriptionally fused to the 3′ end of pilE and assessed the chloramphenicol resistance and competency.

To construct a ΔasRNA7::ermC complement, the genomic region encoding a small RNA upstream of the pilE 5′ UTR corresponding to ΔasRNA7::ermC5 and ΔasRNA7::ermC6 was amplified with primers 10605 (5′-CCGTATGTTAACGGTGTAATCTAAAAAC-3′) and 09449 (5′-GCACAAAAACCGATGTTAATACATTGC-3′) and ligated to a kan gene cassette containing a gonococcal DNA uptake sequence (DUS) in a TA cloning vector. For subsequent transformation into N. gonorrhoeae, the kan::DUS::asRNA7 construct was ligated into the opaE gene within a pBlueScript cloning vector. The pBlueScript-opaE::kan::DUS::asRNA7 construct was then crossed into the opaE locus of N. gonorrhoeae strain MS11. Chromosomal DNA from the ΔasRNA7::ermC6 mutational constructions was then used to transform the opaE::kan::DUS::asRNA7 cells, generating mutants ΔasRNA7::ermC6::opaE::kan1, ΔasRNA7::ermC6::opaE::asRNA7::ermC6::opaE::asRNA7::kan3. Translational efficiencies of WT, ΔasRNA7::ermC5 and ΔasRNA7::ermC6 were determined through transformation efficiency and phenotypic assays.

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Table 1. Primers used to make translational fusions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion 1-HindIII</td>
<td>5’-CCCAAGCTTCCGAAGCCATGATCTTCATTTTGCGCCGAA-3’</td>
<td>5’ UTR-PCR</td>
</tr>
<tr>
<td>Fusion 2</td>
<td>5’-TCAGCGGTTGCGGCCGATTG-3’</td>
<td>5’ UTR-PCR</td>
</tr>
<tr>
<td>Fusion 3-BamHI</td>
<td>5’-ATAGCGATCAAGATATCGTAAAGG-3’</td>
<td>5’ UTR-PCR</td>
</tr>
<tr>
<td>L1-L-KpnI</td>
<td>5’-AAAAAGGTAAGATCGGGATGCTAAGG-3’</td>
<td>Loop 1 deletion</td>
</tr>
<tr>
<td>L1-R-KpnI</td>
<td>5’-AAAAAGGTACATGATCGGGATGCTAAGG-3’</td>
<td>Loop 1 deletion</td>
</tr>
<tr>
<td>EcoRV–Prs</td>
<td>5’-CACAGCAGATCTGGCTATGCTGCCTG-3’</td>
<td>Loop 1 deletion</td>
</tr>
<tr>
<td>L2-L-KpnI</td>
<td>5’-AAAAAGGATCATGATCGGGATGCTAAGG-3’</td>
<td>Loop 2 deletion</td>
</tr>
<tr>
<td>L2-R-KpnI</td>
<td>5’-AAAAAGGTAAGATCGGGATGCTAAGG-3’</td>
<td>Loop 2 deletion</td>
</tr>
<tr>
<td>Fusion 4</td>
<td>5’-TCAGGTTCTTGGCGCTTTG-3’</td>
<td>Loop 2 deletion</td>
</tr>
<tr>
<td>cat3-BamHI</td>
<td>5’-AAGGATCTGAGAAGGAAAATCTACGCTATG-3’</td>
<td>cat gene PCR</td>
</tr>
<tr>
<td>MM13R</td>
<td>5’-GCAAAACGACGGCGAGCTGAGTAATGTA-3’</td>
<td>cat gene PCR</td>
</tr>
<tr>
<td>Fusion 5</td>
<td>5’-AACAGGGAAGTACGGCTCCATGAT-3’</td>
<td>pilE::lacZ fusions PCR</td>
</tr>
</tbody>
</table>

RNA analysis. The conditions employed for RNA extraction, quantitative real-time PCR (qRT-PCR) analysis and primer extension analysis were as described previously (Wachter et al., 2015; Masters et al., 2016). The primer pairs used for loop analysis and translational fusion analysis are found in Table 2; primer pairs for the assessment of the gonococcal insertion mutants have been previously described (Wachter et al., 2015).

Loop formation assay. The assay was utilized to determine whether the 5’ UTR stem–loops are formed under non-denaturing in vitro conditions using qRT-PCR analysis. E. coli, carrying the pilE-containing vector pVD203 was grown to the exponential phase (OD600 reaching 0.5) at which point pilE mRNAs were extracted. pilE-specific, reverse-transcribed cDNAs were then subjected to qRT-PCR reactions utilizing the forward primers that were designed such that each primer resided within each predicted loop sequence. The reverse primers were designed such that they recognized nucleic acid sequences outside of the loop structures and that the amplified products that were produced were of a similar size, 160 bp. If the stem–loop structures are formed due to base pairing following RNA purification under native conditions, then PCRs using the primers within each loop should produce little or no products as base-paired loop sequences are not available for hybridization with the primers. In control experiments, the addition of betaine (a denaturing agent) in the qRT-PCR mix was employed to disrupt loop formation during the qRT-PCR experiments.

Computer modeling analysis. Mfold (Zuker, 2003) and RNAstructure (Bellaousov et al., 2013) web servers were used for analysis of RNA secondary structures within the 5’ UTR of pilE.

RESULTS

Protection of the pilE transcript through loop formation

When the IHF binding site, which is located immediately upstream of the pilE promoter, was deleted, transcription was diminished approximately 10-fold (Hill et al., 1997; Fyfe & Davies, 1998). However, stable residual full-length pilE message is evident when total RNA is assessed by both Northern blotting and primer extension analysis (Hill et al., 1997). However, this residual message does not appear to be translated (Fig. S1, available in the online Supplementary Material). Consequently, we explored the possibility that pilE mRNA structural elements may have contributed to the above-mentioned observations. In silico analysis of the 5’ UTR of the pilE message using the Mfold (Zuker, 2003) and RNA structure (Bellaousov et al., 2013) applications

Table 2. Oligonucleotide primers used in qRT-PCR analysis involving 5’ UTR loops

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop 1</td>
<td>5’-TTGTCGCGAAGCCATGATCTTCATTTTGCGCCGAA-3’</td>
<td>Loop 1-forward</td>
</tr>
<tr>
<td>Loop 2</td>
<td>5’-AAAAAGGTACATGATCGGGATGCTAAGG-3’</td>
<td>Loop 2-forward</td>
</tr>
<tr>
<td>Loop 3</td>
<td>5’-CCCTTCCAATTTGGAATTTTTGATATAGTGATTCCCTTC-3’</td>
<td>Loop 3-forward</td>
</tr>
<tr>
<td>RC3</td>
<td>5’-GTCGGCGACTTTTGGCGCCAATG-3’</td>
<td>Outside the loops-forward</td>
</tr>
<tr>
<td>RC4</td>
<td>5’-GATAGCGGATCAAGATATCGTAAAGG-3’</td>
<td>Loop 1-reverse</td>
</tr>
<tr>
<td>tsp5</td>
<td>5’-CCCAGGCGAGCTGGCAAGG-3’</td>
<td>Loop 2-reverse</td>
</tr>
<tr>
<td>09173</td>
<td>5’-TTGACCTTCCGGCAAGGAAATGAGTGCTCCTCGGAAAC-3’</td>
<td>Loop 3-reverse</td>
</tr>
<tr>
<td>tsp4</td>
<td>5’-CGCCGGCAGAAGTGTGTTTTC-3’</td>
<td>Outside the loops-reverse</td>
</tr>
<tr>
<td>lacZ fusion 1</td>
<td>5’-GTCGGCACATTTTGGCGGCCGAGG-3’</td>
<td></td>
</tr>
<tr>
<td>lacZ fusion 2</td>
<td>5’-GTCGCCATTTTGGCGCCGAAACGAGC-3’</td>
<td></td>
</tr>
<tr>
<td>cat fusion 1</td>
<td>5’-GGAGGATATACCAACAGCTTTTATACCCCATAGCATCAGGTAAGG-3’</td>
<td></td>
</tr>
<tr>
<td>cat fusion 2</td>
<td>5’-GTAACACTATCCCATACACGAGTCCCTTTGTCG-3’</td>
<td></td>
</tr>
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</table>

http://mic.microbiologyresearch.org 2007
revealed the potential for three thermodynamically stable stem–loop structures to form (Fig. 1a). Consequently, transcript levels, and/or protein expression, may be a subject to post-transcriptional regulation by such cis-embedded elements, especially as one of the predicted loops (designated loop 3 or L3) contains the pilE rbs and the AUG start codon. When primer extension analysis was performed using pilE mRNA, several secondary premature 5′ endpoints were observed in addition to the prominent signal for the true transcriptional start site (Fig. 1b; arrows). These secondary 5′ endpoints mapped to AU-rich regions in the predicted loop 2 (Fig. 1a; arrows). The pilE gene of N. gonorrhoeae was reported to possess three promoter sequences (designated P1, P2 and P3), with the sigma70-type P1 being the only active pilE promoter in GC (Fyfe et al., 1995). None of the 5′ endpoints maps to the tsp of these alternative promoters. However, recent analysis of transcription within the pil system has revealed the existence of alternative promoter usage using non-cognate promoter elements (Wachter et al., 2015). Whether alternative promoter usage

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**Fig. 1.** RNA secondary structural analysis. (a) pilE mRNA was analysed using the Mfold algorithm that revealed three potential secondary structural features with highly favourable predicted free energy values within the 5′ UTR of pilE. The loops are designated loops 1, 2 and 3. The rbs is indicated in loop 3. (b) Primer extension analysis of pilE mRNA. The arrows in both panels (b) highlight potential alternative 5′ mRNA endpoints. (c) Schematic diagram indicating the location of the alternative 5′ mRNA endpoints.
accounts for the secondary 5' endpoints has not been explored in the current manuscript. Consequently, the secondary 5' endpoints mapped within the loop 2 of pilE mRNA may be products of either endonucleolytic cleavage or non-cognate promoter usage.

qRT-PCR was used to determine whether these putative pilE-specific RNA loop structures form. In these assays, primer pairs were designed such that the forward primer was located within each putative loop structure, with the reverse primer being located outside of the predicted loop structures. Primer design was such that the amplified products were similar in size (160 bp). Consequently, if base pairing occurred within the predicted loop structures, then it was hypothesized that the loop structures would be less accessible to the forward primer during the amplification process (Fig. 2a). pilE mRNA was prepared following transcription of the pilE gene carried on plasmid pVD203 (Bergström et al., 1986); the qRT-PCR data presented in Fig. 2b indicate the formation of all three loop structures in the pilE 5' UTR mRNA.

The formation of the pilE 5' UTR stem–loops was further investigated through site-directed mutagenesis where the predicted loop sequences were changed such that RNA secondary structure would be disrupted. Mutagenic primers were designed for each loop that would impede complementary base pairing (Fig. 3a), with individual loop stability again being assessed by the qRT-PCR assay. When the loops were mutated, the forward primer was able to gain access to the RNA yielding a product (Fig. 3b), indicating that the previous negative observations were due to pilE 5' UTR loop formation. The data are presented in a log10 scale of pilE expression compared to expression of the internal control amp gene carried on the plasmid. The higher the log value indicates more-amplified product and relates to the loops not forming. A similar qRT-PCR approach was also utilized to examine loop stability when the putative loops were individually deleted (Fig. 3c). Again, the presence of an amplified product indicates loop destabilization. For example, when loop 2 or 3 was deleted, the RNA became accessible to the loop 1 primer; when loops 1 and 3 were deleted, the RNA became accessible to the loop 2 primer; and when loop 1 was deleted, the RNA became accessible to the loop 3 primer. However, in contrast to these observations, when loop 2 was deleted, loop 3 is still able to form.

Overall, these combined experiments indicated that (i) the stem–loops form through complementary base pairing

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**Fig. 2.** Loop formation in pilE mRNA. (a) Schematic representation indicating the relative location of each forward primer. (b) qRT-PCR analysis indicating the relative amount of cDNA using the above-mentioned primers. When the loop primers were used in the qRT-PCR analysis, less-amplified products were observed when compared to the product from the primer located outside of the loop (RC3). The error bars reflect ±sd; n=3. L1 primer vs RC3, P<0.001; L2 primer vs RC3, P<0.001; L3 primer vs RC3, P<0.001.
**Fig. 3.** Stability of the putative loop structures. (a) Nucleotide sequence of the 5’ UTR of pilE (in uppercase letters) and relative position of the primers used in qRT-PCRs; each putative loop sequences is boxed. The loop sequences (boldface uppercase letters) were mutated to alternative sequences (boldface lowercase letters below the sequence) that were predicted to disrupt loop formation. (b) qRT-PCR analysis using constructs containing nucleotide substitutions. (c) qRT-PCR analysis of loop deletion mutants. In (b) and (c), expression levels of pilE transcripts were calculated by subtracting expression of the internal control beta-lactamase gene carried on the plasmid. The error bars reflect ±SD; n=4. In (b), P values of the mutated constructs are compared to the non-mutated pilE construct: Loop 1 primer: L1mut, P<0.001; L2mut, P<0.001; L3mut, P<0.001; Loop 2 primer: L1mut, P<0.001; L2mut, P<0.001; L3mut, P<0.001; Loop 3 primer: L1mut, P<0.001; L2mut, P<0.001; L3mut, P<0.001. In (c), P values of the mutated constructs are compared to the non-mutated pilE construct: Loop 1 primer: L1del, P<0.001; L2del, P=0.004; L3del, P=0.001; Loop 2 primer: L1del, P<0.001; L2del, P<0.001; L3del, P<0.001; Loop 3 primer: L1del, P<0.001; L2del, P<0.001; L3del, P<0.001.
within the pilE 5’ UTR (Fig. 2b), (ii) mutating an individual loop through site-directed mutagenesis also affects the formation/stability of the other loops (Fig. 3b) and (iii) individually deleting any of the loops also disrupts loop stability except for the one exception noted above (Fig. 3c).

The pilE 5’ UTR provides a protective role in maintaining pilE transcript levels

To determine what effect loop formation plays in pilE expression, we focused on the loop deletions and constructed a series of translational fusions, where loop 1 and loop 2 were individually deleted. The deleted pilE 5’ UTR segments were then fused in-frame to one of two truncated reporter genes, beta-galactosidase (lacZ), or, chloramphenicol acetyl transferase (cat), with each reporter gene lacking its own rbs. The deleted constructs were then compared to an equivalent WT construct; relative expression was measured by growth on solid medium, by qRT-PCR analysis and by biochemical analysis. qRT-PCRs were performed using RNAs that were isolated from the different constructs under the same conditions, thus reflecting their relative protective role. Since loop 3 contains the rbs that is needed for translation of the reporter gene, a loop 3 deletion mutant could only be used to determine relative RNA stability. The data presented in Fig. 4a show the growth of each beta-galactosidase fusion strain on solid medium containing the lacZ indicator X-Gal. The data presented in Fig. 4b assess the relative RNA levels by qRT-PCR analysis from the various pilE::lacZ fusions, with Fig. 4c showing the corresponding biochemical analysis. From these combined experiments, we conclude that when loop 1, loop 2 or loop 3 is individually deleted, there is a significant reduction in the amount of RNA compared to WT (for all three deletion constructs, $P<0.001$; $n=3$), which is also reflected at the protein level for loop 1 and loop 2 mutants ($P<0.001$; $n=15$). Moreover, because very little relative RNA is observed with the

Fig. 4. pilE translational fusion analysis. The effects of the 5’ UTR loops on the expression of pilE::lacZ translational fusions in E. coli. (a) Growth of the different pilE::lacZ translational fusion strains (WT, loop 1 deletion and loop 2 deletion) on solid medium containing X-Gal. (b) qRT-PCR analysis of the various deletion strains compared to WT that is set at unity. Error bars reflect ± SD; $n=3$; L1del, $P<0.001$; L2del, $P<0.001$; and L3del, $P<0.001$. The expression levels were compared to a 16S RNA control. (c) Biochemical analysis of beta-galactosidase activity. The error bars reflect ± SD; $n=15$; when compared to WT, L1del ($P<0.001$) and L2del ($P<0.001$).
loop 2 deletion fusion (Fig. 4b) (even though loop 3 still forms; Fig. 3c), this implies that loop 1 is likely to be the critical loop structure for protection of the \( \textit{pilE} \) message. Qualitatively similar results were also obtained from comparable \( \textit{pilE}::\textit{cat} \) translational fusions where the individual loops were deleted, as well as in \( \textit{lacZ} \) translational fusions where the formation of the loop structures was impaired through site-directed mutagenesis. Consequently, these data imply a protective role for the \( \textit{pilE} 5' \) UTR stem–loops.

**Analysis of \( \textit{pilE}::\textit{cat} \) translational fusions in gonococci**

To determine whether the \( \textit{pilE} 5' \) UTR loops play a similar role in the gonococcus, \( \textit{pilE}::\textit{cat} \) translational fusions were placed ectopically on the gonococcal chromosome within the \( \textit{opaE} \) locus. As each fusion construct contained the \( \textit{pilE} \) leader peptide encoding sequence, only mRNA analysis could be performed as any protein product would be secreted from the cell. The data presented in Fig. 5a show the expression levels of the fused \( \textit{cat} \) RNAs produced by the WT and loop deletion constructs. Consistent with the \( \textit{E. coli} \) data, individual loop deletions caused a significant decrease in expression of the reporter gene at the RNA level when compared to WT (\( P<0.001 \) for both deletion fusions; \( n=4 \)).

In the above-mentioned experiment, a WT copy of the \( \textit{pilE} \) gene was also present within the cells (genotype \( \textit{pilE}^+\textit{opaE}::\textit{pilE}::\textit{cat} \); Fig. 5b). Consequently, \( \textit{pilE} \) antisense RNA that originates from the mid-gene intragenic promoter is also being produced within these cells. Consequently, this antisense RNA may bind across the \( 5' \) UTR fusion loops (Masters et al., 2016). When the resident \( \textit{pilE} \) gene was mutated through a kanamycin gene insertion that blocks \( \textit{pilE} \) antisense RNA production across the loops, a twofold to threefold increase in \( \textit{cat} \) RNA level was observed (Fig. 5c; \( P<0.001; n=4 \)). Consequently, this observation suggests that expression of \( \textit{pilE} \) \textit{cis}-antisense RNA may affect stem–loop formation by making the \( \textit{pilE} \) transcript more susceptible to degradation.

![Graph](image)

**Fig. 5.** \( \textit{pilE}::\textit{cat} \) translational fusions in gonococci. (a) qRT-PCR analysis of the various deletion strains compared to WT that was set at unity. Expression levels were compared to a 16S RNA control. The error bars reflect \( \pm SD; n=6 \); when compared to WT, L1del.cat (\( P=0.042 \)) and L2del.cat (\( P<0.001 \)). (b) Schematic representation of the two gonococcal constructs. It shows where the insertion mutations are located on the chromosome at the \( \textit{pilE} \) and \( \textit{opaE} \) loci. The insertion of the kanamycin gene prevents antisense transcription across the loop regions of the \( \textit{pilE} \) from the intragenic promoters. (c) qRT-PCR analysis of the \( \textit{pilE}::\textit{cat} \) expression in these constructs. The \( \textit{opaE}::\textit{pilE}::\textit{cat} \) is normalized to unity. The error bars reflect \( \pm SD; n=4 \).
sRNA predicted to bind to the 5' UTR of the pilE transcript

In many systems, trans regulatory elements are often found adjacent to the gene in question. Therefore, to test this possibility that a regulatory element resides upstream of the pilE promoter, gonococcal strains were constructed such that non-homologous gene inserts encoding erythromycin resistance were placed at regular intervals (six insertional mutants were constructed) upstream of the IHF binding site; a chloramphenicol acetyl transferase (cat) gene lacking its cognate rbs was also fused in-frame downstream of the pilE gene (Fig. 6a). RNA was then isolated from each mutant and pilE mRNA production was assessed by Northern blotting. From the blots presented in Fig. 6b, pilE transcription is apparent in all strains except for the two insertion mutants that are closest to the IHF binding site (oligonucleotide 245; insertions 5 and 6; 63 bp and 18 bp upstream of the IHF binding site; respectively). When an ermC gene cassette was placed 89 bp upstream of the IHF binding site (insert 4), pilE mRNA was observed. The production of pilS-derived sRNA (oligonucleotide 246) did not appear to be affected (Wachter et al., 2015). However, with the use of the more sensitive endpoint PCR amplifying reverse-transcribed cDNAs (real-time PCR assay), pilE message was still apparent in mutants 5 and 6. This result suggests that the pilE RNA is still being transcribed in the insertion mutants 5 and 6, albeit less efficiently when compared to transcription from the other strains, yielding less pilE transcript in the mutants 5 and 6. The difference between the observations with real-time PCR analysis and the Northern blot analysis is likely due to the sensitivity of the two assays. However, the translational efficiency of cells containing these inserts was greatly reduced as these cells displayed neither chloramphenicol resistance nor competence as the cells were non-piliated (Fig. 6c). Competence is measured by the ability of the bacteria to take up exogenous Neisseria-specific DNA via

![Fig. 6](image_url)

**Fig. 6.** Effect of upstream insertions on pilE transcription and translation. (a) Schematic representation of the pilE gene showing the relative positions of the ermC insertions and cat translational fusion. Distance of insertions from the pilE IHF binding site: 1, 562 bp; 2, 277 bp; 3, 162 bp; 4, 89 bp; 5, 63 bp; 6, 18 bp. (b) Transcriptional analysis of pilE with Northern blot utilizing a pilE-specific and pilS-specific probes (probes 245 and 246, respectively; Wachter et al., 2015) and endpoint PCR using pilE reversed-transcribed cDNA templates (real-time PCR) of WT and the ermC insertion cells. (c) Translational analysis as determined by resistance of the gonococcal mutants to chloramphenicol (10 μg·mL⁻¹) and competence for DNA transformation (a positive score reflects a transformation efficiency of approximately 1×10⁻³ transformants per millilitre per microgram DNA; a negative score reflects a transformation efficiency of <1×10⁻⁸ transformants per millilitre per microgram DNA).

![Fig. 7](image_url)

**Fig. 7.** Analysis of asRNA7. (a) Northern blot analysis of total RNA isolated from N. gonorrhoeae. The left panel is probed with a pilE-specific probe 245 (Wachter et al., 2015); the right panel is probed with an oligonucleotide designed to bind to asRNA7. (b) Predicted interactions of asRNA7 and pilE 5' UTR mRNA. asRNA7 has the potential to bind the 5' UTR of pilE and expose the ribosomal binding site. The sites of the ermC insertions 4, 5 and 6 are indicated by arrows on the figure. This predicted interaction would be energetically favourable, with a ΔG=−28.7 kcal mol⁻¹.
DNA transformation and is tightly linked to the piliation status of the organism as mutations causing loss of pilus expression lead to transformation incompetence; in this study, a negative competence score reflected a transformation frequency of $<1 \times 10^{-5}$ transformants per millilitre per microgram of DNA in contrast to a positive competence score of at least $1 \times 10^{-3}$ transformants per millilitre per microgram of DNA (Koomey et al., 1991; Tønjum & Koomey, 1997). Therefore, given the above-mentioned observations, we further explored whether a regulatory element resided between inserts 4 and 6.

The previously described *N. gonorrhoeae* small RNA transcriptome (Wachter & Hill, 2015) was assessed for potential sRNA molecules within this region and a single sRNA (designated asRNA7) was found. An oligonucleotide probe was designed to recognize this antisense sRNA species, and a strong signal was observed at approximately 100 bp on a Northern blot (Fig. 7a). *In silico* hybridization analysis was then performed between the putative asRNA7 and the 5′ UTR of the *pilE* transcript, with complementary binding being predicted with a favourable free energy ($\Delta G = -28.3$ kcal mol$^{-1}$) (Fig. 7b). Therefore, if asRNA7 binds to the 5′ UTR of the *pilE* transcript, such binding could potentially denature the secondary loop structures and expose the ribosomal binding site, thus allowing for translation. Complementation of insertion mutants 5 and 6 with the asRNA7 gene placed within the *opaE* locus (*opaE*:kan:DUS:asRNA7) caused the cells to become piliated, regain competence and express WT levels of *pilE* mRNA (Fig. 8). Consequently, asRNA7 apparently stabilizes the *pilE* transcript in readiness for translation.

**DISCUSSION**

The impetus for the current study was the in silico identification of three putative stem–loop structures in the 5′ UTR of the *pilE* transcript. The loops were shown to form in *pilE* mRNA (Fig. 2) and disruption of these sequences, either by site-directed mutagenesis or by individually removing the loop sequences, destabilized loop stability causing the mRNA to be more susceptible to degradation (Figs 3, 4 and 5). Consequently, the 5′ UTR loops appear to be able to protect *pilE* mRNA from degradation. Similar observations have been made where loop structures within the 5′ UTR of the *ompA*, *rne* and *cspE* mRNA in *E. coli* (Arnold et al., 1998; Uppal et al., 2008; Schuck et al., 2009), as well as within the 5′ UTR of the *ermC* mRNA in *Bacillus subtilis* (Bechhofer & Dubnau, 1987), protect the mRNA, as in each case the presence of a loop prolonged mRNA half-lives. How the *pilE* loop structures protect the RNA is currently under investigation.

Loop structures in a 5′ UTR can determine the fate of transcripts not only by controlling stability but also by influencing translational efficiency (Régnier & Arraiano, 2000; Marzi et al., 2008). The presence of RNA secondary structures in the *pilE* 5′ UTR region may explain why residual *pilE* mRNA remained when the IHF binding site located upstream of the *pilE* promoter was deleted (Hill et al., 1997). However, what was not evident in that study was why this residual mRNA was not translated into PilE polypeptide (Fig. S1). A possible explanation for the lack of translation is that in the construction of the *pilE* IHF deletion mutants, not only was the IHF binding site deleted but also other upstream DNA was removed, causing the asRNA7 gene to be absent as well (Hill et al., 1997). Consequently, without asRNA7, the loop structures would remain, with the *pilE* ribosome binding site still

![Fig. 8. qRT-PCR analysis of asRNA7 constructs.](image)

*Fig. 8.* qRT-PCR analysis of asRNA7 constructs. qRT-PCR analysis of WT, asRNA7 insertion mutant 6 (asRNA7::ermC6) and asRNA7 complements 1, 2 and 3 (asRNA7::ermC6:opaE::asRNA7::kan1, 2 and 3) utilizing primer pairs specific for recA and the 5′ end of *pilE* (Masters et al., 2016). The relative log difference as compared to an external RNA3 control. The error bars reflect ±SD; $n=6$; $P<0.001$. 

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being occluded, thus preventing translation. Likewise, with the insertion mutants 5 and 6, where asRNA7 antisense RNA is also absent, pilE mRNA in each of these mutants is unstable with any residual pilE transcripts also apparently not being translated (Figs 6 and 8). Interestingly, the insertions at the positions 2, 3 and 4 appear to stimulate pilE transcription (Fig. 6b, probe 245). Since these locations are within the vicinity of the pilE-specific, G4-associated asRNA and its promoter region (Cahoon & Seifert, 2013), it could be that these insertions negate any cis-mediated effects caused by G4 asRNA transcription that, in turn, enhances production of the asRNA7 from its promoter resulting in an increase in stabilization of the pilE primary transcript in these mutants. When the asRNA7 gene complements insertion mutants 5 and 6, pilE mRNA is again observed and is translated yielding a pilus + phenotype. Therefore, as the asRNA7 antisense RNA is predicted to bind to the pilE 5′ UTR across loops 1 and 2, it would appear that asRNA7 serves as a small RNA that facilitates mRNA protection, and, after binding, loop 3 presumably opens allowing access to the previously occluded ribosome binding site. Consequently, the pilE transcript could now be translated into PilE polypeptide. A slightly similar scenario has recently been presented regarding an operon involved in Type IV DNA secretion in the gonococcus. In this study, an RNA switch mechanism that involves two putative stem–loop structures contained within the 5′ UTR of the secretion operon has been proposed, one of which occludes the rbs within a putative loop structure; this occluded rbs is then released under certain conditions thus allowing for translation (Ramsey et al., 2015).

In a previous study, it was demonstrated that there exists an inverse relationship between the level of pilE sense RNA levels and antisense RNA production across the pilE gene (Masters et al., 2016). Consequently, a titration model was proposed whereby the presence of pilE antisense transcription helped determine the amount of pilE sense transcript levels. In the analysis of the cat translational fusions in the gonococcus (Fig. 5), elimination of pilE-specific antisense RNA derived from the midgene antisense promoter allowed twofold to threefold more message to be observed, suggesting that pilE antisense transcription may either impede loop formation in the 5′ UTR or alternatively compete with asRNA7 for binding to loops 1 and 2. Therefore, it would seem that there needs to be an orchestrated coordination of antisense RNA production across the pilE locus (both within the pilE gene and upstream with transcription of the asRNA7 gene) in order to obtain optimal transcript levels and to maintain appropriate PilE polypeptide levels. Whether this is achieved by differential promoter strengths, coordinated IHF binding or varying supercoiling fluxes across the pilE locus is currently unknown. Regardless, what has become apparent in this study is that, for a gene where no apparent regulatory protein has been identified, a complex regulatory circuit exists to maintain transcript levels operating in conjunction with a sophisticated translational scheme in order to optimize production of this important virulence determinant in the gonococcus.

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REFERENCES


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