Characterization of *pilE* antisense RNA in *Neisseria meningitidis*

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Abstract

Expression of Type four pili is important for colonization and virulence in *Neisseria meningitidis*, which is a major causative agent of bacterial meningitis and septicaemia. Pili mediate adhesion, twitching motility, DNA uptake, and can be subject to phase and antigenic variation (Av). Pilin expression and Av may be modulated in response to environmental cues; however, the mechanisms of regulation are still unclear.

This work demonstrates the identification of a novel *cis*-encoded RNA on the antisense (AS) strand of *pilE*, which encodes the major pilin subunit. The AS promoter is conserved in different *N. meningitidis* isolates, suggesting that the AS RNA may play an important role in *N. meningitidis* biology. By Northern blot and strand-specific qRT-PCR, the AS transcript was shown to be expressed during specific conditions, namely after overnight growth and in response to salt stress. The AS RNA was found to encompass sequences antisense to the entire *pilE* coding sequence and 5' untranslated region, and extends to a promoter upstream of *pilE* that drives expression of a G4 small RNA that is important for pilin Av.

Noncoding RNAs are increasingly recognized as important regulators of gene expression in bacteria. AS RNAs often modulate expression of the sense mRNA, however in this study, overexpression or deletion of the AS RNA had no significant effect on *pilE* transcript or pilin levels despite its long stretch of complementarity to the *pilE* mRNA. Potential *trans* targets were also investigated by performing RNAseq analysis to identify differentially expressed genes in the absence of the AS RNA. Expression of the AS RNA was found to reduce the frequency of *pilE* variation. The possible influence of the AS RNA on G4 small RNA transcription was investigated by examining its effect on the levels of G4 small RNA and RNA:DNA hybrids. Although technical issues prevented us from obtaining definitive results, our findings suggest the AS RNA forms RNA:DNA hybrids, adding an additional layer of complexity of molecular processes in the *pilE* locus of *N. meningitidis*.

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Abbreviations

Abbreviation	**Full Name**
ATP	Adenosine triphosphate
bp	Base pair
cfu	Colony forming units
ChIP	Chromatin immunoprecipitation
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LOS	Lipooligosaccharide
MLST	Multilocus sequence typing
nt	Nucleotide
OD_{600}	Optical density measured at 600 nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethane sulfonyl fluoride
qRTPCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	RNA sequencing
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulfate
Ser	Serine
Tfp	Type four pili
UTR	Untranslated region

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1 Introduction

1.1 Meningococcal disease and epidemiology

1.1.1 Overview of Neisseria meningitidis

Neisseria meningitidis, also known as the meningococcus, is a human specific Gram negative β-proteobacterium that asymptomatically colonizes the nasopharynx of ~10% of healthy adults (Claus *et al*., 2005; Diallo *et al*., 2016). On rare occasions, for reasons that are not fully defined, meningococci can traverse the nasopharyngeal mucosa to enter the bloodstream where they can survive and multiply to cause bacteremia and meningitis. Both host and bacterial factors contribute to the propensity to cause invasive meningococcal disease.

The genus *Neisseria* consists of only one other member that is regarded as pathogenic – *Neisseria gonorrhoeae*, which colonizes the mucosal surface of the urogenital tract and is the causative agent of gonorrhea. Other members of the genus, such as *Neisseria lactamica* and *Neisseria cinerea*, are generally regarded as non-pathogenic, commensal inhabitants of the nasopharynx but can occasionally cause disease in immunocompromised hosts (Johnson, 1983; Liu *et al*., 2015).

Neisseria are naturally competent for DNA uptake and are able to partake in horizontal genetic exchange – a process involving the incorporation of DNA into the genome by homologous recombination. As a result, *N. meningitidis* genomes demonstrate widespread occurrence of mosaic genes (Maiden, 1993), contributing to the complex intrastrain diversity of the species. *N. meningitidis* also possesses several genetic elements that contribute to genomic plasticity and generation of diversity (Davidsen and Tønjum, 2006). A number of genes encoding surface exposed proteins, lipooligosaccharide synthesis enzymes, and protein glycosylation enzymes are subject to phase variation – a reversible change between on (expressed) and off (not expressed) states, and/or antigenic variation – a gene conversion mediated process which produces different variants of an expressed gene. These processes facilitate pathogenesis by providing mechanisms of immune evasion and phenotypic variation (Rotman and Seifert, 2014).

1.1.2 Typing

Twelve different meningococcal serogroups have been defined based on their capsular polysaccharide. Of these, six (A, B, C, W, Y, and X) are responsible for the majority of disease. Other surface antigens used for serological typing include PorA, PorB, and lipooligosaccharide (LOS), which define a meningococcal isolate's serosubtype, serotype, and immunotype respectively (Frasch *et al*., 1985; Scholten *et al*., 1994). In addition to such serological typing methods, *N. meningitidis* is also characterized using a molecular technique based on indexing genotypes, known as multi-locus sequence typing (MLST) (Maiden *et al*., 1998; Maiden and Frosch, 2001). This approach, which involves the sequencing of seven housekeeping loci, allows for every isolate to be designated a "sequence type" (ST) based on its allelic combination. ST designations can be clustered into clonal complexes, or lineages, providing a useful tool for understanding population structure and epidemiology (Jolley and Maiden, 2014). Today, MLST is regarded as the gold standard for *N. meningitidis* isolate characterization and is used extensively in epidemiological surveillance and in the study of population biology of the meningococcus

(Harrison *et al.*, 2011). The availability of whole genome sequencing (WGS) technology has facilitated the development of a ribosomal MLST (rMLST) method based on indexing variation of the 53 genetic loci encoding ribosomal protein genes (*rps* genes), which allows for a high level of discrimination among closely related isolates (Jolley *et al.*, 2012). More recently, the 50S ribosomal protein L6 (*rplF*) gene has been identified as a genetic target that can be sequenced to accurately discriminate among members of the *Neisseria* genus, providing a single-locus method that can be used in combination with other single-locus tests to rapidly and inexpensively differentiate among species within the genus (Bennett *et al.*, 2014). The highest level of resolution can be attained by whole genome MLST (wgMLST), a gene-by-gene approach which involves the comparison of all the loci of a given isolate to equivalent loci of other isolates, giving rise to a genome-wide MLST profile (Maiden and Harrison, 2016).

1.1.3 Clinical manifestations

At its early stages (4-6 h from disease onset), meningococcal disease can present as non-specific symptoms resembling those of common viral infections, such as upper respiratory tract symptoms, nausea, vomiting, and headaches (Thompson *et al.*, 2006). Invasive meningococcal disease commonly manifests itself as meningitis, which occurs in around 40% of infected individuals (European Centre for Disease Prevention and Control, 2015). Symptoms of meningococcal meningitis include fever, vomiting, headache, and neck stiffness (Ragunathan *et al.*, 2000). Invasive meningococcal disease may also present predominantly as septicemia, which involves rapidly progressive septic shock and often features the appearance of a purpuric rash. This classical sign of meningococcal sepsis arises as a result of the host’s inflammatory response to large quantities of meningococcal LOS (or endotoxin), leading to generalized damage of the microvascular endothelium and can ultimately culminate in multiorgan failure and death (Pathan *et al*., 2003). Pneumonia, a less common clinical manifestation, is more often seen in the elderly and is associated with serogroups W and Y (Ladhani *et al*., 2012; Rosenstein *et al*., 1999). Even with appropriate antibiotic treatment, mortality rates of invasive meningococcal disease are high (10 – 15%) (Sharip *et al*., 2006), and survivors may sustain permanent sequelae such as hearing loss and amputations (Pace and Pollard, 2012).

1.1.4 Epidemiology

The incidence of invasive meningococcal disease varies geographically, ranging from under 1 case per 100,000 in North America and Europe, up to 10 – 1000 cases per 100,000 in Africa during epidemic years (Halperin *et al*., 2012). In particular, large seasonal epidemics of meningococcal disease have been occurring periodically for over 100 years in areas of the African Sahel and sub-Sahel known as the African meningitis belt. The serogroups causing disease also vary by region, with serogroup A predominating in Africa, and serogroup B and C responsible for majority of disease in many other regions (Halperin *et al*., 2012). The implementation of vaccination programs has influenced the epidemiology of meningococcal disease. Vaccination programs involving meningococcal C or quadravalent meningococcal ACWY conjugate vaccines – in which the capsular polysaccharide is conjugated to a carrier protein to generate a T-cell dependent response and thus induce a stronger antibody response compared to polysaccharide vaccines - have contributed to the reduced incidence of disease in many developed countries (Jafri *et al*., 2013), while mass immunization programs with meningococcal A conjugate vaccines across the African meningitis belt has resulted in reduced incidence in both carriage and disease involving serogroup A meningococcus (Collard *et al.*, 2013; Gamougam *et al.*, 2015; Marc LaForce *et al*., 2009).

1.2 Meningococcal pathogenesis

1.2.1 Stages of pathogenesis

Asymptomatic carriage of *N. meningitidis* in the nasopharynx provides a reservoir for meningococcal transmission. Acquisition of *N. meningitidis* occurs through person-to-person transmission by direct contact or by dispersion of respiratory droplets. At the nasopharyngeal mucous membrane, the meningococcus attaches to and interacts with non-ciliated mucosal epithelial cells, allowing multiplication of meningococci and colonization of the mucosal site (Stephens *et al.*, 1983). *N. meningitidis* can traverse the epithelial membrane by transcytosis in a process that is dependent on the host cell microtubule network (Sutherland *et al*., 2010). In the blood, *N. meningitidis* may survive complement-mediated bacterial cell lysis, multiply, and disseminate throughout the body to reach the cerebral capillaries.

Meningococci can adhere to and proliferate in both peripheral and cerebral vasculature, which is comprised of endothelial cells that line the blood vessels, in a process known as vascular colonization (Melican and Dumenil, 2012). The cellular host receptor CD147 (an immunoglobulin superfamily member) interacts with meningococcal Type four pili (see Section 1.3) and is required for the adhesion of meningococci to endothelial cells (Bernard *et al*., 2014). After adhesion, meningococci can then divide and proliferate to form aggregates, known as microcolonies, along the apical surface of endothelial cells (Melican and Dumenil, 2012).

Bacterial adhesion and interaction with endothelial cells elicits signaling events involving the β2-adrenoceptor/β-arrestin signaling pathway (Coureuil *et al*., 2010). Hijacking of this host pathway ultimately leads to two important consequences: 1) cytoskeletal reorganization leading to the formation of microvillus-like protrusions known as “cortical plaques” which enable microcolonies to withstand shear stress in the blood vessels (Eugène *et al*., 2002), and 2) recruitment of the Par3/Par6/PKCζ polarity complex, leading to the depletion of junctional proteins thus locally jeopardizing the integrity of the capillaries and opening a paracellular route for meningococci to cross the blood-brain barrier (Coureuil *et al*., 2009). After crossing the brain vascular endothelium, meningococci can then infect the meninges and cerebrospinal fluid, resulting in meningitis.

1.2.2 Virulence Factors

N. meningitidis possesses a number of virulence factors which enable its adherence to host cells – a process which is essential for meningococcal pathogenesis. Initial adhesion to epithelial cells of the nasopharynx is mediated by type four pili (Tfp), filamentous structures on the bacterial cell surface. Tfp structure and function will be described in greater detail in Section 1.3. After the initial attachment, more intimate adhesion is facilitated by a variety of other outer membrane molecules, such as the opacity proteins Opa and Opc (Virji *et al*., 1993), which bind to carcinoembryonic antigen cell adhesion molecule (CEACAMs) receptors and extracellular matrix proteins, respectively (Unkmeir *et al*., 2002; Virji *et al*., 1994, 1996). Other adhesins include NadA (Capecchi *et al*., 2005) and NspA (Martin *et al*., 1997).

To be able to survive in the bloodstream, *N. meningitidis* employs several mechanisms to avoid host-mediated killing. A major virulence factor is the polysaccharide capsule, which protects the bacterium from opsonization, complement-mediated lysis, and phagocytosis (Geoffroy *et al*., 2003; Kolb-Maurer *et al*., 2001). The capsular polysaccharide forms the basis for polysaccharide and conjugate meningococcal vaccines (except for serogroup B, due to its structural similarities with glycans present on human neuronal-cell adhesion molecules making it an ineffective immunogen) (Romero and Outschoorn, 1997).

In addition to the capsule, LOS also contributes to survival in the blood; mutant strains with disrupted LOS synthesis genes were found to be serum-sensitive (Geoffroy *et al*., 2003). Meningococcal LOS is comprised of lipid A, a core oligosaccharide, and highly variable short oligosaccharides consisting of α-, β-, and γ-chains. The α-chains of many strains contain a terminal lactosamine structure, lacto-N-neotetraose (LNnT), which can be sialylated either endogenously in strains containing genes for sialic acid synthesis, or through acquisition of sialic acid from exogenous sources such as human serum (Mandrell *et al*., 1991). Both LNnT and sialylated LOS mimic host cell-surface structures and may serve to camouflage the bacterial surface from the host (Mandrell *et al*., 1988; Moran *et al*., 1996). Phase variation of the genes encoding glycosyl transferases involved in extension of the α- and β- chains gives rise to antigenic variation of LOS and forms the basis of immunotype classification (Jennings *et al*., 1999). Certain LOS immunotypes (L3, L7, and L9) are associated with invasive meningococcal disease, while others (L1 and L8) are isolated predominantly from carriers, suggesting that the LOS immunotype may be related to virulence or immune evasion (Jones *et al*., 1992). Meningococcal LOS, in particular the lipid A moiety, is a potent agonist of the macrophage CD14/Toll-like receptor 4 (TLR4)-MD-2 receptor and is largely responsible for inducing the inflammatory response associated with meningococcal sepsis (Zughaier *et al.*, 2004).

A surface exposed lipoprotein, factor H binding protein (fHbp), has also been shown to be important for reducing complement-mediated cell lysis by binding human complement factor H (CFH), an inhibitor of the alternative complement pathway (Madico *et al.*, 2006; Schneider *et al.*, 2006). fHbp is capable of inducing bactericidal antibodies and is one of the components of the recently approved 4CMenB (Bexsero®, GSK) and rLP2068 (Trumenba®, Pfizer) vaccines for protection against serogroup B *N. meningitidis* (Brendish and Read, 2015; Martin and Snape, 2013).

Survival in the nasopharynx and bloodstream also requires the ability to acquire iron due to iron being limiting in host bodily fluids as a result of the host's extracellular iron sequestering proteins lactoferrin, which is found mostly in secretions such as mucus, and transferrin, which circulates in human serum (Weinberg, 1975). In order to overcome what is known as the host's "nutritional immunity", *N. meningitidis* possesses a transferrin receptor consisting of TbpA and TbpB (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993), and a lactoferrin receptor consisting of LbpA and LbpB (Fillebeen *et al.*, 1999; Pettersson *et al.*, 1994), which are able obtain iron from the respective host iron binding proteins and transport iron through the outer membrane into the bacterial periplasm. Over half of the clinical meningococcal isolates also express the haemoglobin receptors HmbR and HpuAB which recognize heme containing proteins, suggesting that iron acquisition from these sources plays a role in disease (Lewis and Dyer, 1995; Richardson and Stojiljkovic, 1999; Stojiljkovic *et al.*, 1995).

N. meningitidis expresses the porins PorA and PorB which are β-barrel proteins located in the bacterial outer membrane that associate into trimers and function as pores through which small hydrophilic nutrients can diffuse into the cell (Massari *et al*., 2003). Porins also play additional roles in pathogenesis and host cell signalling. Meningococcal PorA has been shown to bind to C4b-binding protein (C4bp), the major complement pathway inhibitor, thus contributing to serum resistance by enabling *N. meningitidis* to evade complement activation (Jarva *et al*., 2005). Meningococcal PorB is thought to have an anti-apoptotic effect on epithelial cells through its interaction with mitochondria resulting in the stabilization of mitochondrial membrane and enhanced cell survival in the presence of pro-apoptotic stimuli (Massari *et al*., 2000), while interestingly, gonococcal PorB demonstrated the opposite effect by inducing apoptosis (Kozjak-Pavlovic *et al*., 2009). However a recent study found that meningococci lacking both PorA and PorB are equally able to inhibit staurosporine induced apoptosis compared to the parental strain (Peak *et al*., 2016). Expression levels of PorA are phase variable *via* a slipped strand mispairing mechanism due to the presence of a poly-G repeat tract between the -10 and -35 elements of the promoter. Changes in the length of this tract result in altered levels of PorA surface expression (Tauseef *et al*., 2013). As meningococcal carriage can induce a serum bactericidal immune response against PorA (Jordens *et al*., 2004), this process may be involved in facilitating escape of PorA-specific bactericidal antibody responses (Tauseef *et al*., 2013).

1.2.3 Host susceptibility

Variation in a number of host factors is known to contribute to differences in the propensity to develop invasive meningococcal disease and in disease severity. The lack of protective bactericidal antibodies is an important predisposing factor for susceptibility to systemic meningococcal disease (Goldschneider *et al.*, 1969). Disease risk in infants increases with waning levels of maternally acquired antibody, while immune sensitization in the adult occurs as a result of nasopharyngeal carriage of *N. meningitidis* (Goldschneider, 1969). A study involving military recruits showed that development of invasive meningococcal disease was linked to lower bactericidal antibody titers compared to individuals that did not develop disease (Goldschneider, 1969).

In addition to antibody deficiencies, defects in the complement system also lead to marked susceptibility to meningococcal infection due to the importance of complement in clearance of *N. meningitidis* (Ram *et al.*, 2011). The complement system involves three pathways of complement activation: the classical, alternative, and lectin pathways, which all lead to cleavage of C3, giving rise to late events in the complement pathway ultimately resulting in the formation of the membrane attack complex (MAC) which creates pores in the bacterial cell membrane and causes bacterial death. Deficiency in C3, which has a central position in complement activation, results in compromised MAC formation, opsonization, and phagocytosis, and is linked to an increased risk of meningococcal and other bacterial infections (Emonts *et al.*, 2003). Deficiency in components of the MAC, namely C5 to C9, is particularly associated with developing meningococcal disease; affected individuals have a 1000-10,000 fold risk compared with the general population (Fine *et al.*, 1983; Lehner *et al.*, 1992; Rosa *et al.*, 2004). Deficiencies in other components of the complement system that are associated with increased susceptibility to meningococcal disease include properdin and factor D (Fijen *et al.*, 1999; Sprong and Roos, 2006). Using a genome wide association (GWA) approach, it was found that variants in CFH and CFH-related genes (CFHR3, CFHR1) are associated with host susceptibility to meningococcal disease (Davila *et al.*, 2010). As described in Section 1.2.2, CFH is a regulator of the alternative complement pathway and has been known to be exploited by *N. meningitidis* to evade immune-killing *via* its recruitment to the bacterial cell surface by fHbp (Schneider *et al*., 2009). CFHR3 is thought to compete with CFH for binding to fHbp, thereby promoting immune activation and complement-mediated lysis (Caesar *et al*., 2014).

1.3 Type four pili

1.3.1 Tfp structure and biogenesis

Tfp are thin (~60 Å) and long (>1 µm) hair-like projections that are comprised of thousands of protein subunits termed pilins (Craig *et al*., 2006). The pilin subunits are arranged in a helical configuration to form the pilus fiber (Parge *et al*., 1995). In addition to the major pilin PilE, pili also contain minor pilins such as PilX, PilV, and ComP, which are present in low abundance and are involved in the modulation of pilus function (Hélaine *et al*., 2005; Winther-Larsen *et al*., 2001; Wolfgang *et al*., 1999).

Pilins are initially synthesized as pre-proteins with a conserved N-terminal leader sequence that is cleaved by the prepilin peptidase, PilD (Strom *et al*., 1993) (Figure 1.1A). The structure of PilE from the closely related pathogen *N. gonorrhoeae* provides insights into Tfp structure and assembly (Parge *et al*., 1995). The pilin subunit is a ladle shaped protein consisting of a globular head domain and an N-terminal α-helical spine. While the C-terminal half of the α-helix is embedded in the globular head domain, the protruding half of the α-helix is primarily hydrophobic and mediates pilus assembly by forming a hydrophobic helical bundle in the core of the Tfp filament through subunit-subunit interactions (Craig *et al*., 2004) (Figure 1.1B). The globular head domain contains two regions – the αβ-loop and the D-region - that are exposed on the surface of the assembled pilus and thus define the surface chemistry of the pilus (Craig *et al*., 2004; Craig and Li, 2008). These protruding loops in the globular head of the pilin subunit result in the corrugated outer surface of the pilus fiber, characterized by exposed ridges and deep grooves that wind around the filament axis (Craig *et al*., 2006). PilE undergoes a number of post-translational modifications; in strains expressing class I PilE (see Section 1.3.3), the serine residue at position 63 is *O*-glycosylated, while strains expressing class II PilE contain multiple pilin glycosylation sites leading to a highly glycosylated pilus surface in the assembled Tfp fiber (Chamot-Rooke *et al*., 2007; Gault *et al*., 2015). The composition of the pilin glycan can differ between strains and is subject to variation; *Neisseria* possesses a possible repertoire of at least 13 different glycans depending on the phase variation of various pilin glycosylation (*pgl*) genes and on the presence of specific alleles of sugar biosynthesis genes (Børud *et al*., 2011, 2014; Power *et al*., 2004). In addition to being *O*-glycosylated, PilE can also be modified with different phosphorforms such as phosphate, phosphoethanolamine, phosphocholine, or phosphoglycerol (Forest *et al*., 1999; Gault *et al*., 2014). Ser63 and the phospho-modification site Ser68 are both located in the αβ-loop, such that their post-translational modifications protrude from the surface of the assembled pilus fiber (Craig *et al*., 2006).

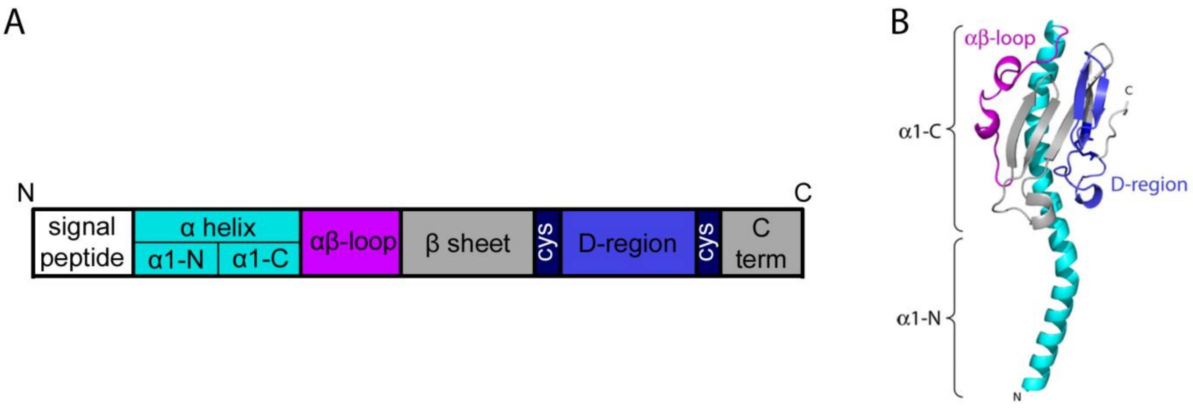


Figure 1.1 Structure of the *N. gonorrhoeae* strain MS11 major pilin subunit, PilE.

(A) Schematic representation of the PilE protein from *N. gonorrhoeae* and the relevant regions. (B) Protein structure of *N. gonorrhoeae* PilE (Protein Data Bank accession no. 1AY2). The conserved α-helix (cyan) consists of two subdomains; α1-N and α1-C. The globular head domain contains two variable regions; the αβ-loop and the D-region. The D-region is located between two cysteine residues at the C-terminal end which form a disulfide bond (Giltner *et al*., 2012). Figure adapted from Craig and Li 2008 and Giltner *et al*., 2012.

Pilus biogenesis in *N. meningitidis* requires a number of Pil proteins involved in pilus fiber assembly, emergence onto the cell surface, and stabilization (Brown *et al*., 2010; Carbonnelle *et al*., 2005) (Figure 1.2). Pilus assembly occurs at the inner membrane and first involves the cleavage of the leader sequence of prepilins by the prepilin peptidase PilD (Strom *et al*., 1993; Strom and Lory, 1993). Assembly of pilin subunits is thought to occur through the incorporation of single pilin monomers into the growing pilus fiber, mediated by interactions between their hydrophobic N-terminal helices (Craig *et al*., 2006) and involves the cytoplasmic ATPase PilF and the inner membrane protein PilG (Carbonnelle *et al*., 2006). A dedicated pilus assembly subcomplex at the inner membrane comprising PilM, PilN, PilO and PilP is also thought to be involved this process (Georgiadou *et al*., 2012). Assembly is accompanied by emergence of the pilus fiber onto the cell surface through a pore in the outer membrane formed by an oligomeric ring of the secretin PilQ (Berry *et al*., 2012; Collins *et al*., 2001). The PilC proteins, which are present as two alleles, PilC1 and PilC2, also play accessory roles in pilus assembly (Jonsson *et al*., 1991; Ryll *et al*., 1997). The retraction ATPase PilT drives depolymerization of the pilus that retracts the fiber at forces that can exceed 80 pN (Merz *et al*., 2000). Pilus stability and counteraction of PilT-mediated pilus retraction involves the proteins PilC, PilG, PilH, PilI, PilJ, PilK and PilW (Carbonnelle *et al*., 2006).

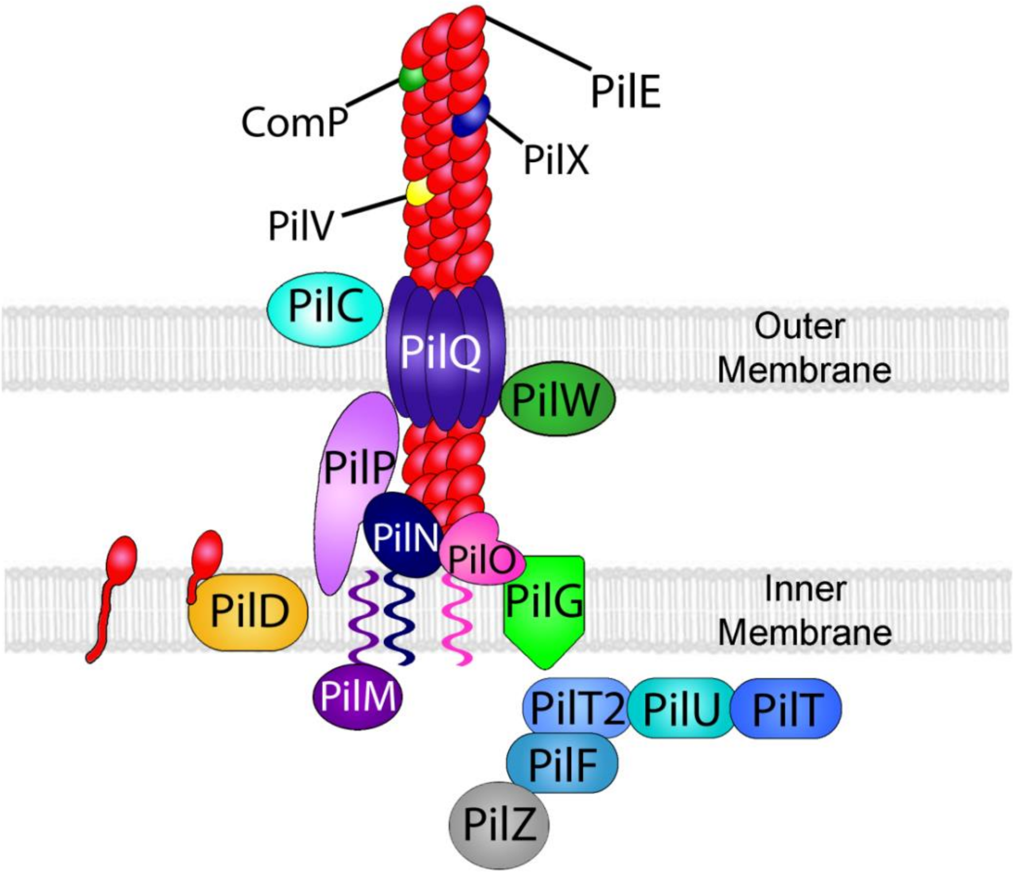


Figure 1.2 Schematic diagram of Tfp biogenesis in *N. meningitidis*.

The major pilin, PilE, is synthesized as a prepilin containing a hydrophobic N-terminal leader sequence which is cleaved by the prepilin peptidase PilD before the mature pilins are assembled into the pilus fiber on the periplasmic face of the inner membrane. Pilus assembly involves a subcomplex comprised of PilN, PilM, PilO and PilP and is powered by the cytoplasmic ATPase PilF. The assembled fiber is secreted through a channel in the outer membrane provided by the PilQ secretin. PilW is an outer membrane protein involved in pilus stabilization. Pilus retraction is powered by the cytoplasmic ATPase PilT, during which pilins are disassembled from the base of the fiber. PilT2 and PilU are paralogs of PilT but cannot substitute for PilT. ComP, PilX, and PilV are minor pilins that are present in low abundance in the Tfp fiber and have important roles in mediating Tfp function. PilC is an accessory assembly factor that is also involved in adhesion to host cells. PilG is a conserved inner membrane protein while PilZ is a cytoplasmic protein of unknown function that interacts with PilF.

1.3.2 Tfp function

The expression of pili appears to be particularly important for virulence. Tfp mediate numerous functions that contribute to colonization and pathogenesis. A key function of Tfp in initializing colonization is the ability to promote adhesion to host surfaces (Stephens and McGee, 1981). Tfp-mediated adhesion to human epithelial and endothelial cells involves the major pilin PilE and the minor pilin PilV, both of which have been reported to function as ligands of β2-adrenoreceptor to mediate binding to endothelial cells and activation of downstream signaling events (Coureuil *et al*., 2010; Lécuyer *et al*., 2012). In *N. meningitidis* strain 8013, it was found that the non-pilin Tfp-associated protein PilC1, but not PilC2, plays a role in cell adhesion (Nassif *et al*., 1994; Rudel *et al*., 1995; Scheuerpflug *et al*., 1999). Although it has previously been proposed that CD46 is the receptor for *Neisseria* Tfp on human epithelial cells (Johansson *et al*., 2003; Källström *et al*., 1997), recent studies have suggested otherwise (Kirchner *et al*., 2005). As such, the identity of the epithelial cell receptor remains controversial. Tfp can also form bundles laterally to promote bacterial aggregation and microcolony formation – processes which require the minor pilin PilX, which was proposed to interact with other PilX molecules protruding from opposing fibers to inhibit retraction of adjacent antiparallel pili and thereby stabilizing microcolonies (Helaine *et al*., 2007; Hélaine *et al*., 2005). However, an alternative theory in which bacterial aggregation is determined by the number of Tfp fibers has also been proposed (Imhaus and Duménil, 2014). Tfp-mediated formation of microcolonies not only promotes surface colonization, but also facilitates the exchange of DNA between cells (Berry and Pelicic, 2015).

Tfp are responsible for twitching motility, enabling the bacterium to translocate across surfaces. This mechanism involves the alternate polymerization and depolymerisation (retraction) of the pilus fiber which allows bacteria to pull themselves towards the site of pilus attachment; the retraction ATPase PilT is indispensable for this function (Merz *et al*., 2000; Wolfgang *et al*., 1998). A single pilus retraction can generate forces exceeding 100 pN (Maier *et al*., 2002), while bundles of 8-10 Tfp filaments can act as retractable units to generate forces in the nN range (Biais *et al*., 2008). In *N. gonorrhoeae*, Tfp retraction forces are involved in interaction with host cells by promoting cortical plaque formation and induction of cytoprotective signaling; infection of epithelial cells with *pilT* mutants was found to trigger apoptotic cell death (Higashi *et al*., 2007).

N. meningitidis is also naturally competent for DNA uptake by virtue of the ability of Tfp to uptake DNA in a process involving DNA binding to the minor pilin ComP and subsequent Tfp retraction (Aas *et al*., 2002; Cehovin *et al*., 2013; Wolfgang *et al*., 1999). ComP is a Tfp-exposed DNA receptor with a preference for the DNA uptake sequence (DUS) which is a species-specific short sequence motif (Goodman and Scocca, 1988), thereby allowing selective uptake of homotypic DNA (Berry *et al*., 2013). After the DNA is pulled through the outer membrane by Tfp retraction, it interacts with another DNA receptor ComE/ComEA which transports the DNA to a translocase machinery that delivers it into the cytoplasm where it can be incorporated into the genome (Chen and Gotschlich, 2001). However, it was recently reported that fully assembled Tfp may not be absolutely required for competence; it was found that only the N-terminal domain of PilE is necessary for transformation competence through a mechanism involving the release of this domain as a soluble form (S-pilin) by specific proteolytic cleavage (Obergfell and Seifert, 2016). S-pilin was hypothesized to form a distinct pseudopilus structure that does not extend beyond the immediate cell surface, and this mechanism was proposed as a means of maintaining competence even when phase or antigenic variation (See Section 1.5) occasionally result in the expression of truncated pilins that cannot be assembled into a Tfp

(Obergfell and Seifert, 2016). The natural competence of *N. meningitidis* provides a driving force for promoting horizontal gene transfer in this genetically diverse organism.

In some strains of *N. meningitidis*, Tfp are also subject to phase variation and antigenic variation, enabling the bacterium to modulate its surface structure to evade host immune responses. Pilin variation will be discussed in detail in Section 1.4.

1.3.3 The pilE *gene*

In *N. meningitidis* and *N. gonorrhoeae*, the major pilin protein is encoded by *pilE*. The *pilE* gene is composed of a highly conserved 5' region which encodes the N-terminal α-helix of the protein (Hagblom *et al.*, 1985). This is followed by a semivariable region which contains several conserved sequence elements designated SV1 to SV5. The 3' region of the gene encodes what is referred to as the hypervariable region of pilin. The greatest degree of sequence variability occurs in between sequences encoding two cysteine residues near the C-terminus (Hagblom *et al.*, 1985) which form a disulfide bond (Figure 1.1B). This region of the protein, known as the disulfide-, or D-region, is surface exposed in the assembled pilus and its sequence may thus influence pilus function (refer to Section 1.3.1).

1.3.4 Class I and Class II PilE

N. meningitidis expresses one of two distinct types of pili which are distinguished by their pilin subunit molecular weight, reactivity to monoclonal antibodies (Diaz *et al.*, 1984), and genetic locus (Wörmann *et al*., 2014). Meningococcal class I pilins are structurally similar to the gonococcal pilins, while the PilE subunits of class II pili have a lower molecular weight than class I PilE and mostly do not bind to the mAb SM1 (Potts and Saunders, 1988; Virji *et al*., 1989). In *N. meningitidis* strains that express class I pili, there is one silent pilin locus containing four to 11 copies of *pilS* located upstream of the *pilE* gene (Potts and Saunders, 1988; Wörmann *et al*., 2014) (Figure 1.3A). In contrast, *N. meningitidis* isolates that express class II pili possess a truncated silent locus containing comparatively fewer copies of *pilS*, while the expressed gene *pilE* is located elsewhere on the chromosome (Aho *et al*., 1997; Perry *et al*., 1988; Wörmann *et al*., 2014) (Figure 1.3B).

It was initially assumed that the *pilE* gene in all pathogenic *Neisseria* had the ability to undergo high frequency gene conversion to alter its sequence, in a phenomenon known as antigenic variation (see Section 1.5). However, while *pilE* variation has been observed *in vitro* in *N. gonorrhoeae* and in class I pilin expressing strains of *N. meningitidis*, *pilE* variation was not detected *in vitro* in the class II pilin strains FAM18 and NMB (Criss *et al*., 2005; Davies *et al*., 2014; Helm and Seifert, 2010). Moreover, in an investigation of the sequence variation of *pilE* from disease-causing *N. meningitidis* isolates, it was found that, while the class I *pilE* genes show considerable variation, class II *pilE* genes were more conserved (Wörmann *et al*., 2014). The region (known as the D-region) encompassed by two conserved cysteine residues located near the C-terminus of the PilE protein is usually highly variable in class I pilin but is strikingly conserved and significantly shorter in class II pilins (13 or 16 amino acids in length compared to 28 to 40 amino acids in class I pilins) (Cehovin *et al*., 2010; Wörmann *et al*., 2014).

Analysis of *pilE* in non-pathogenic *Neisseria* species has revealed that their predicted pilin sequences closely resemble that of class II pilins (Aho *et al*., 2000; Wörmann *et al*., 2014).

A number of non-pathogenic *Neisseria* species including *Neisseria cinerea*, *Neisseria subflava*, and *Neisseria flavescens* possess a second putative pilin gene located in tandem with the initial *pilE* homologue (Aho *et al*., 2005; Wörmann *et al*., 2014); however the precise function of this gene remains to be determined.

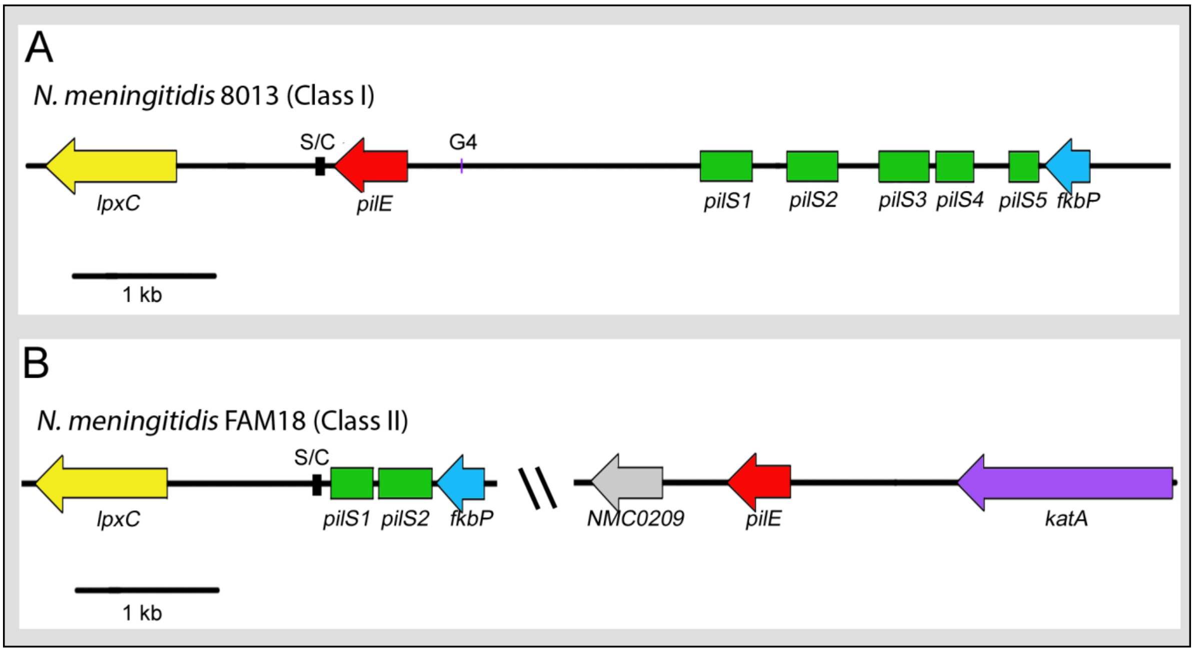


Figure 1.3 Schematic diagram of the *pilE* and *pilS* locus in class I and class II pilin expressing *N. meningitidis.*

(A) pilE locus in *N. meningitidis* 8013 which expresses class I *pilE*. Five silent *pilS* cassettes are located upstream of *pilE*. The *pilS*/*pilE* locus is located between the genes *fkbP* and *lpxC*. G4 and Sma/Cla (S/C) are sequences important for pilin variation. (B) In *N. meningitidis* FAM18 which expresses class II *pilE*, only two *pilS* cassettes are present in the *fkbP*-*lpxC* region. *pilE* is located elsewhere in the chromosome.

1.4 Regulation of *pilE* expression in *N. meningitidis*

Sequence analysis of *N. meningitidis* MC58, which expresses class I pilin, indicates that the upstream region of *pilE* contains a σ^{70} and σ^{54} promoter, while the *N. meningitidis* 8013 *pilE* upstream region only contains a σ^{70} promoter (Carrick *et al*., 1997; Taha *et al*., 1996). σ^{70} promoters are recognized by the bacterial housekeeping σ factor, σ^{70}, also known as RpoD, while transcription from the σ^{54} promoter requires the presence of the alternative σ factor, RpoN (Thöny and Hennecke, 1989). However, *N. meningitidis* does not possess a full-length RpoN due to a deletion of the region that potentially encodes a helix-turn-helix motif (M. Lobanovska, Tang Lab, unpublished) – one of two DNA binding motifs present in bacterial RpoN (Taylor *et al*., 1996), raising the possibility that the *N. meningitidis* RpoN is not functional (Laskos *et al*., 1998). This corroborates the observation that the σ^{54} promoter is not used to transcribe *pilE* in *N. meningitidis,* but is functional when transferred to *Pseudomonas aeruginosa*, in which RpoN is present (Carrick *et al*., 1997).

A number of proteins have been found to play a role in the regulation of *pilE*. It has been reported that CrgA, a transcriptional regulator that belongs to the LysR family, downregulates the expression of *pilE* in *N. meningitidis* in Clone 12 (also known as 2C4-3), a derivative strain of 8013 (Deghmane *et al*., 2002). The *crgA* promoter region harbors a promoter element, CREN (contact regulatory element of *Neisseria*) (Deghmane *et al*., 2000). Upon contact with the host cell, it was shown that *crgA* gene expression is induced in a CREN-dependent manner, which results in the negative regulation of *pilE* at the transcriptional level. CrgA was found to bind to the promoter region of *pilE*, which contains a motif that is typical of a LysR recognition sequence (Deghmane *et al*., 2002; Schell, 1993). It was proposed that the downregulation of bacterial surface structures such as pili and capsule, enable the meningococcus to undergo intimate adhesion to cells (Deghmane *et al*., 2002). However, this model of CrgA mediated downregulation of *pilE* expression has been disputed; Ieva *et al*. (2005) reported that transcription from the *pilE* promoter is not regulated by CrgA in strain MC58. Nevertheless, it was recently shown that CrgA does bind to the promoter region of *pilE,* and that HPr, a phosphocarrier protein of the sugar phosphotransferase system, increases the affinity of CrgA for this binding site in strain 2C4-3 (Derkaoui *et al.*, 2016). Thus, the mechanism of adherence mediated regulation of *pilE* is not yet fully understood.

NafA, a protein which is upregulated and surface expressed after adhesion of *N. meningitidis* to host cells, was also reported to be involved in the regulation of meningococcal piliation (Kuwae *et al.*, 2011). A mutation in *nafA* resulted in the expression of significantly more pili. However there was no difference in *pilE* transcript levels in *nafA* mutants. It was thus hypothesized that NafA controls piliation at the post-transcriptional level by suppressing the excess accumulation of extracellular pilin (Kuwae *et al.*, 2011).

By proteomic analysis, the RNA chaperone Hfq was found to regulate the expression of *pilE* in *N. meningitidis*. Upon deletion of *hfq*, the PilE protein was less abundant in *N. meningitidis* H44/76 (Pannekoek *et al.*, 2009), consistent with a study involving *N. gonorrhoeae*, in which piliation levels were reduced in *hfq* mutants (Dietrich *et al.*, 2009). However, *hfq* deletion in *N. meningitidis* MC58 resulted in the opposite effect, with PilE levels increased by 1.6-fold (Metruccio *et al.*, 2009). It was proposed that the disparity in these observations could be due to strain-specific differences in the regulation of pilin expression (Dietrich *et al.*, 2009). Nevertheless, given the role of Hfq in facilitating interactions between regulatory small RNAs and their target mRNAs (Vogel and Luisi, 2011), this finding suggests the possible involvement of sRNAs in the regulation of *pilE* expression. However, the mechanism by which Hfq regulates pilin expression in *Neisseria* remains to be elucidated. Consistent with the possibility that regulatory small RNAs may be involved in pilin or pilus regulation, it has recently been found that a deletion in polynucleotide phosphorylase (PNPase), a 3'–5' exonuclease that is involved in RNA turnover and the regulation of small RNAs, results in elevated PilE expression, and consequently enhanced aggregation in *N. meningitidis* strain FAM20 *via* a mechanism that was proposed to involve increased pili bundling resulting from the PNPase deletion (Engman *et al*., 2016).

1.5 Pilin antigenic variation

Tfp of *N. meningitidis* expressing class I PilE undergo antigenic variation (Av), in which the DNA sequence of *pilE* is altered, resulting in the intra-strain expression of a pilin protein with a different amino acid sequence and thus potentially changing the antigenicity of the pilus. Pilin Av in *Neisseria* involves the non-reciprocal DNA recombination between one of multiple silent pilin gene copies, *pilS*, and the pilin expression locus *pilE* (Figure 1.3A) leading to the expression of a new variant of *pilE* (Hagblom *et al*., 1985). The silent copies lack ribosome binding sites and the common N-terminal coding sequence of *pilE* and therefore do not express a functional pilin (Haas and Meyer, 1986). Although *pilS* cassettes were previously thought to lack apparent promoters, recent evidence suggests that some *pilS* copies in *N. gonorrhoeae* are actively transcribed to yield both sense and antisense small RNA species. However the function of these transcripts remains to be determined (Wachter *et al*., 2015). During a recombination event, the expression locus receives all or part of a *pilS* sequence, resulting in the generation and expression of a *pilE* of novel sequence (Haas and Meyer, 1986). Studies involving experimental infections in human subjects have demonstrated the specific recombination events occurring during gonococcal infection, showing that recombination of *pilS* with *pilE* occurs *in vivo* to generate new expressed pilins as early as one day after inoculation (Hamrick *et al*., 2001; Swanson *et al*., 1987). Av of PilE in *N. meningitidis* has also been reported to occur both *in vitro* and during human infection (Helm and Seifert, 2010; Omer *et al*., 2011; Tinsley and Heckels, 1986).

Pilin Av is thought to confer several advantages on bacteria in terms of increased survival and virulence in the host. The ability to express antigenically distinct pili may enable pathogenic *Neisseria* to evade the effects of antibodies directed against the pili and thus escape host immune responses (Heckels, 1989). *N. gonorrhoeae* has been observed to switch pilin variants at a higher rate *in vivo* compared to *in vitro*, suggesting that pilus variation is important during survival and invasion in the host (Hamrick *et al*., 2001; Seifert *et al*., 1994), further implying the possibility that increased Av rates are a response to environmental signals encountered *in vivo*. In addition to immune evasion, pilin variation might alter the adherence properties of the pilus. Different pilin variants of the same *N. meningitidis* strain have been found to exhibit distinct levels of adhesion to human epithelial cells, suggesting that the antigenic variation of pilin could play a role in regulating meningococcal adhesion (Nassif *et al*., 1993). Moreover, PilE variation has also been reported to influence serum resistance and the ability to induce host cell response (Hubert *et al*., 2012; Miller *et al*., 2014). *pilE*/*pilS* recombination can also result in pilin phase variation, which occurs when the recombination event introduces a premature stop codon, leading to the expression of a truncated PilE protein (Koomey *et al*., 1987).

As described in Section 1.3.4, while both *N. gonorrhoeae* and class I pilin expressing *N. meningitidis* strains demonstrate highly variable *pilE* resulting from Av, intra-strain *pilE* variation is not thought to occur in class II pilin expressing *N. meningitidis* strains (Cehovin *et al*., 2010; Wörmann *et al*., 2014). *N. meningitidis* MC58, which expresses class I pili, was found to have an Av frequency of 0.03 events per cell with a rate of 1.6 x 10^{-3} events per cell per generation (Helm and Seifert, 2010), while no pilin antigenic variation was detected in *N. meningitidis* FAM18, which expresses class II pili (Helm and

Seifert, 2010). It should be noted that in spite of the purported functions of pilin Av in pathogenesis, both class I and class II pilin expressing strains of *N. meningitidis* are capable of causing human disease; in fact, strains expressing class II *pilE* often belong to so-called “hypervirulent” clonal complexes (*i.e.* groups of sequence types) that display high disease to carriage ratios or are associated with epidemic disease (Wörmann *et al*., 2014)

1.5.1 Current understanding of mechanisms of antigenic variation

Although pilin Av has been more extensively studied in *N. gonorrhoeae* compared to *N. meningitidis*, the exact mechanism by which this occurs has yet to be fully understood in either organism. RecA, a DNA recombination and repair protein belonging to a family of ATPases that catalyze ATP-dependent strand exchange between ssDNA and heterologous duplex DNA (Radding, 1981), is necessary for pilin Av in *N. gonorrhoeae*, providing evidence that this process is mediated by homologous recombination (Koomey *et al*., 1987). The mechanism by which RecA promotes strand exchange during homologous recombination involves a three stage process: RecA first binds to ssDNA to form an active nucleoprotein filament; next, the nucleoprotein aligns and pairs with homologous dsDNA; finally, during branch migration, one strand of the duplex DNA is gradually displaced by the incoming ssDNA (Brenner *et al*., 1987). RecX, the negative regulator of RecA, is involved in controlling the length of the RecA nucleoprotein filament and in turn optimizes RecA filament length to stimulate pilin antigenic variation (Gruenig *et al*., 2010).

Pilin Av has previously been shown to require a *cis*-acting DNA element located in the intergenic region upstream of *pilE*, consisting of a 16 base-pair guanine-rich sequence

($G_3TG_3TTG_3TG_3$) that conforms to a guanine quartet (G4) motif (Cahoon and Seifert, 2009; Kline *et al*., 2007) (Figure 1.3A). The G4 motif is also present in the *pilE* locus of class I *pilE* expressing *N. meningitidis* strains but not in the *pilE* locus of class II *pilE* expressing strains or in commensal *Neisseria* (Cahoon and Seifert, 2009). This sequence motif is able to form a four-stranded quadruplex structure *in vitro* (Cahoon and Seifert, 2009) that is stabilized by hydrogen bonding between guanines within G-quartets (a planar array of guanines) and from stacking of the G-quartets (Maizels and Gray, 2013). Formation of the G-quadruplex structure was found to be necessary for pilin antigenic variation in *N. gonorrhoeae,* as point mutation of any 11 of the 12 GC base pairs in this motif that inhibited G4 structure formation was found to abolish Av (Cahoon and Seifert, 2009). Moreover, growth of *N. gonorrhoeae* on N-methyl mesoporphyrin IX (NMM), which specifically binds G4 DNA but not duplex DNA demonstrated significantly decreased pilin Av (Cahoon and Seifert, 2009). Quadruplex formation is predicted to require the transcription of a noncoding RNA from an upstream promoter that initiates transcription within the G4 sequence (Cahoon and Seifert, 2013). Mutations which inactivate the promoter or alteration of the direction and orientation of the promoter prevent Av, while expression of the noncoding RNA in *trans* does not rescue a promoter mutation (Cahoon and Seifert, 2013). It was therefore proposed that transcription of this noncoding RNA is required to open the DNA duplex for G-quadruplex formation (Figure 1.4).

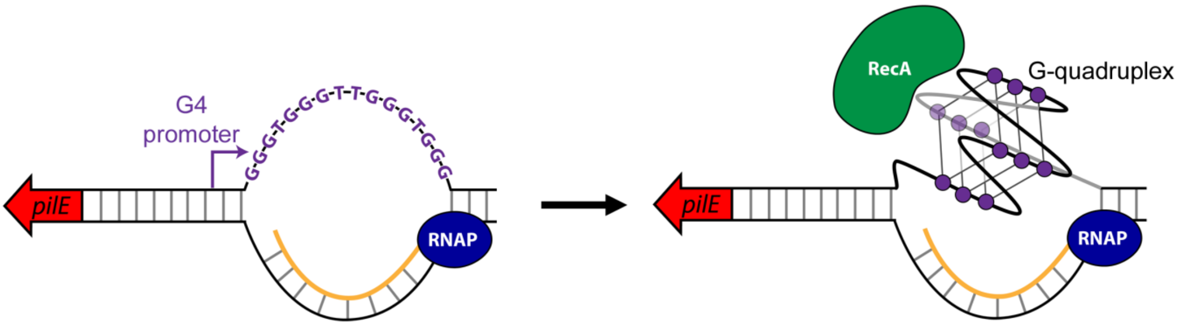


Figure 1.4 Proposed model of the role of G4 transcription in Av .

(A) Schematic diagram of the *pilE* locus of *N. gonorrhoeae* illustrating the proposed model of the role of G4 transcription in Av. Transcription of the G4 sequence by RNA polymerase (RNAP) has been proposed to result in the formation of a G-quadruplex structure by the single stranded G-rich strand of DNA which recruits proteins (such as RecA) involved in the recombination process (Cahoon and Seifert, 2013).

The structure of the *pilE* G-quadruplex has been resolved by nuclear magnetic resonance (NMR) and was found to adopt a three-layered all parallel-stranded G-quadruplex fold and is able to bind *E. coli* RecA and promotes strand exchange mediated by RecA *in vitro* (Kuryavyi *et al.*, 2012). The finding that RecA interacts specifically with the *pilE* quadruplex, but not other G-quadruplex structures, coupled with the fact that the G-quadruplex forming sequence is only present at one location in the *N. gonorrhoeae* genome (upstream of *pilE*), suggests that RecA may play a role in pilin Av *via* its recruitment to the *pilE* locus by the G-quadruplex to facilitate recombination between *pilE* and *pilS* (Kuryavyi *et al.*, 2012).

In addition to RecA, homologues of *E. coli* RecQ and RecO are necessary for pilin antigenic variation in *N. gonorrhoeae* (Mehr and Seifert, 1998). In particular, deletion of the “helicase and RNase D C-terminal” (HRDC) domains of RecQ decreased the frequency of pilin Av (Killoran *et al.*, 2009) and also resulted in a decrease in G-quadruplex structure binding and unwinding *in vitro*, suggesting a possible role for this enzyme in unwinding the *pilE* G4 structure (Cahoon *et al*., 2013).

In *E. coli*, homologous recombination occurs primarily through the RecBCD pathway and also *via* the RecF pathway, both of which require RecA (Kowalczykowski *et al*., 1994). RecQ and RecO are part of the RecF pathway, suggesting that pilin antigenic variation in *N. meningitidis* occurs through a RecF-like pathway. Subsequent mutational studies revealed that the *recJ* and *recR* genes, which are also part of the RecF-like pathway, are involved in pilin Av (Sechman *et al*., 2005; Skaar *et al*., 2002). However, the RecBCD pathway is not involved in pilin Av in *N. gonorrhoeae* (Mehr and Seifert, 1998). The mismatch correction (MMC) system, which repairs DNA mismatches and small insertion-deletions that arise following DNA replication or recombination, has also been reported to modulate gene conversion events at *pilE* (Criss *et al*., 2010). The MutS protein of the MMC system has an inhibitory effect on pilin Av due to its ability to limit recombination between homeologous (*i.e*. partially homologous) DNA by preventing RecA from performing branch migration on mismatched homologues during strand exchange (Rotman and Seifert, 2015).

Another conserved DNA sequence in the *pilE* locus is also required for efficient pilin recombination. This sequence, called the Sma/Cla repeat due to it being flanked by *Cla*I and *Sma*I sites (Figure 1.3A), is located downstream of both *N. gonorrhoeae* and *N. meningitidis pilE* and bears sequence similarity to several recombinase-binding sites (Meyer *et al*., 1984; Wainwright *et al*., 1994). On further investigation of the *N. gonorrhoeae* proteins that bind to the Sma/Cla sequence, Wainwright *et al*. (1997) found that only pathogenic *Neisseria* harbor the Sma/Cla repeat and the Sma/Cla binding proteins, suggesting that their function is important for pathogenesis. However, the identities of these proteins have yet to be elucidated. Notably, both the Sma/Cla repeat and the consensus G4 sequence are absent from the meningococcal class II pilin expression locus, possibly explaining the lack of antigenic variation in class II pilin expressing strains (Aho *et al*., 1997; Helm and Seifert, 2010; Wörmann *et al*., 2014).

1.5.2 Regulation of antigenic variation

The impact of several environmental conditions on the rate of pilin Av has been studied in *N. gonorrhoeae*. The frequency of pilin Av in *N. gonorrhoeae* was only altered under iron limiting conditions, while conditions such as carbon source, temperature, aromatic amino acid availability, and oxygen availability were not found to significantly alter pilin Av (Serkin and Seifert, 2000). In low iron conditions, pilin antigenic variation frequency was increased. This could act as an environmental signal for increased recombination events and thus gonococcal pathogenesis *in vivo* (Serkin and Seifert, 2000). However, it is not known whether pilin Av in *N. meningitidis* responds similarly to environmental cues.

1.6 Noncoding regulatory RNAs in bacteria

Noncoding RNAs with regulatory functions were first discovered in *E. coli* plasmid systems: the antisense RNAs, RNA I and CopA, produced by the ColE1 and R1 plasmids respectively were found to regulate plasmid replication (Stougaard *et al*., 1981; Tomizawa *et al*., 1981). It has since become evident that noncoding regulatory RNAs are present in all domains of life. Today, noncoding regulatory RNAs are increasingly recognized as important regulators of gene expression in bacteria and in eukaryotes. RNA regulators may be advantageous compared to protein regulators for a number of reasons: they are faster to produce and less costly to the cell, their regulatory effect can also be fast, allowing the bacterium to rapidly respond to environmental signals, and regulatory RNAs are generally less stable than protein regulators, allowing them to be rapidly cleared when no longer needed (Waters and Storz, 2009). In addition, many RNAs act at the post transcriptional level, targeting mRNAs that have already been transcribed (Gripenland *et al*., 2010). Regulatory RNAs employ various mechanisms to modulate a wide range of physiological responses. Some regulatory RNA elements are mRNA leader sequences that affect expression in *cis*, and some mediate gene regulation through protein binding, however, the majority of noncoding regulatory RNAs modulate gene expression through base-pair interactions, as described in further detail below.

1.6.1 trans-*acting regulatory RNAs*

Trans-acting regulatory RNAs (sometimes known as small RNAs, sRNAs) regulate the expression of their target mRNAs, which may be encoded at a distance from the *trans*-acting RNA, through interactions involving short regions of imperfect complementarity. A number of *trans*-acting RNAs require the RNA chaperone Hfq to effectively carry out their regulatory function. By facilitating the base-pairing interactions between sRNAs and their targets, Hfq modulates the stability and translation rate of the target mRNA (Vogel and Luisi, 2011). The majority of *trans*-acting RNAs are expressed under specific growth conditions or as a result of specific environmental stresses, such as iron limiting, oxidative stress, or glucose starvation, suggesting a role in stress response in a number of different bacteria such as *E. coli*, *Vibrio cholerae* and *Pseudomonas aeruginosa* (Gottesman *et al*., 2006; Gottesman and Storz, 2011). A few *trans*-acting RNAs are involved in the regulation of virulence genes. For example, RNAIII regulates the expression of several virulence factors and also represses the synthesis of Rot, a transcriptional repressor of exoprotease and exotoxin genes in *Staphylococcus aureus* (Boisset *et al*., 2007), while four Qrr sRNAs are involved in control of quorum sensing and derepression of virulence genes in *V. cholerae* (Hammer and Bassler, 2007). Moreover, mutation in *hfq* was found to decrease virulence in several pathogens such as *Listeria monocytogenes* and *P. aeruginosa* (Christiansen *et al*., 2004; Sonnleitner *et al*., 2003).

Trans-acting RNAs act on their targets through a number of different mechanisms. Base-pairing with the 5'- UTR of the target mRNA may result in occlusion of the ribosome binding site and consequently inhibition of translation (Figure 1.5A), or may lead to sRNA-mRNA duplex degradation by RNases such as RNase E (Waters and Storz, 2009) (Figure 1.5B). Hfq/sRNA mediated degradation of mRNAs involves the formation of ribonucleoprotein complexes in which the sRNA and mRNA associate with RNase E through Hfq (Morita *et al*., 2005). *Trans*-acting RNAs can also act positively by preventing the formation of inhibitory secondary structures that sequester the ribosome binding site (Hammer and Bassler, 2007; Prévost *et al*., 2007) (Figure 1.5C).

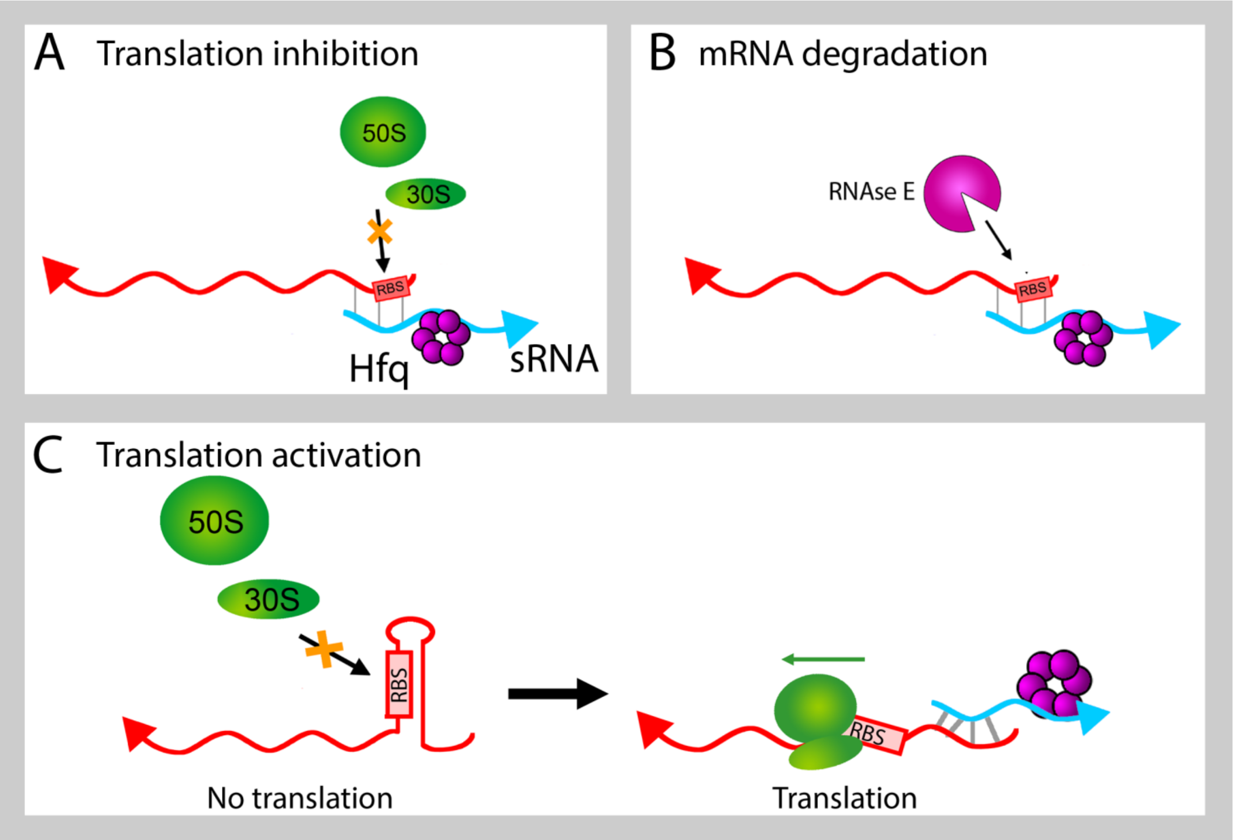


Figure 1.5 Mechanisms of gene regulation by *trans*-acting regulatory RNAs.

(A) Trans-acting regulatory RNAs (light blue) may base pair with the 5'- UTR of their target mRNA to prevent ribosome binding to the ribosome binding site (RBS) and inhibit translation. The RNA chaperone Hfq may be involved in sRNA stabilization and/or facilitating binding to the mRNA. (B) Base pair interactions between the *trans*-acting regulatory RNA with the target mRNA may also result in RNase E mediated degradation of the sRNA:mRNA duplex. (C) *Trans*-acting regulatory RNAs can also activate translation by preventing the formation of secondary structures that sequester the ribosome binding site.

1.6.2 Antisense RNAs

In contrast to *trans*-acting regulatory RNAs, antisense RNAs, also known as *cis*-encoded RNAs, are encoded on the opposite strand of their target and are therefore fully complementary to their targets over an extended sequence stretch. Though not as extensively studied as *trans*-acting RNAs, antisense transcripts are increasingly being identified across the bacterial kingdom due to advances in whole genome transcriptomic methods such as tiling arrays and RNA sequencing (RNAseq) (Thomason and Storz, 2010). Deep sequencing of the *E. coli* transcriptome has revealed widespread antisense transcription of about 1000 antisense RNAs (Dornenburg *et al*., 2010). In the human pathogen *Helicobacter pylori*, it was reported that ~46% of all open reading frames are associated with at least one antisense RNA (Sharma *et al*., 2010).

Antisense RNAs mediate their regulatory function by a number of different mechanisms. Antisense RNAs can regulate transcription of the genes encoded on the opposite strand by transcription interference or transcription attenuation (Sesto *et al*., 2013). In the former, sense transcription is physically and directly suppressed by the process of antisense transcription when the two promoters are convergent, which may result in the premature termination of one or both transcription processes (André *et al*., 2008) (Figure 1.6A). In transcription attenuation, base pairing of the antisense RNA to the mRNA can induce the formation of a terminator structure in the target mRNA, resulting in premature termination (Stork *et al*., 2007) (Figure 1.6B).

Through perfect base-pairing interactions with the target mRNA, antisense RNAs can also affect the stability of the target mRNA by either promoting or blocking cleavage by RNases. In some cases, formation of the antisense RNA – mRNA duplex can lead to codegredation by RNases such as RNase III, a double stranded endoribonuclease (Figure 1.6C), while in other cases could result in the triggering of specific cleavage of the sense transcript to produce two stable independent transcripts (Figure 1.6D), as is the case in GadY mediated positive regulation of *gadXW* in *E. coli* (Opdyke *et al*., 2004, 2011). In addition, base-pairing to form the antisense RNA – mRNA duplex may prevent recognition of a cleavage site by RNase E, which cleaves single stranded RNA, resulting in stabilization of the mRNA (Sakurai *et al*., 2012) (Figure 1.6E).

Finally, in a manner similar to the mechanism of many *trans*-acting regulatory RNAs, for antisense RNAs containing the sequence complementary to the 5'- UTR of the target mRNA, regulation of the target can occur in a similar mechanism, resulting in the blocking of ribosome binding and inhibition of translation (Kawano *et al*., 2007) (Figure 1.6F).

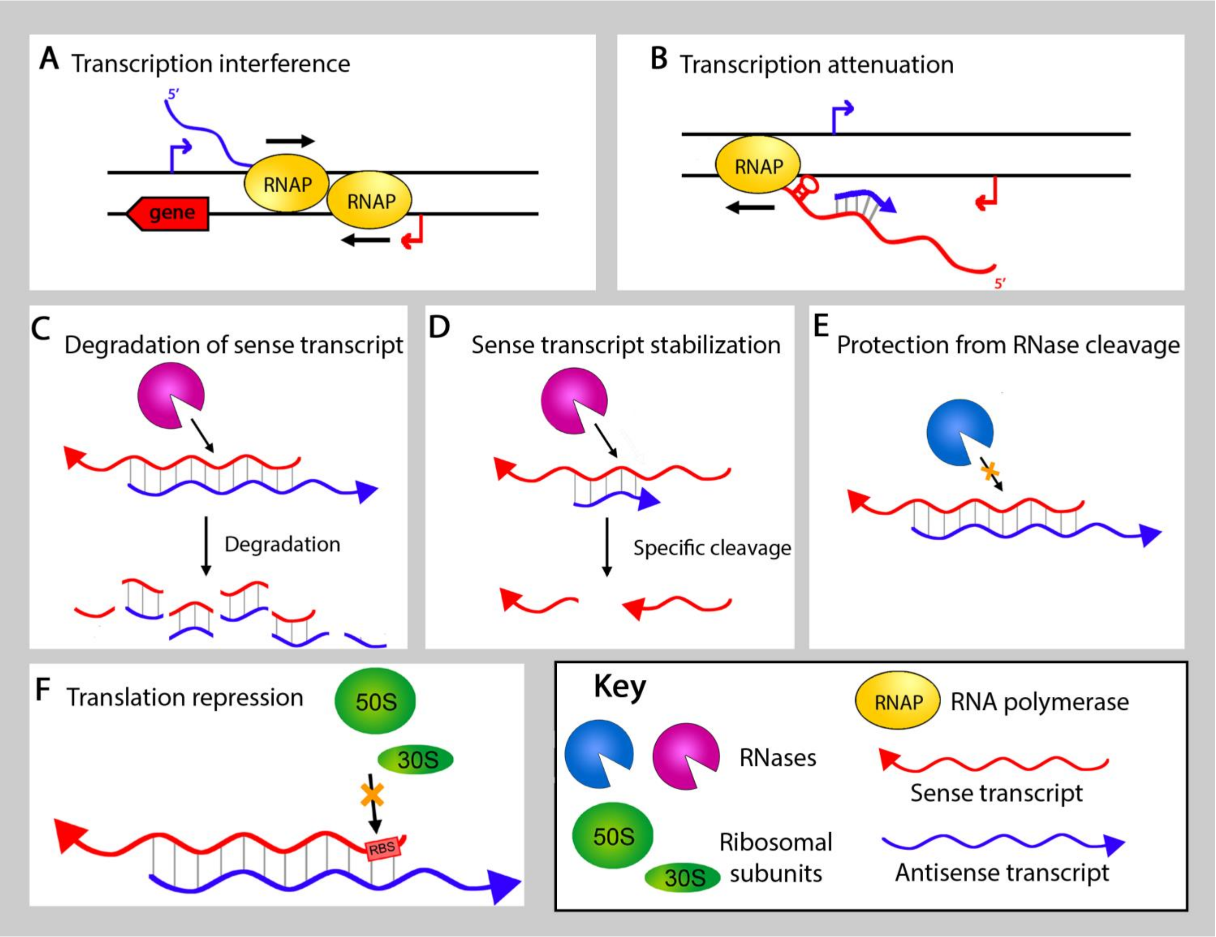


Figure 1.6 Mechanisms of gene regulation by antisense RNAs.

(A) Transcriptional interference involves the physical inhibition of transcription due to antisense transcription. (B) Transcription attenuation occurs when binding of the antisense RNA to the growing mRNA induces transcription termination as a result of the formation of a terminator structure in the target mRNA. Base-pairing between the antisense RNA and the sense RNA can either (C) target the RNA:RNA duplex for degradation by RNases, (D) promote a specific cleavage event to produce stable sense transcripts, or (E) protect RNase recognition sites on the sense transcript from RNase cleavage. (F) Antisense RNAs may also inhibit translation by binding to the ribosome binding site of the target mRNA.

1.7 Noncoding regulatory RNAs in *Neisseria meningitidis*

As described in Section 1.6, many *trans*-acting regulatory RNAs rely on the RNA chaperone Hfq for efficient stabilization and annealing to the mRNA targets. Hfq belongs to the Sm and Sm-like protein family which includes proteins involved in nuclear pre-mRNA splicing in eukaryotes (Møller *et al.*, 2002) and is widely conserved in bacteria; approximately half of bacterial genomes available in the NCBI database were found to possess a *hfq* homolog (Sun *et al.*, 2002). An analysis of the changes in the transcriptional profile of *N. meningitidis* incubated in blood found that Hfq is upregulated, suggesting that Hfq plays a role in meningococcal bloodstream survival (Echenique-Rivera *et al.*, 2011). Studies have also found that Hfq is involved in the stress response and virulence of *N. meningitidis*. Hfq mutants exhibit reduced sensitivity to stress in *ex vivo* and *in vivo* models (Fantappiè *et al.*, 2009), while a transposon insertion in *hfq* was found to attenuate virulence (Sun *et al.*, 2000). Two separate studies involving proteomic analyses of *N. meningitidis* lacking Hfq reported that the expression of proteins involved in various functions including metabolism, amino acid biosynthesis, and oxidative stress response, was affected by the mutation (Fantappiè *et al.*, 2009; Pannekoek *et al.*, 2009). Thus, these findings suggest the possible presence of a network of sRNAs involved in virulence and stress adaptation in *N. meningitidis*.

Although Hfq is the best characterized RNA chaperone protein in bacteria, *N. meningitidis* also possesses an RNA chaperone with structural and functional similarities to the *E. coli* RNA chaperone, FinO (Chaulk *et al.*, 2010). FinO, which is expressed from the F family of plasmids, is highly specific and is involved in the facilitation of RNA-RNA interactions between FinP and *traJ* mRNA which results in TraJ translation in *E. coli* (Arthur *et al.*, 2003). A homologue of FinO, which is encoded on the *N. meningitidis* chromosome, displays RNA chaperone activity and could potentially be involved in the modulation of sRNA-mediated processes in *N. meningitidis* (Chaulk *et al*., 2010).

The first regulatory sRNA recognized in *N. meningitidis* was identified using a bioinformatic approach, in which intergenic regions were searched for putative binding sites of the iron-responsive transcriptional regulator Fur, and rho-independent terminators, in order to identify putative Fur-regulated sRNAs (Mellin *et al*., 2007). A novel sRNA, termed NrrF, is a homolog of the *E. coli* Fur-regulated sRNA RyhB, and is involved in the regulation of succinate dehydrogenase genes in *N. meningitidis* (Mellin *et al*., 2007; Metruccio *et al*., 2009). Succinate dehydrogenase is an iron-containing enzyme and its mRNA levels are downregulated in many bacteria in response to iron starvation (Massé and Gottesman, 2002; Salvail and Massé, 2012). In low-iron conditions, Fur activates NrrF transcription, which acts in *trans* to repress the *sdhA* and *sdhC* transcripts (Mellin *et al*., 2007). NrrF has also been identified in *N. gonorrhoeae* (Ducey *et al*., 2009).

An Hfq-dependent sRNA, AniS, has been identified in *N. meningitidis* (Fantappiè *et al*., 2011) and was found to depend on the global anaerobic transcriptional regulator FNR. In low oxygen concentrations, FNR binds the *aniS* promoter and activates *aniS* transcription, which subsequently downregulates mRNA targets NMB1468 and NMB0214 which were identified through transcriptome profiling (Fantappiè *et al*., 2011). NMB1468 and NMB0214 respectively encode a lipoprotein of unknown function and the PrlC oligopeptidase whose exact function is as yet unknown. As such, the rationale of AniS in downregulating these two targets in response to oxygen limitation has not been defined.

Numerous studies suggest that there is a large unexplored network of sRNAs that play a role in regulation in *N. meningitidis*. Analysis of the total transcriptome of *N. meningitidis* using a tiling array revealed that small intergenic RNAs, antisense RNAs, and transcripts with extended 5'- and 3'- UTRs were differentially expressed during incubation in human blood (Del Tordello *et al*., 2012). These transcripts represent noncoding RNA elements that may regulate genes involved in adaptation to human blood (Del Tordello *et al*., 2012). In *N. gonorrhoeae*, differential RNAseq (dRNAseq) analysis has resulted in the identification of 253 novel transcripts which may include undiscovered regulatory antisense and *trans*-acting RNAs (Remmele *et al*., 2014). Recent analysis of transcriptional profiling data of *N. meningitidis* exposed to stress signals such as heat shock, oxidative stress, iron and carbon source limitation, has revealed a set of 98 putative sRNAs that were differentially expressed (Fagnocchi *et al*., 2015). Four of these sRNAs were identified to play a role in *N. meningitidis* infection in the *in vivo* infant rat model and contribute to meningococcal bacteremia (Fagnocchi *et al*., 2015). In particular, Bns1 was identified as a blood-induced sRNA which regulates genes involved in energy metabolic processes, glutamate metabolism and purine biosynthesis, and is important for meningococcal fitness during infection (Fagnocchi *et al*., 2015). Furthermore, a recent study involving transposon insertion site sequencing (Tn-seq) analysis has identified a total of 33 intergenic regions containing sRNAs which were considered important for cell colonization (Capel *et al*., 2016).

1.8 Project Aims

Inspection of the nucleotide sequence of the *pilE* locus in *N. meningitidis* strain 8013 resulted in the identification of a putative σ^{70} consensus promoter on the antisense strand of *pilE*. This putative promoter has high sequence similarity to the bacterial consensus promoter sequence but no clear ribosome binding site or open reading frame was identified in its vicinity. Thus this putative promoter could potentially initiate the transcription of an RNA antisense to *pilE*. Given the importance of Tfp in meningococcal pathogenesis, it is intriguing to consider the possible role of a regulatory RNA in mediating *pilE* expression or Tfp function. The aims of this project were therefore to:

1. Determine whether the putative *pilE* antisense promoter is functional in *N. meningitidis*.
2. Investigate the possible role of the antisense RNA in mediating *pilE* expression or pilin function, and/or pilin variation.

2 Materials and Methods

2.1 Strains, plasmids and growth conditions

Strains and plasmids are described in Table 2.1. *E. coli* was grown overnight on Luria-Bertani (LB) media at 37°C containing carbenicillin (100µg/ml), kanamycin (50µg/ml) or erythromycin (200µg/ml) when required. *N. meningitidis* was grown overnight at 37°C, 5% CO_2 on brain heart infusion (BHI, Oxoid) agar supplemented with 5% heat denatured horse serum and 0.1% starch, or in liquid BHI. Erythromycin (2µg/ml), kanamycin (100 µg/ml) or tetracycline (2.5 µg/ml) was added for selection.

Overnight liquid cultures of *E. coli* were set up by inoculating LB broth with single colonies from plate cultures and incubating at 37°C overnight with shaking (180 rpm). For analysis of transcript levels during growth, liquid cultures of *E. coli* were grown by inoculating overnight cultures into LB broth in a 1:100 dilution and incubating at 37°C overnight with shaking. For liquid cultures of *N. meningitidis*, strains were grown overnight on BHI agar. Bacteria were harvested from plates, resuspended in PBS and quantified using A_{260nm} to measure the amount of DNA in a lysed sample. A volume equivalent to $1x10^9$cfu was inoculated into 25 ml of BHI media in 125 ml conical flasks (Corning), and cultures were shaken at 180 rpm at 37°C. OD_{600} readings were taken at intervals to determine the growth phase, and samples were taken at various time points for RNA and protein analysis. For stress experiments, liquid cultures of *E. coli* or *N. meningitidis* were grown for approximately 3 h in LB or BHI media respectively, to an OD_{600}= 0.5-0.6 (mid-log phase) and subjected to acid stress (HCl pH 2.5), envelope stress (5% Triton X-100 or 5% ethanol), oxidative stress (0.15% H_2O_2), salt stress (0.5 M NaCl or 0.5 M KCl), osmotic stress (6% sucrose), or temperature stress (10°C) for 10 minutes.

Table 2.1 Strains and plasmids used in this study.

Strain or Plasmid	Description	Reference or source
E. coli		
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi*-1 *hsdR17*	
N. meningitidis		
8013	Serogroup C, ST-18	Rusniok *et al*., 2009
WT_ery	Erythromycin resistance cassette upstream of AS promoter	This study
Mut_ery	Erythromycin resistance cassette upstream of AS promoter, AS promoter mutation	This study
WT_ery(RecA6)	WT_ery with IPTG inducible RecA	This study
Mut_ery(RecA6)	Mut_ery with IPTG inducible RecA	This study
*pilE*WT	Erythromycin resistance cassette upstream of *pilE* promoter	This study
*pilE*Mut	Erythromycin resistance cassette upstream of *pilE* promoter, *pilE* promoter mutation	This study
8013Δ*rnhA*	Kanamycin resistance cassette replacing *rnhA* gene	This study
8013Δ*rnhB*	Kanamycin resistance cassette replacing *rnhB* gene	This study
8013Δ*rnc*	Kanamycin resistance cassette replacing *rnc* gene	This study
8013Δ*rne*	Kanamycin resistance cassette replacing *rne* gene	This study
WT_eryΔ*rnhA*	WT_ery with kanamycin resistance cassette replacing *rnhA* gene	This study
Mut_eryΔ*rnhA*	Mut_ery with kanamycin resistance cassette replacing *rnhA* gene	This study
WT_eryΔ*rnhB*	WT_ery with kanamycin resistance cassette replacing *rnhB* gene	This study
Mut_eryΔ*rnhB*	Mut_ery with kanamycin resistance cassette replacing *rnhB* gene	This study
WT_eryΔ*rnc*	WT_ery with kanamycin resistance cassette replacing *rnc* gene	This study
Mut_eryΔ*rnc*	Mut_ery with kanamycin resistance cassette replacing *rnc* gene	This study
WT_eryΔ*rne*	WT_ery with kanamycin resistance cassette replacing *rne* gene	This study
Mut_eryΔ*rne*	Mut_ery with kanamycin resistance cassette replacing *rne* gene	This study
G4W	Kanamycin resistance cassette downstream of G4 sequence	This study

G4M	Kanamycin resistance cassette downstream of G4 sequence, G4 promoter mutation	This study
ASWT_G4M	WT_ery with kanamycin resistance cassette downstream of G4 sequence, G4 promoter mutation	This study
ASMut_G4M	Mut_ery with kanamycin resistance cassette downstream of G4 sequence, G4 promoter mutation	This study
Plasmid		
pGEM-T	Cloning and expression vector, carbenicillin resistance	Promega
pCR.2.ITOPOery	Cloning and expression vector, erythromycin resistance	R. Exley, unpublished
pEGFP-N2	Cloning and expression vector, kanamycin resistance	Clontech
pEGFP-N2(Insert1)	*N. meningitidis* 8013 AS promoter region cloned into pEGFP-N2	This study
pEGFP-N2(Insert1_M1)	*N. meningitidis* 8013 AS promoter region cloned into pEGFP-N2. Mutation to -35.	This study
pEGFP-N2(Insert1_M2)	*N. meningitidis* 8013 AS promoter region cloned into pEGFP-N2. Mutation to -10	This study
pEGFP-N2(Insert1_M3)	*N. meningitidis* 8013 AS promoter region cloned into pEGFP-N2. Mutation to -35 and -10.	This study
pEGFP-N2 (AS_lpx_ery)	Construction of WT_ery	This study
pEGFP-N2 (AS_lpx_ery_M3)	Construction of Mut_ery	This study
pUC19	Cloning and expression vector, carbenicillin resistance	Yanisch-Perron *et al*., 1985
pUC19(*pilE*_prom_WT)	Construction of *pilE*WT	This study
pUC19(*pilE*_prom_Mut)	Construction of *pilE*Mut	This study
pUC19(*rnhA*)	Construction of Δ*rnhA* mutants	This study
pUC19(*rnhB*)	Construction of Δ*rnhB* mutants	This study
pUC19(*rnc*)	Construction of Δ*rncA* mutants	This study
pUC19(*rne*)	Construction of Δ*rne* mutants	This study

2.2 Genetic techniques

2.2.1 Polymerase Chain Reaction (PCR)

PCR was carried out using Herculase II Fusion DNA polymerase (Agilent) or Q5 High Fidelity DNA Polymerase (New England Biolabs) according to manufacturers' protocol using 1 µl of purified plasmid or genomic DNA as template per 50 µl reaction. For colony PCR, lysates were first prepared by resuspending single colonies of *E. coli* or *N. meningitidis* in 10 or 100 µl of PBS respectively and boiling for 10 minutes. PCR was performed with Taq polymerase (Sigma Aldrich) according to manufacturer's directions using 1 or 5 µl of *E. coli* or *N. meningitidis* lysate respectively. All PCRs were performed using Mastercycler® pro thermocyclers (Eppendorf).

2.2.2 Plasmid and genomic DNA isolation

Plasmid DNA was isolated from liquid cultures of *E. coli* grown overnight in LB broth using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer's directions. Genomic DNA was isolated from *N. meningitidis* using the Wizard® Genomic DNA Purification Kit (Promega) with slight modifications from the manufacturer's protocol. Specifically, approximately half a loop of bacteria was scraped from BHI agar plates containing strains grown overnight and resuspended in 600 µl nuclei lysis buffer and incubated for 5 minutes at 80°C. After addition of 200 µl of protein precipitation solution and incubation at 4°C for at least 5 minutes, DNA was precipitated with isopropanol; the DNA pellet was washed with 70% ethanol and resuspended in water.

2.2.3 *Gel electrophoresis and purification of DNA*

DNA was analyzed by electrophoresis in 1.2% (w/v) agarose gels (Invitrogen) in 1x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) containing 1x SYBR Safe DNA stain (ThermoFisher). DNA samples were separated alongside Hyperladder™ 1 kb (Bioline) and gels were imaged in a Syngene gel bioimaging system using Genesnap software. DNA fragments were extracted and purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer's directions.

2.2.4 *Restriction endonuclease digestion and ligation*

Restriction endonuclease digestion was performed using restriction enzymes and buffers (New England Biolabs) according to manufacturer's directions. Ligation reactions were carried out using Quickstick ligase (Bioline) at room temperature for 5 minutes.

2.2.5 *Gibson assembly*

DNA fragments were PCR amplified using primers designed using the NEBuilder® assembly tool (New England Biolabs) and were assembled using Gibson Assembly® Master Mix (New England Biolabs) for 1 hour at 50°C according to manufacturer's protocols.

2.2.6 *Transformation*

Chemically competent *E. coli* cells were prepared as described previously (Inoue *et al.*, 1990). Briefly, 120 ml of *E. coli* cultures were grown to an OD_{600} of approximately 0.5 and harvested by centrifugation. Cells were resuspended in RF1 (15% w/v glycerol, 100 mM RbCl, 50 mM $MnCl_2$, 30 mM $CH_3COO^-K^+$, 10 mM $CaCl_2$, pH 5.8), pelleted and resuspended in RF2 (15% w/v glycerol, 10 mM RbCl, 10 mM MOPS, 75 mM $CaCl_2$, pH 6.8), and stored at -80°C until required. Transformation was performed by incubating chemically competent cells with plasmid DNA on ice for 30 minutes before heat shock at 42°C for 45 seconds followed by 2 minutes on ice. LB broth was added and cells were allowed to recover for 1 hour at 37°C with shaking. The cells were then spread onto solid LB media containing the appropriate antibiotics.

N. meningitidis strains grown overnight on BHI agar were resuspended in PBS and 10 μl of the suspension spotted onto BHI agar. Ten microliters of PCR product, restriction endonuclease digested DNA fragment, or genomic DNA (gDNA) was added to each spot as donor DNA. After incubation at 37°C for 5 h, bacterial spots were collected by scraping and spread onto solid BHI media containing the appropriate antibiotics. Backcrossing for strain construction was performed by using gDNA from sequence confirmed strains obtained from the initial transformation as donor DNA.

2.3 Plasmid and strain construction

2.3.1 pEGFP-N2(Insert1)

A 595 bp fragment including the AS promoter was amplified from *N. meningitidis* 8013 gDNA using primers *pilE*_AS-F/*pilE*_AS-1R and cloned into pEGFP-N2 using *Eco*RI and *Xma*I to generate the plasmid pEGFP-N2(Insert1). Site directed mutagenesis of the -35 sequence (TTGATT →TcccTT) or the -10 sequence (TATAAT →TcacAT) or both were performed using primer pairs SDM_mut1_F/R, SDM_mut2_F/R or SDM_mut3_F/R respectively to obtain pEGFP-N2(Insert1_M1), pEGFP-N2(Insert1_M2) and pEGFP-N2(Insert1_M3).

2.3.2 *AS promoter mutant in* N. meningitidis

A 752 bp fragment adjacent to the aforementioned 595 bp region containing the AS promoter was amplified from 8013 gDNA using primers lpxC_fragment_F/R. The PCR product was cloned adjacent to an erythromycin resistance cassette into pEGFP-N2(insert1) using *Sac*I and *Nhe*I to obtain plasmid pEGFP-N2(AS_lpx_ery). Site-directed mutagenesis (QuickChange Kit, Agilent) was carried out using primers SDM_mut3_F/R to obtain plasmid pEGFP-N2(AS_lpx_ery_M3). Plasmids were digested with *Hin*dIII and *Sac*I, and the purified inserts (Appendix 1 – DNA fragments used for strain construction) were used to transform *N. meningitidis* 8013 to obtain the strains WT_ery and Mut_ery respectively. Three sets of single colonies from independent transformations were kept as frozen stocks both as individual colonies and as pooled stocks for use in experiments. For the construction of strains WT_ery(RecA6) and Mut_ery(RecA6), genomic DNA from *N. meningitidis* 8013 containing the *recA6* allele (gift from Professor Hank Seifert) was used to transform the strains WT_ery and Mut_ery respectively. Single colonies of the transformants obtained were confirmed by Western blot analysis of whole cell extracts for RecA expression in the presence or absence of IPTG.

2.3.3 *pilE promoter mutant in* N .meningitidis

Downstream (549 bp) and upstream (922 bp) fragments were amplified from *N. meningitidis* 8013 using primers *pilE*_prom_ery_LHS_F/R and *pilE*_prom_ery_RHS_F/R respectively and cloned flanking an erythromycin resistance cassette by Gibson Assembly

(New England Biolabs) to obtain plasmid pUC19(*pilE*_prom_WT). Site-directed mutagenesis (QuickChange Kit, Agilent) was carried out to mutate the -10 (TATAAT →TcacAT) and -35 (CAAACT → CgggCT) elements of the *pilE* promoter using primers *pilE*_promoter_SDM3_F/R to obtain plasmid pUC19(*pilE*_prom_Mut). Plasmids were digested with *Bam*HI and *Sca*I and the purified inserts (Appendix 1 – DNA fragments used for strain construction) were used to transform *N. meningitidis* 8013 to obtain the strains *pilE*_WT and *pilE*_Mut respectively. Two sets of single colonies from independent transformations were kept as individual frozen stocks to be used in experiments.

2.3.4 RNase mutants

RNase mutants were constructed by gene replacement as follows: the plasmids pUC19(*rnhA*), pUC19(*rnhB*), pUC19(*rnc*) and pUC19(*rne*) were constructed by amplifying 500 bp fragments upstream and downstream of the respective genes in *N. meningitidis* 8013 (see Table 2.2 for primers) and cloning the fragments flanking a kanamycin resistance cassette into pUC19 by Gibson assembly. DNA fragments were amplified from the plasmids or directly from the Gibson assembly mix using primers M13F/R and used to transform *N. meningitidis* 8013 to obtain the strains 8013Δ*rnhA*, 8013Δ*rnhB*, 8013Δ*rnc* and 8013Δ*rne*. RNase mutants of WT_ery and Mut_ery were similarly constructed by using the respective fragments to transform WT_ery and Mut_ery strains. Single colonies obtained from transformation were checked by PCR and sequencing to confirm deletion of the gene and the sequence of the flanking region. gDNA from sequence confirmed single colonies was isolated and used for backcrossing. Transformants were checked by PCR and pooled (~10-15 colonies) as frozen stocks.

2.3.5 *G4 promoter mutant*

For the construction of the G4 promoter mutant strain G4M, a 249 bp fragment of DNA containing the G4 sequence was synthesized (Integrated DNA Technologies). This fragment, termed "G4prom_mut_mid", harbors mutations in both the -10 sequence (TAGCAT→ccGtcc) and the -35 sequence (TTGAGA→ccactc) of the G4 promoter. Fragments of size 500 bp upstream and downstream of this region were amplified (see Table 2.2 for primers) and the fragments were cloned flanking a kanamycin resistance cassette into pUC19 by Gibson assembly. For the construction of the isogenic control strain G4W, a 749 bp fragment containing the G4 sequence and a 500 bp fragment downstream were amplified and cloned flanking a kanamycin resistance cassette into pUC19 by Gibson assembly. DNA fragments (Appendix 1 – DNA fragments used for strain construction) were amplified directly from the respective Gibson assembly mixes using M13F/R and use to transform *N. meningitidis* to obtain G4M and G4W respectively. Genomic DNA from WT_ery and Mut_ery were used to transform G4M to construct the strains ASWT_G4M and ASMut_G4M respectively. Sequences of transformants were confirmed as above and gDNA was used for backcrossing to obtain pooled stocks of strains as described in Section 2.3.4.

Table 2.2 Oligonucleotides used for strain construction.

Oligonucleotide	**Sequence (5′-3′)**	**Description**
*pilE*_AS-F	GGGGGAATTCCGCGCCTGTCAGATAAACC	Amplification of *pilE* AS. EcoRI site underlined.
*pilE*_AS-1R	GGGGCCCGGGCCGAAGCCATCCTTTTGGC	Amplification of *pilE* AS. XmaI site underlined.
Ery_HindIII_F	GGGGAAGCTTCCGATACCCCCGATGACG	Amplification of Ery. HindIII site underlined.
Ery_SacI_R	GGGGGAGCTCGAATTCGCCCTTCCCGGGG	Amplification of Ery. SacI site underlined.
*lpxC*_fragment_F	GGGGGAGCTCCCCTGTCGCCGTCATTCC	Amplification of *lpxC* fragment. SacI site underlined.
*lpxC*_fragment_R	GGGGGCTAGCGCGTTCAGCTCAATCAGCG	Amplification of *lpxC* fragment. NheI site underlined.

SDM_mut3_F	CACTTACCGCT**CCC**TTTATTTAAAATTTATGGT**CAC**ATTTACCTTAGC	Site directed mutagenesis to mutate -35 and -10 of putative AS promoter. Mutations in bold.
SDM_mut3_R	GCTAAGGTAAAT**GTG**ACCATAAATTTTAAATAAA**GGG**AGCGGTAAGTG	Site directed mutagenesis to mutate -35 and -10 of putative AS promoter. Mutations 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	CGTCGCCGTCCAGCTCGACCAG	Sequencing of AS fragment in pEGFP-N2.
*lpxC*_frag_R2	GGAAAAAATAGAAAGCGTTATCC	Amplification and sequencing of *lpxC* in
Ery_F_R	GCACGAGCTCAAGCTTCG	Sequencing of WT_ery and Mut_ery.
*pilE*_prom_SDM3_F	CGATACCTGTGTAT**GTG**AAAGCAAGATTGGTATCAAAG**CCC**GTGTTTTGAG	Site directed mutagenesis to mutate -35 and -10 of *pilE* promoter. Mutations in bold
*pilE*_Prom_SDM3_R	CTCAAAACAC**GGG**CTTGATACCAATCTTGCTTT**CAC**ATACACAGGTATCG	Site directed mutagenesis to mutate -35 and -10 of *pilE* promoter. Mutations in bold.
*pilE*_LHS_F	agtgaattcgagctcggtacAATCTACATCCCGTCATTCCCAC	Gibson assembly of pUC19(*pilE*_prom_WT)
*pilE*_promE_LHS_R	cgggaagggcCATCGCGGCAGGTTTCCG	Gibson assembly of pUC19(*pilE*_prom_WT)
*pilE*_promE_ERY_F	tgccgcgatgGCCCTTCCCGGGGACCAT	Gibson assembly of pUC19(*pilE*_prom_WT)
*pilE*_promE_ERY_R	gcagttgaatCCGATACCCCCGATGACGATG	Gibson assembly of pUC19(*pilE*_prom_WT)
*pilE*_promE_RHS_F	ggggtatcggATTCAACTGCCGTTTGCACAGG	Gibson assembly of pUC19(*pilE*_prom_WT)
*pilE*_RHS_R	aggtcgactctagaggatccTCGGTCTTGCCGTTGGCG	Gibson assembly of pUC19(*pilE*_prom_WT)
*rnhA*_LHS_F	attcgagctcggtacccgggGATGAATAAGTTCTTGGCAACATG	Gibson assembly of pUC19(*rnhA*)
*rnhA*_LHS_R	gcatgcatcgGCGCAGTTTTCCTGACTG	Gibson assembly of pUC19(*rnhA*)
*rnhA*_Kan_F	aaaactgcgcCGATGCATGCCAACAGATAAAAC	Gibson assembly of pUC19(*rnhA*)
*rnhA*_Kan_R	caaaaccgttCAACCATCATCGATGAATTGTG	Gibson assembly of pUC19(*rnhA*)
*rnhA*_RHS_F	atgatggttgAACGGTTTTGTCCATTATTGTGCCTTTG	Gibson assembly of pUC19(*rnhA*)
*rnhA*_RHS_R	tgcatgcctgcaggtcgactGCCCGCCGGTACGGTATC	Gibson assembly of pUC19(*rnhA*)
*rnhB*_LHS_F	attcgagctcggtacccgggGGCGGGAATCTAGGTCTG	Gibson assembly of pUC19(*rnhB*)
*rnhB*_LHS_R	gcatgcatcgCAGGCCTTGTTTTAAACCG	Gibson assembly of pUC19(*rnhB*)
*rnhB*_Kan_F	acaaggcctgCGATGCATGCCAACAGATAAAAC	Gibson assembly of pUC19(*rnhB*)
*rnhB*_Kan_R	catactgaccCAACCATCATCGATGAATTGTG	Gibson assembly of pUC19(*rnhB*)
*rnhB*_RHS_F	atgatggttgGGTCAGTATGTGCATGATG	Gibson assembly of pUC19(*rnhB*)
*rnhB*_RHS_R	tgcatgcctgcaggtcgactCGCCGAAGACGAACAAATC	Gibson assembly of pUC19(*rnhB*)
*rnc*_LHS_F	attcgagctcggtacccgggGGCAACAAACGCCTCCAG	Gibson assembly of pUC19(*rnc*)
*rnc*_LHS_R	gtttttctaaGGCGGCGCGTGAATATGC	Gibson assembly of pUC19(*rnc*)
*rnc*_Kan_F	acgcgccgccTTAGAAAAACTCATCGAGCATC	Gibson assembly of pUC19(*rnc*)
*rnc*_Kan_R	gaagacggtaAATACAAGGGGTGTTATGAG	Gibson assembly of pUC19(*rnc*)

*rnc*_RHS_F	cccttgtattTACCGTCTTCCGCAAAAAAC	Gibson assembly of pUC19(*rnc*)
*rnc*_RHS_R	tgcatgcctgcaggtcgactTCTGAAGGTTTCAGTCTTG	Gibson assembly of pUC19(*rnc*)
*rne*_LHS_F	attcgagctcggtacccgggTGCTACCGCATACTGGCC	Gibson assembly of pUC19(*rne*)
*rne*_LHS_R	gcatgcatcgACCCGGCAAGGCTGAACC	Gibson assembly of pUC19(*rne*)
*rne*_Kan_F	cttgccgggtCGATGCATGCCAACAGATAAAAC	Gibson assembly of pUC19(*rne*)
*rne*_Kan_R	aagaatgttaCAACCATCATCGATGAATTGTG	Gibson assembly of pUC19(*rne*)
*rne*_RHS_F	atgatggttgTAACATTCTTTTCATGATCACCTC	Gibson assembly of pUC19(*rne*)
*rne*_RHS_R	tgcatgcctgcaggtcgactCGAACATCCGTTTCAGCAAAAAG	Gibson assembly of pUC19(*rne*)
*rnhA*_check_F	TATTCCAAATTACATCATTATTGTG	Checking *rnhA* construct
*rnhA*_check_R	TCAAACCCCTTCAAACCGAG	Checking *rnhA* construct
*rnhB*_check_F	TAATCCACTATATCGTCATTCC	Checking *rnhB* construct
*rnhB*_check_R	AGCCATCGAGCGCGAAATC	Checking *rnhB* construct
*rnc*_check_F	GCTTAATCAGCTCCAACAGG	Checking *rnc* construct
*rnc*_check_R	GTCAAGGTTGGGGGCGATG	Checking *rnc* construct
*rne*_check_F	ATGTGGTATCGTTCGCCTTG	Checking *rne* construct
*rne*_check_R	GGCAACCCCTATCAAGCTG	Checking *rne* construct
R_Kan_int	CGCATCAACCAAACCGTTAT	internal Kan primer
Kan_F_R	GACGTTTCCCGTTGAATATGG	Sequencing upstream of Kan
AS_Term(LHS)_F	gctcggtacccggggatcctGAATGACGAAGCCATCCATAC	Gibson assembly of G4W/G4M
AS_Term(LHS)_R	gcatgcatcgAACTTATCGGGAAAGCGG	Gibson assembly of G4W/G4M
AS_Term(KAN)_F	ccgataagttCGATGCATGCCAACAGATAAAAC	Gibson assembly of G4W/G4M
AS_Term(KAN)_R	tcggtacggaCAACCATCATCGATGAATTGTG	Gibson assembly of G4W/G4M
AS_Term(MID)_F	atgatggttgTCCGTACCGACAAGGCTAG	Gibson assembly of G4W/G4M
AS_Term(MID)_R	cgtggggtatctcTATTGGCATGGGGCATCG	Gibson assembly of G4W/G4M
AS_Term(RHS)_F	catgccaataGAGATACCCCACGCCAATAAAAAAGTGCCG	Gibson assembly of G4W/G4M
AS_Term(RHS)_R	ctatgaccatgattacgccaTCGCCGGGCCATTCGCCG	Gibson assembly of G4W/G4M
AS_term_seq_F	GATAAGTTTCCGTACCGACAG	Checking/Sequencing of G4W/G4M

2.4 SDS-PAGE and Western Blotting

Whole cell extracts were prepared from stressed or un-stressed cultures of *E. coli* or *N. meningitidis* at different time points by harvesting bacterial cell pellets and resuspending in

1 ml of 1x SDS-PAGE loading buffer (50 mM TrisHCl pH 6.8, 2% w/v SDS, 0.1% bromophenol blue, 10 % glycerol, 100 mM *β*-mercaptoethanol) per unit OD_{600}. Proteins were separated by SDS-PAGE in 1x SDS PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.5) alongside molecular weight markers (All Blue, Biorad) and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham) by semi-dry transfer for Western blotting. PilE was detected using SM1 (Virji *et al.*, 1989) (final dilution1:10,000) and IRDye 800CW goat-anti-Mouse IgG (Li-Cor; 1:10,000). GroEL loading control was detected using α-GroEL antibody (gift from Professor Jörgen Johansson; 1:8000) and IRDye 680 goat-anti-rabbit IgG (Li-Cor; 1:10,000). RecA was detected using α-RecA antibody (Abcam; 1:6,000) and IRDye 680 goat-anti-rabbit IgG (Li-Cor; 1:10,000). Bands were visualized and quantified using an 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 a ratio relative to the normalized PilE value of the first lane.

2.5 RNA Isolation

Total RNA was isolated from *E. coli* using Fast RNA Blue Kit (MP Biomedicals) followed by DNase treatment (Roche) or from *N. meningitidis* using RNeasy midi kit and on-column DNase treatment (Qiagen) or using TRIzol® reagent (ThermoFisher). RNA was further treated with DNase (Roche) when necessary followed by acid phenol/chloroform extraction.

2.6 Northern Blotting

RNA (10-20μg) was separated by electrophoresis on 1.5% agarose formaldehyde gels in HEPES buffer, transferred onto Hybond-N membranes and crosslinked by UV light. Probes were generated by end-labelling 50 nt oligonucleotides (Table 2.3) or a PCR product generated using primers 8013_tmRNA_F/R with γ-ATP (Perkin Elmer) using polynucleotide kinase (Roche). Membranes were incubated in hybridization buffer (GE Healthcare) and hybridized with labelled probes overnight at 64°C. After washing membranes with wash buffer 1 (2x SSC, 0.1 % SDS) and wash buffer 2 (0.5x SSC, 0.1% SDS), signals were detected using the Fuji FLA-5000 laser imaging system, and quantified using AIDA image analyzer software.

Table 2.3 Oligonucleotides used for Northern blotting.

Oligonucleotide	**Sequence (5'-3')**	**Description**
Insert1_probe	GCCGCCGCCAACGGCAAGACCGACGACAAAATCAACACCAAGCACCTGCC	Probe for AS transcript from pEGFP-N2(Insert1)
(AS)*pilE*-1	ACCGATGGTCAAATACATTGCATAATGCCGATGGCGTAAGCTTGAGGCAT	AS probe for Northern Blot.
*pilE*_probe	GGCTGATTTTTGACCTTCAGCCAAAAGGATGGCTTCGGAAACTTGTGCGC	*pilE* probe for Northern Blot.
8013_tmRNA_F	GGTTGCGAAGCAGATGCG	Amplification of *N. meningitidis* 8013 *tmRNA* PCR probe for Northern Blot.
8013_tmRNA_R	CCAGTCAATGTAAGATGACG	Amplification of *N. meningitidis* 8013 *tmRNA* PCR probe for Northern Blot.
*comP*_probe	TCGGCAAACTTGGCCATTTGGTAGATGTTTGTTTAAACCTCCCATTCTGC	*comP* probe for Northern Blot
*pilX*_probe	CTTGATGGTCTGATTATCGTCCAGGGGATTTTTCAAAATAAACTGTTTGG	*pilX* probe for Northern Blot
*pilV*_probe	GTCTGCGCGTTGTGCAGCAGGGTCGTCCTGACTTCCGACAGGCGGACGCG	*pilV* probe for Northern Blot
G4 Probe	AAGAAAACGGAAATTTTTAAAAATTAAATTTAAAATTTCCCACCCAACCC	Probe for G4 small RNA

2.7 Quantitative RT-PCR

First-strand cDNA was synthesized from 3 μg total RNA using random hexamers (Roche) and Superscript III reverse transcriptase (Invitrogen) in the presence of actinomycin to avoid spurious second-strand cDNA synthesis. Control reactions were set up in the absence of reverse transcriptase. After RNase H (Invitrogen) treatment for 20 minutes at 37°C, cDNA was purified and quantitative real time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and monitored using a StepOnePlus Real Time PCR system. To quantify cDNA, primer-pair specific standard curves were generated alongside the real-time PCR reaction using serially diluted genomic DNA from *N. meningitidis* 8013 as the template. Results were expressed as the amount of transcript calculated from the respective standard curves standardized to the tmRNA control. Experiments were performed in triplicate.

2.8 Strand specific quantitative RT-PCR

First-strand cDNA was synthesized from 5 μg total RNA using AS or *pilE* specific primers carrying a 5′ tag sequence not present in the *N. meningitidis* genome (Table 2.4). RNAs were reverse transcribed in the presence of actinomycin to avoid spurious second-strand cDNA synthesis. After RNase H treatment for 20 minutes 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 the average R value (calculated as 2^{-Ct}, where Ct 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 tmRNA control. The efficiencies of each primer pair was evaluated by performing a 10-fold dilution series experiment and determining the slope of the standard curves. The primer pairs were found to have efficiencies ranging from 85-100%.

Table 2.4 Oligonucleotides used for qRT-PCR.

Oligonucleotide	**Sequence (5′-3′)**	**Description**
Strand-Specific qRT-PCR		
ssQRTPCR_tag_F	CCGTCTAGCTCTCTCTAATCG	ssRTPCR tag.
ssRTPCR_AS_tag	CCGTCTAGCTCTCTCTAATCCGTAAGCTTGAGGCATTTCC	cDNA synthesis of ssRTPCR of AS. Tag sequence underlined.
ssQRTPCR_AS_F	CGCCAAAATGCCGACGATG	ssRTPCR of AS.
ssRTPCR_*pilE*_tag	CCGTCTAGCTCTCTCTAATCGGATGGCTTCGGAAACTTGTG	cDNA synthesis of ssRTPCR of *pilE*. Tag sequence underlined.
ssQRTPCR_*pilE*_F	CGAGCTGATGATTGTGATTGC	ssRTPCR of *pilE*.
ssRTPCR_tmRNA_tag	CCGTCTAGCTCTCTCTAATCCCATTGACCGACTGCTGC	cDNA synthesis of ssRTPCR of *tmRNA*. Tag sequence underlined.
DIP and non strand-specific qRTPCR		
QRT_AP_F	GATGGCTTCGGAAACTTGTG	DIP/qRTPCR (region AP)
QRT_AP_R	CGAGCTGATGATTGTGATTGC	DIP/qRTPCR (region AP)
QRT_A_F	GCCTCAAGCTTACGCCATCG	DIP/qRTPCR (region A)
QRT_A_R	GCTTCCTCAAACCACACCC	DIP/qRTPCR (region A)
QRT_B2_F	AATTTCCGTTTTCTTGGAAAGTG	DIP/qRTPCR (region B2)
QRT_B2_R	TCCGTACCGACAAGGCTAG	DIP/qRTPCR (region B2)
QRT_C_F	CTGAAAGTCCGGGATTCTAG	DIP/qRTPCR (region C)
QRT_C_R	CTGTTGCTCCCGATAAATTCC	DIP/qRTPCR (region C)
QRT_D_F	GAATGACGGGATGTAGGTTC	DIP/qRTPCR (region D)
QRT_D_R	CAACGACAAAAAGATCGACAC	DIP/qRTPCR (region D)
QRT_16S_F	GGTACCTGAAGAATAAGCACC	DIP/qRTPCR (16S)
QRT_16S_R	CTGCTTAAGTAACCGTCTGC	DIP/qRTPCR (16S)
QRT_tmRNA_F	GGTTGCGAAGCAGATGCG	DIP/qRTPCR (tmRNA)
QRT_tmRNA_R	CCAGTCAATGTAAGATGACG	DIP/qRTPCR (tmRNA)

2.9 Primer Extension

RNA (10 µg) extracted from *N. meningitidis* strains following NaCl stress was mixed with labelled probe (*pilE*_AS-1R) and denatured at 80°C for 5 minutes, the primer was extended by AMV reverse transcriptase in extension buffer (25mM Tris-HCl pH 8.3, 30mM NaCl, 15mM $MgCl_2$, 1.25mM DTT, 1mM dNTPs) at 42°C for 1 hour. Products were denatured at 80°C for 3 minutes 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 labelled *pilE*_AS-1R primer according to standard methods (Sambrook and Russell, 2001). Products were separated on a 5% denaturing polyacrylamide gel. Signals were detected as described above in Section 2.6.

2.10 Walking RT-PCR

Walking RT-PCR was performed according to published protocols (West *et al*,, 2006). Total RNA was used as a template for the synthesis of separate cDNAs using primers R1 to R4.5 (Table 2.5). Reverse transcription was carried out using Superscript III reverse transcriptase (Invitrogen) according to 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 1 kb DNA ladder (Bioline) as 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 RiboMAX large scale RNA production system (T7) (Promega).

Table 2.5 Oligonucleotides used for walking RT-PCR and antigenic variation assays.

Oligonucleotide	Sequence (5´-3´)	Description
Walking RT-PCR		
RT_F	AGCTGGCAGATGAATCATCG	Amplification of cDNA for mapping of 3'end
RT_R1	CCCTTCAAAAAGGTTTTACCC	Amplification of cDNA for mapping of 3'end
RT_R2	CAAACTTGATACCAATCTTGCT	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	CCAACCCACCCTATGCTAC	Amplification of cDNA for mapping of 3'end
RT_4.5	CCAAGAAAACGGAAATTTTTAAAAA	Amplification of cDNA for mapping of 3'end
RT_R5	CCGTTTGCACAGGAACACC	Amplification of cDNA for mapping of 3'end
RT_R6	GAATCCCGGACTTTCAGATAA	Amplification of cDNA for mapping of 3'end
T7_iv_temp_F	GGGGGAATTCTAATACGACTCACTATAGGAGCCTTGAAGCGCAGTCG	Amplification of template for in vitro transcription (T7 promoter underlined)
iv_temp_R	GCGGAGCGGTTTCTGTTGC	Amplification of template for in vitro transcription
Antigenic variation assay		
*pilE*_AS-F	GGGGGAATTCCGCGCCTGTCAGATAAACC	Amplification of *pilE.*
*pilE*_F	CGATGGCGTAAGCTTGAGG	Amplification of *pilE.*

2.11 Antigenic variation assays

For antigenic variation assays on solid media with WT_ery and Mut_ery, strains were grown overnight on solid BHI media from frozen stocks. For each replicate, four single colonies (progenitors) from each strain were picked and restreaked onto BHI agar. Fifteen colonies from each progenitor were passaged twice on BHI agar and single colonies were picked for preparation of single colony lysates. Lysates were prepared by resuspending single colonies in 100 µl PBS and boiling for 10 minutes.

For antigenic variation assays with WT_ery and Mut_ery strains containing the *recA6* allele performed on solid media, experiments were carried out as described above with the following modifications: strains were grown from frozen stocks on BHI agar containing 1 mM IPTG and progenitors were restreaked onto BHI agar containing 1 mM IPTG. The final two passages of fifteen colonies were carried out on BHI agar without IPTG.

Antigenic variation assays in liquid media were performed with 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 colonies of each strain, to ensure that each experiment started with a homogenous population of cells containing the same *pilE* sequence. Strains grown overnight on solid BHI media 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 hours (see Fig. S2). Ninety six colonies of each strain from each condition were passaged onto BHI agar and incubated overnight. Lysates were prepared as described above.

pilE was amplified from single colonies using primers AS_F and *pilE*_F and Herculase II Fusion DNA polymerase (Agilent). Products were sequenced and compared to *pilE* progenitor sequence, and changes were mapped to potential *pilS* donors. Each variant *pilE* was considered to result from a single Av event. The assay with 8013 and 8013ΔG4 was performed once. Av assays with WT_ery(*recA6*) and Mut_ery(*recA6*) were performed three times.

2.12 Cell Culture and Adhesion Assay

The human lung epithelia carcinoma cell line, A549, was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum and incubated at 37°C, 5% CO_2. One day prior to the adhesion assay, confluent monolayers were trypsinized and seeded into 24-well plates at a concentration of 5 x 10^5 cells per well. *N. meningitidis* strains grown overnight on BHI agar were resuspended in PBS and diluted in DMEM to achieve an inoculum with a multiplicity of infection (MOI) of 50 per cell. Bacteria (in a 1 ml volume) were added to each well and incubated at 37°C, 5% CO_2 for 1.5 hours. The medium was removed and cells were washed three times with PBS to remove non-adherent bacteria and lysed with PBS-1% saponin. The number of bacteria in the inoculum and cell-associated bacteria was quantified by plating dilutions onto BHI agar. Adhesion was calculated as the percentage of the number of cell-associated bacteria to the number of bacteria in the inoculum. Results are the average of four replicate experiments.

2.13 DNA transformation efficiency assays

N. meningitidis strains grown overnight on BHI agar were resuspended in PBS, quantified as described above and diluted to a concentration of 10^9 bacteria/ml. Ten microliters of the suspension were spotted onto BHI agar and 1 μg of 8013Δ*pilE* (M. Wörmann, Tang Lab, unpublished) genomic DNA was added to each spot as donor DNA. After incubation at 37°C for 5 h, bacterial spots were collected by scraping, resuspended in PBS, quantified, and diluted to 10^8 cells/ml. Serial dilutions were prepared and plated onto BHI agar and BHI agar containing kanamycin to quantify total bacteria and transformants respectively. The transformation efficiency was calculated as the percentage of transformants compared with the total number of cfu. Results are the average of duplicate experiments each performed with three sets of WT_ery and Mut_ery strains obtained from three independent transformations.

2.14 NaCl survival assays

Liquid cultures of *N. meningitidis* strains were grown as previously described to an OD_{600} of ~0.6 and subjected to NaCl stress (0.5 M, 10 minutes) where indicated. Cultures were serially diluted, spread onto BHI agar and cfu were enumerated following overnight incubation at 37°C. Survival in NaCl stress was expressed as the ratio of cfu after 10 minutes stress to cfu at time 0 min. Results are the average of duplicate experiments.

2.15 DNA immunoprecipitation (DIP)

DIP was performed as described in Skourti-Stathaki *et al*. (2011) with modifications. Specifically, the strains WT_ery, Mut_ery, and WT_eryΔ*rnhA* were grown to OD_{600} 0.5 – 0.6 and subjected to NaCl stress (0.5 M, 10 minutes) where indicated. Genomic DNA containing RNA/DNA hybrids was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer's protocols with an additional incubation with proteinase K (Qiagen) for 1 h at 50°C after protein precipitation. RNaseH sensitivity analysis was performed on the isolated genomic DNA (diluted to a concentration of 960 ng/µl in 100 µl) by the addition of 20 U of RNase H for 3 hours at 37°C (New England Biolabs).

Three hundred microlitres of ChIP IP buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100) was added to the genomic DNA followed by sonication (Diagenode Bioruptor). Sonicator settings are as follows: medium setting, 30 sec on/ 30 sec off interval, 5 min total sonication time. Sonicated genomic DNA was pre-cleared with 50 µl protein A agarose beads (Millipore) in 3 ml of ChIP IP buffer including protease inhibitors (0.8 µg/ml pepstatin A, 1 µg/ml leupeptin, 0.5mM PMSF) for 1 h at 4°C. An aliquot of precleared genomic DNA was removed (input) and used in the subsequent qPCR analysis. Immunoprecipitation was performed using an antibody which recognizes RNA/DNA hybrids, purified from S9.6 hybridoma cell lines (Boguslawski *et al*., 1986). Samples were divided into two fractions: one for a no antibody control and one for immunoprecipitation. The antibody (14.44 µl) was added to the latter and immunoprecipitation was carried out overnight at 4°C.

Washing and elution steps were performed as described for ChIP (Kadener *et al*., 2002). Briefly, beads were washed once each with Wash Buffer A (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), Wash Buffer B (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100) and Wash Buffer C (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% DOC, 1% NP-40), followed by three washes with Wash Buffer D (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA/DNA hybrids were eluted twice with 250 µl Elution Buffer (100 mM $NaHCO_3$, 1 % SDS). Samples were digested with 11 µg proteinase K (Qiagen) for 2 h at 45°C, followed by DNA purification (Promega Gel and PCR Cleanup Kit).

The input, immunoprecipitated, and non-immunoprecipitated DNAs were used as templates for qPCR. DNA was quantified by standard curve method using primer-pair specific standard curves generated alongside the real-time PCR using serially diluted genomic DNA from *N. meningitidis* 8013 as the template. Amount of immunoprecipitated RNA/DNA hybrid at a particular region was calculated as the percentage compared with the input after subtracting the background signal from the “no antibody” control. DIP signals were normalized either to 16S RNA or tmRNA.

2.16 RNAseq

WT_ery and Mut_ery strains were grown in triplicate in BHI liquid media as described above. After 12 hours of growth, bacteria were harvested and total RNA isolated using TRIzol® reagent, DNaseI treated and purified by acid phenol/chloroform extraction as above. Half of each RNA sample was treated with terminator-5'-phosphate-dependent exonuclease (TEX) (Epibio) to deplete processed RNAs, allowing detection of transcription start sites (TSS), as described in Sharma *et al.*, 2010. All samples were then purified by acid phenol/chloroform extraction using phase lock gel tubes (5prime). cDNA library preparation and Illumina sequencing were carried out by the Wellcome Trust Centre for Human Genetics (Oxford). cDNA library preparation and Illumina sequencing by WTCHG was performed as follows: TEX treated and untreated RNA were ribodepleted before conversion to cDNA. Second strand synthesis involved incorporation of dUTP. The cDNA was end-repaired, A-tailed, and adapter ligated. Prior to amplification, samples underwent uridine digestion. The prepared libraries were size selected, multiplexed and quality checked before paired-end sequencing on HiSeq Rapid 2500.

Data obtained from Illumina sequencing was aligned to the 8013 reference genome (Accession number NC_017501.1) and quality checked using FastQC. Transcriptome mapping and transcript abundance quantification was performed using the Rockhopper program (McClure *et al.*, 2013) and visualized using Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013). Differential gene expression and statistics were computed using the DESeq2 workflow (Love *et al.*, 2014). Transcription start sites (TSS) were predicted using the program TSSpredator (Dugar *et al.*, 2013).

2.17 Statistical Analysis

Statistical analyses of results from strand-specific qRT-PCR, NaCl survival and bacterial adherence assays were performed using Student's *t*-test. Statistical analysis of differences between multiple strains/conditions for non strand specific qRTPCR was performed using two-way ANOVA (analysis of variance) and Turkey's multiple comparison test. Antigenic variation assay results were analyzed by fitting generalized linear models (GLMs) (McCullagh and Nelder, 1989). Binomial GLMs were fitted with the response variable being defined as a sequence of ordered pairs (Number of variant, Number of same) from *pilE* sequences and the predictor variables were: strain (WT_ery or Mut_ery), NaCl induction (yes or no), and assay (1, 2, or 3). R statistical software was used, specifically the function GLM (Venables and Ripley, 2002). In terms of goodness-of-fit the model proved to be satisfactory (Goodness-of-fit statistic = 5.8392 on 6 degrees of freedom, *p*-value = 0.4414).

3 Results - Identification and characterization of the *pilE* AS RNA

3.1 Identification of a putative AS promoter in the *pilE* locus

Visual inspection of the sequence of *N. meningitidis* strain 8013 *pilE* locus revealed the presence of a putative promoter on the antisense (AS) strand, five nucleotides downstream of the *pilE* coding region (Figure 3.1A). This sequence bears similarity to the bacterial RpoD σ^{70} promoter consensus sequence. To determine its prevalence, the genomes of more than 200 *N. meningitidis* strains available in the pubMLST database of meningococcal isolates were examined, including strains expressing Class II *pilE* in which *pilE* is located at a site distinct from the *pilS* locus (Figure 3.1B). The putative promoter sequence is conserved amongst *N. meningitidis* strains across different clonal complexes (Representative strains shown in Figure 3.1C). In class II pilin expressing strains, this putative promoter sequence is conserved on the antisense strand downstream of *pilS1* (Figure 3.1B) but is not found downstream of *pilE*. In the two genomes *of N. gonorrhoeae* examined, the sequence is conserved apart from three substitutions, two of which occur in the putative -10 TATA box sequence (CCTAAT instead of TATAAT) (Figure 3.1C).

No sequences resembling a Shine-Dalgarno ribosome binding site were identified within proximity to a putative ATG start codon within 350 nucleotides of this promoter. Therefore, we hypothesized that this promoter drives transcription of a noncoding antisense (AS) RNA.

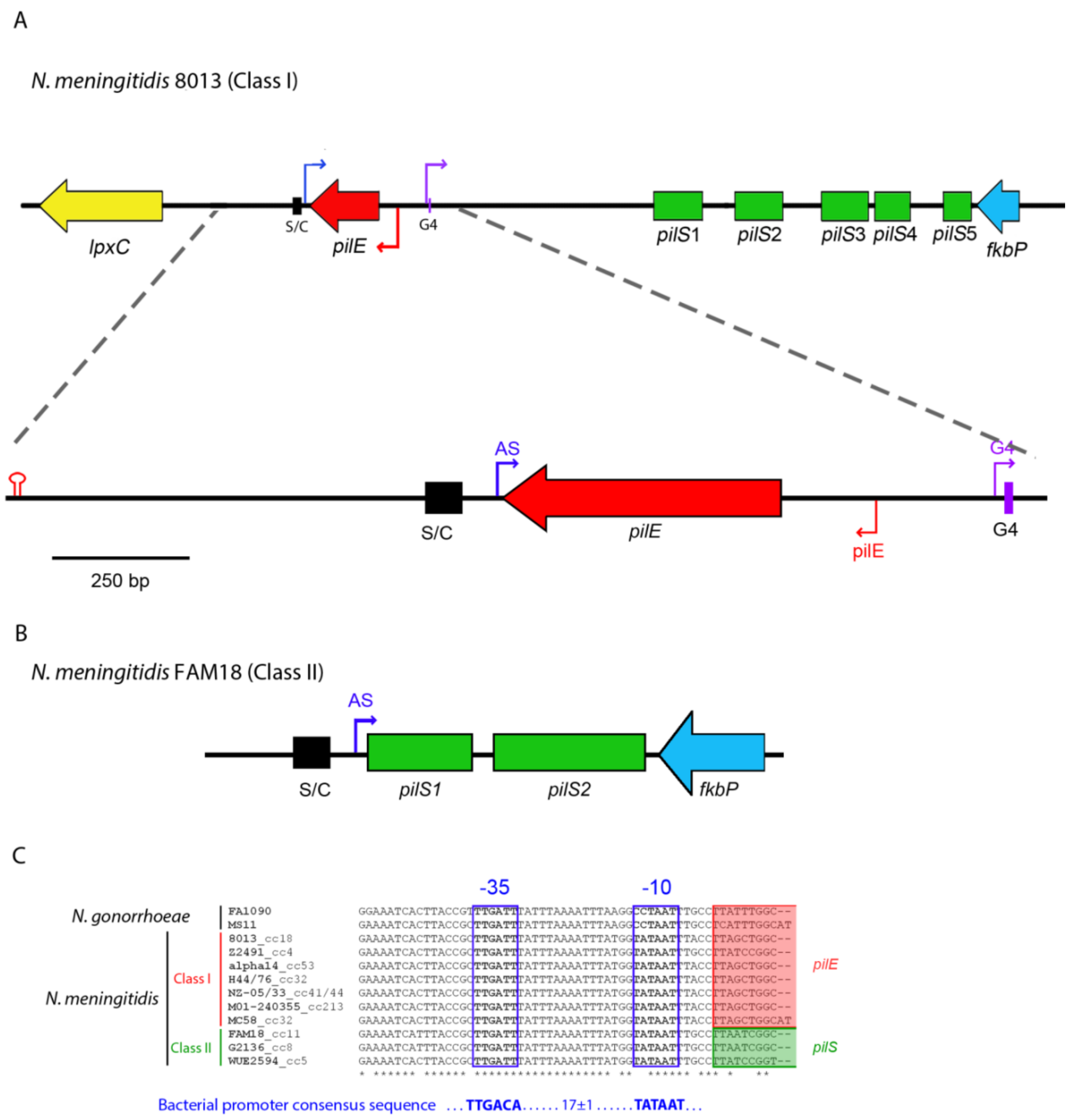


Figure 3.1 Location of a promoter sequence in the class I *pilE* locus in *N. meningitidis*.

(A) Schematic diagram of the pilin locus in *N. meningitidis* 8013, which expresses class I pilin. The *pilE/pilS* locus is flanked by *lpxC* and *fkbP* which are not related to Tfp biology. Sma/Cla (S/C) and G4 are DNA elements in the *pilE* locus that are important for pilin antigenic variation in *N. gonorrhoeae*. 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) Schematic diagram of the pilin locus in *N. meningitidis* FAM18, which expresses class II pilin. Although the *pilE* gene is located at a site distinct from the *pilS* locus, the putative AS promoter sequence is conserved downstream of *pilS1*. (C) Multiple sequence alignment of the region corresponding to the AS promoter in *N. meningitidis* strains from different clonal complexes (cc) expressing class I (red) or class II (green) pilin, and in *N. gonorrhoeae*. The putative -10 and -35 sequences are boxed in blue. *pilE* sequences are shaded in red, *pilS* 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.

3.2 *E. coli* as a heterologous system to study promoter activity

To determine whether the AS promoter is functional, a reporter system was used to study promoter activity in *E. coli*. A 595 bp region of DNA from *N. meningitidis* strain 8013, including the Sma/Cla sequence and 386 bp of *pilE* coding sequence but not the *pilE* promoter sequence was introduced into the vector pEGFP-N2 to obtain pEGFP-N2(Insert1) and transformed into *E. coli*. pEGFP-N2 only harbors a CMV early immediate promoter for transcription in eukaryotic but not prokaryotic cells. Thus, transcription of the AS region would entirely be driven by the activity of the putative AS promoter in *E. coli*.

E. coli strain DH5α harboring pEGFP-N2(Insert1) was grown for 3 hours to $OD_{600} \approx 0.5 – 0.6$ in LB liquid media. As the expression of regulatory small RNAs is often induced in response to environmental stress, bacterial cultures were subjected to various stresses in an attempt to determine the conditions under which transcription from the AS promoter is active. The cultures were subjected to acid (HCl, pH 2.5), NaCl (0.5 M), temperature (10°C), envelope (5% Triton X-100 or 5% ethanol), or oxidative stress (0.15% H_2O_2) for 10 minutes to determine whether transcription from the AS promoter is induced by the stress condition. The presence of a transcript was detected by Northern blot using a 50 nt oligonucleotide probe that hybridizes 39 nt downstream of the predicted -10 sequence (Insert1_probe, Table 2.3). Exposure of bacteria to hypertonic conditions led to increased levels of the AS RNA, indicating that the promoter is activated by salt stress (Figure 3.2A).

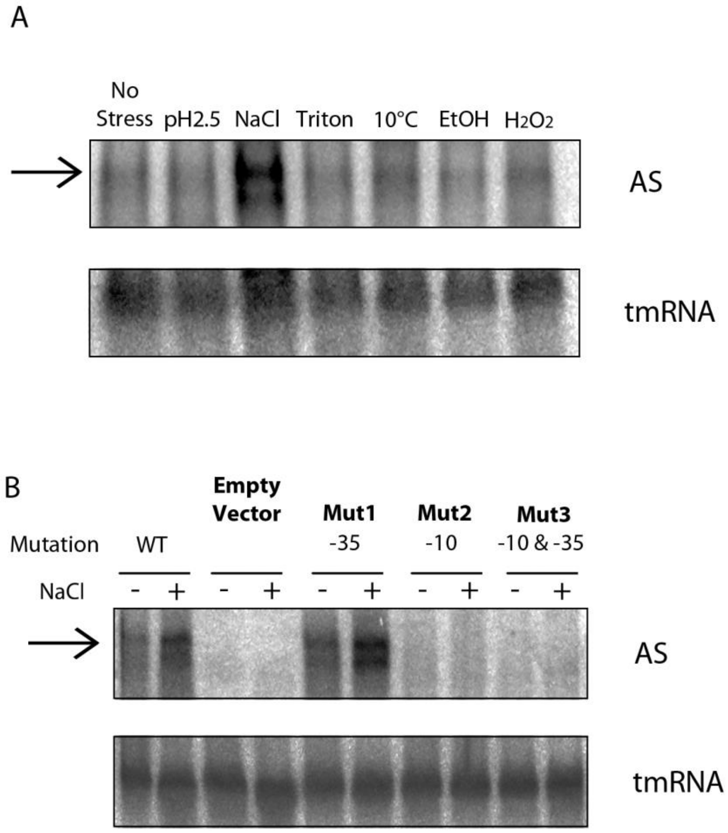


Figure 3.2 AS transcription is induced by NaCl stress in *E. coli*.

(A) pEGFP-N2(Insert1) was transformed into *E. coli*, and the bacteria 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 messenger RNA (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 (Mut1), the -10 (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.

To identify mutation(s) that would abolish promoter activity, site directed mutagenesis of each promoter element was carried out. Nucleotide changes were made to the -35 sequence (TTGATT→TcccTT), the -10 sequence (TATAAT→TcacAT), or both to obtain the plasmids pEGFP-N2(Insert1_M1), pEGFP-N2(Insert1_M2) and pEGFP-N2(Insert1_M3), respectively. *E. coli* strains DH5α harboring each plasmid were grown in LB liquid media and subjected to NaCl stress as described above. Northern blot analysis indicates that transcription was abolished upon mutation of only the -10 sequence and when both the -10 and -35 sequences were changed; altering the -35 sequence alone had no effect (Figure 3.2B). Furthermore, no signal was observed for the empty vector control, confirming that the bands observed were indeed due to transcription from a functional AS promoter. These results indicate that the AS promoter is functional in a heterologous *E. coli* system and that expression is induced by NaCl stress. Therefore, we next investigated the functionality of the AS promoter in *N. meningitidis*.

3.3 Characterization of *pilE* antisense RNA in *N. meningitidis*

3.3.1 The AS transcript is upregulated in NaCl stress

The expression of the AS RNA in wild-type *N. meningitidis* in response to different stress conditions was analyzed by Northern blotting. Strain 8013 was grown overnight on solid BHI and used to inoculate liquid BHI media. After 3 hours growth at 37°C with agitation, bacteria were subject to stress by addition of NaCl or KCl (0.5 M final concentration), sucrose to 6%, HCl to adjust the pH to 2.5, or hydrogen peroxide to 0.15% for 10 minutes. Total RNA was isolated and subjected to Northern blot analysis. The oligonucleotide probe ((AS)*pilE*-1) consists of a 50 nt sequence at the 5’ UTR of the *pilE* mRNA which is not present at any *pilS* cassette and is therefore specific for the AS RNA. Using this probe, a single transcript was detected in bacteria treated with NaCl or KCl, indicating that the AS RNA is expressed in response to salt stress in wild-type *N. meningitidis* (Figure 3.3A), similar to *E. coli*. The concentration of salt necessary to induce expression of the AS RNA was determined by growing strain 8013 in liquid media as described and adding NaCl at concentrations ranging from 0-0.5 M. As shown in Figure 3.3B, AS RNA levels were increased at salt concentrations greater than 0.4 M; the AS RNA cannot be detected or is barely detectable in the absence of stress (Figure 3.3A and B).

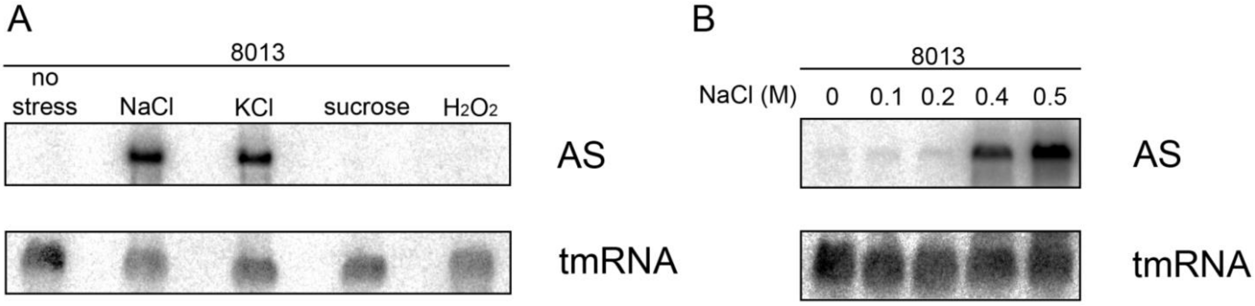


Figure 3.3 The AS transcript is upregulated in salt stress in *N. meningitidis*.

(A) Northern blot analysis of total RNA from *N. meningitidis* strain 8013 subjected to different stresses (indicated). The AS transcript was detected using a 50 nt strand specific probe ((AS)*pilE*-1) that hybridizes specifically to the AS RNA 533 nt downstream of the AS promoter. tmRNA was used as loading control. A transcript corresponding to the AS RNA was detected following exposure of bacteria to NaCl and KCl. (B) Analysis of AS RNA expression in *N. meningitidis* following 10 minute incubation with different concentrations of NaCl. AS RNA levels are increased at higher salt concentrations (*i.e*. 0.4 and 0.5M NaCl).

3.3.2 Mutation of the AS promoter in N. meningitidis

An isogenic pair of strains with an intact or a mutated AS promoter were constructed in *N. meningitidis* 8013 (Figure 3.4). The control strain, WT_ery, carries an intact AS promoter and the erythromycin antibiotic resistance marker divergent to the AS promoter (Figure 3.4B), while the AS promoter mutant strain, Mut_ery, carries mutations to the -35 sequence (T<u>TGA</u>TT→TcccTT) and the -10 sequence (T<u>ATA</u>AT→TcacAT) of the AS promoter and is otherwise isogenic with WT_ery (Figure 3.4C). Expression of the AS RNA is similar in WT_ery and *N. meningitidis* 8013 at various stages of growth, with low or no AS expression during lag, log, stationary, and late stationary phase (Appendix 2 – Detection of AS RNA in 8013 and WT_ery

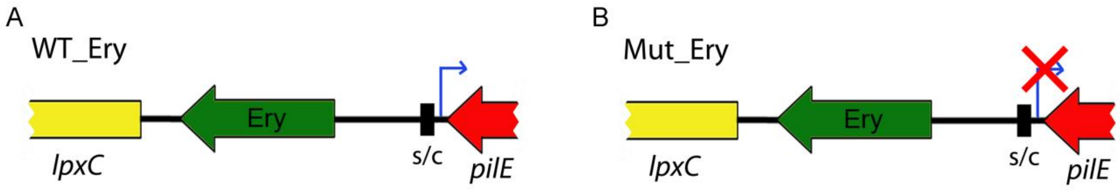


Figure 3.4 Construction of WT_ery and Mut_ery.

Schematic diagram of the *pilE* locus of (A) WT_Ery and (B) Mut_Ery, both constructed in 8013. The erythromycin resistance cassette comprising promoter and terminator sequences was inserted downstream of the *pilE* coding sequence and Sma/Cla repeat (S/C).

Growth curves of WT_ery and Mut_ery grown in BHI liquid were performed to examine whether the AS has any impact on bacterial fitness. The results demonstrate that both strains grow at similar rates (Figure 3.5A), suggesting that the AS RNA is not essential for growth and that absence of the AS RNA does not result in any growth defect. Northern blot analysis of total RNA isolated from WT_ery and Mut_ery at different growth phases (Figure 3.5B) demonstrate that the AS transcript is detected in WT_ery but not in Mut_ery after overnight growth (21 hrs, OD_{600}=1.9). In agreement with results using wild-type strain 8013, exposure of WT_ery to high salt (0.5M NaCl) significantly increased the abundance of the AS transcript (by approximately 280-fold estimated from qRTPCR analysis; p = 0.0003), as shown by both Northern blot (Figure 3.5C) and strand specific qRTPCR analyses (Figure 3.5D). No AS transcript was detected in Mut_ery, confirming that the predicted promoter is indeed responsible for AS RNA expression and that the changes to the promoter abolished its activity.

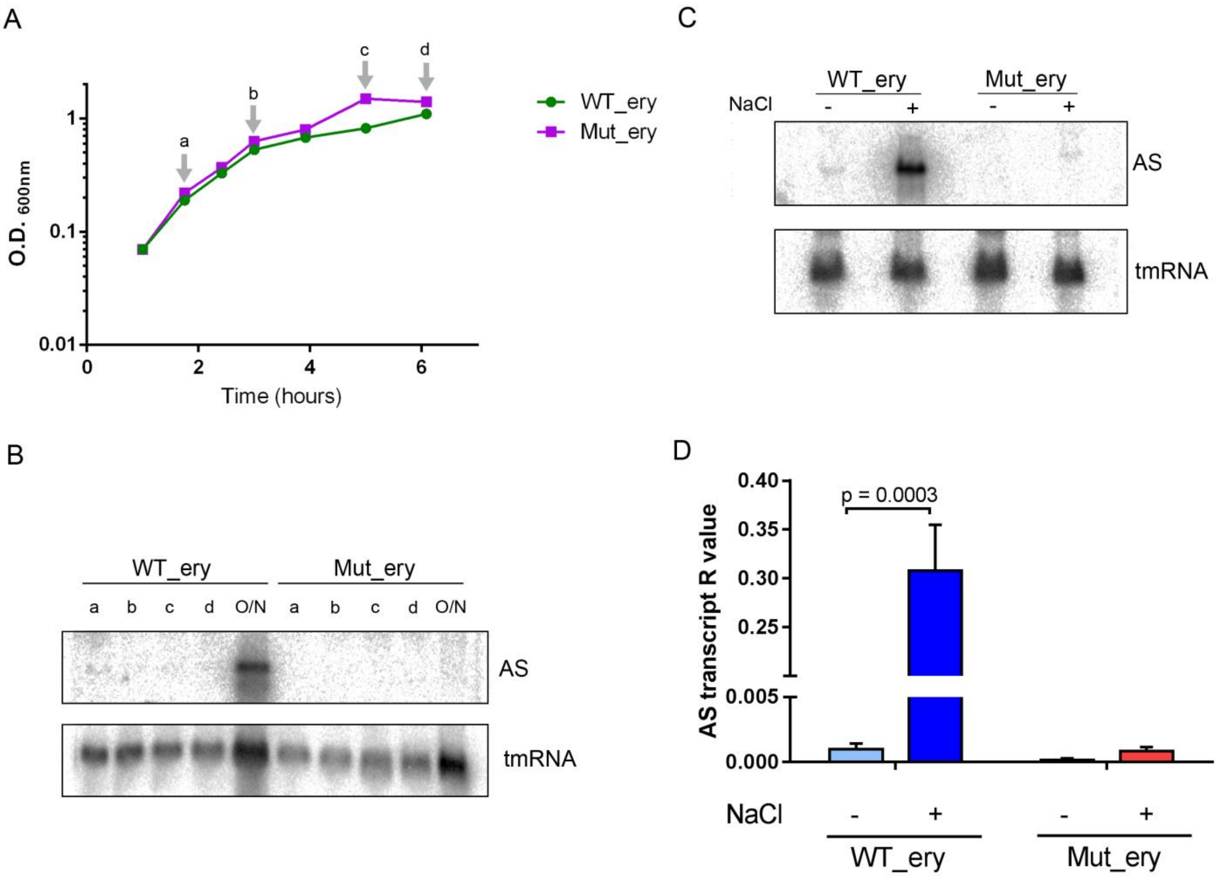


Figure 3.5 Expression of the AS transcript in WT_ery and Mut_ery.

(A) Growth curves of WT_ery and Mut_ery grown at 37°C in BHI broth. Arrows indicate times at which RNA was extracted for Northern blot anaylsis of AS RNA expression. (B) Northern blot analysis of total RNA isolated at different time points during growth (a, 1.75 hrs; b, 3 hrs; c, 5 hrs; d, 6 hrs and overnight (O/N) 21.5 h). (C) Representative Northern blot analysis of total RNA from *N. meningitidis* strains WT_ery and Mut_ery with and without NaCl stress. (D) Strand-specific qRT-PCR analysis of AS transcript levels in WT_ery and Mut_ery strains with or 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 in NaCl stress in WT_ery, while no transcript was detected in Mut_ery.

3.3.3 Characterization of the 5' and 3' ends of the AS RNA

Primer extension was performed to map the 5′ end of the AS transcript. Reverse transcription was carried out using the labelled probe *pilE*_AS-1R. The resulting cDNA primer extension product was run alongside dideoxy chain termination sequencing reaction products on a 5% denaturing polyacrylamide gel to determine its size. The 5' end was 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 (Figure 3.6A).

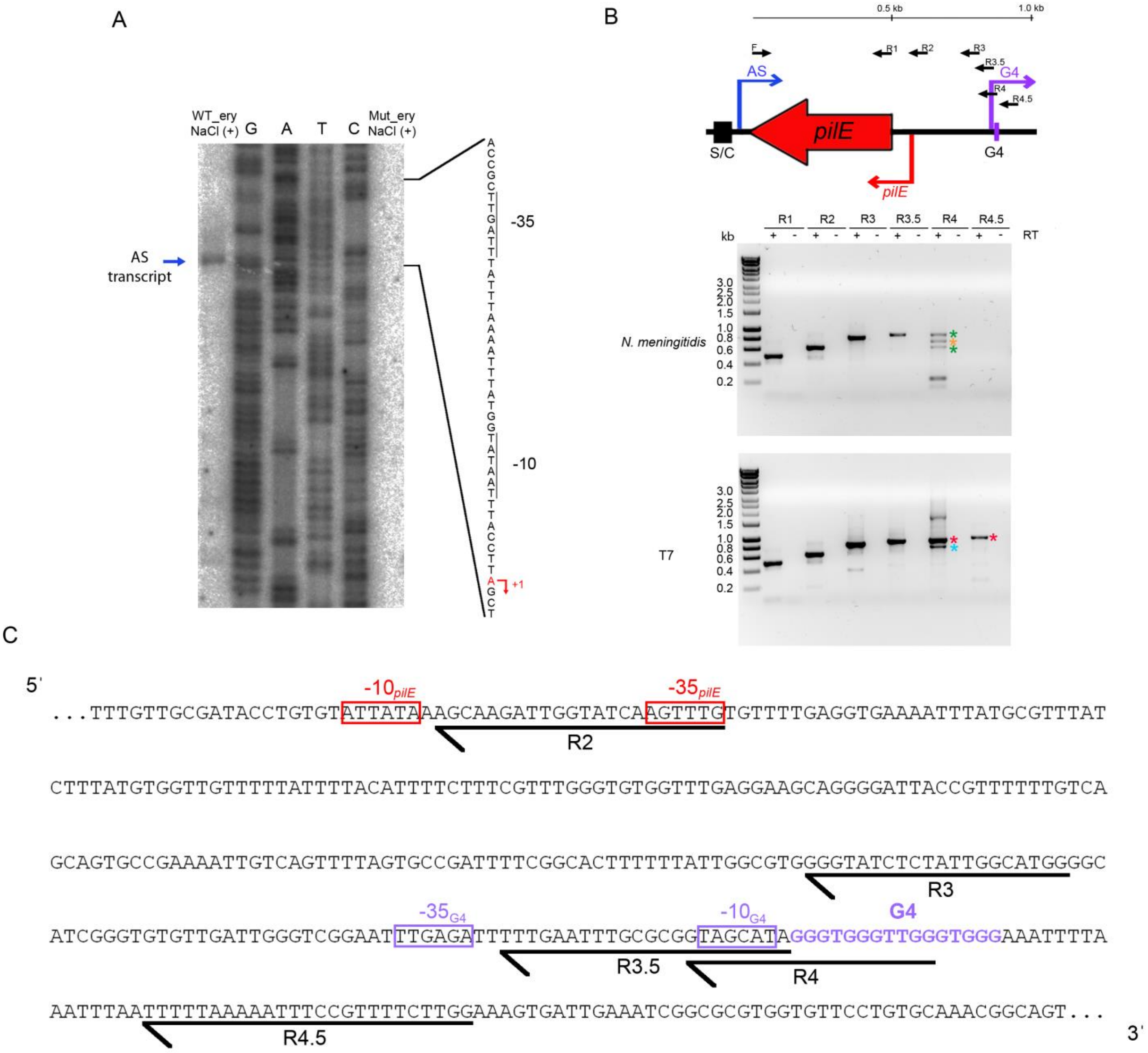


Figure 3.6 Characterization of the 5' and 3' ends of the AS transcript.

(A) Primer extension analysis of the transcriptional start site of the AS RNA in WT_ery following salt stress. The -10 and -35 sequences are indicated and the transcriptional start site is shown (red arrow). No product was obtained using RNA from Mut_ery. (B) Mapping of the AS 3' end by walking RT-PCR. Arrows correspond to the hybridization positions of primers F and R1-R4.5. The scale bar indicates distance from the AS transcriptional start site. Agarose gel analysis of PCR products amplified using cDNAs from *N. meningitidis* WT_ery total RNA (top panel) or *in vitro* T7-synthesized AS RNA (lower panel) as a template. The presence or absence of reverse transcriptase is indicated by (+) or (-). A full size 888 nt product obtained from amplification with primers F and R4 was detected using *in vitro* synthesized RNA (red asterisk), but not total RNA from *N. meningitidis*, indicating that the *in vivo* transcript terminates before or within the sequence corresponding to primer R4. Green asterisks indicate non-specific products amplified from ribosomal RNA, orange and blue asterisks indicate truncated PCR products as ascertained by sequence analysis. (C) Sequence of region at 3' end of the AS RNA and location of primers R2-R4.5. The G4 sequence is indicated in purple. The promoters of *pilE* and the G4 associated sRNA are depicted in the red and purple boxes respectively.

The 3′ end of the transcript was mapped by RT-PCR using a set of reverse primers which hybridize at various intervals within the region corresponding to the *pilE* promoter and G4 sequence (Figure 3.6B). Reverse transcription was carried out using each reverse primer on total RNA from WT_ery cultures treated with NaCl to induce AS RNA expression, followed by PCR of the individual cDNA using the forward primer AS_RT_F and the respective reverse primer. The presence of a PCR product demonstrates that the AS RNA extends to beyond the sequence complementary to the reverse primer. To ensure that absence of PCR product is indeed due to the absence of the cDNA and not a result of inefficient PCR, the same PCR was performed using the AS RNA synthesized *in vitro* with T7 RNA polymerase using a ~1.4 kb DNA template.

Results indicate that using RNA recovered from the *N. meningitidis* as the template, a PCR product of expected size was obtained with primers F+R3.5 but not with F+R4. A product of 888 nt was amplified from *in vitro* synthesized RNA with primers F+R4 (Figure 3.6B, red asterisk), demonstrating that the absence of this product when meningococcal RNA was used as the template is not due to unsuccessful amplification conditions. This result indicates that the 3′ end of the AS transcript in *N. meningitidis* lies between the site of annealing of primers R3.5 to R4, which corresponds to the promoter of the G4-associated sRNA (Cahoon and Seifert, 2013) (Figure 3.6B). To verify this, the smaller sized products obtained from reactions using primers F+R4 were sequenced. The products obtained from *N. meningitidis* RNA correspond to non-specific amplification from ribosomal RNA (Figure 3.6B, green asterisks) and a truncated PCR product of 727 nt and 733 nt (Figure 3.6B, orange asterisk and blue asterisk respectively). Thus, results from RT-PCR and sequencing demonstrate that 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 (Figure 3.6C).

3.3.4 Mutation of the pilE *promoter*

Given the converging directions of the *pilE* and AS promoters and the long stretch of exact complementarity between the *pilE* mRNA and the AS RNA, we next considered whether *pilE* expression modulated the regulation of AS RNA levels, either through transcriptional interference or though base-pairing interactions between the *pilE* mRNA and AS RNA. To determine whether the presence of the *pilE* transcript has an effect on AS RNA expression, an isogenic pair of strains was constructed in *N. meiningitidis* 8013. *pilE*WT carries an intact *pilE* promoter sequence with an erythromycin resistance cassette located 351 bp upstream of the *pilE* promoter and divergent to the promoter, while *pilE*Mut carries mutations to the -10 (TATAAT→TcacAT) and -35 (CAAACT→CgggCT) elements of the *pilE* promoter in addition to the erythromycin resistance cassette (Figure 3.7A).

Total RNA was isolated from strains grown in BHI liquid then either untreated or treated with NaCl stress to induce AS RNA expression. Abolition of *pilE* promoter activity was confirmed by Northern blot analysis of total RNA isolated from NaCl stressed and unstressed liquid cultures of *pilE*WT and *pilE*Mut (Figure 3.7B). A band corresponding to the *pilE* transcript was detected in *pilE*WT using a 50 nt oligonucleotide probe (*pilE* probe) that hybridizes specifically to the *pilE* transcript 109 bp downstream from the *pilE* promoter. No *pilE* transcript was detected in *pilE*Mut and furthermore, Western blot analysis of whole cell extracts of the strains demonstrates that PilE protein is not expressed by *pilE*Mut (Figure 3.7C).

Interestingly, the intensity of the *pilE* transcript signal was noticeably reduced by Northern blot analysis when *pilE*WT was treated with NaCl stress compared to unstressed *pilE*WT. Despite the reduced *pilE* transcript levels in NaCl treated *pilE*WT, there is no observable difference in PilE protein levels in the NaCl treated *pilE*WT compared to unstressed *pilE*WT (Figure 3.7C).

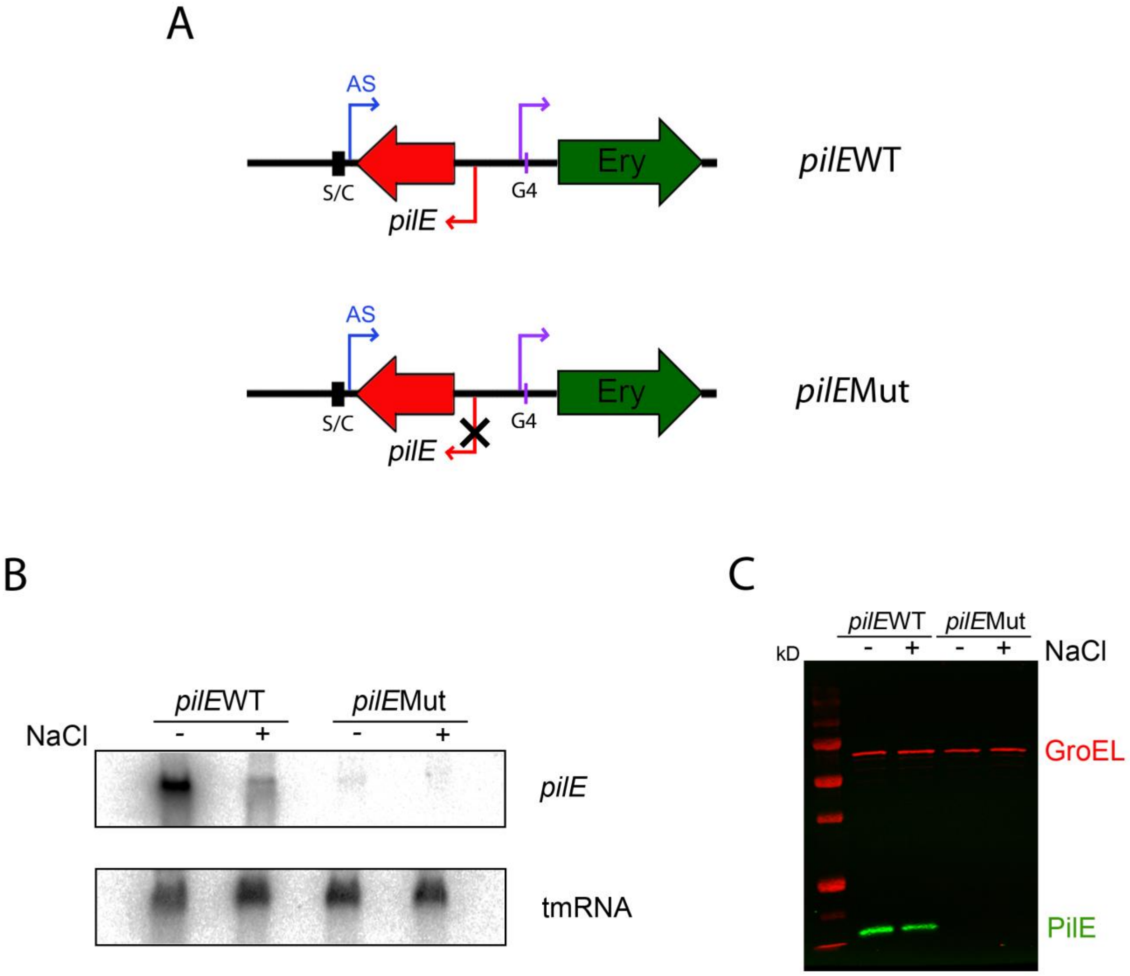


Figure 3.7 Construction of *pilE*WT and *pilE*Mut.

(A) Schematic diagram of *pilE*WT and *pilE*Mut. *pilE*WT carries an intact *pilE* promoter and an erythromycin resistance cassette transcribed divergently to *pilE* while *pilE*Mut carries mutations in the -35 and -10 elements of the *pilE* promoter in addition to the erythromycin resistance cassette. (B) Representative Northern blot analysis of total RNA isolated from *pilE*WT and *pilE*Mut strains with (+) and without (-) NaCl stress using a 50 nt oligonucleotide probe specific for the *pilE* transcript (*pilE* probe). tmRNA was used as a loading control. No *pilE* transcript was detected in *pilE*Mut. (C) Western blot analysis of whole cell lysates from *pilE*WT and *pilE*Mut strains with (+) and without (-) NaCl stress. No PilE protein (green) was detected in *pilE*Mut. GroEL (red) was used as a loading control.

Northern blot analysis was performed on *pilE*WT and *pilE*Mut with and without NaCl stress using the probe (AS)*pilE*-1 in order to determine whether the *pilE* transcript plays a role in mediating AS transcription. Levels of AS transcript were increased when both *pilE*WT and *pilE*Mut strains were treated with NaCl stress (Figure 3.8, lanes 2 and 4). However, AS transcript levels were noticeably higher in *pilE*Mut compared to *pilE*WT in the absence of NaCl stress (Figure 3.8), suggesting that the *pilE* transcript may downregulate AS transcription or impair the stability of the AS transcript.

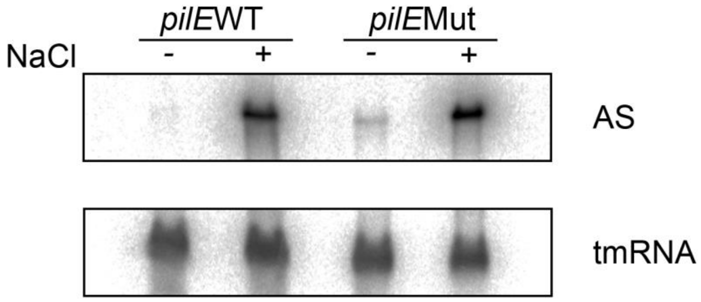


Figure 3.8 Expression of the AS transcript in *pilE*WT and *pilE*Mut.
Representative Northern blot analysis of total RNA isolated from *pilE*WT and *pilE*Mut strains with (+) and without (-) NaCl stress using a 50 nt oligonucleotide probe specific for the AS transcript ((AS)*pilE*-1). tmRNA was used as a loading control. The AS transcript is upregulated by NaCl stress in *pilE*WT and *pilE*Mut.

3.3.5 Involvement of RNases in regulation of the AS transcript

In bacteria, RNA turnover is a key process in the post transcriptional control of gene expression; rapid production and degradation of coding and noncoding RNAs allows the cell to adapt rapidly to changing environments. RNases are enzymes which are involved in the processing, decay, and quality control of RNAs to monitor and control RNA levels to meet the requirements of the bacterial cell (Arraiano *et al*., 2010). Therefore, we aimed to determine if RNAs were involved in influencing the levels of the AS RNA.

3.3.5.1 Constructing RNase mutants in *N. meningitidis*

Strains with mutations in various RNases were generated to investigate ther role in influencing AS transcript levels. The RNases chosen for this study are: RNaseHI and RNaseHII (encoded by *rnhA* and *rnhB* respectively), RNaseIII (encoded by *rnc*) and RNaseE (encoded by *rne*). RNaseHI and RNaseHII are endoribonucleases which selectively degrade RNA hybridized with DNA (Crooke *et al*., 1995; Kogoma, 1986). RNaseIII, an endoribonuclease specific for double stranded RNA (dsRNA), is involved in the maturation of rRNA, and is also responsible for the degradation of mRNA targets in sRNA-mRNA hybrids as part of sRNA mediated gene regulation (Saramago *et al*., 2014). Finally, RNaseE is an endoribonuclease involved in mRNA turnover and stable RNA maturation in bacteria (Bandyra *et al*., 2013).

RNaseIII is known to be involved in clustered, regularly interspaced, short palindromic repeat (CRISPR)-mediated adaptive immunity in *Neisseria*; the 3'-ends of CRISPR RNAs (crRNAs) are processed by RNAse III in *N. meningitidis* (Zhang *et al*., 2013). In addition, a number of mRNAs in *N. meningitidis* contain short insertion sequences featuring terminal inverted repeats known as Correia elements (Correia *et al*., 1988), or *nemis* (*Neisseria* *m*iniature *i*nsertion *s*equences) (Mazzone *et al*., 2001), which form RNA hairpins that can be processed by RNaseIII (De Gregorio *et al*., 2002, 2003). However to our knowledge, the roles of RNaseE and the RNaseH enzymes have not been specifically investigated in *N. meningitidis*.

RNase mutants were constructed in wild type *N. meningitidis* 8013, WT_ery, or Mut_ery. For *rnhA*, *rnhB* and *rne*, mutants were generated by replacing the coding sequence (leaving ~15 nt of the regions encoding the 5'- and 3'- termini of each gene) of the RNase genes with a kanamycin resistance cassette containing its own constitutive promoter and two predicted *rrnB* terminators (Figure 3.9A, B and D). The *rnc* gene is the first gene in an operon, with no predicted promoter sequences in the 47 nt intergenic region between *rnc* and the downstream gene NMV1712. Therefore, *rnc* mutants were generated by replacing the entire coding sequence with just the kanamycin resistance cassette without any additional promoters or terminators in an attempt to allow expression of the downstream gene(s) (Figure 3.9C). Growth curves of 8013Δ*rnhA*, 8013Δ*rnhB*, 8013Δ*rnc* and 8013Δ*rne* show that there is no difference in the growth rates of the strains in BHI liquid at 37°C compared to wild-type strain 8013 (Figure 3.9E).

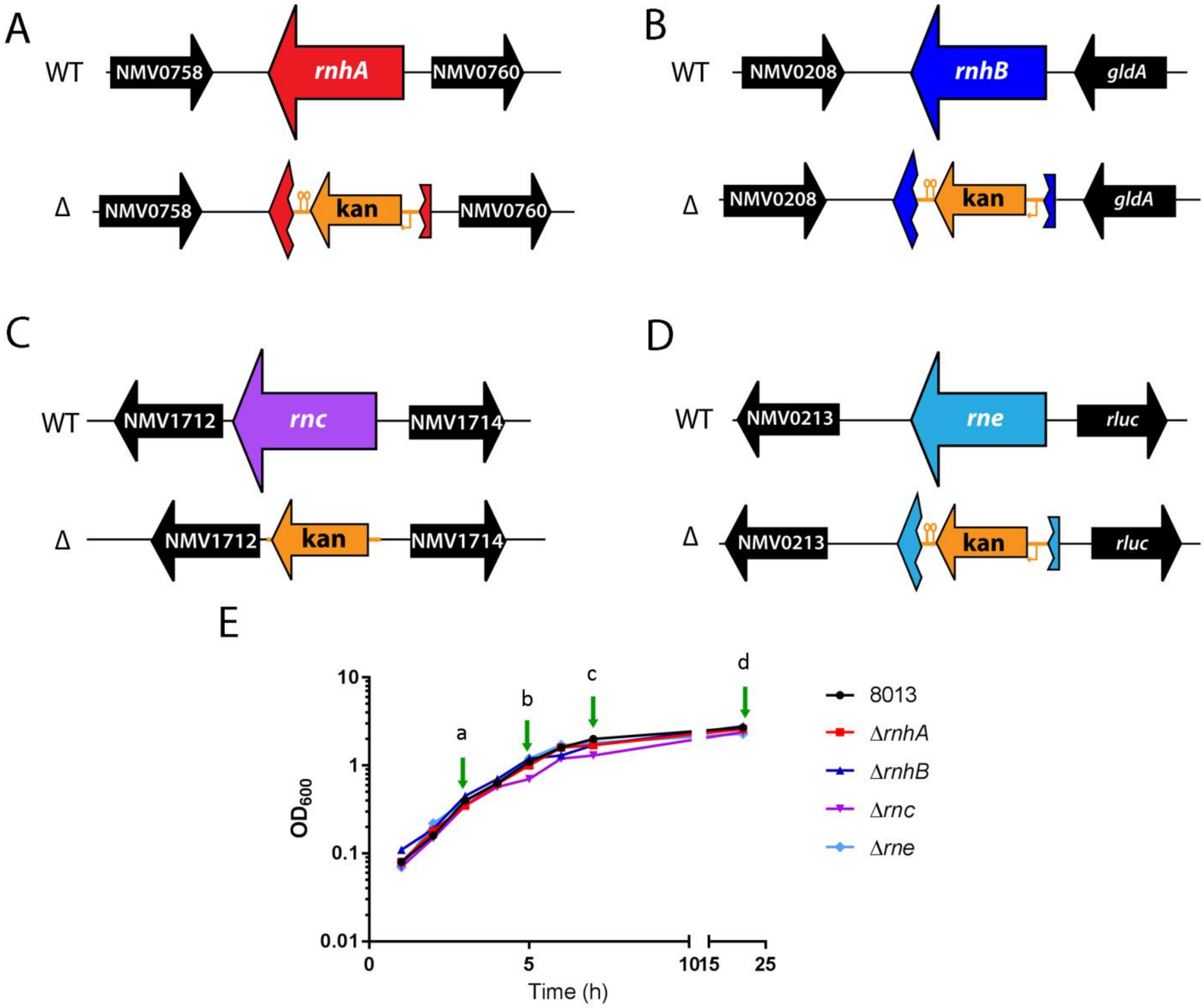


Figure 3.9 Construction of RNase mutants in *N. meningitidis* 8013.

Schematic diagrams of the loci of (A) *rnhA*, (B) *rnhB*, (C) *rnc*, and (D) *rne* in *N. meningitidis* 8013 and the respective mutants generated by replacement of the genes with a kanamycin resistance cassette. RNase mutations were introduced in strains 8013, WT_ery and Mut_ery. Figures not to scale. (E) Growth curves of 8013, 8013Δ*rnhA*, 8013Δ*rnhB*, 8013Δ*rnc* and 8013Δ*rne* show no difference in growth rates.

3.3.5.2 RNaseHI and RNaseHII

First the involvement of the two RNaseH enzymes, RNaseHI and RNaseHII, encoded by *rnhA* and *rnhB* respectively, on AS RNA levels were examined. Total RNA was isolated from *N. meningitidis* 8013, 8013Δ*rnhA* and 8013Δ*rnhB* at different growth phases, and analyzed by Northern blot. Consistent with previous results, the AS transcript was detected only after overnight growth in wild-type *N. meningitidis* 8013. This expression pattern was also observed for 8013Δ*rnhB* (Figure 3.10A). However, in 8013Δ*rnhA*, the AS transcript was detected during all growth phases, albeit at lower levels during mid-exponential, late-exponential and early stationary phases (Figure 3.10A, lanes 5-7) compared to after overnight growth (Figure 3.10A, lane 8). Similarly, Northern blot with WT_eryΔ*rnhA* also demonstrates a low level of AS expression in the absence of NaCl stress and upregulation of expression in response to NaCl stress (Figure 3.10B). In WT_eryΔ*rnhB*, the AS transcript was also increased in NaCl stress but was not detected in the absence of NaCl stress. Therefore, the results indicate that RNaseHI is involved in maintaining the lower levels of the AS RNA in the absence of stress.

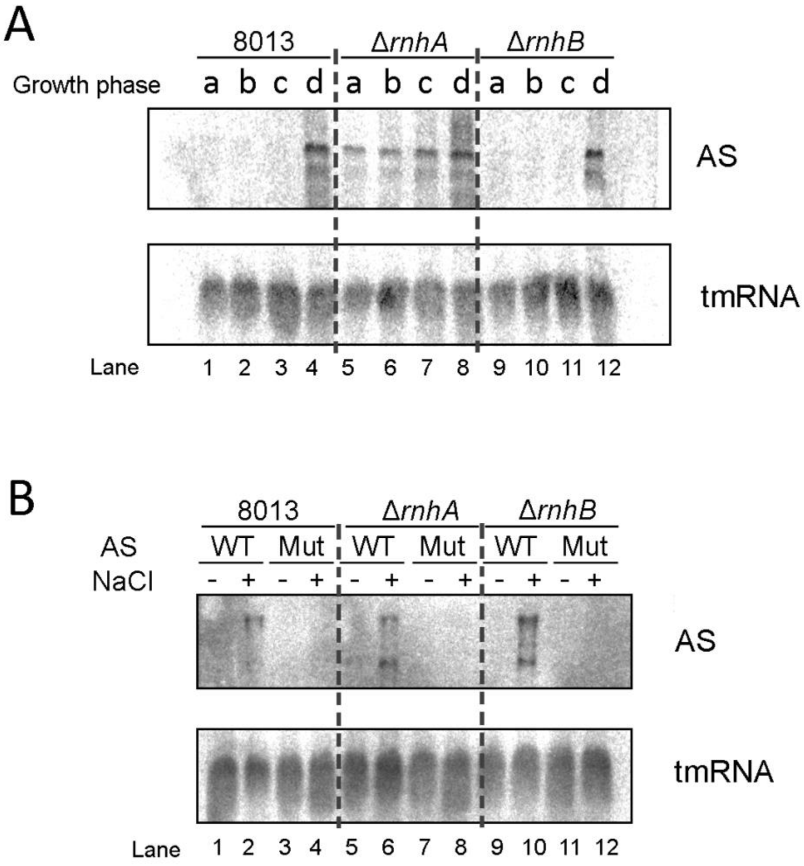


Figure 3.10 Expression of the AS transcript in RNaseHI and RNaseHII mutants.

(A) Northern blot analysis of total RNA from *N. meningitidis* 8013, 8013Δ*rnhA* and 8013Δ*rnhB* at different growth phases using a probe specific for the AS RNA. Growth phases (a – d) correspond to labelled points on the growth curves in Figure 3.9E. (B) Northern blot analysis of total RNA isolated from WT_ery and Mut_ery (lanes 1-4), WT_eryΔ*rnhA* and Mut_eryΔ*rnhA* (lanes 5-8), and WT_eryΔ*rnhB* and Mut_eryΔ*rnhB* (lanes 9-12) with and without NaCl stress. The AS transcript is only detected in WT_ery and WT_eryΔ*rnhB* after NaCl stress.

3.3.5.3 RNaseIII and RNaseE

We next investigated the potential involvement of the RNases RNaseIII and RNaseE, which are encoded by *rnc* and *rne* respectively. Expression of the AS transcript was also examined in 8013Δ*rnc* and 8013Δ*rne* during different growth phases. 8013Δ*rne* showed a similar expression pattern as 8013, with the AS transcript only detected after overnight growth (Figure 3.11A). In 8013Δ*rnc*, a band corresponding to a smaller product was detected during exponential (lane 5) and early stationary (lane 7) phase and after overnight growth (lane 8) (cyan arrow, Figure 3.11A). This product is also present in unstressed WT_eryΔ*rnc* (Figure 3.11B, lane 5). However, NaCl stress results in the expression of a product of similar size to that seen in WT_ery (blue arrow, Figure 3.11B, lanes 2 and 6). These results suggest a role for RNaseIII in preventing the formation of a smaller sized AS product.

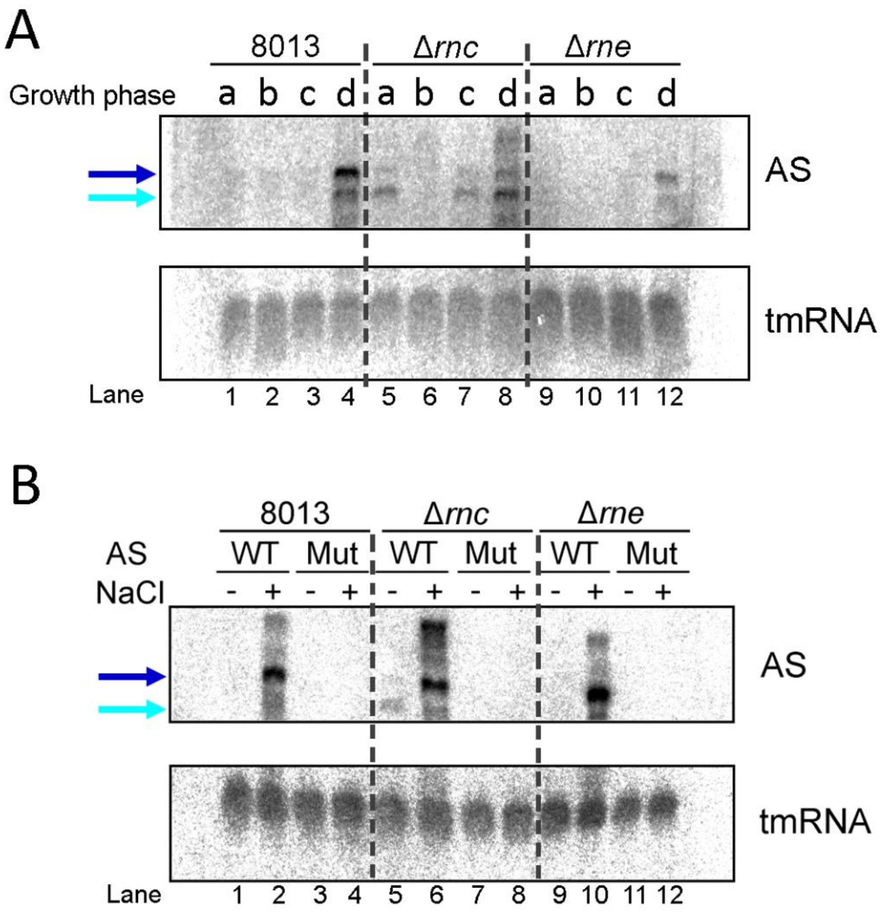


Figure 3.11 Expression of the AS transcripts in RNaseIII and RNaseE mutants.

(A) Northern blot analysis of total RNA from *N. meningitidis* 8013, 8013Δ*rnc* and 8013Δ*rne* at different times during growth using a probe specific for the AS RNA. Growth phases (a – d) correspond to labelled points on the growth curves in Figure 3.9E. Blue and cyan arrows point to the different sized AS RNA products referred to in the text. (B) Northern blot analysis of total RNA isolated from WT_ery and Mut_ery (lanes 1-4), WT_eryΔ*rnc* and Mut_eryΔ*rnc* (lanes 5-8), and WT_eryΔ*rne* and Mut_eryΔ*rne* (lanes 9-12) with and without NaCl stress.

3.4 Summary

This chapter describes the identification and characterization of a novel antisense RNA in the *pilE* locus of *N. meningitidis*. The sequence of the AS promoter is 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. Conservation of the AS promoter sequence across different *N. meningitidis* strains expressing class I pilin is particularly striking given the sequence variability of the adjacent *pilE* gene. This conservation suggests that the AS promoter has a function in *N. meningitidis*. 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 de*via*te from the consensus sequence, and thus may result in a non-functional promoter. This is consistent with a recent transcriptomic study of *N. gonorrhoeae* which did not detect *pilE* AS transcription (Remmele *et al*., 2014). However, it is also possible that the AS RNA was not detected during transcriptomic studies of *N. gonorrhoeae* due to the absence of stress conditions that induce AS RNA expression. Nevertheless, possible species specific differences in the expression of the AS RNA would not be surprising given that *N. gonorrhoeae* inhabits a physiologically distinct niche within human hosts compared to the meningococcus. Thus, while the AS RNA may potentially function as a noncoding regulatory RNA in *N. meningitidis*, there may not be a similar requirement for this function in *N. gonorrhoeae*.

To study the activity of this promoter, the AS RNA was examined using *E. coli* as a heterologous host. Using this system, it was found that the transcription from this promoter is induced in NaCl stress. In addition, mutation to the -10 sequence of the promoter was found to be sufficient to abolish transcription. Salt stress induction of AS expression was also observed in *N. meningitidis,* suggesting that the molecular mechanisms of this response are common to *E. coli* and *N. meningitidis*. Although the concentration of salt required for AS RNA induction was higher than would normally be faced by the meningococcus *in vivo,* the transcript was also found to be upregulated after overnight growth, which may represent environments that meningococci would encounter *in vivo,* for example during conditions of nutrient depletion, high cell density and oxygen limitation (Llorens *et al*., 2010).

Although AS RNA expression is undetectable or at low levels in the absence of stress, the levels of transcript increased significantly after short exposure to NaCl stress; such a regulated response to environmental stress is a feature common to many sRNAs (Gottesman *et al*., 2006). sRNAs have long been known to be involved in bacterial stress response. Well characterized examples of *trans*-encoded sRNAs include DsrA and RprA which are upregulated by low temperature and cell surface stress respectively to stimulate the translation of RpoS, a general stress response sigma factor in *E. coli* (McCullen *et al*., 2010; Repoila *et al*., 2003). Expression of the sRNA RyhB in *E. coli* is induced in iron-limiting conditions, resulting in the downregulation of iron-storage proteins and non-essential iron-containing proteins (Massé and Gottesman, 2002). A handful of *cis*-encoded antisense RNAs have been shown to play a role in adaptation to stress conditions. For example, GadY, encoded on the opposite strand of *gadX*, is upregulated during stationary phase and results in the upegulation of *gadX*, which encodes a transcriptional regulator of the acid response in *E. coli* (Opdyke *et al*., 2004). Transcription of antisense RNA encoded on the opposite strand of known acid-repressed genes has also been found to be induced by acid stress in *Helicobacter pylori* (Sharma *et al*., 2010). The possible role of the AS RNA in NaCl stress will be explored in Section 4.3.

To further understand of the mechanisms of AS RNA regulation in terms of its stability and degradation, we investigated the influence of a number of RNases on AS transcript levels. The AS RNA was detected in the absence of NaCl stress in strains lacking RNAseHI, suggesting that RNaseHI may be involved in degradation of the AS RNA. The possible involvement of RNaseHI in the regulation of AS transcript expression or stability is an intriguing prospect. As the substrates of RNaseHI are RNA molecules that are part of RNA:DNA hybrids, this result raises the possibility that the AS transcript may associate with DNA. The involvement of the AS RNA in RNA:DNA hybrids will be discussed in Section 5.4.2. In addition, it was also observed that deletion of *rnc* results in the appearance of a smaller sized product detected by the probe (AS)*pilE*-1 during exponential and early stationary phases of growth. This suggests that RNaseIII may contribute to the stability of the AS transcript so that the absence of RNaseIII results in cleavage into a smaller product. This counterintuitive result suggests that RNaseIII may not be directly responsible for AS RNA cleavage. Given the enzyme’s role in sRNA-mediated cleavage of target mRNAs, it is conceivable that other sRNAs or mRNAs may be directly or indirectly involved in the regulation of AS RNA stability.

The sequence and length of the AS RNA can provide an indication of its possible function. Utilizing primer extension and walking RTPCR, the transcriptional start site of the AS RNA was mapped to 8 nt downstream of the -10 sequence, and was found to extend to the G4 promoter. The AS RNA is complimentary to the entire *pilE* coding region and 3'-UTR, raising the possibility that the AS RNA could be involved in mediating *pilE* regulation or PilE expression through base-pairing. In addition, the increased levels of AS RNA levels detected in the absence of *pilE* transcript further suggest possible AS RNA – *pilE* mRNA interactions. Moreover, as the AS RNA also encompasses the G4 promoter, it could also potentially influence G4 promoter activity or G4 sRNA expression, and consequently play a role in modulating PilE antigenic variation. The next chapter will focus on exploring the possible functions of the AS RNA in *pilE* expression or pilin function.

4 Results - Investigating the function of *pilE* antisense RNA in *N. meningitidis*

In the previous chapter, we characterized a novel AS RNA in the *pilE* locus of *N. meningitidis* which is expressed in NaCl stress and after overnight growth. Antisense RNAs, also known as *cis*-encoded regulatory RNAs, can regulate the transcription, RNA stability, or translation of the sense transcript (Thomason and Storz, 2010). Having identified this novel RNA, we postulated that it could play a role in mediating Tfp functions such as adhesion to host cells or competence for DNA uptake (Aas *et al*., 2002; Stephens and McGee, 1981), or be involved in regulating the expression of Tfp-related genes. In order to investigate the possible function(s) of the AS RNA, a number of different assays were conducted using Mut_ery, which lacks a functional AS promoter, and the isogenic strain WT_ery, to determine any differences in phenotype or gene expression that can be attributed to the presence of the AS transcript.

4.1 The AS transcript is not involved in adhesion of *N. meningitidis* to epithelial cells

Given the position of the AS transcript in the *pilE* locus, it is possible that the transcript modulates Tfp-related function by influencing the expression or processing of Tfp associated genes, in particular *pilE*, as the *pilE* transcript is complementary to the AS RNA.

To examine whether the AS affects the ability of Tfp to mediate bacterial attachment to host cells, adhesion assays were performed with the epithelial cell line, A549, challenged with WT_ery and Mut_ery. The lung carcinoma cell line A549 were chosen due to its respiratory origin and the fact that it is a common epithelial cell line used to study meningococcal adhesion and host-cell interactions (Hey *et al*., 2013; Virji *et al*., 1992). A549 cells were infected with WT_ery or Mut_ery at an MOI of 50 and incubated for 1.5 h. After removal of non-adherent bacteria by washing the monolayer with PBS, cells were lysed and cell-associated bacteria were enumerated by plating dilutions onto BHI agar. Adhesion was calculated as the number of cell-associated bacteria as a percentage of the inoculum. Each assay was performed with two sets of strains obtained from independent transformation events to rule-out effects of any off target mutations in single colonies obtained following transformation. There was no significant difference in relative percentage of cell adhesion in WT_ery and Mut_ery (Student's *t*-test, $p = 0.1154$). The results demonstrate that WT_ery and Mut_ery do not differ significantly in their ability to adhere to epithelial cells, indicating that the AS transcript is not involved in mediating adhesion in this assay (Figure 4.1). However, it should be noted that there was a large amount of variability within and between assays, making it difficult to detect subtle differences between strains. Such inter assay variability is common and may be due to differences in adhesion efficiencies due to expression of PilE proteins with altered sequences as a result of Av (Nassif *et al*., 1993), phase variation of other adhesins such as Opa proteins (Kupsch *et al*., 1993), or variability in the condition or growth phase of the cell line.

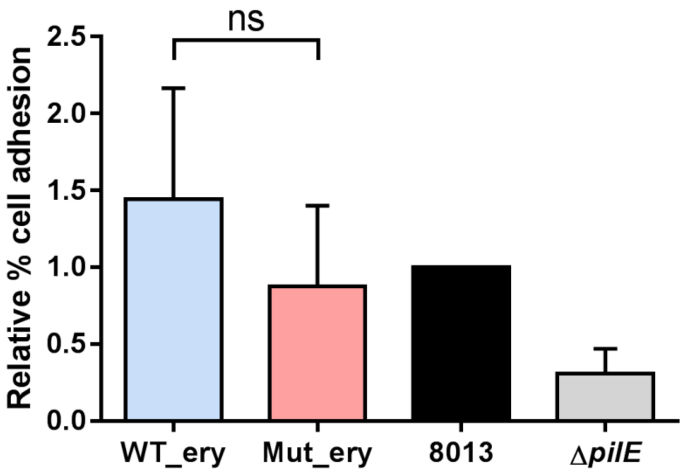


Figure 4.1 Loss of the *pilE* AS RNA does not affect adhesion to epithelial cells.
Confluent A549 epithelial cells were infected at a multiplicity of infection (MOI) of 50 in DMEM and incubated at 37°C, 5% CO_2 for 1.5 hours. The medium was removed and cells were washed three times with PBS to remove non-adherent bacteria then lysed with PBS-saponin. Adhesion was calculated as the percentage of the number of cell-associated bacteria to the number of bacteria in the inoculum. Relative cell association for each assay was calculated as the % cell association as a ratio to that of 8013. Each assay involved two technical replicates performed on two sets of strains obtained from independent transformation events. The assays were performed in triplicate. Data shown is the average of three replicates. Error bars show the S.D.

4.2 The AS transcript is not involved in transformation efficiency

Another important function mediated by Tfp is the ability to take up extracellular DNA. DNA uptake by *N. meningitidis* is proposed to involve the following steps: DNA is first bound by the minor pilin ComP (Cehovin *et al*., 2013), followed by retraction of the Tfp fiber by PilT (Wolfgang *et al*., 1998) which pulls the DNA through the outer membrane, and finally transport of DNA into the cytoplasm by ComE (Chen and Gotschlich, 2001; Gangel *et al*., 2014) where it can integrate into the chromosome. DNA uptake and transformation drives horizontal gene transfer in the meningococcus, contributing to the generation of genetic diversity and bacterial evolution (Chen and Dubnau, 2004). The effect of the AS transcript on natural competence was investigated by comparing transformation efficiency of WT_ery and Mut_ery. Each strain was incubated with equal amounts of purified genomic DNA from the strain 8013Δ*pilE*, in which the *pilE* coding sequence is replaced by a kanamycin resistance cassette. Thus, transformation efficiency, calculated as the percentage of total number of cfu on solid media containing kanamycin, gives a combined measure of the ability to uptake DNA and for homologous recombination to occur in the *pilE* locus. The results show that there is no significant difference in transformation efficiency between the two strains (Student's *t*-test, p = 0.5335), indicating that the AS transcript is not required for DNA transformation (Figure 4.2).

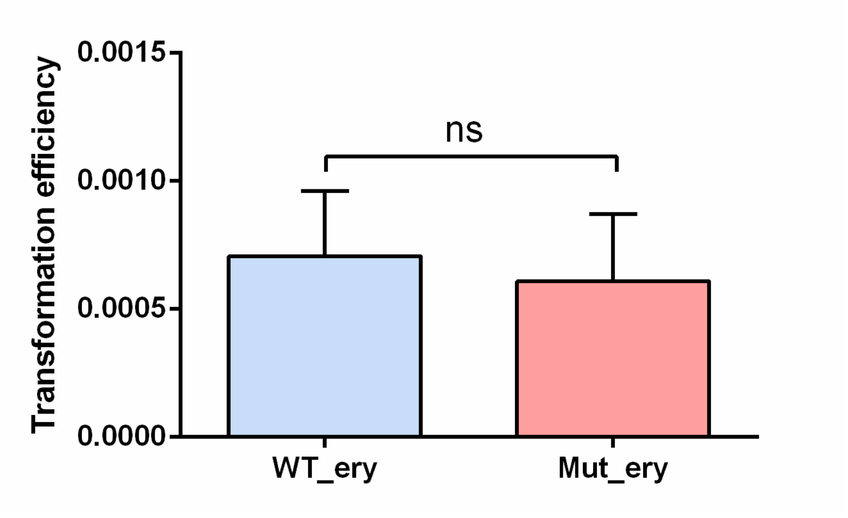


Figure 4.2 Loss of the *pilE* AS RNA does not affect transformation efficiency.

The DNA transformation efficiencies were assayed for WT_ery and Mut_ery by incubating 10^7 cells of each strain with 1 µg of 8013Δ*pilE* genomic DNA. After incubation for 5 h, bacterial spots were collected and spread onto BHI agar and BHI agar containing kanamycin to quantify total bacteria and the number of transformants respectively. The transformation efficiency was calculated as the percentage of transformants to the total number of bacteria. Assays were performed twice, with three sets of strains obtained from independent transformation events.

4.3 Absence of the AS transcript does not affect survival in NaCl

Since expression of the AS transcript is induced in response to NaCl stress (Figure 3.3), it is possible that the AS could play a role during survival in high concentrations of NaCl. NaCl survival assays were performed with WT_ery and Mut_ery to determine whether absence of the AS transcript affects survival of bacteria in NaCl stress. Liquid cultures of WT_ery and Mut_ery were grown in BHI broth to an OD_{600} of 0.6 (designated t = 0 min) and subjected to NaCl stress (0.5 M) for 10 minutes. A parallel assay was also performed without NaCl stress (*i.e.* 10 minutes incubation without NaCl starting at t = 0 min). Cultures were then diluted and spread onto solid media for enumeration. Survival in NaCl stress was calculated as the ratio of cfu at t = 10 min to that at t = 0 min. Results show that there is no significant difference between the survival rate of WT_ery and Mut_ery after 10 min of NaCl stress (Student's *t*-test, $p = 0.9999$) (Figure 4.4). Moreover, there is no significant difference in the ratios of survival rates of NaCl treated compared to untreated bacteria between both strains (Student's *t*-test, $p = 0.6581$) (not shown). Thus there is no evidence that the AS transcript plays a role in bacterial survival in NaCl stress.

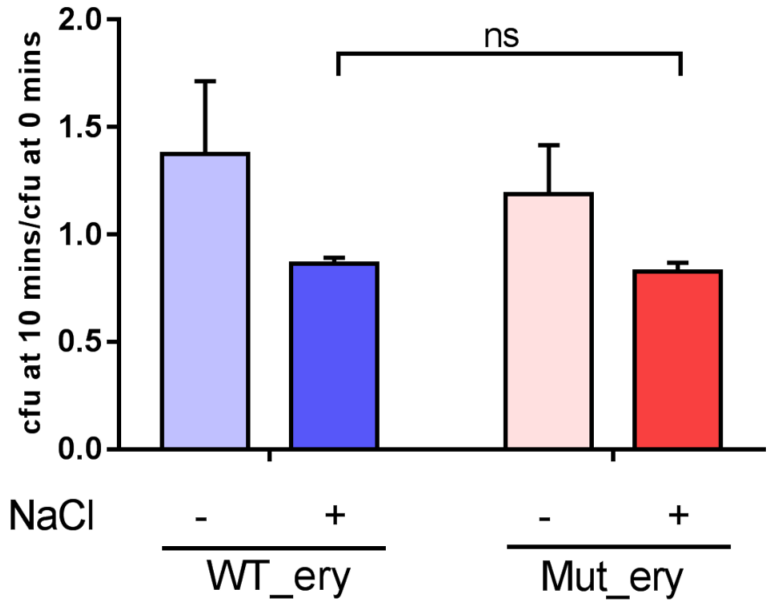


Figure 4.3 The *pilE* AS RNA does not affect bacterial survival in NaCl stress.

NaCl survival assays were performed with WT_ery and Mut_ery to determine if the AS transcript is involved in survival in NaCl stress. Strains were grown in liquid media to an OD_{600} of 0.6 and were either treated with NaCl (final concentration 0.5M , 10 min) or untreated, then incubated at 37°C with agitation. Bacterial cultures were enumerated at t = 0 min and t = 10 min. Survival in NaCl stress is expressed as the ratio of cfu at t = 10 min to that at t = 0 min. Assays were performed twice.

4.4 The AS transcript does not regulate *pilE* or PilE

Although phenotypic assays for Tfp-mediated functions indicated that loss of the AS RNA did not significantly impact bacterial adhesion to epithelial cells or in transformation efficiency, this does not rule out the possibility that the AS RNA regulates *pilE* transcript levels or PilE protein expression. The AS RNA is complementary to the entire *pilE* coding sequence and 5′ UTR (Figure 3.6), so the AS RNA could mediate *pilE* or PilE regulation through base pairing interactions. Therefore it was next determined whether *pilE* transcript or pilin protein levels are affected by the presence of the AS transcript.

Northern blot analysis was performed on total RNA prepared from WT_ery and Mut_ery at different growth phases using a *pilE* specific oligonucleotide probe (*pilE*_probe). This strand specific probe was designed to hybridize to the conserved N-terminal coding region of *pilE* which is not present in *pilS* cassettes (Haas and Meyer, 1986). Analysis of total RNA isolated from the strains at different growth phases revealed that *pilE* mRNA appears to be expressed at similar levels throughout growth in both WT_ery and Mut_ery (Figure 4.4A), and is detected even after overnight growth, when AS levels are highest (Figure 3.8B), suggesting that increased AS expression after overnight growth does not appreciably alter levels of *pilE* transcript.

Total RNA was prepared from WT_ery and Mut_ery with and without brief incubation in high salt concentrations (0.5 M, 10 minutes) to examine the levels of *pilE* transcript when expression of the AS RNA is induced by NaCl stress. In the absence of NaCl stress, both Northern blot analysis and strand-specific qRT-PCR indicated a trend towards elevated *pilE* mRNA levels in Mut_ery compared to WT_ery (Figure 4.4B and C) but this was not significant (p=0.1223, Student's *t*-test). Following NaCl stress (which induces an increase in AS RNA levels, Figure 3.8C), an approximately five fold reduction of *pilE* transcript levels was observed, but this was independent of the AS transcript as the reduction of *pilE* levels in response to NaCl stress 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 considered the possibility that the AS RNA might be involved in post-transcriptional regulation of PilE translation. Therefore, changes in pilin protein levels were investigated. Western blot analysis was performed on whole cell lysates using anti-pilin antibody and fluorescence analysis to quantify the relative amounts of pilin in whole cell lysates from WT_ery and Mut_ery either during growth (Figure

4.4D) or following salt stress (Figure 4.4E). Mutation of the AS promoter and NaCl stress did not lead to a detectable change in PilE protein levels under these conditions (Table 4.1). Therefore, there is no evidence that the AS RNA affects *pilE* transcript or pilin protein levels in the meningococcus.

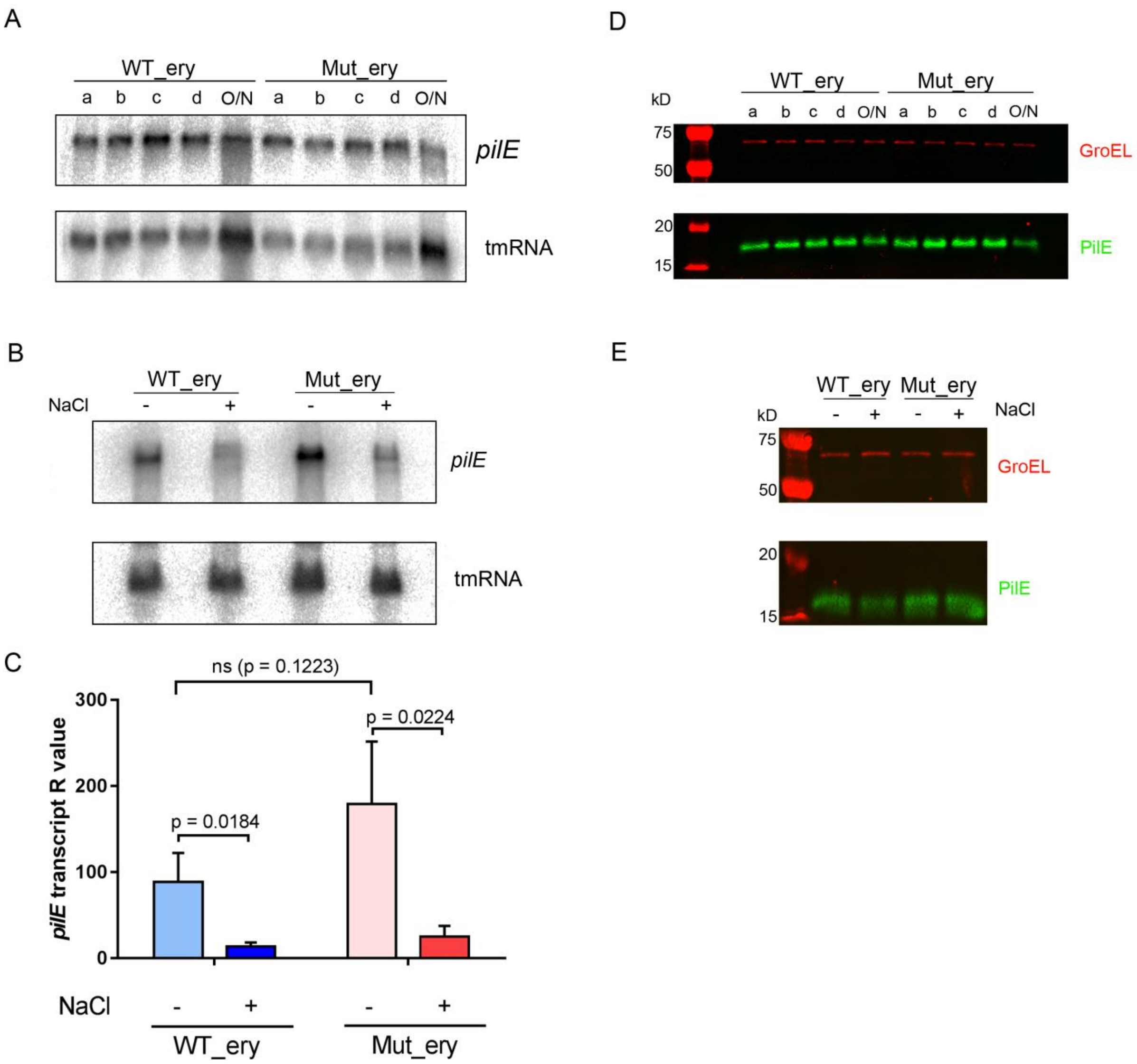


Figure 4.4 Effect of the AS RNA on *pilE* mRNA and PilE protein levels in *N. meningitidis*.

(A) Northern blot analysis of RNA from WT_ery and Mut_ery at different growth phases. *pilE* transcript was detected using *pilE*_probe. tmRNA was used as a loading control. Growth phases labelled a–d correspond to labelled points on the growth curves in Figure 3.8A (B) Detection of *pilE* transcript in *N. meningitidis* with and without NaCl stress by Northern blot analysis. (C) Strand-specific qRT-PCR 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 Section 2.8. (D) Western blot analysis of whole cell lysates of WT_ery and Mut_ery at different growth phases. PilE (green), GroEL loading control (red). (E) Western blot analysis of WT_ery and Mut_ery with and without NaCl stress. PilE (green), GroEL loading control (red).

Table 4.1 Quantification of PilE detected by Western blot.

PilE band intensities were quantified using an Odyssey® Sa Infrared Imaging System, normalized to the signal of the corresponding GroEL band and expressed as a ratio to the normalized PilE value of the first lane.

Replicate	**PilE**			
	WT_ery		**Mut_ery**	
	No NaCl	**NaCl**	**No NaCl**	**NaCl**
1	1	0.41	1.104	1.803
2	1	0.853	1.163	1.349
3	1	1.601	1.808	1.931
Average	1	0.955	1.358	1.694

4.5 The AS transcript is not involved in expression of minor pilins

Although the AS transcript does not appear act in *cis* to influence levels of *pilE* transcript or the major pilin protein PilE, we considered the possibility that it could play a role in the regulation of minor pilins in *trans*. Minor pilins are encoded in the genome distinct from the *pilE/pilS* locus, and are present in the Tfp fiber at low levels. PilX, PilV, and ComP are involved in specific Tfp-mediated functions such as bacterial aggregation, host cell signaling, and DNA uptake (Hélaine *et al*., 2005, Coureuil *et al*., 2010, Wolfgang *et al*., 1999). The minor pilins share structural features with PilE and can contain stretches of sequence identity with *pilE*, which are thus also complementary to the AS RNA. *comP* and *pilX* share 46.12% and 45.27 % sequence identity with *pilE*, with short stretches of identity of around 5 – 8 nt. *pilV* shares the highest sequence identity with *pilE* (50.0 %), with stretches of identity up to 15 nt long. Therefore, the AS RNA might act in *trans* to regulate these target mRNAs through base pairing interactions.

The effect of the AS transcript on the expression of minor pilins was analyzed by Northern blot. Total RNA from WT_ery and Mut_ery without and with NaCl stress to induce AS

RNA expression was analyzed using oligonucleotide probes specific for *pilX*, *pilV*, or *comP* (Table 2.3). Results indicate that although all three minor pilins appear to be downregulated in response to NaCl stress, this downregulation occurs in both WT_ery and Mut_ery, suggesting that the AS transcript does not play a role in this response (Figure 4.5).

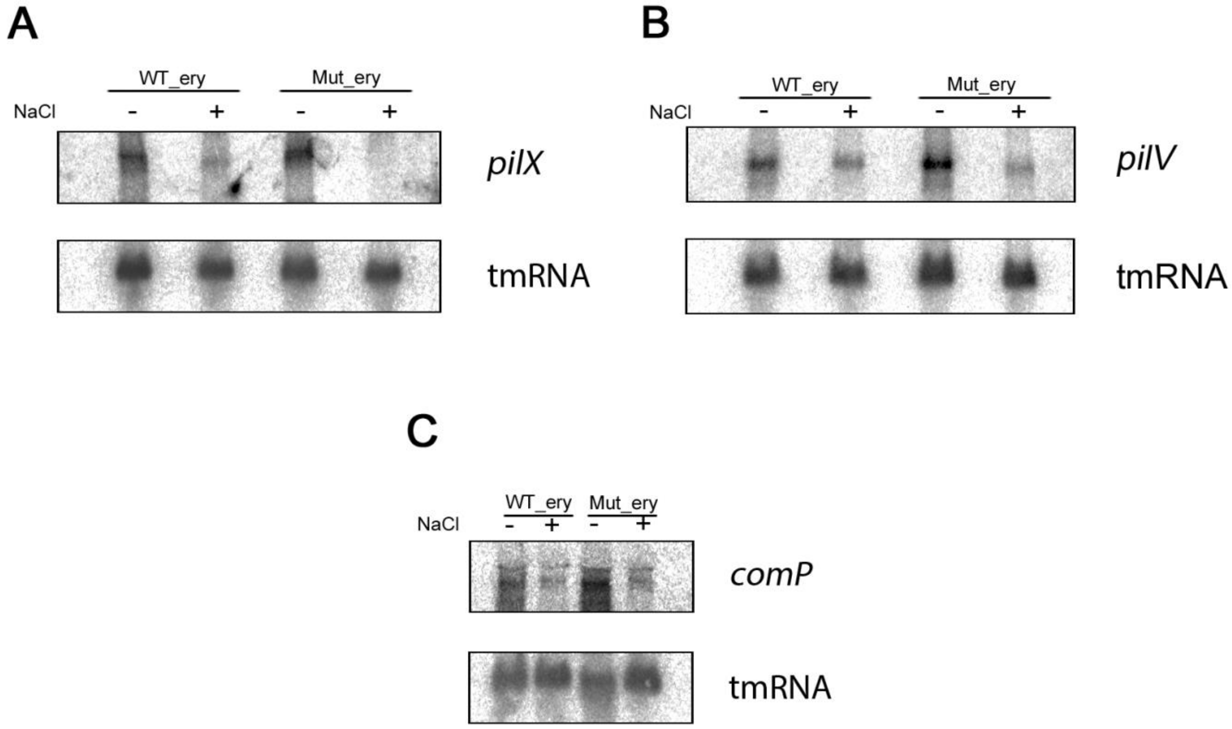


Figure 4.5 The AS RNA is not involved in the expression of minor pilins.

Northern blot analysis of total RNA from WT_ery and Mut_ery with and without NaCl stress. (A) *pilX*, (B) *pilV*, and (C) *comP* transcripts were detected using 50 nt oligonucleotide probes *pilX*_probe, *pilV*_probe, and *comP*_probe respectively. tmRNA was used as a loading control.

4.6 Identification of potential *trans* targets

4.6.1 Global analysis of gene expression in WT_ery and Mut_ery by RNAseq

Since there was no evidence to suggest that the AS RNA is involved in the regulation of minor pilin transcript levels, RNAseq was utilized as a strategy to identify other potential *trans* targets of the AS RNA on a global scale. Other studies have previously utilized tiling arrays, in which a large number of probes are used to investigate the whole genome, to probe the transcriptional profile of *N. meningitidis* in an *ex vivo* model of human whole blood infection (Echenique-Rivera *et al*., 2011). However due to its advantages over tiling arrays, such as its large dynamic range and ability to map transcriptional start sites at single nucleotide resolution (Mortazavi *et al*., 2008), RNAseq has become the method of choice for transcriptomics and differential gene expression in a number of different bacterial pathogens including *H. pylori*, *Salmonella* Typhi, and *N. gonorrhoeae* (Perkins *et al*., 2009; Remmele *et al*., 2014; Sharma *et al*., 2010). Therefore, we performed RNAseq analysis to compare the transcriptomes of WT_ery and Mut_ery to identify genes that are differentially expressed in the absence of the AS RNA.

First, we investigated the optimal growth conditions for AS RNA expression and subsequent RNAseq analysis. Although NaCl stress induces the expression of the AS RNA, examining the transciptome at a specific growth phase during which the AS RNA is expressed may be more physiologically relevant than utilizing a high salt concentration to induce AS RNA expression. In order to select an optimal time point for AS RNA expression, total RNA isolated from WT_ery and Mut_ery at different time points was analyzed on agarose gels (Figure 4.6A and B) and by strand specific qRTPCR (Figure 4.6C) to determine the quality of RNA and AS RNA expression, respectively. Figure 4.6A and B illustrate diminishing RNA quality and yield after 15 h and 20 h of growth, likely reflecting RNA degradation and low levels of transcription as the bacterial population enters stationary phase. Thus, although the AS transcript is expressed after overnight (21.5 h) growth (Figure 3.5B), this time point is not ideal for isolating RNA for RNAseq analysis. Strand-specific qRTPCR results show that after 12 h incubation, the AS RNA is upregulated in WT_ery compared to Mut_ery (Figure 4.6C). Therefore, this time point was selected as the optimal time point for RNA isolation and RNAseq analysis.

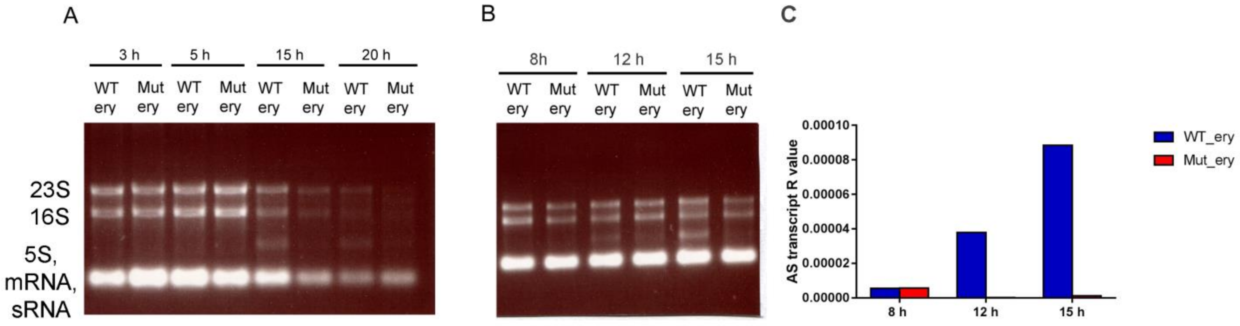


Figure 4.6 AS transcript expression and RNA quality at different time points during growth. Assessment of RNA quality by agarose gel analysis of total RNA isolated from WT_ery and Mut_ery at (A) 3 h, 5 h, 15 h and 20 h and (B) 8h, 12 h, and 15 h of growth. RNA was isolated from equal numbers of total bacteria. (C) Total RNA from WT_ery and Mut_ery was isolated after 8 h, 12 h and 15 h of growth and analyzed for AS transcript expression by strand specific qRTPCR. Results are the R values normalized to that of tmRNA loading control.

Total RNA was therefore isolated from WT_ery and Mut_ery grown in triplicate in liquid BHI media for 12 hours. cDNA library preparation and paired-end sequencing on HiSeq Rapid 2500 were carried out by the Wellcome Trust Centre for Human Genetics. RNAseq reads were mapped to *N. meningitidis* 8013 (NC_017501.1) using Rockhopper software (McClure *et al*., 2013). A total of 80,096,203 reads from WT_ery and 74,541,557 reads from Mut_ery were mapped to the genome, and a total of 2052 transcripts were detected. A transcript was considered to be expressed if it had at least 10 reads per kilobase per million reads (RPKM) (Mortazavi *et al*., 2008). Transcripts with RPKM < 10 were not considered for differential gene expression analysis due to the poor reproducibility of lowly expressed genes (Bullard *et al*., 2010). Out of the detected transcripts, a total of 1935 was considered to be expressed in WT_ery and 1909 in Mut_ery.

4.6.2 Differential gene expression analysis

A number of different programs are available for the identification of differentially expressed genes from RNAseq data. The DESeq2 workflow was chosen for its relatively high sensitivity among the methods that control the false discovery rate, especially for smaller fold changes (Love *et al*., 2014). Transcript count data from Rockhopper software was thus analyzed using the DESeq2 workflow to determine which transcripts were differentially expressed in Mut_ery compared to WT_ery to identify potential *trans* regulated targets of the AS RNA.

The DESeq2 algorithm performs the analysis as follows: a generalized linear model (GLM) is fitted for each gene by modeling read counts as following a negative binomial distribution, which returns coefficients indicating gene expression strength and the $\log_2$ fold change between WT_ery and Mut_ery. Variability between replicates is modeled on the assumption that genes of similar expression strength have similar dispersion, which aids in avoiding potential false positives. A key feature of DESeq2 is its ability to overcome heteroscedasticity (*i.e.* a phenomenon where fold-change ratios tend to exhibit higher variance at lower mean counts) by shrinking $\log_2$ fold change estimates toward zero such that shrinkage is stronger when counts are low, thus removing exaggerated $\log_2$ fold change values for low counts. These shrunken fold change estimates are regarded as allowing a more reliable basis for quantitative conclusions (Love *et al.*, 2014). An independent filtering step is performed which omits genes with mean normalized counts below a filtering threshold. Hypothesis tests for differential expression are then performed for each gene in the filtered subset using a Wald test and adjusted for multiple testing using the procedure of Benjamini and Hochberg (Benjamini and Hochberg, 1995; Love *et al.*, 2014)

A total of 304 transcripts were found to be differentially expressed with an adjusted *p* value of < 0.05 (red points, Figure 4.7). Of these, 21 transcripts had a $\log_2$ fold change value < -1, while one transcript had a $\log_2$ fold change value > 1 (Figure 4.8, Table 4.2). Thus, a total of 22 transcripts were found to be differentially expressed by at least twofold in Mut_ery compared to WT_ery.

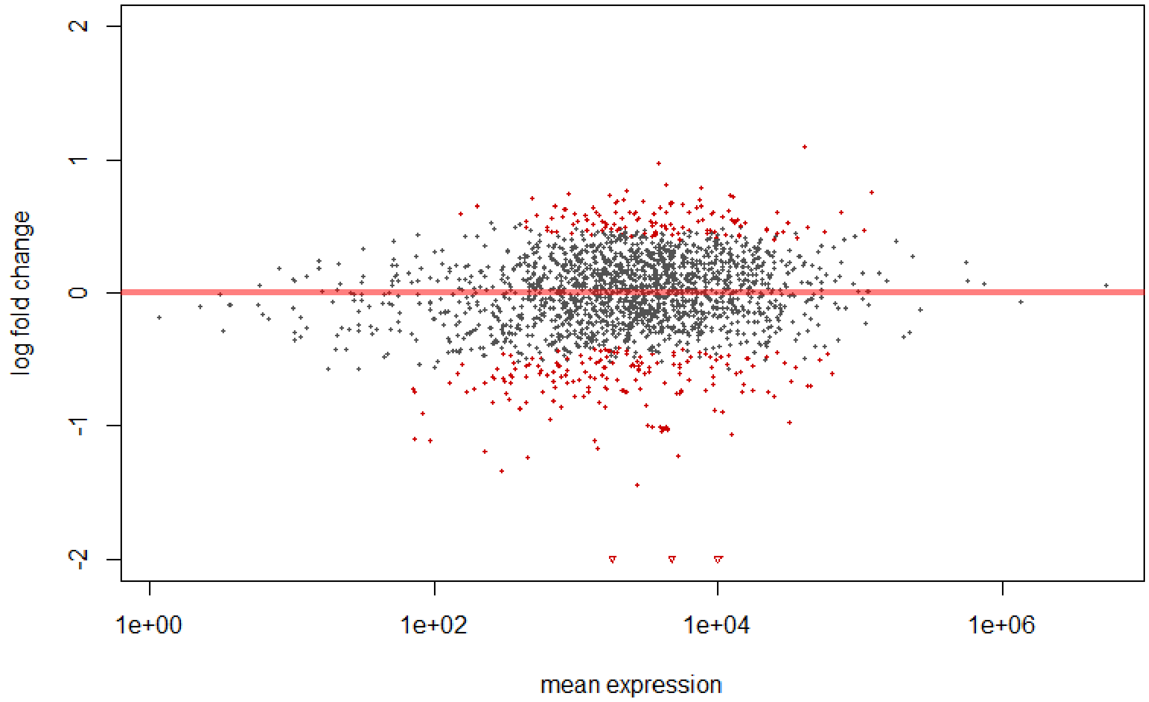


Figure 4.7 MA-plot of the $\log_2$ fold change of each transcript (M-values) against the mean of normalized counts (A-values).

Count values for each transcript from Rockhopper analysis were analysed using the DESeq2 workflow for differential expression. Empirical Bayes shrinkage was performed for the values of the logarithmic fold change $\log_2$(Mut_ery/WT_ery) in order to overcome heteroscedasticity and plotted over the average expression. Points colored red indicate that the adjusted *p* value is less than 0.05. Three points plotted as open triangles pointing down indicate the values of $\log_2$ fold change fall outside the window shown.

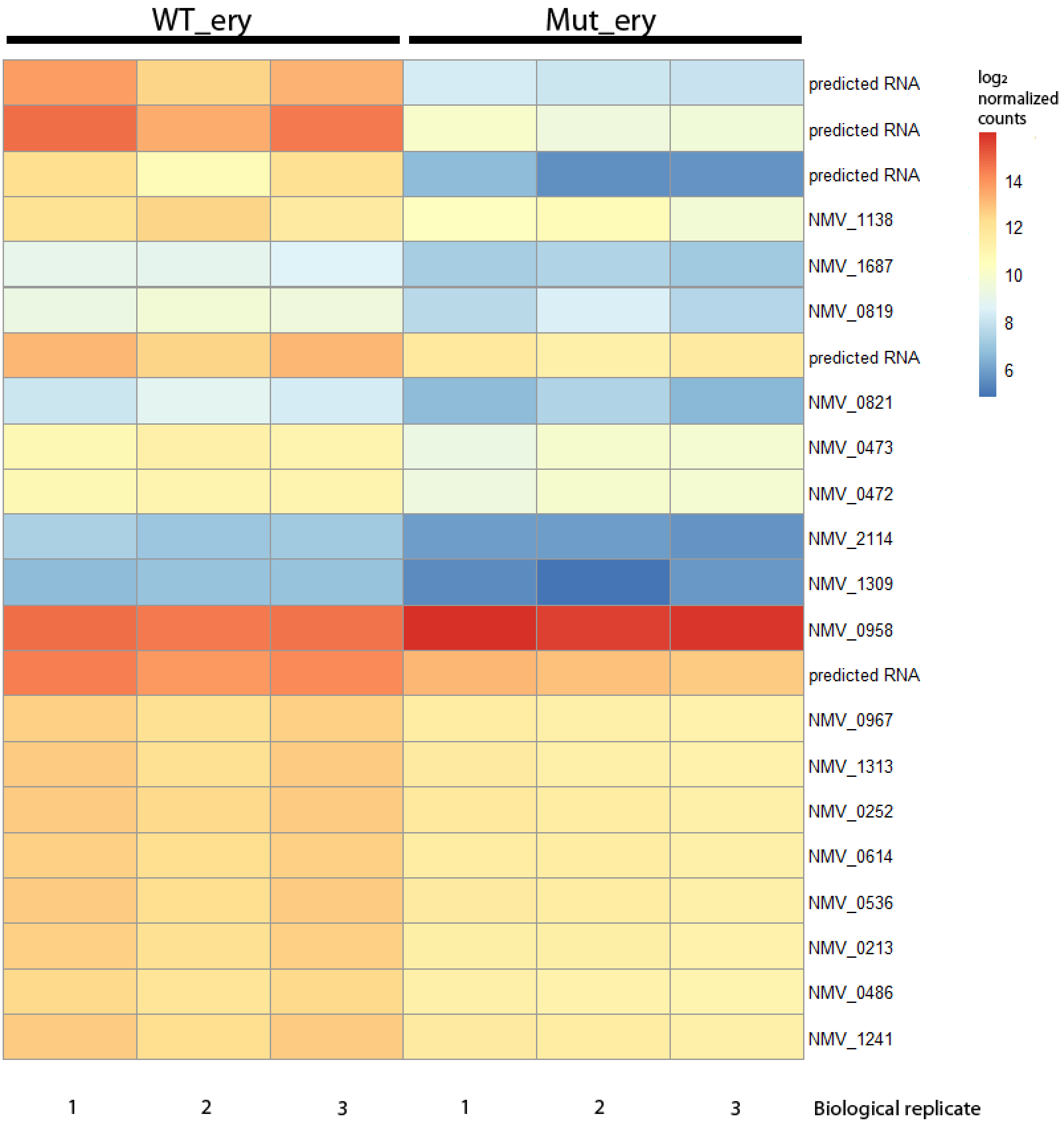


Figure 4.8 Heatmap of expression data of transcripts differentially expressed by greater than twofold.

The $\log_2$ normalized counts of the transcripts that are differentially expressed by at least twofold (adjusted p value < 0.05) were plotted in a heatmap to visualize the changes in expression level between WT_ery and Mut_ery across three biological replicates. Detailed description of each transcript and protein product can be found in Table 4.2.

Table 4.2 Differentially expressed transcripts in Mut_ery compared to WT_ery.

Rank	Transcription Start	Transcription Stop	Strand	Name	Gene ID	Product	$\log_2$FoldChange	pvalue	padj
1	20817	20971	+	-	predicted RNA	antisense: *pilS3*	-4.1527	4.14E-87	8.50E-84
2	17001	17412	+	-	predicted RNA	antisense: *pilE*	-3.6343	6.00E-62	6.16E-59
3	17571	17709	+	-	predicted RNA	antisense: *pilE*	-2.1454	3.05E-15	2.09E-12
4	1150173	1153112	+	-	NMV_1138	putative type III restriction-modification system enzyme Res	-1.4450	4.78E-12	1.64E-09
5			+	*lnt*	NMV_1687	apolipoprotein N-acyltransferase (ALP N-acyltransferase)	-1.3454	1.82E-13	9.36E-11
6	797557	798120	+	-	NMV_0819	hypothetical protein	-1.2431	1.13E-09	1.54E-07
7	1591565	1591813	+	-	predicted RNA	antisense: NMV_1640	-1.2302	1.82E-11	5.33E-09
8			+	-	NMV_0821	putative prophage DNA primase	-1.1882	6.09E-08	5.43E-06
9	450535	451905	+	*prpC*	NMV_0473	2-methylcitrate synthase (methylcitrate synthase; citrate synthase 2)	-1.1721	2.11E-10	4.33E-08
10	449318	450450	+	-	NMV_0472	putative methylisocitrate lyase (2-methylisocitrate lyase)	-1.1104	9.01E-11	2.05E-08
11			+	-	NMV_2114	hypothetical protein	-1.1094	1.11E-07	9.08E-06
12			-	-	NMV_1309	putative DNA-binding phage protein	-1.1039	1.54E-06	1.05E-04
13	960629	961982	+	*porA*	NMV_0958	major outer-membrane protein P.IA (protein IA; PIA; porin)	1.0947	3.55E-12	1.46E-09
14	1922252	1921738	-	-	predicted RNA	-	-1.0703	3.41E-10	5.83E-08
15	972325	971188	-	-	NMV_0967	IS1655 transposase	-1.0410	1.47E-09	1.77E-07
16	1305525	1303713	-	-	NMV_1313	IS1655 transposase	-1.0309	1.70E-09	1.87E-07
17	237829	236340	-	-	NMV_0252	IS1655 transposase	-1.0259	9.40E-10	1.38E-07
18	603385	601664	-	-	NMV_0614	IS1655 transposase	-1.0239	8.49E-11	2.05E-08
19	522278	523269	+	-	NMV_0536	IS1655 transposase	-1.0202	1.37E-09	1.75E-07
20	2139091	2138975	-	-	NMV_5S_1	ribosomal RNA 5S	-1.0092	3.24E-10	5.83E-08
21	468161	466837	-	-	NMV_0486	IS1655 transposase	-1.0074	1.82E-09	1.87E-07
22	1259215	1258224	-	-	NMV_1241	IS1655 transposase	-1.0056	1.81E-09	1.87E-07

The three most differentially expressed transcripts are located in the *pilE* locus on the antisense strand of *pilE* (Figure 4.9A). Transcripts 2 and 3 correspond to the *pilE* antisense RNA as expected, due to mutation of the AS promoter in strain Mut_ery, which confirms the absence of the AS RNA in Mut_ery (Figure 4.9A). The most differentially expressed transcript (transcript 1) corresponds to a transcript antisense to *pilS3* (Figure 4.9A). *pilS3* is one of five *pilS* cassettes containing sequence homology with *pilE* located upstream of the *pilE* coding region. However, due to the high sequence identity between *pilS3* and *pilE* (96.81% identity with *pilE,* compared to 87.92%, 90.42%, 89.14% and 86.96% identity with *pilE* for *pilS1, 2, 4* and *5,* respectively), and the disappearance of this transcript in Mut_ery, it is likely that this could be due to sequence reads from the AS RNA mapping to *pilS3* (Figure 4.9B). Moreover, the shapes of the cDNA coverage plots of transcripts 1 and 3 appear highly similar (Figure 4.9A), further suggesting that they correspond to sequence reads mapping to both regions. Visual inspection of the region downstream of *pilS3* did not reveal the presence of any obvious putative promoter sequences. Nevertheless, the possibility of antisense transcription of *pilS3* cannot be excluded. Although the *pilS* cassettes have previously been assumed to be untranscribed, a recent study showed that both sense and antisense transcription occurs at many *pilS* cassettes in *N. gonorrhoeae* (Wachter *et al*., 2015).

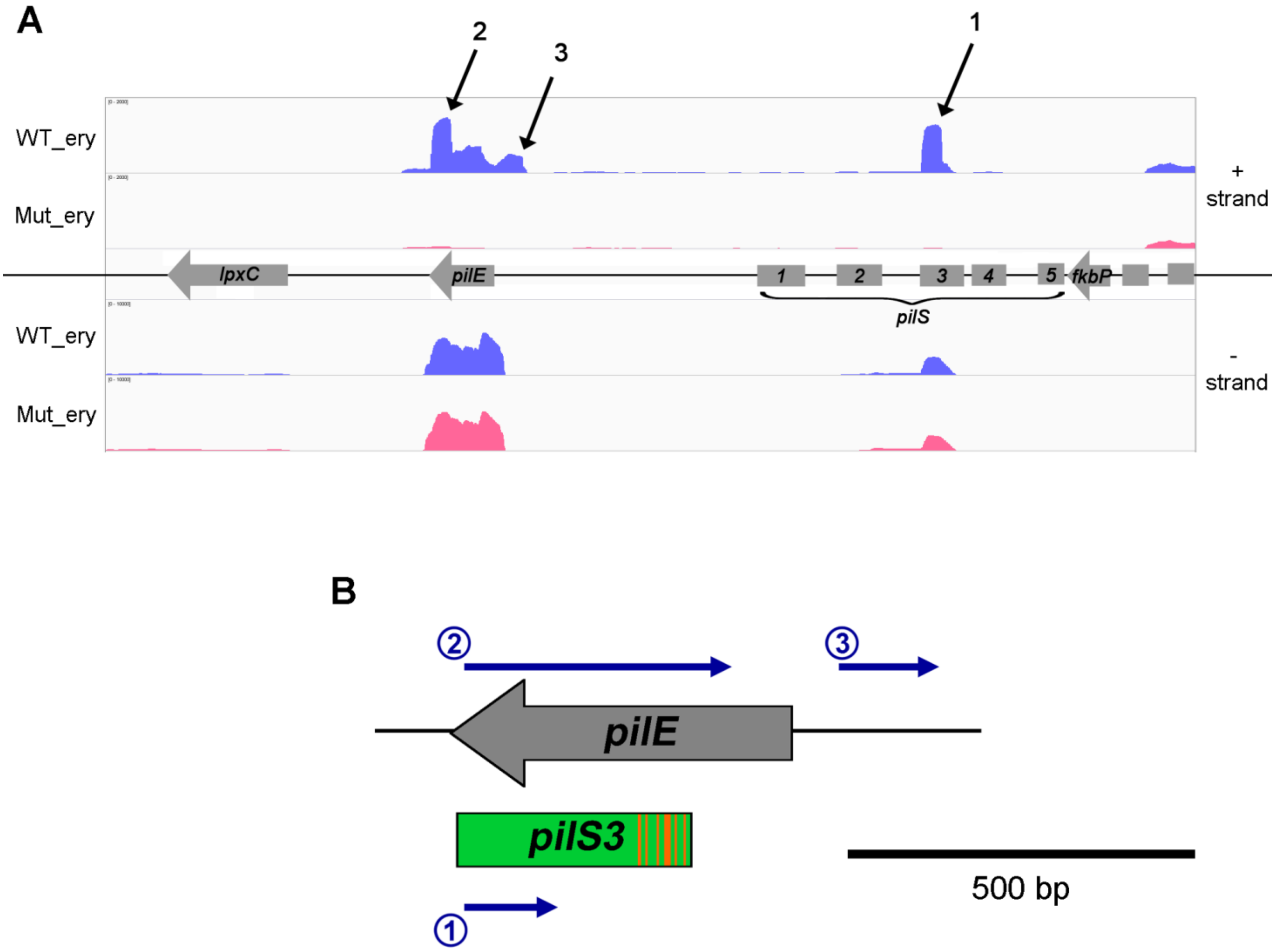
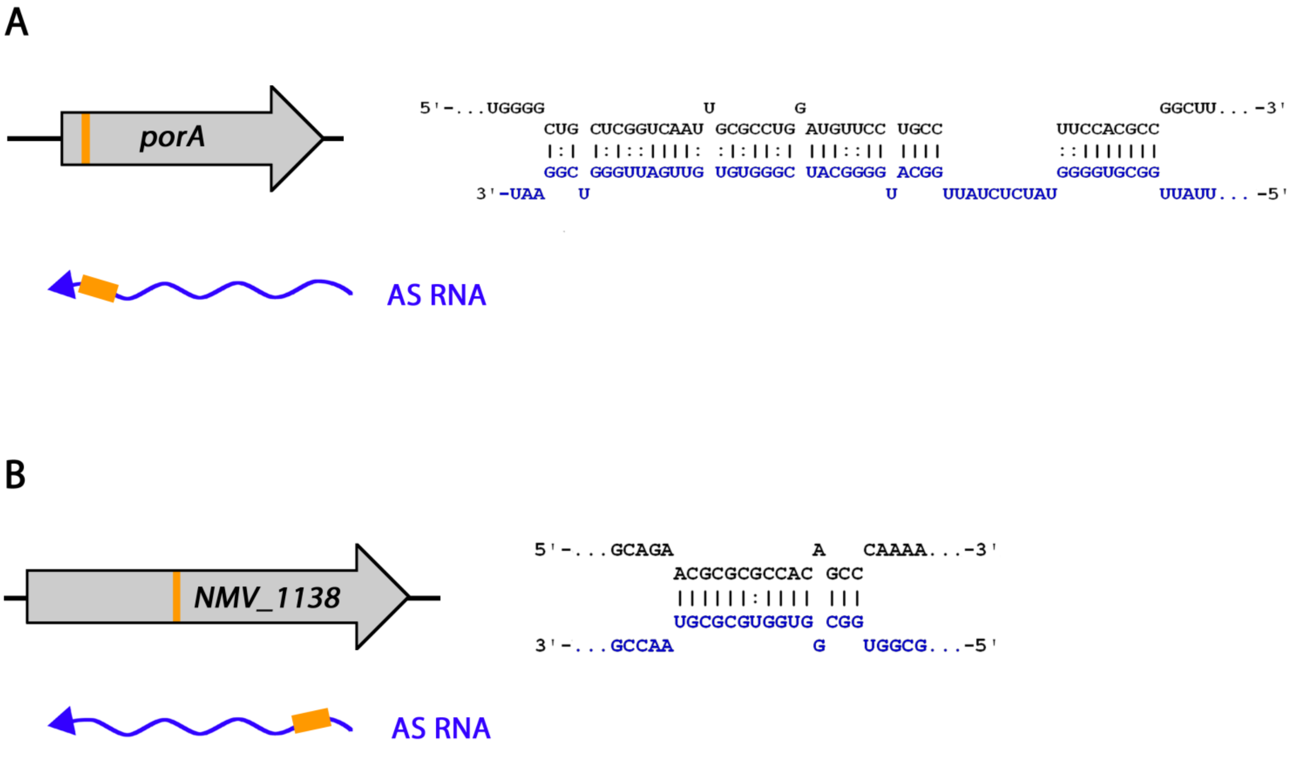


Figure 4.9 Visualization of differentially expressed genes in the *pilE* locus.

(A) cDNA reads from WT_ery and Mut_ery were mapped to the *N. meningitidis* 8013 genome using Rockhopper software and the mapped reads were visualized using Integrative Genomics Viewer. Labelled arrows point to the top three differentially expressed transcripts in Mut_ery compared to WT_ery. The two strands of DNA are shown. (B) Schematic diagram illustrating the sequence homology between *pilS3* and *pilE*. Regions of sequence identity with *pilE* are shown in green while mismatched regions are colored in orange. Labelled blue arrows indicate the top three differentially expressed transcripts in Mut_ery compared to WT_ery, with (1) corresponding to a transcript antisense to *pilS3*, and (2) and (3) corresponding to *pilE* antisense transcripts. Lengths of transcripts (*i.e.* transcriptional start and stop sites) were estimated by the Rockhopper program. Diagram is drawn to scale.

Notably, the *pilE* transcript was not found to be differentially expressed, consistent with Northern blot and strand specific RTPCR data above (Figure 4.4). Of the remaining 22 most differentially expressed transcripts (Table 4.2), their corresponding genes do not appear to be functionally related with each other, nor do they have obvious association with pilin or Tfp biogenesis. *Trans*-acting sRNAs frequently regulate their target RNAs through sRNA-mRNA binding followed by nuclease mediated degradation of the mRNA (Gottesman and Storz, 2011). However sRNAs can also positively regulate their targets through mRNA stabilization (Papenfort and Vanderpool, 2015). In the list of the top 22 most differentially expressed transcripts, only one, *porA*, is upregulated in Mut_ery compared to WT_ery, suggesting that the AS RNA may act in *trans* to destabilize the *porA* transcript. PorA is a phase variable outer membrane protein that functions as a pore through which small hydrophilic nutrients can diffuse into the cell (Massari *et al*., 2003). The putative RNA:RNA interaction site was predicted using the IntaRNA program, which is a method that takes into account the accessibility of target sites in addition to the hybridization energy to predict the location of interactions between sRNAs and target mRNAs (Busch *et al*., 2008). The predicted regions of interaction between the AS RNA and *porA* mRNA is shown in Figure 4.10A and the combined energy score of the interaction (calculated as the sum of the free energy of hybridization and the free energy required for accessibility of the interaction sites) is -20.16 kcal/mol. The predicted site of interaction is located at the 5' end of the *porA* mRNA, which is common for bacterial sRNA-mediated regulation (Gottesman and Storz, 2011). However, further experiments are necessary to validate these predicted interactions and to determine whether *porA* is indeed a target.

The remaining putative transcripts were downregulated in Mut_ery, suggesting a role for the AS RNA in positive regulation of *trans* targets. There is at least a 12-fold difference in AS RNA levels in WT_ery compared to Mut_ery, while the highest ranked (non-AS RNA) differentially expressed gene, NMV_1138, shows a 2.7 fold downregulation in Mut_ery. Predicted sites for the putative interaction between the AS RNA and NMV_1138 transcript are shown in Figure 4.10B with a combined energy score of -15.10 kcal/mol. However, as with *porA*, this result requires validation. Previous studies involving whole-genome microarrays to detect global changes in transcript levels as a result of sRNA overexpression found that strongly regulated targets can exhibit fold-changes of around fivefold, while putative targets showing at least twofold change were generally considered for further investigation (Corcoran *et al.*, 2012). A number of IS1655 transposases were identified to be differentially expressed, however, inspection of the nucleotide sequences of these genes found them to be identical and it is therefore not possible to conclude whether the sequencing reads for this gene reflect transcripts from different loci.



```
5'-...UGGGG              U       G                               GGCUU...-3'
           CUG CUCGGUCAAU GCGCCUG AUGUUCC UGCC          UUCCACGCC
           |:| |:|::||||: :|:||:| |||::|| ||||          ::|||||||
           GGC GGGUUAGUUG UGUGGGC UACGGGG ACGG          GGGGUGCGG
     3'-UAA   U                          U    UUAUCUCUAU         UUAUU...-5'
```

```
5'-...GCAGA           A   CAAAA...-3'
           ACGCGCGCCAC GCC
           ||||||:|||| |||
           UGCGCGUGGUG CGG
3'-...GCCAA           G   UGGCG...-5'
```

Figure 4.10 Predicted interaction sites between AS RNA and putative target mRNAs. Interaction sites (orange) between the AS RNA (blue) and the putative targets (A) *porA* and (B) NMV_1138 were predicted using the program IntaRNA (Busch *et al.*, 2008). Diagrams are not to scale.

4.7 Summary

This chapter describes the attempts made to identify the possible functions of the AS RNA by comparing phenotypes of strains with the WT AS promoter, and an isogenic strain in which AS transcript is abolished by mutation of the promoter. Since the AS RNA is located in the *pilE* locus, phenotypic assays for Tfp mediated functions were first performed to investigate the role of the AS RNA. 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 Streptococcus by targeting and destabilizing the polycistronic mRNA from the pilus biogenesis operon encoding the pilus genes *cpa*, *sipA1*, *tee1*, *srtC1* and *111* (Liu *et al*., 2012). Here, it was found that the AS RNA is not involved in bacterial adhesion to epithelial cells or in transformation efficiency. Fully assembled Tfp are required for adhesion and transformation in *N. meningitidis* (Aas *et al*., 2002; Stephens and McGee, 1981). As Tfp are complex multiprotein organelles involving multiple genes for pilus biogenesis (Georgiadou *et al*., 2012), it is probable that these phenotypic assays may not be sensitive enough to detect any functional consequences of subtle changes in levels of Tfp proteins. Slight changes in pilin levels may not necessarily lead to observable differences in bacterial adhesion. Moreover, our assays did not utilize antigenic variation-deficient strains to account for the possibility that pilin Av can contribute to different adherence properties. Av can result in the expression of variant PilE proteins with altered adhesion efficiencies either due to different primary amino acid sequences affecting the surface charge of the Tfp (Craig *et al*., 2004; Nassif *et al*., 1993) or may also be due to the creation or loss of glycosylation sites on the pilin subunit (Virji *et al*., 1993). Therefore, although the AS RNA did not appear to impact Tfp function, we further investigated this by examining whether the AS RNA influenced pilE transcript or PilE protein levels.

Hence, the possibility that the AS RNA acts in *cis* to regulate *pilE* expression was investigated. AS RNAs have perfect complementarity to the sense transcript and therefore can regulate the expression of genes encoded on the opposite DNA strand (Georg and Hess, 2011). In most cases this requires RNA:RNA interaction and can result in transcription attenuation, sense transcript degradation or stabilization, or inhibition of translation. In other cases, antisense transcription can physically interfere with and suppress sense transcription (Georg and Hess, 2011; Sesto *et al*., 2013).

Northern blot and strand specific qRTPCR results demonstrate that *pilE* mRNA is detected throughout growth *in vitro* in both the wild-type strain and the strain lacking the AS RNA, suggesting that the AS RNA is not required for *pilE* mRNA stabilization. Furthermore, no significant AS RNA-dependent change was detected in *pilE* transcript levels, either in the mutant or when the AS was induced, suggesting that the AS RNA does not destabilize the *pilE* transcript or inhibit *pilE* transcription. The finding that AS RNA induction does not appear to affect *pilE* transcript levels is surprising, given its extensive complementarity with the *pilE* transcript. However in this project we did not investigate at a molecular level whether such RNA:RNA hybrids are indeed formed during upregulation of AS RNA expression.

Next, we considered that the AS RNA might alter pilin at the translational level. Mapping of the 3' end of the AS RNA showed that the transcript includes the 5′ UTR of the *pilE* transcript, enabling pairing interactions with this region. Noncoding regulatory RNAs can pair with the 5' UTR of mRNAs to affect ribosome binding and consequently repress translation (Lalaouna *et al*., 2013). Western blot analysis showed no difference in the amount of pilin in whole cell lysates in the presence or absence of the AS RNA. Notably, it was observed that there was no decrease in pilin protein levels detected in cell lysates even when a decrease in the amount of *pilE* transcript was detected by Northern blot following salt stress. Upon expression, pilins are inserted into the inner membrane before cleavage by a pre-pilin peptidase and assembly into Tfp (Craig *et al*., 2006). In this way, the levels of pilin present in the Tfp may not change significantly even if translation of PilE is reduced. Moreover, as PilE is a relatively stable protein (data not shown), any changes in protein expression as a result of *pilE* transcript downregulation after 10 minutes of NaCl stress may not be detectable by Western blotting. As such, further work is required to rule out any influence of AS RNA on pilin expression.

The expression levels of genes encoding minor pilins in the presence and absence of the AS RNA were also investigated. Although no effect of the AS RNA was found, an interesting observation was made with regards to regulation of the minor pilins. Like the *pilE* transcript, the *pilX*, *pilV* and *comP* transcripts were also observed to be downregulated in response to NaCl stress. Previous studies have reported that consistent with this finding, piliation and pilin levels in *N. gonorrhoeae* are also reduced during growth in 300 mM KCl or NaCl (Larribe *et al*., 1997). However, the mechanism of this response and possible biological function of salt regulation of Tfp components remain unknown. Respiratory droplets are a potential biologically significant high-salt environment encountered by the meningococcus; concentrations of solutes in these droplets can increase by up to 15 times due to evaporation (Parienta *et al*., 2011; Yang *et al*., 2012).

RNAseq was carried out as a means to identify potential *trans* regulated targets of the AS RNA by analyzing differences in transcript abundance at a global level by comparing strains with and without a functional AS promoter. Differential expression analysis of RNAseq data has generated a list of potential *trans* regulated targets. Although only the transcripts that show a $\log_2$ fold change of > 1 or < -1 are presented in this chapter, it is possible that *bone fide* mRNA targets regulated by the AS RNA on a more subtle level (*i.e*. a smaller fold change) may have been overlooked. Given the high throughput nature of

RNAseq and the large volume of data generated, it is not feasible to experimentally validate every differentially expressed transcript. Nevertheless, this method has validated that the AS RNA is expressed in WT_ery but not in Mut_ery, and corroborated our previous results demonstrating that the AS RNA does not affect transcript levels of *pilE*, or that of the minor pilins. Future work could attempt to investigate and confirm promising potential targets by qRTPCR analysis and to explore the mechanism of these RNA:RNA interactions using heterologous GFP reporter systems (Corcoran *et al*., 2012; Urban and Vogel, 2007).

In this chapter, it was found that the AS RNA does not play a significant role in Tfp function, or in *pilE* or pilin expression despite being perfectly complementary to the *pilE* mRNA. Furthermore, the AS RNA does not appear to regulate the minor pilins in *trans*, and further investigation is needed to validate putative *trans* targets identified by RNAseq. The next chapter will focus on another important property of Tfp, antigenic variation; in particular, the potential involvement of the AS RNA in influencing the molecular processes taking place in the *pilE* region that are important for antigenic variation to occur.

5 Results - Role of the AS transcript in antigenic variation

The preceding chapter demonstrated that the *pilE* AS RNA does not appear to influence Tfp functions such as adhesion and transformation, or to modulate levels of *pilE* transcript or PilE protein in *N. meningitidis*. We next considered whether the AS transcript could impact class I PilE antigenic variation (Av). Av is an interesting property of PilE and is thought to be involved in immune evasion and influence Tfp adherence and induction of host cell responses (see Section 1.5) (Heckels, 1989; Miller *et al*., 2014).

Class I pili of *N. meningitidis* and *N. gonorrhoeae* are a paradigm of Av. Av involves the non-reciprocal recombination between a silent *pilS* cassette and the expressed *pilE* gene to generate a *pilE* gene of a novel sequence (Haas and Meyer, 1986). A transposon-based genetic screen and subsequent targeted screen reported that a G-rich sequence located upstream of *pilE* was necessary for Av to occur (Kline *et al*., 2007; Sechman *et al*., 2005). Following this, it was found that the activity of a promoter adjacent to the G-rich sequence was required for the transcription of a G4-associated small RNA that is essential for pilin antigenic variation in *N. gonorrhoeae* (Cahoon and Seifert, 2013). As described in Section 1.5.1, this small RNA is proposed to act in *cis*, by forming an RNA:DNA hybrid with the C-rich DNA strand and thus promoting the formation of the G-quadruplex structure on the G-rich strand of DNA to initiate gene conversion. It was suggested that RecA, a DNA recombination and repair protein which is essential for Av, is recruited to the *pilE* locus by interacting with the G-quadruplex to facilitate *pilE/pilS* recombination (Kuryavyi *et al*., 2012)

Mapping of the *pilE* AS RNA (Section 3.3.3) demonstrated that the 3' end of the transcript extends to the promoter region of the G4-associated sRNA. As overlapping transcription units can affect the downstream transcription through promoter occlusion (Palmer *et al*., 2009), we therefore investigated whether the AS RNA had any impact on *pilE* Av.

5.1 Antigenic variation assays on solid media

Antigenic variation arises *via* recombination between one or more silent *pilS* cassettes and the expressed gene *pilE* giving rise to an altered *pilE* sequence, resulting in either the expression of a PilE protein with a different amino acid sequence or a truncated or extended but non-functional PilE protein due to a frameshift, an occurrence known as phase variation. In *N. gonorrhoeae*, phase variation has been used as a surrogate measure for Av; the absence of assembled Tfp gives rise to a “nonpiliated colony phenotype” with distinct characteristics (*i.e.* size, shape, and color) compared to piliated colonies (Sechman *et al*., 2005; Swanson *et al*., 1971). However, piliated and nonpiliated colonies of *N. meningitidis* cannot be easily discerned by colony morphology, probably due to the presence of the capsule. Moreover, such pilus-dependent colony morphology based assays only detect a subset of Av events and ignore events resulting in expression of full length PilE protein with altered sequence, and does not account for other non-Av processes (such as *pilE* gene deletion) which give rise to nonpiliated colonies (Criss *et al*., 2005). To address these issues, RT-PCR-based Av assays have been designed, using *pilS* and *pilE* specific primers to detect and quantify recombination events (Rohrer *et al*., 2005; Serkin and Seifert, 1998). However these assays also underestimate Av frequency as they are do not detect Av events that result in short sequence changes which can be as subtle as a single nucleotide change (Criss *et al*., 2005).

Therefore, to ascertain whether the AS RNA impacts PilE Av, we used an unbiased sequence-based Av assay to compare the frequency of PilE Av in WT_ery and Mut_ery. Assays were initially performed only on solid media using a method adapted from Criss *et al*. (2005) (Figure 5.1A). Specifically, bacteria were plated to BHI agar from frozen stocks. After overnight incubation at 37°C, four single colonies (progenitors) were restreaked onto BHI agar and incubated overnight, and lysates of the progenitors were prepared by resuspending in PBS followed by boiling. Fifteen single colonies from each progenitor were restreaked and incubated overnight (passage 1). The 15 colonies were passaged overnight once more (passage 2), after which, single colonies were picked from each, and single colony lysates prepared as above. The *pilE* gene was amplified from the single colony lysates by PCR using primers AS_F and *pilE*_F (Table 2.4), sequenced, and the sequence compared with the sequence of its progenitor. "Variant *pilE* sequences" were defined as *pilE* sequences containing regions which could be mapped to a donor *pilS* sequence, regardless of the length of the *pilS* sequence incorporated. With this approach, single nucleotide changes could be detected and were considered as Av events (not point mutants) as all single nucleotide changes could be attributed to a donor *pilS*; no other point mutations were detected in *pilE* across all the colonies sequenced.

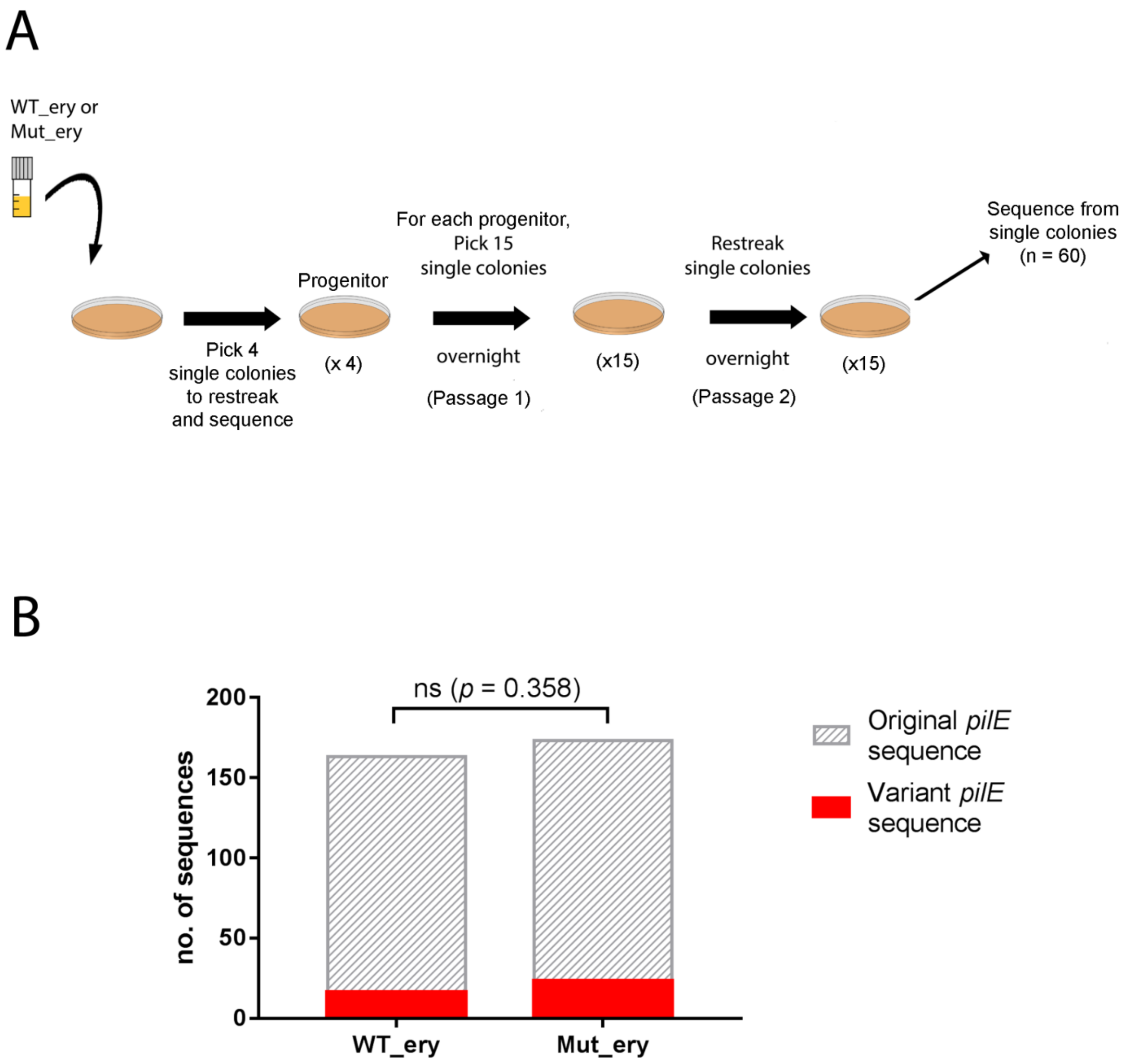


Figure 5.1 PilE Av in WT_ery and Mut_ery on solid media.
(A) Assays involved the passage of strains several times on solid BHI media to allow Av to occur. The *pilE* gene was sequenced to detect any change in *pilE* sequence. (B) Av assay results from three biological replicates indicate that there is no significant difference between the frequencies of variant *pilE* sequence detected in WT_ery and Mut_ery (*p* = 0.358). Results were analyzed by fitting generalized linear models.

Results of the Av assay indicate that Av frequency of WT_ery was 9.82% and that of Mut_ery was 13.29% out of a total of 165 and 174 progeny analyzed respectively, with no statistically significant difference in frequencies between the two strains (*p* = 0.358, Figure 5.1B, Table 5.1). Analysis of the *pilE* sequences obtained from the assay revealed that four *pilE* sequences could not be determined due to mixed reads, suggesting that more than one *pilE* sequence may be present within a single colony. Such events were excluded from the analysis.

Table 5.1 Av assay with WT_ery and Mut_ery on solid media

Strain	Experiment Number	Number of Progeny Analyzed	Number of Variant Sequences Detected
WT_ery	1	15	2
	2	13	1
	3	15	1
	4	15	1
	5	15	2
	6	15	1
	7	15	0
	8	15	3
	9	15	2
	10	15	3
	11	15	0
	Total (% colonies analyzed)		**16 (9.82)**
Mut_ery	1	15	4
	2	15	2
	3	10	1
	4	15	0
	5	15	4
	6	15	2
	7	15	3
	8	15	3
	9	15	1
	10	14	1
	11	15	1
	12	14	1
	Total (% colonies analyzed)		**23 (13.29)**

5.2 Antigenic variation assays on solid media with inducible RecA strains

To address the issue of unreadable *pilE* sequences, the Av assay was modified with the aim of i) controlling when Av occurred, using strains containing an inducible RecA, and ii) obtaining single (non-mixed) colonies for sequencing. RecA is a DNA recombination and repair protein which is necessary for pilin Av (Koomey *et al.*, 1987). Strains WT_ery(RecA6) and Mut_ery(RecA6) were constructed by homologous recombination using genomic DNA from 8013(RecA6) in which the native *recA* gene has been replaced with a *N. gonorrhoeae recA6* allele under the control of a lac promoter (Seifert, 1997). Therefore, these strains only express RecA in the presence of IPTG (Seifert, 1997). In the revised assay, bacteria were initially grown in the presence of IPTG to allow Av to occur, followed by two passages on BHI media without IPTG to ensure that no further recombination occurred, followed by sequencing of *pilE* (Figure 5.2A).

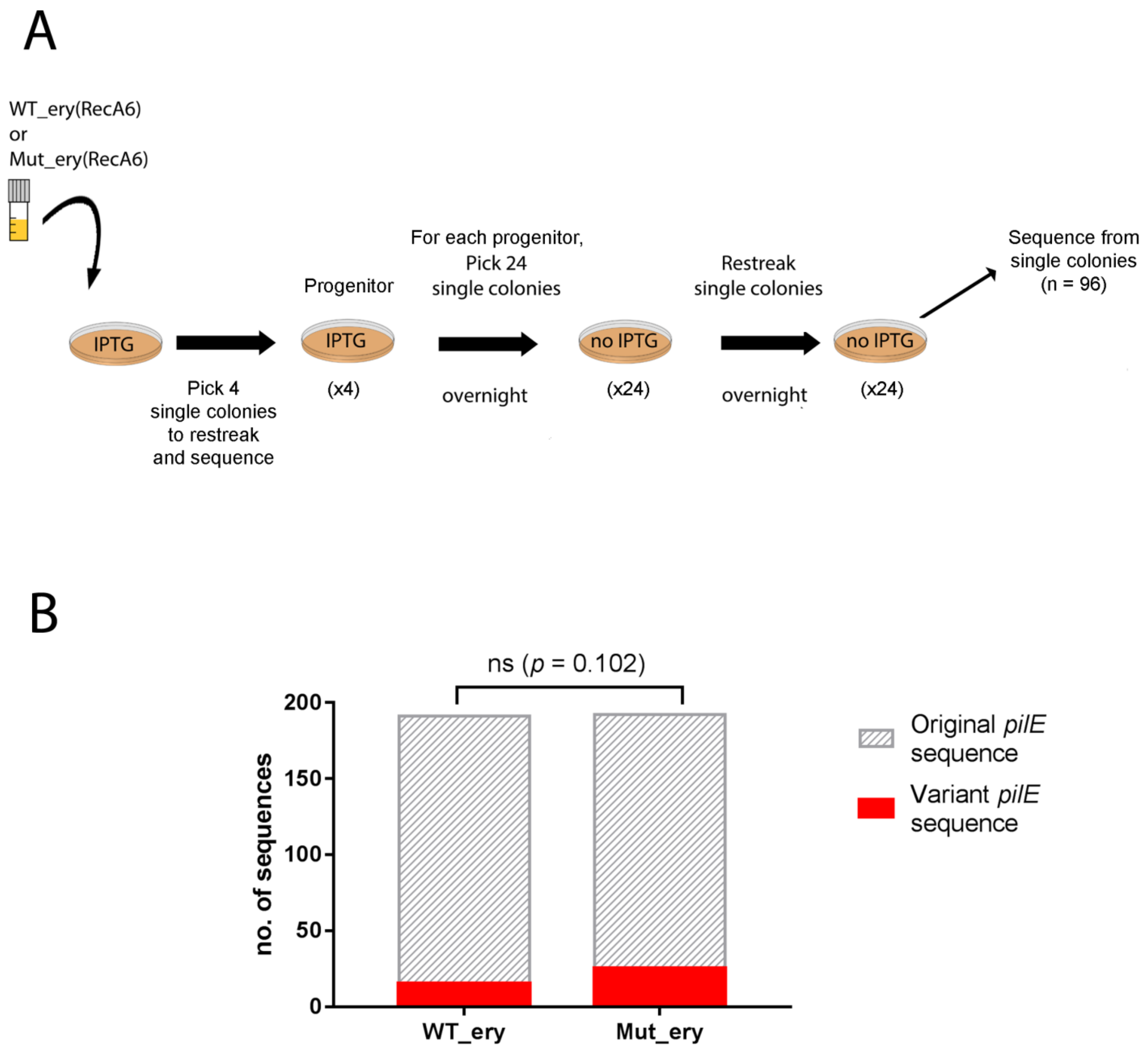


Figure 5.2 Av assay with WT_ery(RecA6) and Mut_ery(RecA6) on solid media.

(A) Workflow of Av assay with WT_ery(RecA6) and Mut_ery(RecA6) on solid media. Bacteria were grown from frozen stocks on BHI agar containing 1 mM IPTG to induce expression of RecA to allow for Av. After overnight incubation at 37°C, four single colonies (progenitors) were restreaked onto BHI agar containing IPTG and incubated overnight. Single colony lysates of progenitors were prepared by resuspending in PBS followed by boiling. Twenty-four single colonies were restreaked from each progenitor onto BHI agar without IPTG and incubated overnight. Colonies were passaged overnight once more, then lysates were prepared. The *pilE* gene was amplified and sequenced. (B) Av assay results from two biological replicates indicate that there is no significant difference between the frequencies of variant *pilE* sequence detected in WT_ery(RecA6) and Mut_ery(RecA6) (p = 0.102). Results were analyzed by fitting generalized linear models.

In this assay, there was no significant difference (*p* = 0.102) in the frequency of Av in WT_ery(RecA6) and Mut_ery(RecA6) after two biological replicates and sequencing of a total of 191 and 192 progeny, respectively. Although not statistically significant, the trend of the results is consistent with the previous Av assay; *i.e*. Mut_ery(RecA6) demonstrated slightly higher Av frequency compared to WT_ery(RecA6), suggesting that the presence of the AS RNA may reduce Av frequency.

Table 5.2 Av assay with WT_ery(RecA6) and Mut_ery(RecA6) on solid media.

Strain	Experiment Number	Number of Progeny Analyzed	Number of Variant Sequences Detected
WT_ery (RecA6)	1	24	2
	2	24	0
	3	24	3
	4	24	2
	5	24	0
	6	24	0
	7	23	4
	8	24	4
	Total (% colonies analyzed)		**15 (7.85%)**
Mut_ery (RecA6)	1	24	2
	2	24	3
	3	24	1
	4	24	5
	5	24	1
	6	24	4
	7	24	5
	8	24	4
	Total (% colonies analyzed)		**25 (13.02%)**

From this and the previous Av assays, it was noticed that some progenitors possessed a *pilE* sequence different to that of the parental strain, *N. meningitidis* 8013 (NC_017501.1), indicating that either the frozen stocks of the strains were already mixed, or that Av had occurred during the overnight growth on solid media from frozen stocks. The *pilE* sequence of the progenitor colony can influence the rate of Av; it has previously been reported that the starting *pilE* sequence of the parental strain influences the frequency at which *pilS* cassettes recombine into *pilE* in *N. gonorrhoeae* (Rohrer *et al*., 2005). Although both assays did not demonstrate a significant difference in Av frequency between WT_ery and Mut_ery, we continued to investigate this phenotype by modifying the Av assay to control for the initial *pilE* sequence at the start of the assay, and also to include induction of AS RNA expression.

5.3 Analysis of *pilE* variation during growth in liquid media

To address the aforementioned issues, the Av assay was modified as follows (Figure 5.3A):

1. Assays were carried out using frozen stocks made from single colonies of WT_ery(RecA6) and Mut_ery(RecA6) expressing the same *pilE* sequence as that of *N. meningitidis* 8013 (NC_017501.1). This was to ensure that the initial *pilE* sequence was identical in WT_ery and Mut_ery.
2. Strains were grown in liquid media to mid-log phase (OD_{600} ~0.5 – 0.6) before the addition of IPTG to induce RecA expression an attempt to standardize the period of time during which *pilE/pilS* recombination could occur.
3. Two independent cultures were set up for each strain: one culture was subjected to NaCl stress (0.5 M, 10 minutes) at mid-log phase while the other was untreated. NaCl stress induces the expression of the AS RNA (Section 3.3.1) so this might allow detection of effects of AS overexpression on Av.

4. After 10 minutes of incubation with IPTG and NaCl, liquid cultures were spread onto BHI agar containing IPTG to allow Av to occur during overnight growth.
5. For each strain/condition, ninety-six single colonies were then restreaked into BHI plates without IPTG and incubated overnight to prevent further *pilE*/*pilS* recombination to obtain single colonies for sequencing.
6. 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 calculated, as described in the previous Av assays.

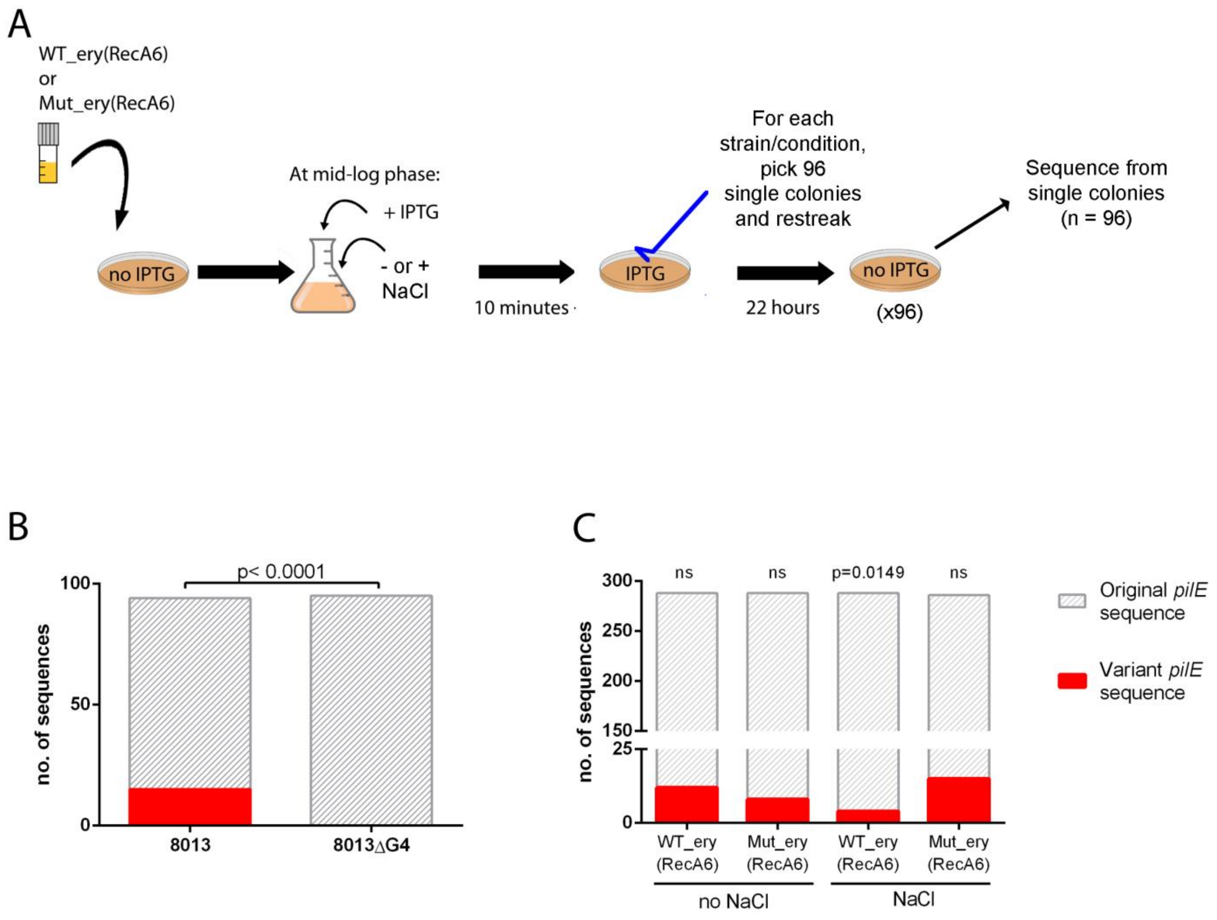


Figure 5.3 Effect of AS induction by salt stress on Av.

(A) Workflow of Av assay with WT_ery(RecA6) and Mut_ery(RecA6) involving liquid media and NaCl stress. Refer to main text for detailed description of protocol. (B) Av assays were performed with wild-type *N. meningitidis* strain 8013 and isogenic mutant with a kanamycin resistance cassette inserted into the guanine quartet (ΔG4) sequence. *pilE* sequences obtained were compared to the potential *pilS* donor sequences and the number of variation events was calculated. No Av was detected in the strain with the G4 mutation. (C) Av assays were performed in triplicate 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).

To validate that *pilE* sequence variation resulted from *pilE*/*pilS* recombination in the liquid culture assay, the assay was first performed using wild-type 8013 and 8013ΔG4, which has a kanamycin resistance marker with transcriptional terminators inserted into the G4 sequence. The G4 sequence is essential for PilE Av in *N. gonorrhoeae* (Cahoon and

Seifert, 2013). The assay was performed in the absence of NaCl stress or IPTG. *pilE* variation was detected in 8013 while no Av events occurred in 8013ΔG4 ($p < 0.0001$, Figure 5.3B), indicating that the G4 sequence is required for Av in the *N. meningitidis*, and that Av can be detected and quantified using this method.

Table 5.3 Av assay with liquid media.

Strain	NaCl	Replicate	Number of Progeny Analyzed	Number of Variant Sequences Detected
WT_ery (RecA6)	no NaCl	1	96	5
		2	96	3
		3	96	4
		Total (% colonies analyzed)		**12 (4.17 %)**
Mut_ery (RecA6)	no NaCl	1	96	2
		2	96	3
		3	96	3
		Total (% colonies analyzed)		**8 (2.78%)**
WT_ery (RecA6)	+ NaCl	1	96	0
		2	96	1
		3	96	3
		Total (% colonies analyzed)		**4 (1.39%)**
Mut_ery (RecA6)	+ NaCl	1	96	7
		2	96	4
		3	94	4
		Total (% colonies analyzed)		**15 (5.24%)**

Table 5.3 and Figure 5.3B show results from three independent assays (involving a total of 1150 *pilE* sequences). Statistical analysis was performed with advice from Dr Daniel Lunn (University of Oxford Statistics Department). The results were analyzed by fitting binomial generalized linear models (GLMs) (McCullagh and Nelder, 1989) using R statistical software, specifically the function GLM (Venables and Ripley, 2002). By considering assay number (*i.e*. replicate 1, 2, and 3) as a third predictor variable - the first two being the strain (WT_ery or Mut_ery) and NaCl induction (yes or no) - we avoided

pooling data from different replicates which could lead to misleading conclusions, and were able to rule out any effects arising due to replicate-to-replicate differences. Our chosen model also introduced an interaction term to consider the interaction between strain and NaCl induction (*i.e.* the combined effect of strain and NaCl induction). The model demonstrated a goodness-of-fit statistic of 5.8392 on six degrees of freedom, giving a *p*-value of 0.4414, which is not significant at the five percent level; therefore there is no evidence against this model.

The results indicate that on their own, neither the presence of NaCl nor the mutation resulted in a statistically significant change in the levels of Av. However, there was a significantly reduced level of antigenic variation when WT_ery was induced with NaCl ($p = 0.0149$) but this was not observed for Mut_ery, indicating that the AS RNA transcript or transcription influences *pilE* Av. The direction of these results are consistent with the trend observed in the previous two Av assays (Figure 5.1 and Figure 5.2), in which the presence of the AS RNA or the induction of AS RNA expression results in a reduced rate of Av compared to when the AS RNA is absent.

5.4 Mechanism of action of the AS RNA on Av

As induction of AS expression reduced Av frequency, the mechanism by which this occurs was examined. Work in Section 3.3.3 (Figure 3.6) demonstrated that the 3' end of the AS RNA extends to the promoter of the G4 sRNA. Transcription of the G4 sRNA is essential for Av in *N. gonorrhoeae*, in a process leading to the formation of a G-quadruplex structure on the non-template DNA strand that recruits proteins involved in recombination

(Cahoon and Seifert, 2013) (Figure 1.4A); however, this sRNA has not been directly detected by Northern blot (Cahoon and Seifert, 2013).

The G4 sRNA has not previously been studied in *N. meningitidis*. The -10 sequence of the putative G4 promoter differs from that of *N. meningitidis* by a single nucleotide (TAGCAT in *N. meningitidis* instead of TAGAAT in *N. gonorrhoeae*). However, as the G4 sequence is also present in the same locus upstream of *pilE* in *N. meningitidis* 8013 and our results showed abolition of Av in a strain with an interrupted G4 sequence, Av in *N. meningitidis* also occurs in a G4-dependent mechanism as in *N. gonorrhoeae*. One potential mechanism by which the AS RNA could interfere with Av is by affecting transcription of the G4 sRNA which occurs on the same DNA strand (Figure 1.4B).

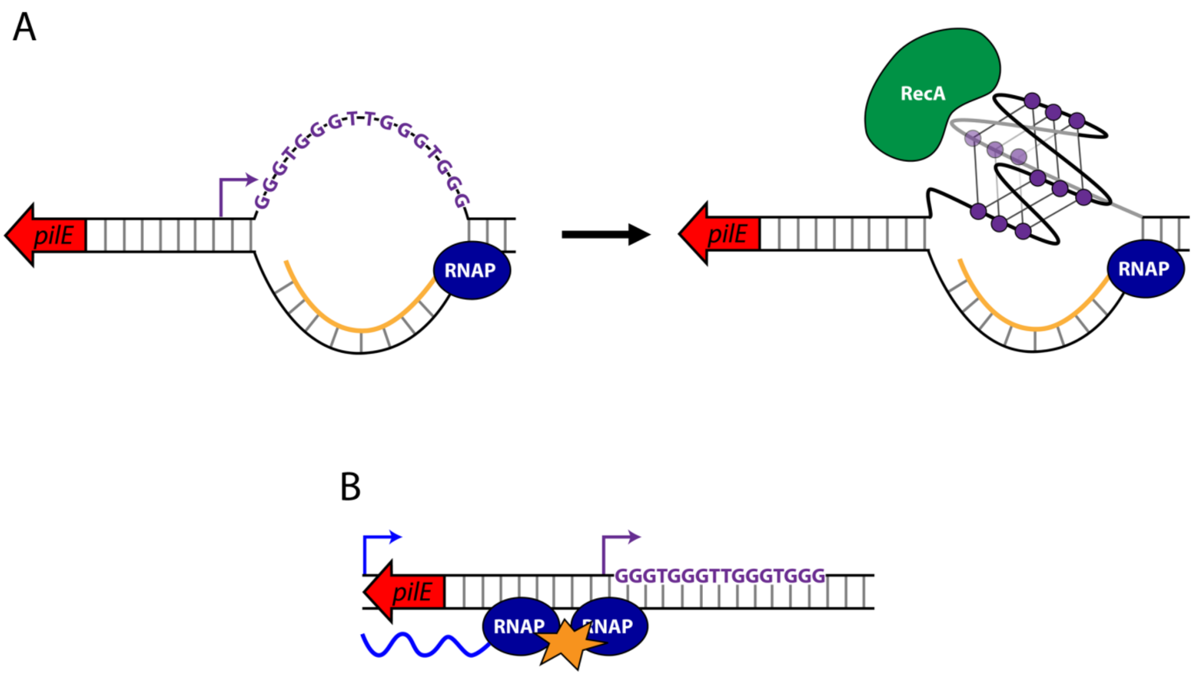


Figure 5.4 Proposed model of the role of G4 transcription in Av and proposed mechanism of the AS RNA.

(A) Schematic diagram of the *pilE* locus of *N. gonorrhoeae* illustrating the proposed model of the role of G4 transcription in Av. Transcription of the G4 sequence by RNA polymerase (RNAP) has been proposed to result in the formation of a G-quadruplex structure by the single stranded G-rich strand of DNA which recruits proteins involved in the recombination process (Cahoon and Seifert, 2013). (B) Schematic diagram of the *pilE* locus of *N. meningitidis* illustrating the possible mechanism by which AS may affect Av. Based on our results showing the decrease in Av as a result of AS overexpression, we speculated that transcription of the AS could interfere with G4 transcription and therefore prevent G-quadruplex formation.

Two approaches were undertaken in parallel to test the hypothesis that the AS RNA modulates Av through interference with G4 transcription (Figure 1.4B). The first approach involved investigating whether G4 RNA transcript levels were altered by the presence or absence of the AS RNA by examining the transcriptional profile of the *pilE* locus. Differential RNAseq and RTPCR were performed to measure transcript levels across the *pilE* locus The second approach involved the detection of any changes in levels of RNA:DNA hybrids in the G4 regions as these structures are speculated to be formed during G4 transcription by the pairing of the newly transcribed G4 sRNA with the template strand of DNA.

5.4.1 Transcriptional profile of the pilE locus

5.4.1.1 Differential RNAseq (dRNAseq) analysis

The transcriptional landscape of *N. meningitidis* strain 8013 in the presence and absence of a functional AS promoter was investigated by performing differential RNAseq (dRNAseq) – an RNAseq approach that is selective for the 5' end of primary transcripts, in order to identify transcriptional start sites (TSS) in the *pilE* locus. Together with downstream analysis of dRNAseq data using the TSSPredator program, this method would enable the detection of TSS in an automated and unbiased manner. Total RNA was first isolated from three biological replicates of WT_ery and Mut_ery grown for 12 h in BHI broth. Two cDNA libraries were prepared from each sample: one from untreated total RNA, and the other from total RNA treated with terminator exonuclease (TEX), which specifically degrades processed RNAs with a 5'-monophosphate but not RNAs with 5' tri-phosphates (Sharma *et al.*, 2010). Figure 5.5A demonstrates successful TEX treatment as indicated by the disappearance of two bands corresponding to the 16S and 23S RNAs in the TEX+ samples due to ribosomal RNAs being 5' processed. Primary transcripts (including most mRNAs and noncoding regulatory RNAs) which contain a 5' tri-phosphate and 5S RNA (which is not 5' processed) are present in both TEX- and TEX+ samples, as seen by the intense bands of higher electrophoretic mobility (Figure 5.5A). In this way, TEX+ cDNA libraries are enriched for primary transcripts compared to TEX- cDNA libraries. Data from TEX- samples were analyzed for differential gene expression and discussed in Section 4.6.

TEX- and TEX+ cDNA libraries were paired-end sequenced on a HiSeq Rapid 2500 by the Wellcome Trust Centre for Human Genetics (University of Oxford) and reads mapped to the 8013 reference genome using the Rockhopper program (McClure *et al*., 2013). Sequencing of TEX– and TEX+ libraries resulted in the characteristic enrichment of cDNA reads at TSS in TEX+ samples (Sharma *et al*., 2010). To identify TSS in an automated and unbiased fashion, mapped reads were analyzed using the TSSpredator program (Dugar *et al*., 2013). Annotation of TSS by TSSpredator is a two-step process whereby potential start sites are first identified as locations where a significant number of reads start, followed by determining whether the enrichment factor of each potential TSS (calculated as the ratio of the expression height at that position of the TEX treated to that of the untreated sample) reaches a value above a given threshold (set at an enrichment factor of 2) (Dugar *et al*., 2013).

Visual comparison of the mapped reads at the *pilE* locus from TEX+ and TEX- samples reveals an obvious pattern of cDNA enrichment at primary 5' ends of the AS RNA and *pilE* mRNA in the TEX+ libraries (Figure 5.5B). This confirms that both the AS RNA and *pilE* mRNA are primary transcripts (as opposed to processed/cleaved transcripts).

TSSpredator analysis of the reads mapping to the *pilE* locus revealed a number of predicted TSS. (Figure 5.5B), including TSS corresponding to the *pilE* transcript and to the AS transcript (Figure 5.5B, orange asterisk); the latter was only detected in WT_ery but not Mut_ery, as expected. There was an additional predicted TSS detected in both WT_ery and Mut_ery located upstream of the AS promoter (Figure 5.5B, green asterisk). It is likely that this TSS, annotated as an “orphan” TSS by the TSSpredator program due to the lack of an annotated gene, is likely a result of a promoter in the erythromycin resistance cassette in that locus in WT_ery and Mut_ery, however it should be noted that no transcription from this promoter has been previously detected by Northern blot or by strand-specific qRTPCR (Figure 3.5). Nonetheless, the possibility that this TSS represents another sRNA in the *pilE* locus cannot be excluded.

TSS were also detected for *pilS* in both the sense and antisense direction. However, it is not possible to distinguish whether these TSS had resulted from reads mapped to *pilE* or the AS transcript as they share significant sequence identity (See Section 4.6.2). Notably, no TSS was detected for the putative G4 RNA, suggesting that the G4 RNA is not expressed at these conditions (*i.e.* after 12 h growth) or is expressed at undetectable levels.

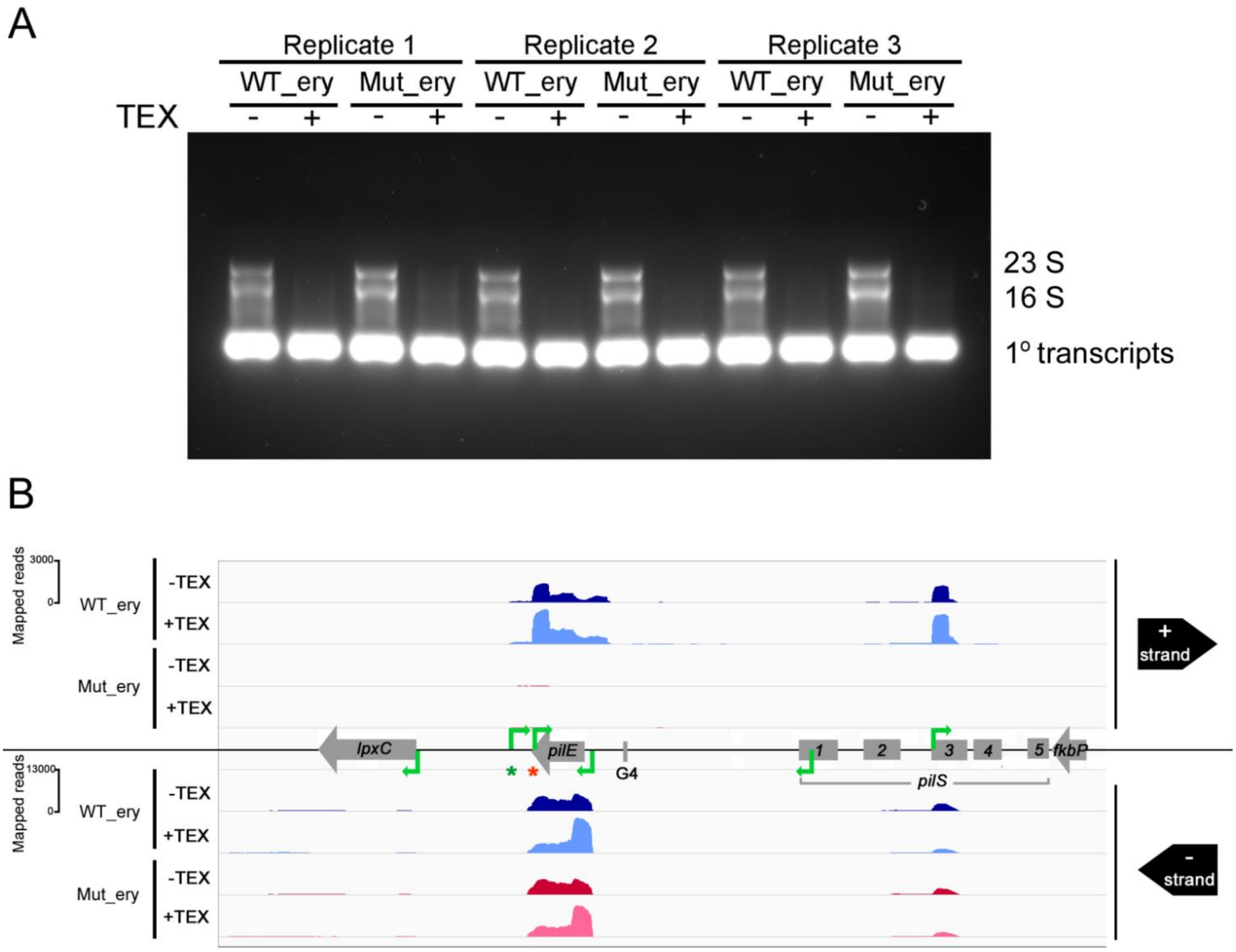


Figure 5.5 Detection of TSS in the *pilE* locus of WT_ery and Mut_ery by differential RNAseq.

(A) Agarose gel analysis of RNA from WT_ery and Mut_ery used for differential RNAseq. Libraries for dRNAseq were prepared from untreated total RNA (-) or total RNA treated (+) with terminator exonuclease (TEX). Successful TEX treatment is demonstrated by the disappearance of the two bands corresponding to 23S and 16S RNA which are 5' processed. (B) *pilE* region of the *N. meningitidis* 8013 genome with mapped dRNAseq reads from -/+ TEX libraries of WT_ery and Mut_ery. Mapped transcripts were visualized using Integrative Genomics Viewer (IGV) and TSS (green arrows) were predicted using TSSpredator. TSS of the *pilE* AS RNA is indicated by the orange asterisk. The “orphan” TSS is indicated by a green asterisk. Results shown are from one representative replicate out of three biological replicates.

5.4.1.2 Detection of the G4 small RNA

Although the G4 sRNA was not detected by dRNAseq analysis, other methods were used to detect the transcript in bacteria grown to mid-log phase (OD_{600} ~0.5-0.6) in the presence and absence of NaCl stress. Initially, Northern blotting was employed to detect the G4 transcript using an oligonucleotide probe (G4_probe) specific for the G4 sequence. As a control, total RNA from 8013ΔG4, which contains a kanamycin resistance cassette interrupting the G4 sequence, was analyzed alongside the samples. No bands corresponding to the G4 transcript were detected, even following overnight exposure of the membrane (Figure 5.6), indicating the G4 transcript is not expressed under these conditions or that levels are too low for detection by Northern blotting. This result is consistent with previous studies in *N. gonorrhoeae* which were also unable to detect the G4 transcript by Northern blot analysis (Cahoon and Seifert, 2013).

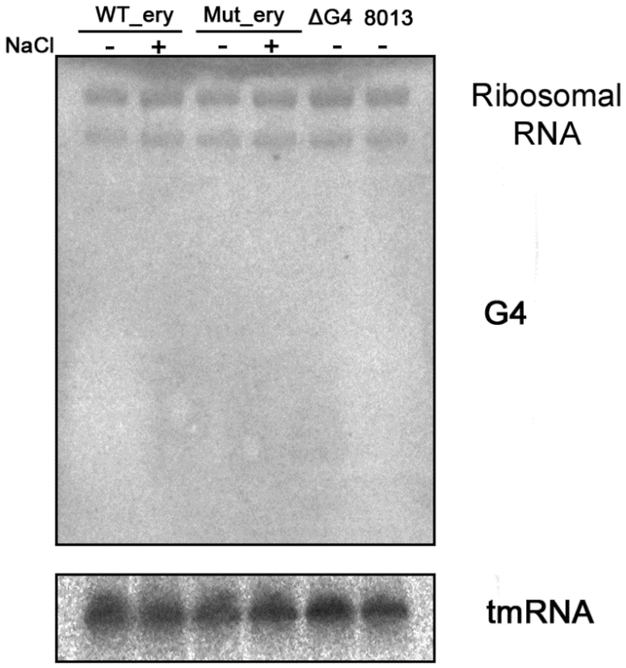


Figure 5.6 Detection of G4 small RNA by Northern blotting.

Total RNA from WT_ery and Mut_ery untreated (-) and treated (+) with NaCl stress (0.5 M, 10 minutes), and 8013ΔG4 and 8013 were analyzed by Northern blot using an oligonucleotide probe specific for the G4 RNA (G4 probe). No signal corresponding to the G4 transcript was detected. Double bands in all samples are likely due to background signal of ribosomal RNAs. tmRNA was used as a loading control.

Next, quantitative RTPCR (qRTPCR) was utilized as this is a more sensitive method for the detection of the G4 transcript in *N. gonorrhoeae* (Professor Hank Seifert, personal communication). First strand cDNA synthesis was first performed using random primers followed by qPCR analysis using specific primer pairs (Table 2.4). Three pairs of primers were designed to examine transcript levels at different regions of the *pilE* locus. Primer pair AP is specific for the region coding for the 5'-end of *pilE* and would detect the *pilE* transcript and AS transcript. Primer pair A amplifies the region upstream of the *pilE* promoter and would therefore only detect the AS transcript. Primer pair B2 is specific for the region downstream of the G4 promoter and partially encompasses the G4 sequence (Figure 5.7A). The abundance of transcript detected by each primer pair was normalized to that of the tmRNA loading control, and results were expressed as the average normalized transcript levels from three biological replicates. Statistical analysis of comparisons between different strains/conditions was conducted using two-way ANOVA and Tukey's multiple comparisons test.

Transcript levels at regions AP and A were first analyzed to determine whether results were in accordance with that previously obtained by strand-specific qRTPCR analysis of the AS and *pilE* transcripts (Figure 3.5D and Figure 4.4C). Results of qRTPCR analysis with primer pair AP show no alteration in overall transcript levels in the AP region in response to NaCl stress (Figure 5.7B). This is consistent with previous data showing that while the AS RNA is upregulated by NaCl stress, the *pilE* transcript is downregulated. However, there is an observable but not statistically significant decrease in transcript levels detected by primer pair AP when Mut_ery is subject to NaCl stress, possibly reflecting the downregulation of *pilE* in NaCl stress (See to Section 4.4). AS transcript upregulation by NaCl stress was detected specifically by primer pair A (Figure 5.7C) in WT_ery (140 to 300 fold compared to WT_ery without NaCl stress, across three replicates), although at non-statistically significant levels, likely due to the variability in the level of AS RNA induction with NaCl. As expected, no transcript was detected by primer pair A for Mut_ery (Figure 5.7C). qPCR with primer pair B2 revealed that there is a low level of transcript detected at the G4 region compared to the negative controls (*i.e.* no reverse transcriptase, Appendix 3). However there is no significant difference across strains or conditions (Figure 5.7D). Therefore, these results are consistent with the presence of a G4 RNA in *N. meningitidis*, and suggest that the AS RNA does not affect expression of the G4 sRNA, as induction of AS RNA expression does not result in any change in transcript levels detected at the G4 region.

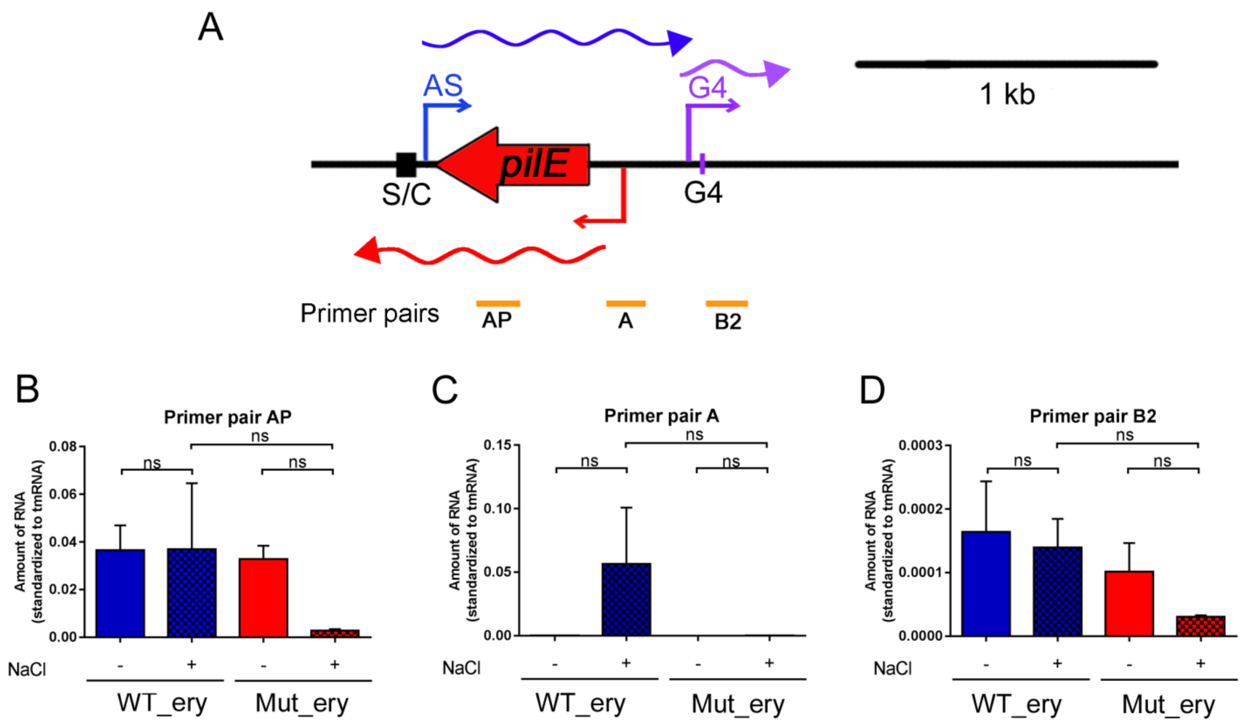


Figure 5.7 Detection of transcripts in the *pilE* locus in WT_ery and Mut_ery by qRTPCR. (A) Schematic diagram of the *pilE* locus of *N. meningitidis* 8013 and the locations of the amplicons of primer pairs AP, A, and B2 used for qRTPCR. RNA isolated from WT_ery and Mut_ery grown in liquid cultures and unstressed(-) or stressed(+) with NaCl (0.5 M, 10 minutes) was analyzed by qRTPCR using the primer pairs AP (B), A (C), and B2 (D). Results are expressed as the transcript abundance normalized to the tmRNA loading control. Results are the average of three biological replicates. Statistical analysis was conducted using two-way ANOVA and Tukey's multiple comparisons test.

5.4.1.3 Detection of transcripts in a G4 sRNA promoter mutant

As qRTPCR was the only method with which we were able to detect a transcript corresponding to the G4 region, a strain with mutations in the promoter of the G4 sRNA was constructed to abolish G4 sRNA transcription to confirm whether the primer pair B2 specifically detects the G4 transcript. The strain, G4M, contains a kanamycin resistance cassette for selection (located 180 bp downstream of and directed away from the G4 sequence to avoid interfering with G4 transcription) and mutations to the predicted -10 sequence (TAGCAT→ccGtcc) and -35 sequence (TTGAGA→ccactc) of the G4 promoter. These mutations have previously been shown to abolish G4 transcription in *N. gonorrhoeae* (Cahoon and Seifert, 2013). The isogenic control strain G4W contains the kanamycin resistance cassette and an intact G4 promoter (Figure 5.8A).

The transcript levels at the *pilE* loci of the two strains were investigated by qRTPCR as above. qPCR with primer pair AP revealed no significant difference in transcript levels between the strains in the AP region, as expected (Figure 5.8B), while the AS transcript is induced by NaCl stress in both G4W and G4M (Figure 5.8C), indicating that mutation of the G4 promoter does not influence NaCl induced expression of the AS RNA. Surprisingly, using primer pair B2, it was found that there was increased transcript levels in the G4 region in response to NaCl stress in G4W and in G4M, despite mutations to the G4 promoter in G4M (Figure 5.8D). There are two possible explanations to this result: firstly, it was possible that the mutations made to the -10 and -35 elements were not sufficient to abolish transcription. Alternatively, the G4 promoter could be inactive in G4M but the transcripts detected by the primer pair B2 could have originated from an upstream promoter, such as the AS promoter.

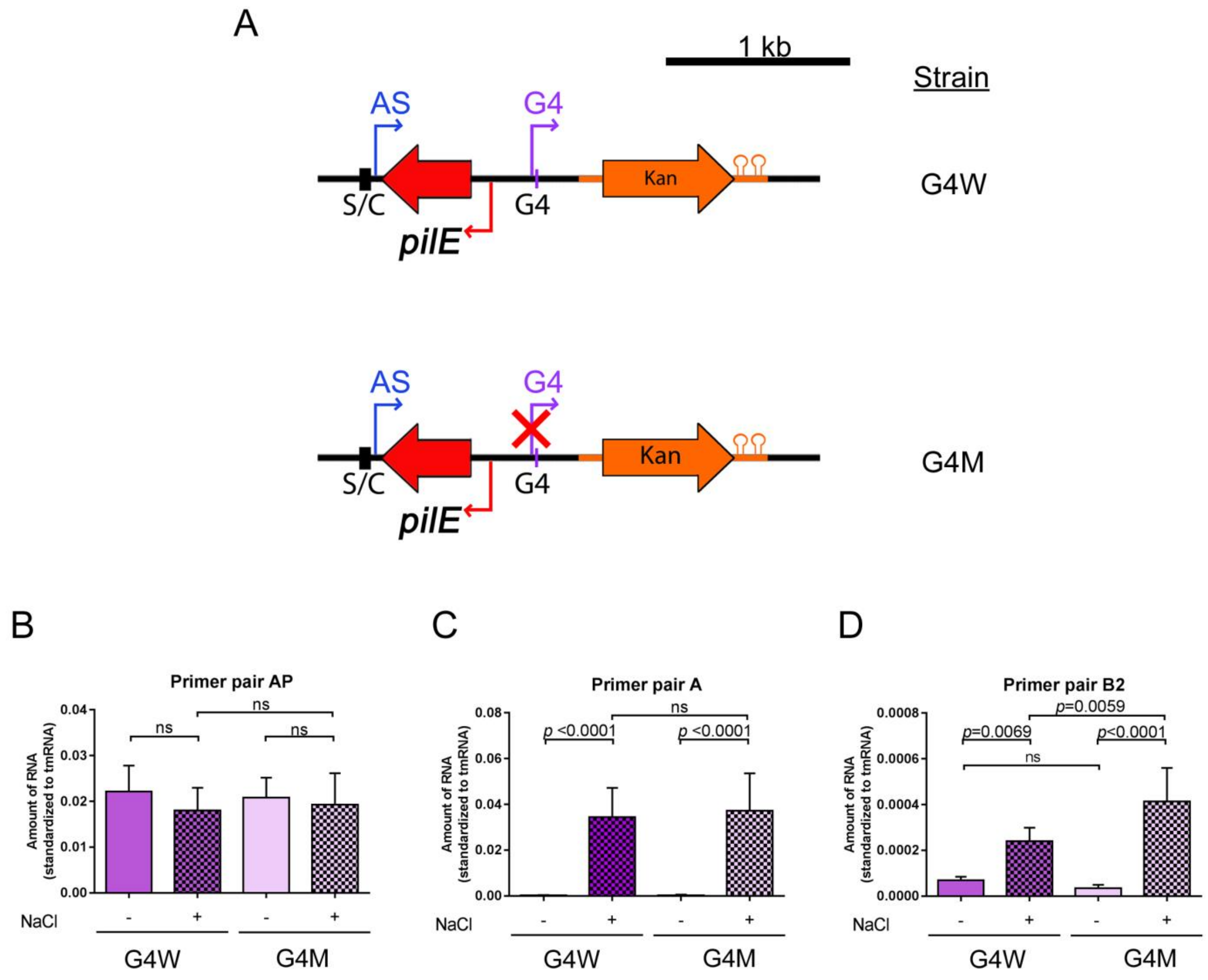


Figure 5.8 Detection of transcripts in the *pilE* locus of G4W and G4M by qRTPCR.

(A) Schematic diagram of strains G4W and G4M. G4W carries an intact G4 promoter and a kanamycin resistance cassette downstream of and in the same direction as the G4 promoter followed by two *rrnB* terminators. G4M carries the same kanamycin resistance cassette in the same position as G4W but carries mutations to the -10 and -35 elements of the G4 promoter. RNA isolated from G4W and G4M grown in liquid cultures and unstressed(-) or stressed(+) with NaCl (0.5 M, 10 minutes) was analyzed by qRTPCR using the primer pairs AP (B), A (C), and B2(D). Results are expressed as the level of transcript normalized to the tmRNA loading control. Results are the average of six biological replicates. Statistical analysis was conducted using two-way ANOVA and Tukey's multiple comparisons test.

5.4.1.4 Investigating read-through of AS RNA into the G4 region

To ascertain if the transcript detected by primer pair B2 in G4M originated from the AS promoter (*i.e*. read-through of the AS transcript into the G4 region), the G4 mutation was introduced into the strains WT_ery and Mut_ery, generating ASWT_G4M and ASMut_G4M (Figure 5.9A). Total RNA from ASWT_G4M and ASMut_G4M was analyzed by qRTPCR as described. qPCR analysis with primer pairs AP and A (Figure 5.9B and C) show a similar transcription pattern as with WT_ery and Mut_ery (Figure 5.7B and C), confirming that mutation of the G4 promoter does not affect AS RNA transcription or its induction by NaCl stress. On analysis of the G4 region using primer pair B2, it was found that while NaCl stress induces upregulation of transcript levels in ASWT_G4M, no transcript was detected in the presence of NaCl in ASMut_G4M. This demonstrates that the transcript detected by primer pair B2 in the G4 promoter mutant strains originate from the AS promoter, and that AS transcripts that have read through into the G4 region.

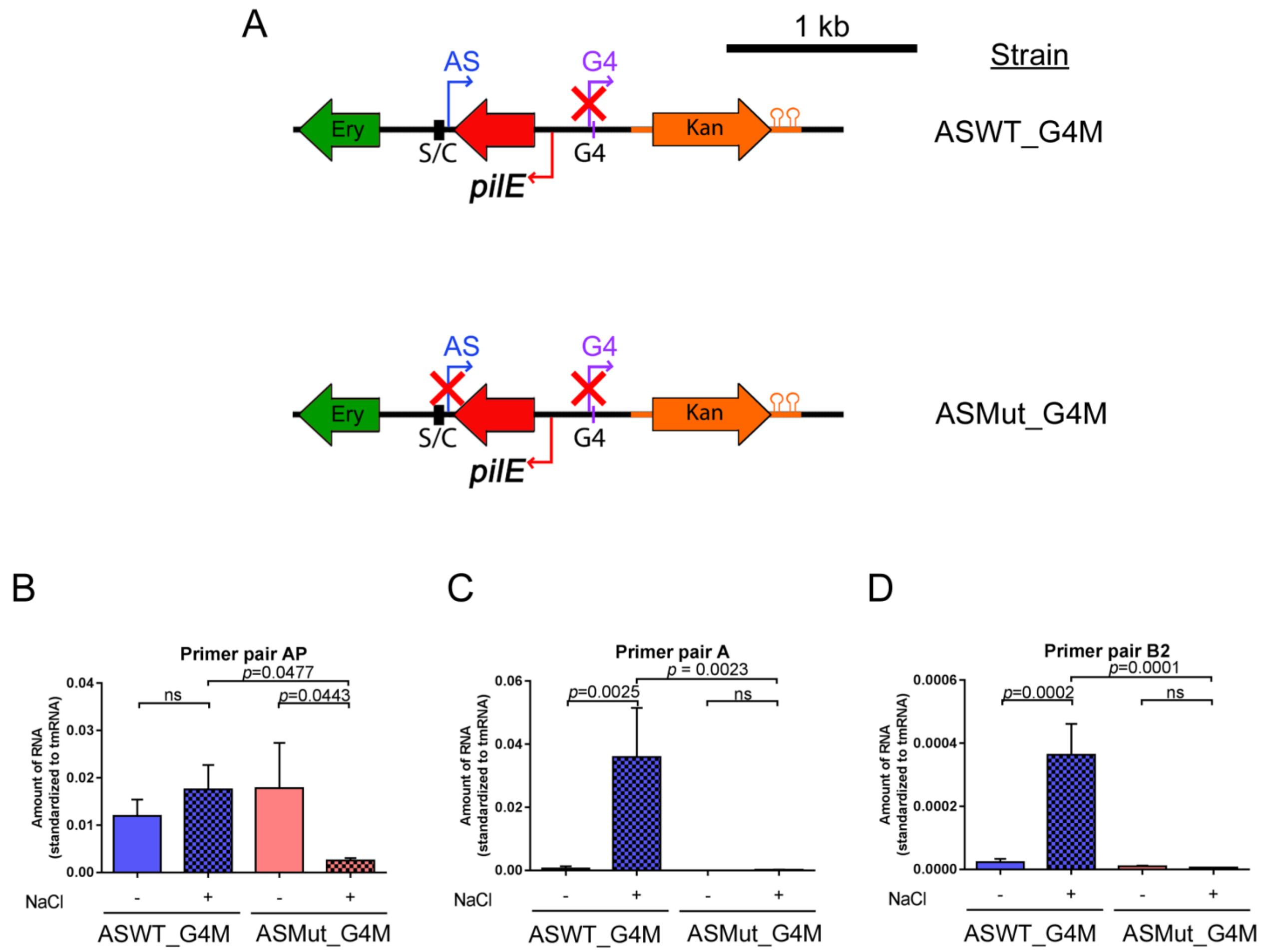


Figure 5.9 Detection of transcripts in the *pilE* locus of ASWT_G4M and ASMut_G4M by qRTPCR.

(A) Schematic diagram of ASWT_G4M and ASMut_G4M. ASWT_G4M carries an intact AS promoter and an erythromycin resistance cassette upstream of and divergent to the AS promoter, while ASMut_G4M carries mutations to the -35 and -10 elements of the AS promoter in addition to the erythromycin resistance cassette. Both strains carry mutations to the -10 and -35 elements of the G4 promoter. RNA isolated from G4W and G4M grown in liquid cultures and unstressed(-) or stressed(+) with NaCl (0.5 M, 10 minutes) was analyzed by qRTPCR using the primer pairs AP (B), A (C), and B2(D). Results are expressed as the amount of transcript normalized to the tmRNA loading control. Results are the average of three biological replicates. Statistical analysis was conducted using two-way ANOVA and Tukey's multiple comparisons test.

Therefore, using qRTPCR analysis, we have detected transcripts in the G4 region and found evidence of transcript readthrough from the AS promoter to the G4 region (Figure 5.9D). On abolition of AS transcription by mutating the AS promoter, transcripts are still detected at the G4 region, likely due to the activity of the G4 promoter (Figure 5.7D). A comparison between the strains ASMut_G4M and ASMut_G4W should be made to confirm this; however the latter strain has not been generated as yet.

Because of the runthrough of AS RNA transcription to the G4 region, we were not able to determine whether AS induction or mutation affects transcription of the G4 RNA from the G4 promoter. Due to the technical limitations of qRTPCR, it was not possible to determine the origin of the transcripts detected by primer pair B2 in Figure 5.7D as both the AS readthrough transcript and G4 RNA contain the B2 amplicon. We additionally undertook a parallel approach to investigate the role of the AS RNA in Av and its possible influence on R-loop formation in the *pilE* locus.

5.4.2 Detection of R-loops

The second approach used to investigate the possible mechanism of action of the AS RNA in influencing Av involved the detection of RNA:DNA hybrids, also known as R-loops. It has been proposed that transcription of the G4 transcript, which is necessary for Av results in the formation of an RNA:DNA hybrid between the nascent transcript and template strand of DNA, freeing the single stranded non-template strand of DNA to form a three dimensional G quadruplex structure (Cahoon and Seifert, 2013) (Figure 1.4A). Based on the Av results showing that induction of AS RNA expression results in a lowered frequency of PilE Av (Figure 5.3C), we hypothesized that transcription of the AS RNA could interfere with R-loop formation at the G4 region, thus inhibiting G-quadruplex formation and consequently inhibiting Av (Figure 1.4B).

To test this hypothesis, DNA immunoprecipitation (DIP) was used to detect changes in levels of R-loops across the *pilE* locus in the presence and absence of the AS transcript (Figure 5.10). This method involves the immunoprecipitation of RNA:DNA hybrids from fragmented genomic DNA with S9.6, a mAb which recognizes RNA:DNA duplexes (Gift from Dr Natalia Gromak, Boguslawski *et al*. 1986), followed by qPCR analysis of the immunoprecipitated DNA with primer pairs specific for regions spanning the *pilE* region (Figure 5.7A) in order to determine the approximate location of the RNA:DNA hybrids. This method has previously been used to map R-loops in eukaryotic systems (Skourti-Stathaki *et al*., 2001, Groh *et al*., 2014). Although R-loops are known to exist in bacteria (Massé and Drolet, 1999, Gowrishankar and Harinarayanan 2004), there have been no previous reports studying R-loops in *Neisseria* or their role in Av. We therefore utilized this method to investigate the presence of R-loops in the *pilE* locus in addition to the possible effect of AS RNA on G4 specific R-loops which could be involved in Av.

DIP was performed on genomic DNA isolated from WT_ery and Mut_ery grown in liquid cultures with and without NaCl stress (0.5 M, 10 min) at mid-log phase (OD_{600} = 0.5 – 0.6). As a control, gDNA from each strain was first divided into two tubes, one of which was treated *in vitro* with RNaseH, which specifically degrades RNA in R-loops (Stein and Hausen, 1969), while the other was left untreated, before proceeding with immunoprecipitation, in order to ensure that the DIP signal detected was specific for R-loops. In addition, the strain WT_ery(Δ*rnhA*) was analyzed in order to examine the effects of mutating the gene encoding RNaseHI, an enzyme which degrades RNA hybridized to DNA (Kogoma, 1986), on R-loop levels.

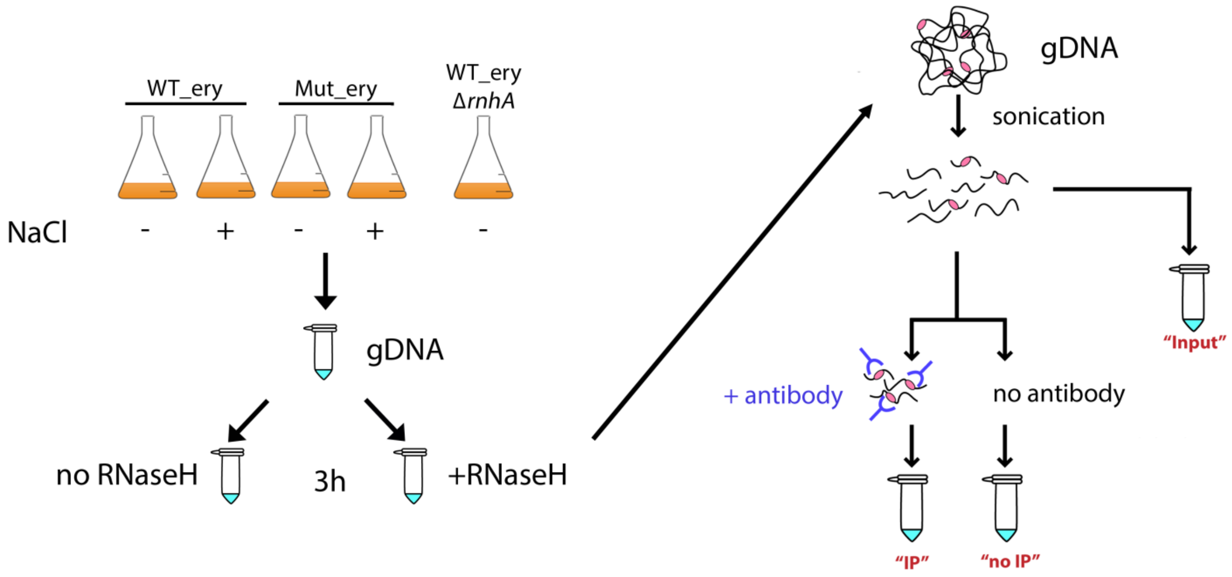


Figure 5.10 Schematic diagram of DNA immunoprecipitation (DIP).

WT_ery, Mut_ery and WT_ery(Δ*rnhA*) were grown in BHI liquid to OD_{600} = 0.5 – 0.6 and stressed (+) or not stressed (-) with NaCl (0.5M, 10 min). Genomic DNA was isolated and divided into two tubes: one was treated with RNaseH while the other was not. Both tubes were then sonicated to fragment the DNA sample. An aliquot was removed (as the Input), while the remainder was either immunoprecipitated with the mAb S9.6 (IP) or treated the same but without the mAb (no IP). The immunoprecipitated DNA was then subjected to qPCR analysis. The DIP signal was calculated as (IP- noIP)/Input.

To investigate the variability in the efficiency and yield of the immunoprecipitated DNA samples, the levels of R-loops in the regions of DNA encoding 16S RNA and tmRNA were determined. 16S RNA and tmRNA were chosen as potential "loading controls" based on the hypothesis that transcription of these RNAs would involve R-loops as transcription levels are relatively high and transcription of these RNAs is not coupled to translation (Gowrishankar and Harinarayanan, 2004). Moreover, as both 16S RNA and tmRNA are housekeeping RNAs, transcription levels and hence R-loop levels are not expected to differ across the strains and conditions tested. However, results indicate that the DIP signals in these regions are indeed variable (Figure 5.11), indicating sample-to-sample variation in pulldown efficiency and yield which may contribute to inconsistent DIP results across samples and replicates.

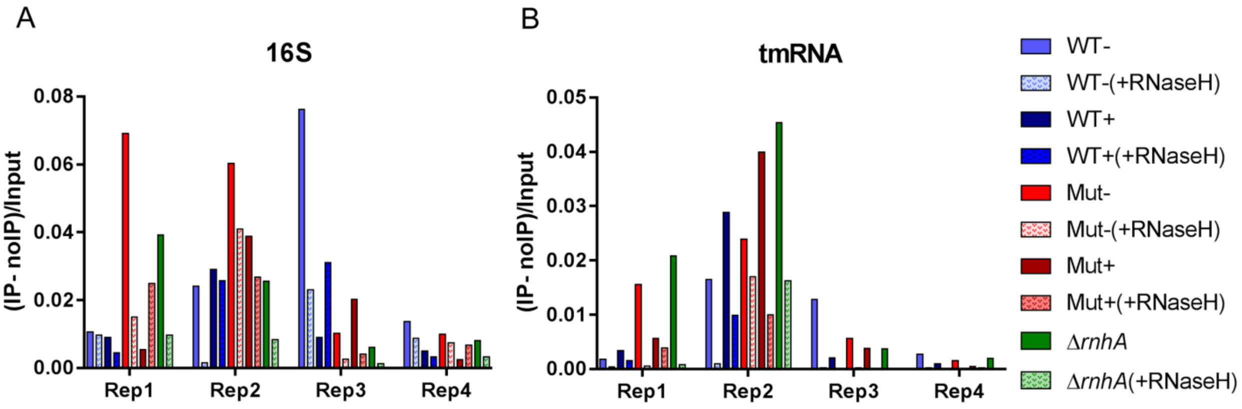


Figure 5.11 Detection of R-loops in regions encoding 16S RNA and tmRNA.
Four biological replicates of DIP analysis of WT_ery, Mut_ery, and WT_ery(Δ*rnhA*) in the presence (+) and absence (-) of NaCl stress, with and without in vitro RNaseH treatment using primer pairs specific for (A) 16S and (B) tmRNA. Strains are shown in the key.

The results from four biological replicates are shown separately in Appendix 4B - E. Regions C and D were only analyzed once in replicate 1 (Appendix 4B), while the rest of the replicates only focused on primer pairs AP, A and B2 due to the greater relevance of these regions in Av.

DIP signals for each replicate were normalized to either 16S RNA (Figure 5.12B - D) or tmRNA (Figure 5.12E - G) and expressed as the average normalized values of the four replicates for each primer pair. Normalized DIP signals for primers AP (panels B and E) and B2 (*i.e.* G4 region) (panels D and G) show no significant differences between the strains WT_ery and Mut_ery with or without NaCl stress whether signals were normalized to 16S RNA or tmRNA. This suggests that the presence or absence of the AS RNA does not appear to influence R-loop levels in these regions. Levels of R-loops detected in region A, which corresponds to a region transcribed by the AS RNA but not by the *pilE* mRNA, show an increase in WT_ery stressed with NaCl compared to unstressed WT_ery or to Mut_ery. This effect is consistent with the upregulation of the AS transcript in NaCl stress, suggesting that the AS transcript may be involved in a RNA:DNA hybrid. This increase is statistically significant using values normalized to 16S (Figure 5.12A) but not when using values normalized to tmRNA (Figure 5.12B). Therefore, further refinement of the method is necessary to validate this preliminary result.

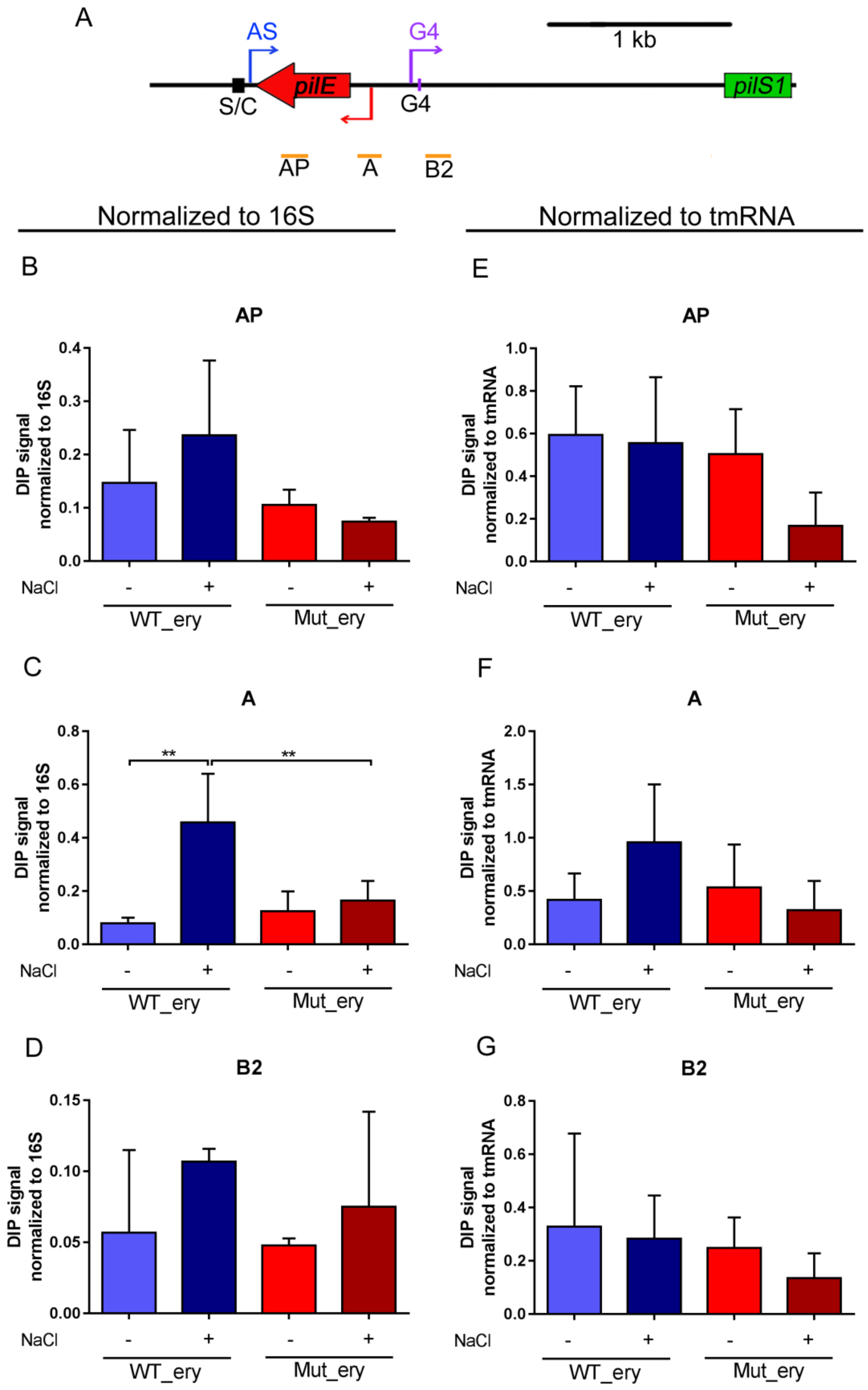


Figure 5.12 Detection of RNA:DNA hybrids in the *pilE* region of WT_ery and Mut_ery normalized to different loading controls.

(A) Primer pairs AP, A, and B2 used for DIP. DIP signals for primers AP, A, and B2 were normalized to the respective DIP signals of 16S (B - D) or tmRNA (E -G). Results shown are the average of four biological replicates.

Despite the technical challenges, a number of observations can be made from the preliminary DIP analysis. Firstly, R-loops can be detected across the *pilE* locus at varying levels; regions AP and A show a higher DIP signal compared to B2 (Figure 5.12, Appendix 4), likely reflecting their respective levels of transcription. Moreover, in most samples, the DIP signals are lower in samples that were treated with RNase H *in vitro,* confirming that the signals detected are indeed due to RNA:DNA hybrids which are removed by RNase H treatment (Appendix 4). Secondly, WT_ery(Δ*rnhA*) did not demonstrate a consistent increased DIP signal as might have been expected based on the role of RNaseHI in resolving RNA:DNA hybrids (Crooke *et al*., 1995; Kogoma, 1986). *N. meningitidis* possesses two RNaseH genes, *rnhA* and *rnhB.* Therefore, it is possible that mutation of *rnhA* alone may not be sufficient to lead to increased R-loop levels due to functional redundancy. Finally, it can also be observed that there is no consistent pattern of R-loop enrichment in the different strains/conditions for any primer pair across the four biological replicates. This lack of reproducibility is likely due to variability in the efficiency and yield of DIP (Figure 5.11) or could result from there being no significant differences between the strains/conditions.

5.5 Summary

This chapter describes the investigation of the role of the AS RNA in influencing pilin Av. Transcription of the G4-associated sRNA is required for Av of *pilE* in *N. gonorrhoeae* and is proposed to result in the formation of a G-quadruplex structure that recruits proteins involved in recombination (Cahoon and Seifert, 2013, Figure 1.4A). Mapping experiments revealed that the 3' end of the AS transcript overlaps with the promoter of the G4-associated RNA and could therefore affect G4 transcription and thus PilE Av.

To determine whether the AS RNA influences Av, Av assays were initially performed by passaging WT_ery and Mut_ery on solid BHI media followed by analysis of the *pilE* sequence of individual colonies to determine the frequency of variant *pilE* sequences. Similar Av assays have been performed previously to determine the Av frequency of wild-type *N. meningitidis* (Helm and Seifert, 2010). A modified Av assay was subsequently also performed in which the expression of RecA was induced to control when Av occurs in order to obtain more reproducible results. Although consistent differences in Av frequencies between WT_ery and Mut_ery were observed, these differences were not statistically significant. This could be due to low AS RNA expression levels in WT_ery during these assays; our previous experiments investigated the expression of the AS RNA in liquid cultures, but not from strains grown on solid media. We therefore speculated that induction of AS expression by NaCl stress would allow for detection of any effect of the AS RNA on pilin sequence variation.

Therefore, Av assays were performed, during which AS expression was induced by the addition of NaCl. In AS inducing conditions, a significant decrease in *pilE* variation was observed in the wild-type strain, but this was not observed in the AS promoter mutant. This indicates that, in contrast to the G4-associated sRNA which is necessary for Av, AS transcript or transcription reduces the rate of PilE Av. We thus sought to determine the mechanism by which this occurs. Overlapping transcription units can influence the expression of the downstream transcript, through promoter occlusion (Orosz *et al.*, 1991). Therefore, we hypothesized that increased transcription of the AS RNA could interfere with the transcription of the G4-associated sRNA and consequently reduce the efficacy or frequency of G-quadruplex formation and thus affect *pilE* variation frequency (Figure 1.4B).

To test this proposed mechanism of action, two approaches were adopted. The first approach was to determine whether AS transcription affected levels of any putative G4 transcript in *N. meningitidis*. The second approach involved the hypothesis that G4 transcription and G-quadruplex formation involved the generation of RNA:DNA hybrids (Figure 1.4A, left panel) and subsequently the investigation of whether AS transcription influences RNA:DNA hybrid levels in the G4 region.

Although initially studied in *N. gonorrhoeae,* the G4 sRNA has yet to be detected by direct methods in *N. gonorrhoeae*. Instead, the requirement of G4 transcription for Av has been shown indirectly by promoter mutagenesis (Cahoon and Seifert, 2013). More recent investigation has found that the *N. gonorrhoeae* G4 sRNA can be detected by qRTPCR (Professor Hank Seifert, personal communication). In *N. meningitidis* strain 8013, the sequences of the -10 and -35 elements of the G4 sRNA promoter are almost identical to that of *N. gonorrhoeae*, except for a single nucleotide difference in the -10 element (TAGCAT in *N. meningitidis* instead of TAGAAT in the gonococcus). Given that this single nucleotide difference results in a sequence that is less similar to the bacterial consensus -10 sequence (TATAAT), it is possible that the G4 sRNA promoter may be less active or inactive in *N. meningitidis,* and/or G4 sRNA expression may occur in different conditions than in *N. gonorrhoeae*. Therefore, it was important to first identify a method by which the G4 transcript could be detected in *N. meningitidis* to determine whether its levels are altered as a result of AS transcription. As the G4 RNA could not be detected by Northern blot or by dRNAseq, qRTPCR was used as a method to quantify levels of G4 transcript and transcript levels of other transcription units in the *pilE* locus.

Using qRTPCR, it was found that induction or absence of AS RNA expression did not result in a significant change in transcript levels detected at the G4 region. Transcript levels at the G4 region did not appear to be correlated with results from the Av assays.

Indeed, contrary to our hypothesis, G4 transcript levels were not found to be reduced during NaCl stress even though Av frequency is reduced when AS RNA expression is induced in WT_ery. However, it was not possible to determine whether these transcripts were indeed from the G4 sRNA or a result of read-through of the AS RNA. Moreover, this is further compounded by the possibility that G4 transcription may affect the propensity for AS read-through due to the obstruction of RNA polymerase. It is plausible that the two transcriptional processes – AS and G4 transcription – may mutually affect each other in a manner that is not as straightforward as our proposed mechanism. Detection of AS read-through into the G4 region also raises the intriguing question of whether read-through transcription is capable of performing the function of the G4 sRNA in promoting Av. Future work will address this by investigating the rates of pilin variation in strains lacking a functional G4 promoter (*i.e.* ASWT_G4M and ASMut_G4M) to determine whether Av still occurs despite the absence of the G4 sRNA and thereby implying that the AS read-through transcript is capable of facilitating the formation of the G-quadruplex structure that is necessary for PilE Av.

Since the possible effect of AS on G4 transcription could not be definitively determined due to the difficulty in differentiating G4 transcript from AS read-through, R-loop detection by DIP provided an alternative strategy to elucidate the mechanism by which the AS transcript reduces the frequency of pilin variation. R-loops are known to contribute to DNA damage in eukaryotes by exposing single stranded DNA which is susceptible to lesions (as a result of DNA-damaging agents and deaminases), transcription-associated mutagenesis, or transcription-associated recombination (Skourti-Stathaki and Proudfoot, 2014) but also play a functional role in facilitating mammalian immunoglobulin class switch recombination to generate diverse antibody isotypes (Roy *et al.*, 2008). R-loops have also been shown to be involved in driving genome instability in bacteria by promoting double strand breaks and genome rearrangement (Wimberly *et al*., 2013) and by impairing replication fork progression (Gan *et al*., 2011). DIP has previously been used for the detection of R-loops in yeast and in mammalian cells (El Hage *et al*., 2010; Skourti-Stathaki *et al*., 2011). To our knowledge there have been no reports of this method being used to investigate prokaryotic R-loops. Although prokaryotic R-loops have primarily been studied using plasmid systems, genomic R-loops have recently been studied in *E. coli* utilizing a bisulfite-sensitivity assay which involves treatment with sodium bisulfite which converts cytosine residues on the exposed single stranded DNA of an R-loop to uracil, followed by whole-genome sequencing to identify regions containing R-loops by locating C→T mutations (Leela *et al*., 2013).

DIP analysis revealed the novel finding that R-loops can be detected across the *pilE* locus. However, it was difficult to obtain consistent and reproducible DIP signals for the four biological replicates performed. In contrast to eukaryotic systems in which transcriptional units may be kilobases apart, the *pilE* locus of *N. meningitidis* contains at least three (AS, *pilE,* and G4) promoter elements within a span of a single kb. When performing DIP using sonicated gDNA with fragment size of 200-300 nt, it may be difficult to obtain a consistent and sufficient level of resolution when utilizing primer pairs spaced ~300 nt apart to analyze such a small, yet transcriptionally active region. Thus, limited resolution may be a potential explanation for poor reproducibility. Moreover, given the transient nature of transcription processes, it may be difficult to consistently analyze levels of R-loops resulting from transcription in a non-synchronous bacterial culture despite efforts taken to ensure that cells were harvested at the same growth phase.

In order to standardize DIP signals across all replicates to obtain more comparable results, DIP signals were normalized to loading controls. However, as global R-loop levels have not been extensively examined in bacteria, there was no precedent in the selection of loading controls for normalization. Initially, 16S RNA was chosen as a potential loading control due to it being highly transcribed and not coupled to translation – factors that are thought to contribute to R-loop formation (Gowrishankar and Harinarayanan, 2004; Massé *et al.*, 1997). Furthermore, transcription of the *rrnB* operon, which encodes 16S and 23S RNA, has been shown to result in R-loop formation in plasmid system (Massé *et al.*, 1997). tmRNA was also chosen as a potential loading control for similar reasons and because tmRNA levels were not found to vary across the strains and conditions used.

Although normalized DIP signals demonstrated greater consistency, there did not appear to be any effect of AS expression or mutation on the level of R-loops detected at the G4 region. Nonetheless, this data does indicate the presence of additional R-loops in the *pilE* locus. There was a trend suggesting that induction of AS expression in WT_ery may result in increased levels of R-loops. The presence of R-loops in the *pilE* locus, together with the possibility that AS transcription in particular leads to R-loop formation give rise to the intriguing prospect of the potential involvement of R-loops in the mechanism of PilE Av. Therefore, we can conclude that induction of AS RNA expression results in decreased pilin Av frequency. However, the possible involvement of the R-loops resulting from the expression of AS RNA in modulating Av remains to be elucidated. Future work involving RNase H deletion and/or overexpression could aid in determining the potential role of R-loops in pilin variation.

6 Discussion

In this work, I identified and characterized a novel RNA antisense to *pilE* in *N. meningitidis* strain 8013. The AS RNA was found to encompass the entire *pilE* coding region and 5'-UTR, extending to the G4 sRNA promoter which is essential for pilin Av (Cahoon and Seifert, 2013). As *cis*-encoded AS RNAs have exact complementarity to the sense transcript, they can regulate the expression of the gene encoded on the opposite DNA strand, either through RNA:RNA interactions or through transcriptional interference (Georg and Hess, 2011). However, we found that overexpression of the AS RNA or mutation of its promoter did not have an effect on *pilE* transcript levels, PilE protein levels, or on Tfp-mediated functions such as adhesion and transformation.

It is increasingly apparent that *cis*-encoded antisense RNAs are abundant in bacteria. Technological advances in unbiased genome-scale approaches to transcriptomics has resulted in the discovery of pervasive antisense transcription in many bacterial species including *H. pylori*, *E. coli*, *Synechocystis* spp. PCC6803, and *Staphylococcus aureus* (Lasa *et al.*, 2011; Mitschke *et al.*, 2011; Sharma *et al.*, 2010; Thomason *et al.*, 2015). Antisense transcription has been suggested to mediate important regulatory functions of the cognate sense RNAs such as influencing sense RNA transcription, altering mRNA translation, targeting of the dsRNA for degradation by RNases, or stabilization of sense RNA (Georg and Hess, 2011). However, despite the hundreds of antisense TSS now being detected in a number of bacterial genomes, functional roles have only been assigned to a limited number of AS RNAs. For this reason, and also due to their low abundance and the lack of promoter sequence conservation between species, it has been suggested that majority of bacterial AS RNAs are non-functional but are instead products of spontaneously arising promoter sequences (Raghavan *et al.*, 2012). However there is recent evidence suggesting that many of these AS transcripts are indeed functional. Co-immunoprecipitation experiments in *E. coli* have revealed that a large number of AS RNAs bind to the RNA chaperone Hfq *in vivo*, indicating potential regulatory roles (Bilusic *et al*., 2014). In addition, a novel technique involving the immunoprecipitation of double stranded RNA (dsRNA) has also revealed more than 316 potentially functional AS RNAs in *E. coli* that are present in higher levels in an RNase III mutant strain, suggesting that there is a significant number of biologically relevant AS RNAs which base pair with their cognate sense RNAs to form dsRNAs that are processed or degraded by the action of RNases (Lybecker *et al*., 2014).

In this work, a number of different methods (Northern blot, qRTPCR and RNAseq) demonstrated that *pilE* transcript levels are not influenced by the AS RNA. This finding is somewhat unexpected as one might assume that the AS RNA would be able to pair with *pilE* mRNA to form a dsRNA that can be targeted for stabilization or degradation. However, one limitation of these methods is that they only provide time- and population-averaged transcription data and do not indicate whether such dsRNAs are formed within a cell. A single cell approach may be necessary to determine whether both sense and antisense transcription are occurring in the same cell or if AS transcription is only occurring stochastically in a subpopulation of cells. It may also be possible that no regulatory effects on *pilE* were observed due to the lower levels of AS RNA relative to the *pilE* transcript, such that stoichiometric co-degradation of the AS RNA and *pilE* mRNA may not result in a noticeable decrease in *pilE* levels.

Since the AS RNA did not appear to act in *cis* to modulate *pilE* or pilin levels, we also considered the possibility that the AS RNA might act in *trans* to regulate the stability of expression of a target mRNA encoded elsewhere on the genome. RNAseq analysis resulted in a list of 22 transcripts that were differentially expressed in the isogenic strains Mut_ery compared to WT_ery, with the highest magnitude fold-change value of 2.7 corresponding to NMV_1138 which encodes a putative type III restriction-modification enzyme. None of the potential *trans* targets have yet been fully validated. A parallel investigation was also performed, involving *in silico* prediction of putative mRNA targets based on potential RNA:RNA interactions using sRNA target prediction algorithms (Wright *et al*., 2014), followed by assessment of putative targets by co-expressing the AS RNA and translational fusions of the target mRNA and green fluorescent protein (GFP) utilizing a two-plasmid-system in *E. coli*, with GFP expression levels as a reporter of translational control (Corcoran *et al*., 2012; Urban and Vogel, 2007). However, we were not able to identify any positively or negatively regulated targets by this method (data not shown). Nevertheless, the GFP-fusion approach is a useful tool which can be used to validate the potential mRNA targets identified by RNAseq and provides a straightforward method to investigate regions of potential RNA:RNA interactions by mutational analysis and compensatory base-pair exchange (Corcoran *et al*., 2012). Interestingly, none of the top 22 differentially expressed transcripts determined by RNAseq were identified as putative targets by the target prediction algorithms. This observation, together with the negative results of GFP-fusion experiments, suggests that such algorithms may not be particularly accurate or reliable predictors of putative target mRNAs, as has been suggested by Li *et al*. (2012).

In this study the *pilE* AS RNA was found to play a role in modulating pilin Av; induction of AS RNA expression resulted in a reduction of Av frequency. Pilin Av in *N. meningitidis* occurs *via* a non-reciprocal DNA recombination event between *pilS* and *pilE* (Hagblom *et al*., 1985). This process requires transcription of the G4 sRNA (Cahoon and Seifert, 2013) and involves a number of recombination proteins such as RecA and members of the RecF-like pathway (Koomey *et al*., 1987; Mehr and Seifert, 1998). However the precise molecular mechanisms of this process and the mechanisms behind the regulation of Av are still poorly understood, especially in *N. meningitidis*.

In addition to the pathogenic *Neisseria*, several other pathogens also undergo recombination-mediated antigenic variation. Members of the spirochete genus *Borrelia*, of which four species (*Borrelia burgdorferi, Borrelia garinii, Borrelia afzelii, and Borrelia valaisiana*) cause Lyme disease, express a surface-exposed lipoprotein, VlsE, which undergoes antigenic variation during infection of mammalian hosts (Zhang and Norris, 1998). Closely resembling in the *pilE*/*pilS* system in *Neisseria*, the *vls* locus in *Borrelia* contains a number of silent cassettes which can recombine into the expressed *vlsE* locus to result in sequence variation (Zhang *et al*., 1997). Unlike in *Neisseria*, this process is not dependent on RecA (Liveris *et al*., 2008) but does require the RuvAB Holliday junction branch migrase (Dresser *et al*., 2009; Lin *et al*., 2009). Although the exact mechanism by which *vls* recombination occurs is not well defined, it has been suggested that G-quadruplex formation at the *vls* locus may be involved in this process (Walia and Chaconas, 2013); however there is no indication of any involvement of a G4 associated sRNA.

Members of the *Trypanosoma* genus are eukaryotic parasites that cause sleeping sickness and Chagas disease and also evade the host immune response through high frequency recombination-based antigenic variation of the variant surface glycoprotein VSG. Trypanosomes possess a vast repertoire of transcriptionally silent VSG genes and pseudogenes in addition to the active VSG located at one of about 15 bloodstream expression sites (BES), of which only one is active in each cell (Berriman *et al*., 2005). Switching of the active VSG occurs *via* different mechanisms, the most important of which is mediated by gene conversion whereby the active VSG is replaced by a VSG sequence copied from the silent archive (Marcello and Barry, 2007) and involves the eukaryotic homolog of RecA, RAD51 (McCulloch and Barry, 1999). Although the route by which lesions are formed in the BES to initiate VSG switching by homologous recombination is not yet clear, it has been suggested that VSG switch initiation involves replication-transcription clashes within the BES which may lead to DNA breaks and DNA rearrangements (Devlin *et al*., 2016).

In this study, a model was proposed in which transcription of the AS RNA interferes with transcription of the G4 sRNA, thereby preventing G-quadruplex formation and reducing the rate of Av. In order to investigate the possible mechanism by which the AS RNA influences Av in *N. meningitidis*, we undertook two complementary approaches in parallel. The first approach was to investigate whether overexpression of the AS RNA affected G4 transcript levels. Due to the low level of G4 transcription, qRTPCR was the only method that was able to detect transcripts at the G4 region. However, a major shortcoming of this technique was its inability to differentiate between the G4 sRNA and the AS RNA readthrough into the G4 region, making it impossible to conclude whether G4 sRNA levels were directly affected by AS transcription. To address this, attempts were made to insert a transcriptional terminator upstream of the G4 promoter with the aim of terminating AS RNA transcription before it could interfere with the G4 promoter or read through into the G4 region (not shown). However, despite multiple attempts, it was not possible to generate this strain; for reasons still unknown, transformants that acquired the inserted terminator contained point mutations in the G4 sequence that would disrupt the G-quadruplex structure.

A question that arises from our results is that of whether AS readthrough into the G4 region would result in G-quadruplex formation and the initiation of pilin Av. In other words, can AS readthrough complement the function of the G4 sRNA? Although it has been shown that mutation of the G4 sRNA promoter results in a complete block in pilin

Av in *N. gonorrhoeae* (Cahoon and Seifert, 2013), this does not rule out the possibility that the AS RNA could function to mediate Av in the absence of the G4 sRNA in *N. meningitidis* for two reasons: firstly, it is possible that the AS RNA is not present in *N. gonorrhoeae*; antisense transcription of *pilE* was not observed in *N. gonorrhoeae* by dRNAseq (Remmele *et al.*, 2014) and the AS promoter sequence is not identically conserved in the *N. gonorrhoeae pilE* locus. Secondly, it is also possible that the AS RNA is not transcribed or very lowly transcribed in *N. gonorrhoeae* under normal culture conditions as is the case in *N. meningitidis*, and is only induced during a specific stress conditions. Nevertheless, in the presence of an intact G4 promoter, it is unlikely that induction of AS RNA expression results in G-quadruplex formation as our experiments show that induction of the AS RNA by NaCl stress results in decreased Av. Therefore, future work should investigate the possibility of G-quadruplex formation by the AS readthrough transcript by examining pilin Av frequencies in strains lacking the G4 sRNA promoter in the presence and absence of AS transcription and readthrough.

The second approach we used to investigate the mechanism of action of the AS RNA was to determine whether the AS RNA influenced the levels of R-loops present in the G4 region, based on the speculation that G-quadruplex formation would be accompanied by the formation of a RNA:DNA hybrid during G4 sRNA transcription. Although it has been implied that transcription of the G4 sRNA involves the formation of an R-loop for the stabilization of the G-quadruplex structure (Cahoon and Seifert, 2013), this has not previously been demonstrated experimentally. This hypothesis is consistent with G-rich regions on the non-template DNA strand being conducive for R-loop nucleation and expansion (Roy and Lieber, 2009). However, to date, there have been no reports describing R-loops being directly involved in the *pilE/pilS* recombination process.

From our DIP experiments, we were able to detect a low level of R-loops at the G4 region, however, due to the technical limitations of the method, we were unable to conclusively determine whether AS transcription influenced levels of R-loops in the G4 region. Nevertheless, the DIP results demonstrate that R-loops are present in the *pilE* locus, which leads to the intriguing possibility that these structures may play a role in *pilE*/*pilS* recombination. Based on these results, and also on the observation that the AS transcript is upregulated in strains lacking RNase HI, our experiments strongly suggest that the AS RNA is indeed involved in an R-loop. This finding is consistent with the fact that transcription of noncoding RNA is prone to R-loop formation; the coupling of transcription and translation is one of the processes employed by bacteria to prevent R-loop formation by rendering the nascent transcript unavailable for annealing with upstream DNA (Gowrishankar and Harinarayanan, 2004; Leela *et al.*, 2013).

In bacteria, R-loops can occur either during replication where they are formed from RNA pimers, or during transcription by RNA polymerase where they can arise by 5'–end invasion of the DNA duplex and reannealing of the nascent transcript with upstream DNA after exiting the RNA polymerase (Kogoma, 1997). The formation of excessive R-loops is toxic to cells, giving rise to transcription-replication conflicts and genome instability. During DNA replication, transcriptional R-loops can interfere with DNA replication fork progression, resulting in DNA double strand breaks (DSBs), DNA rearrangements, and recombination events (Dutta *et al.*, 2011; Gan *et al.*, 2011). In non-replicating cells, R-loops can also promote the formation of DSBs by initiating replication forks that collapse when they encounter a single stranded nick in template DNA, which can ultimately result in genome rearrangements or point mutations (Wimberly *et al.*, 2013). Bacteria employ a number of strategies to prevent R-loop formation or to remove R-loops in order to mitigate their potential detrimental consequences. As mentioned above, as transcription is coupled to translation in bacteria, the presence of ribosomes on the nascent transcript prevents the formation of transcriptional R-loops (Massé and Drolet, 1999). Furthermore, in *E. coli*, the transcription termination factor Rho binds to nascent untranslated transcripts that are not engaged with ribosomes and terminates transcription by promoting RNAP disassociation from RNA in a process known as Rho-dependent transcription termination which prevents the transcript from invading and annealing to upstream DNA (Leela *et al.*, 2013). If mechanisms to prevent R-loop formation fail, bacterial cells utilize a number of factors which remove or resolve these structures. The most important and best characterized enzyme involved in the removal of R-loops is RNase HI which degrades RNA in RNA:DNA duplexes (Horiuchi *et al.*, 1984), while RecG is a DNA translocase with RNA/DNA helicase activity and is able to unwind R-loops (Hong *et al.*, 1995).

Although R-loops are also generally considered deleterious in eukaryotic cells, they play a key functional role in immunoglobulin class switch recombination (CSR) which occurs at the at the highly repetitive switch (S) region of the *IgH* locus in mammalian B cells to change the isotype of the antibody molecule produced. Transcription through the G-rich S regions promotes R-loop formation, exposing the single stranded non-template DNA to the activity of activation-induced cytidine deaminase (AID) which deaminates cytosines to uridines (Chaudhuri *et al.*, 2007). These generated mismatches can be processed by base excision repair or mismatch repair pathways which eventually leads to the generation of DSBs in the S regions that are acted upon by non-homologous end joining pathways that ligate DSBs between two S regions (Chaudhuri *et al.*, 2007). In contrast, not much is currently known about the possible functional roles of chromosomal R-loops in bacteria. Thus, in light of the known role of R-loops in a eukaryotic recombination system, the novel finding that AS transcription is associated with R-loop formation raises the possibility of its involvement in *pilE/pilS* recombination.

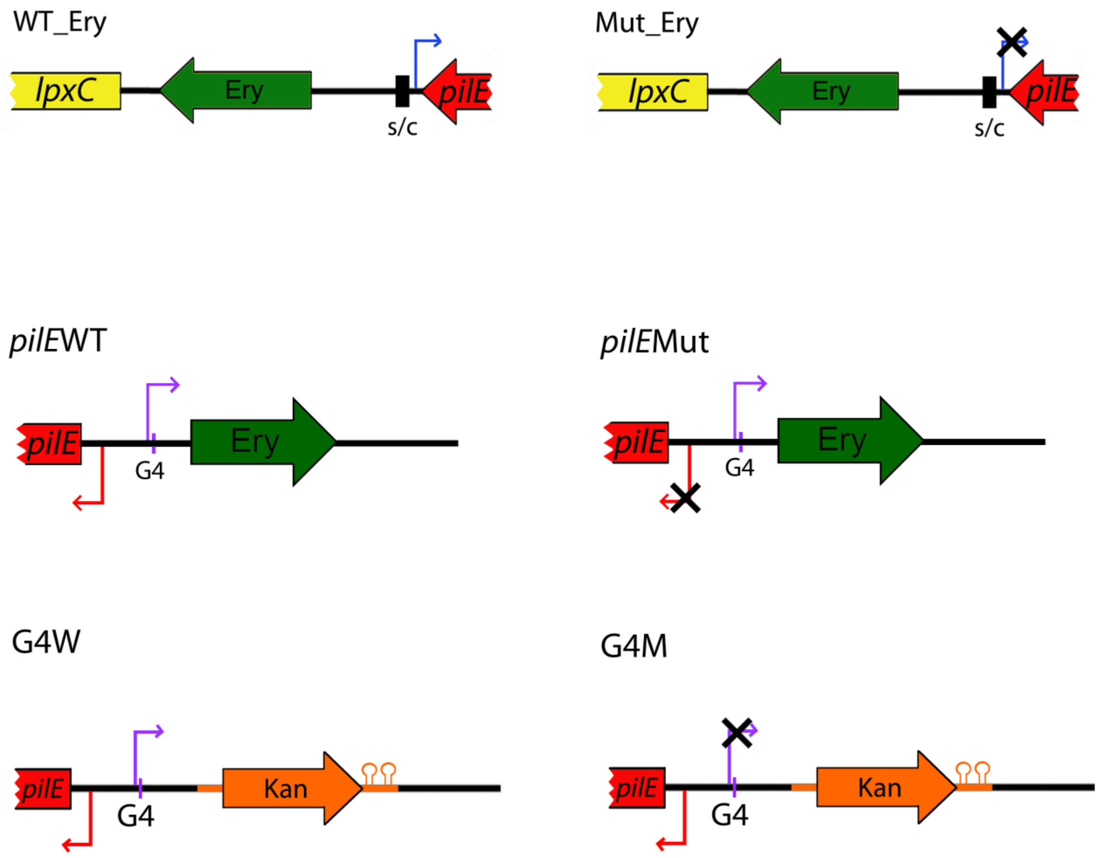
Therefore, further work could explore the possible role of R-loops in pilin Av by investigating the effect of RNAseH or RecG mutation on the rate of Av. One could speculate that R-loop formation due to AS transcription could play a role in exposing single stranded DNA (ssDNA) to act as a substrate for RecA to initiate homologous recombination (Kowalczykowski *et al*., 1994). However, given that pilin Av still occurs in the AS promoter mutant, it is evident that R-loop formation by AS RNA transcription is not a requirement in the recombination mechanism. In an alternative hypothesis, R-loops in the *pilE* locus may instead cause replication fork stalling and subsequent DNA damage, ultimately promoting DNA recombination (Gan *et al*., 2011). However, as our experiments show that induction of AS expression leads to reduced Av, it is unlikely that R-loops formed by the AS RNA modulates Av through promoting DNA recombination. *In vitro* plasmid-based systems may prove useful in understand the possible role of R-loops in transcription-replication conflicts in the *pilE/pilS* locus. In addition, nothing is known about the role (if any) of R-loops formed by *pilE* transcription in *pilE/pilS* recombination. It would therefore be interesting to investigate whether abolition of both sense and antisense transcription of *pilE* (thereby precluding any R-loop formation on either strand at the *pilE* locus) affects the rate of pilin Av. Thus, the potential involvement of R-loops in *pilE/pilS* recombination is currently a completely unexplored frontier.

This work has shown that expression of the AS RNA is induced upon high levels (0.4 – 0.5 M) of NaCl stress. Although this level of salt is nonphysiological, Na and Cl ion concentrations have been reported to increase up to almost 300 mM in airway surface liquid in certain chronic inflammatory airway diseases such as cystic fibrosis or rhinitis (Vanthanouvong *et al*., 2006). The AS RNA is also expressed when bacteria are in late stationary phase after overnight growth. The competitive conditions of nutrient depletion, high cell density, and oxygen limitation that are characteristic of stationary phase growth may resemble the environments that meningococci would encounter *in vivo* (Llorens *et al*., 2010) and can provide insight into the physiological influence of the microenvironment on pilin Av. In *N. gonorrhoeae*, an increase in Av frequency was observed when bacteria were grown under iron-starved conditions, but Av rates were not found to be affected by temperature, carbon source, or oxygen availability (Serkin and Seifert, 2000). Given the purported advantages that pilin Av confers onto the pathogenic *Neisseria*, our finding that NaCl-induced AS RNA expression reduces Av raises the intriguing question of why reduction of Av may be beneficial to the meningococcus. A possible speculation could be that inhibiting Av may release the DNA repair and recombination enzymes that were sequestered to the *pilE/pilS* locus, allowing them to perform more critical functions which may be required when the bacterium encounters stressful environments.

The mechanism by which AS RNA induction occurs in response to NaCl stress and after overnight growth remains unclear. In *E. coli*, the RpoS sigma factor regulates genes in stationary phase and in response to stresses such as osmotic stress (Battesti *et al*., 2011). However *Neisseria* lacks an identifiable RpoS homolog (Chiang and Schellhorn, 2010) and has been suggested to utilize RpoH, a heat-shock sigma factor, in the regulation of stress response genes (Gunesekere *et al*., 2006)). In addition, it is also not known whether transcriptional activation or derepression processes are involved in AS RNA expression. Therefore, the potential role of transcription factors and repressors such as the histone-like nucleoid structuring (H-NS) protein (which is known to repress basal expression of many RpoS-dependent genes during exponential growth (Battesti *et al*., 2011) may worth investigating.

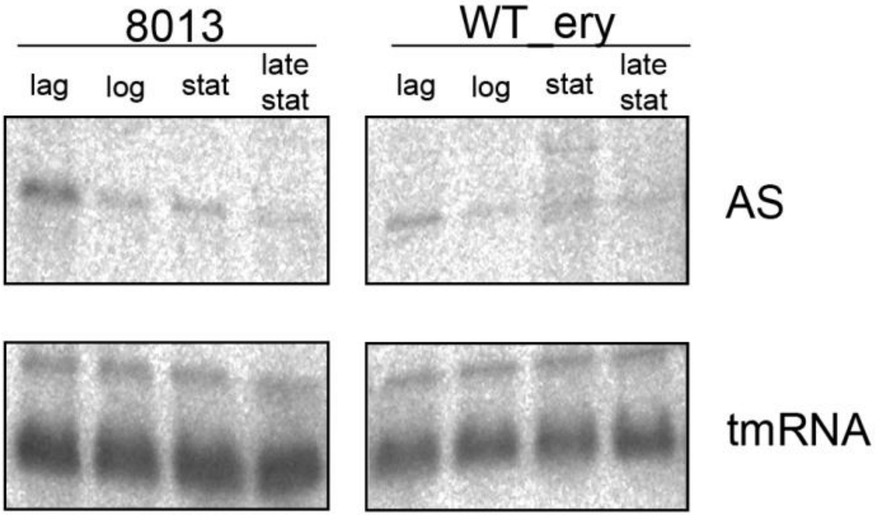
Finally, in this study we utilized dRNAseq to investigate the transcriptional profile of the *pilE* region. However, in addition to providing a picture of the local transcriptional landscape of the *pilE* locus, dRNAseq analysis has also produced a wealth of TSS data on a transcriptome-wide scale. Preliminary analysis has found that a total of 431 TSS were detected in WT_ery; this relatively low number of TSS possibly reflects the low level of transcription in bacteria at 12 h of growth. Therefore, future work could utilize this dataset for the global analysis of the subset of genes potentially important for stationary phase growth and putative stationary phase-induced noncoding regulatory RNAs in *N. meningitidis*.

Appendix 1 – DNA fragments used for strain construction



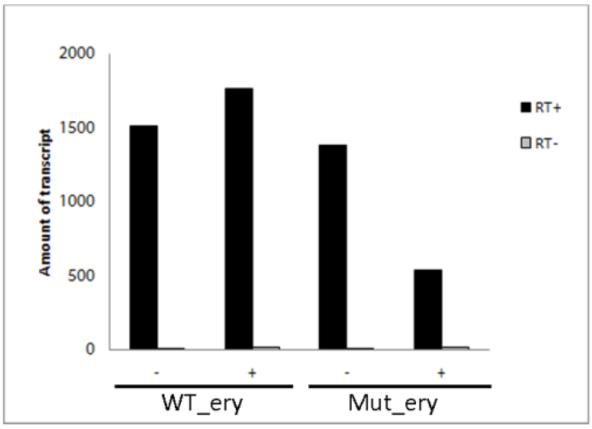
DNA fragments used for the construction of strains WT_ery, Mut_ery, *pilE*WT, *pilE*Mut, G4W, and G4M. Figures are not drawn to scale.

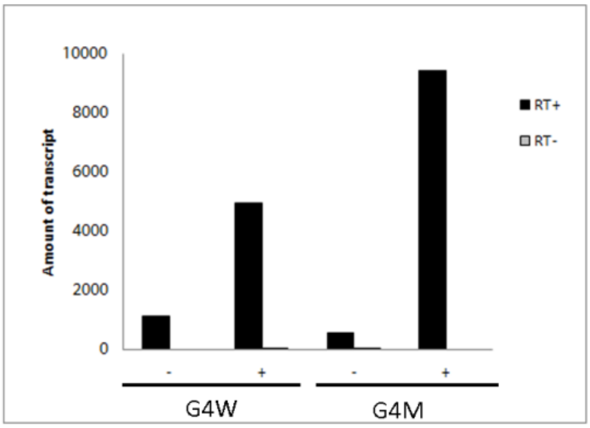
Appendix 2 – Detection of AS RNA in 8013 and WT_ery

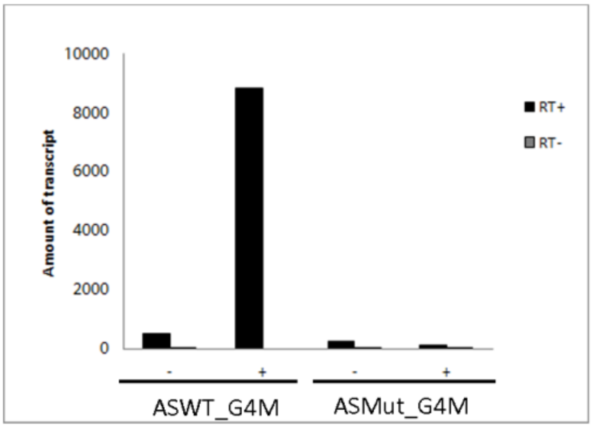


Northern blot analysis of total RNA isolated from *N. meningitidis* 8013 and WT_ery at different growth phases. The AS transcript was detected using the oligonucleotide probe (AS)*pilE*-1.

Appendix 3 – qRTPCR results with primer pair B2

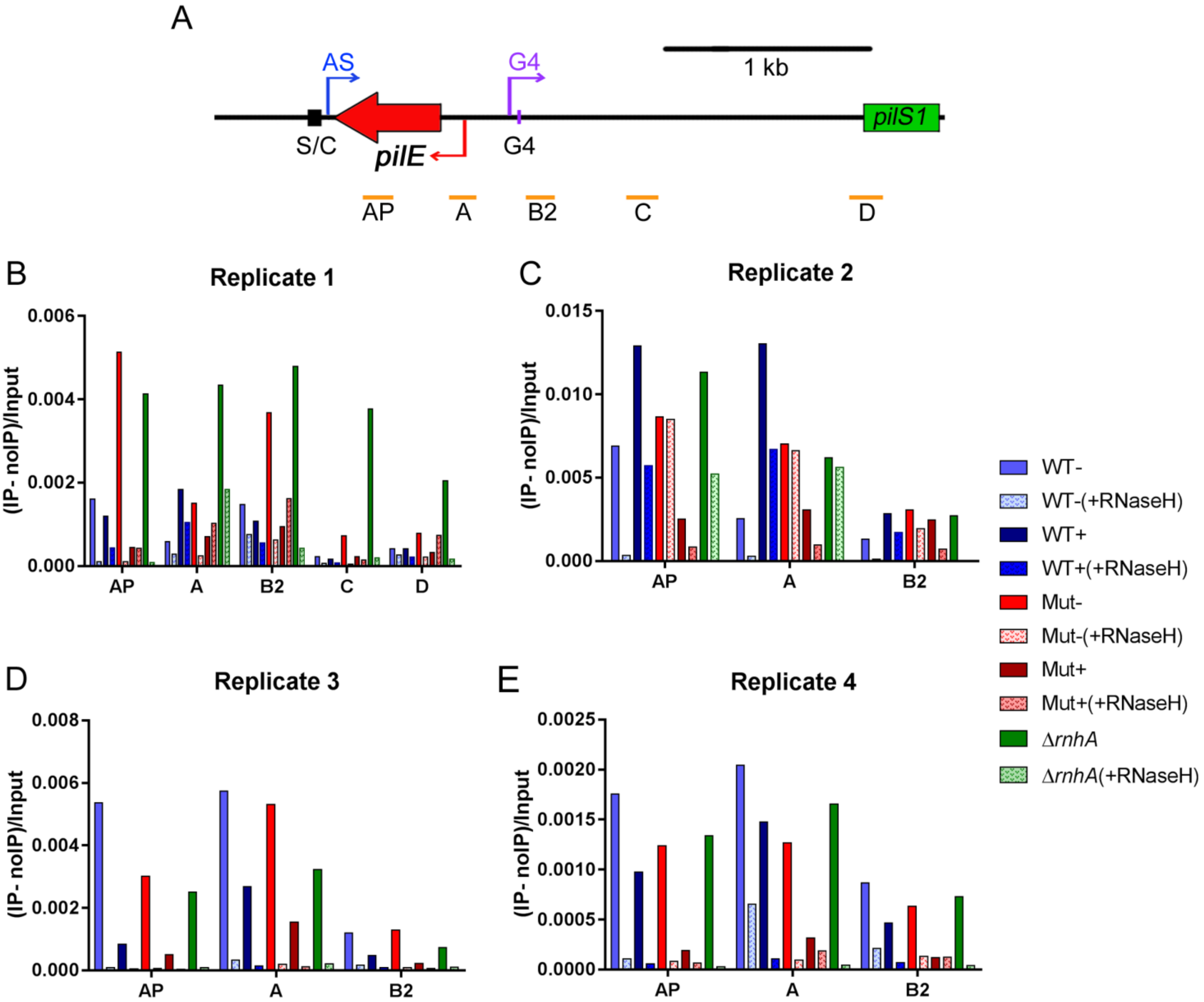






RNA isolated from WT_ery, Mut_ery, G4W, G4M, ASWT_G4M and ASMut_G4M grown in liquid cultures and unstressed (-) or stressed (+) with NaCl (0.5 M, 10 minutes) was analyzed by qRTPCR using the primer pair B2. Assays were performed with (RT+) and without (RT-) reverse transcriptase. The amount of transcript was quantified using standard curves were generated alongside the real-time PCR reaction using serially diluted genomic DNA from *N. meningitidis* 8013 as the template. Three biological replicates were performed; data shown are from a single representative replicate.

Appendix 4 – DIP results from individual replicates



(A) Primer pairs used for qPCR of immunoprecipitated DNA for DIP analysis. (B) – (E) Four biological replicates of DIP analysis of WT_ery, Mut_ery, and WT_ery(Δ*rnhA*) in the presence(+) and absence(-) of NaCl stress, with and without in vitro RNaseH treatment.

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