The Genetics of *Neisseria* Species

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Abstract

Neisseria gonorrhoeae and *Neisseria meningitidis* are closely related organisms that cause the sexually transmitted infection gonorrhea and serious bacterial meningitis and septicemia, respectively. Both species possess multiple mechanisms to alter the expression of surface-exposed proteins through the processes of phase and antigenic variation. This potential for wide variability in surface-exposed structures allows the organisms to always have subpopulations of divergent antigenic types to avoid immune surveillance and to contribute to functional variation. Additionally, the *Neisseria* are naturally competent for DNA transformation, which is their main means of genetic exchange. Although bacteriophages and plasmids are present in this genus, they are not as effective as DNA transformation for horizontal genetic exchange. There are barriers to genetic transfer, such as restriction-modification systems and CRISPR loci, that limit particular types of exchange. These host-restricted pathogens illustrate the rich complexity of genetics that can help define the similarities and differences of closely related organisms.

INTRODUCTION

Members of the genus *Neisseria* are Gram-negative β-proteobacteria, commonly found as commensals on animal and human mucosal surfaces, such as the nasopharynx or the genital epithelium. Most members of the *Neisseria* exist as small diplococci of approximately 0.6–1 microns that can also occur as monococci or, occasionally, tetrads. The two clinically relevant and most studied species are *Neisseria gonorrhoeae* (the gonococcus), the sole causative agent of the sexually transmitted infection gonorrhea, and *Neisseria meningitidis* (the meningococcus), which can cause cerebrospinal meningitis and septicemia. Other human commensal species include *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria cinerea*, *Neisseria subflava*, *Neisseria flavescens*, *Neisseria perflava*, *Neisseria mucosa*, *Neisseria elongata*, and *Neisseria sicca*.

Gonorrhea is the second most prevalent sexually acquired infection in the United States, with more than 300,000 reported cases per year (27) and an estimated 106 million cases worldwide (198). *N. gonorrhoeae* is an exclusively human pathogen that primarily colonizes the urogenital epithelia. Symptomatic infection is characterized by a robust inflammatory response and a purulent discharge composed almost entirely of gonococci and recruited polymorphonuclear leukocytes (PMNs; neutrophils) (36, 149). The purulent exudate from the urogenital tract is usually observed in infected males, but is less often noticed in infected females. Gonococci can ascend to the upper genital tract, leading to serious diseases, such as epididymitis in men and cervicitis, endometriosis, and pelvic inflammatory disease in women (a major cause of infertility). In rare cases, gonococci can disseminate to the blood stream from the initial site of infection, causing disseminated gonococcal infection (DGI) and the associated complications of arthritis and endocarditis (27, 198). *N. gonorrhoeae* can also infect the eyes of newborns as they pass through the birth canal, resulting in ocular gonorrhea, which is a leading cause of infectious blindness in the developing world (202).

The meningococci, first recognized in the nineteenth century, are also restricted to humans. Meningococcal disease affects fewer than 1,000 people per year in the United States but up to tens of thousands of people a year in epidemics around the world, with the highest rates occurring in sub-Saharan Africa (67, 197). Ten to twenty percent of children and young adults are carriers for *N. meningitidis* worldwide, but the majority of those carriers do not experience invasive disease (31, 170). Invasive disease can present as septicemia or meningitis and is a serious infection with 10% mortality (50% if untreated) and neurological problems in up to 20% of survivors (133). Determining why some strains are hyperinvasive and identifying vaccine targets are active areas of meningococcal research.

The human commensal *Neisseria* are also restricted to the human nasopharynx and are closely related to *N. meningitidis* and *N. gonorrhoeae*, although they rarely cause disease in immunocompetent people. *N. lactamica*, the closest relative to the pathogenic species, is often used in studies comparing pathogenic with nonpathogenic *Neisseria*. *N. lactamica* typically colonizes children (55) and may protect against *N. meningitidis* colonization (50). Many of the virulence traits associated with the pathogenic *Neisseria* are also present in the commensal species, such as iron scavenging and immunoreactive surface proteins (101, 159), presumably because they are required for colonization, growth, and survival of the organisms within the human host. Interestingly, some known virulence factors are not absolutely required for virulence. For example, although the capsule is considered a major virulence factor of meningococci, the unencapsulated *N. meningitidis* strain α14 can still cause invasive disease (143). The determination of which genes (or combinations of genes) are responsible for virulence is an active field of investigation.

Like many bacteria, the *Neisseria* contain a repertoire of genetic elements that enable increased genomic plasticity. There are conserved genetic systems in the pathogenic *Neisseria* that likely contribute to host colonization and survival, such as DNA transformation and variation of

displayed antigens. The pathogenic *Neisseria* use their basic recombination and replication machinery to create a system of complex intrastrain diversity that enhances spread and colonization. The pathogenic *Neisseria* have very low linkage disequilibrium (154), a measurement of how closely related chromosomal loci are relative to chance (68), which means there must be extensive recombination between strains in mixed infections. Thus, *N. gonorrhoeae* is essentially nonclonal, whereas *N. meningitidis* shows localized clonality during outbreaks but also does not exist in long-term, clonal lineages.

Although most gonococcal strains still respond to some regime of antibiotic treatment, resistance to currently available antimicrobials is common enough to complicate treatment and multidrug-resistant strains continue to emerge (5, 94, 203). Antibiotic resistance is presently less of an issue in meningococci than in gonococci, but this is likely to change considering the over-prescription of antibiotics that occurs worldwide. There are 13 different meningococcal serogroups, classified by capsule, but disease-causing isolates are primarily types A, B, and C (67, 170). There are highly effective capsular-based vaccines available against meningococcal serotypes A and C (67), and a new multicomponent serotype B vaccine has been approved in many countries but not in the United States (115). There are no known effective vaccine strategies for gonococci. Therefore, studying how the mechanisms of antigenic variation and horizontal gene transfer relate to immune system evasion is of prime importance for the development of novel vaccine strategies.

This review discusses the genome organization of the *Neisseria* and mechanisms of horizontal genetic exchange. The extensive phase and pilin antigenic variation (pilin Av) processes are the second focus of this review, and these systems illustrate a series of genetic processes that have allowed the *Neisseria* to become successful pathogens.

GENOME ORGANIZATION

The *Neisseria* typically contain an ~2.2-Mbp circular chromosome. However, both *N. meningitidis* and *N. gonorrhoeae* are polyploid, containing two to five genome equivalents per growing monococcal cell, with an estimated two chromosomal copies per coccal cell unit (185). However, these organisms are still genetically haploid and cannot be made to carry two different alleles in the same genomic locus (186). Interestingly, the nonpathogenic *N. lactamica* is monoploid (186), leading to the possibility that polyploidy is a virulence trait. In the bacteria *Deinococcus radiodurans*, polyploidy is used to repair radiation-induced chromosomal damage via homologous recombination (37). The presence of extra template DNA could facilitate the processes of antigenic variation and other recombination-mediated gene exchanges in *Neisseria*, but this remains to be directly demonstrated.

Elements and Islands

One of the more salient features of the *Neisseria* is the amount and variety of repetitive DNA sequences present in their genomes (101). Hundreds of copies of repeat elements are present, which play diverse roles, such as serving as phage integration sites or alternative terminators, and these DNA elements can be used as a means to trace strain lineages and divergence.

Repeat elements. The most abundant repeated sequence in neisserial genomes is a nonpalindromic 10-bp sequence known as the DNA uptake sequence (DUS) (56). There are approximately 2,000 10-mer DUSs found in neisserial chromosomes, amounting to approximately one every kilobase of DNA (101), although they are not evenly spaced. The DUSs function in mediating species-specific DNA transformation (discussed below).

A 20-bp sequence called dRS3 (duplicated repeat sequence 3) is the second most abundant repeat, appearing 200 times in *N. gonorrhoeae* (101) and up to 700 times throughout the *N. meningitidis* genome (12, 119, 101). Inverted dRS3 sequences flank different 50–150-bp RS (repeat sequence) elements, together known as a neisserial intergenic mosaic element (NIME) (119). The dRS3 can act as a site for phage integration (12, 85) and has been mapped to points of genome rearrangements (139, 163).

The Correia repeat (CR) is a 26-bp sequence found hundreds of times in the *N. gonorrhoeae* and *N. meningitidis* genomes (21, 33, 34, 99). The CRs often flank a 100–150-bp DNA cassette as terminal inverted repeats, collectively known as a Correia repeat enclosed element (CREE) (99) or a *Neisseria* miniature insertion sequence (NEMIS) (106) (described below).

Insertion sequences, minimal mobile elements, and Correia repeat enclosed elements. Like many bacteria, the *Neisseria* contain insertion sequences (ISs), which are small transposable elements capable of movement within the chromosome. ISs only encode genes for transposition and are usually flanked by inverted repeats. The *Neisseria* species contain on the order of 10–50 ISs in total (101), comparable to other bacterial species. IS elements are at sites of major inversion events in *N. meningitidis* (12, 20, 139) and define differences between *N. gonorrhoeae* strains (163). Different IS element profiles of *N. meningitidis* have been used to track the separation of the meningococci from *N. gonorrhoeae* and *N. lactamica* (139).

Minimal mobile elements (MMEs) demonstrate the genomic flexibility of the *Neisseria*. An MME is a cassette flanked by protein-encoding genes whose contents differ between strains or species. The cassettes can be exchanged and incorporated by homologous recombination in the conserved flanking regions (138, 158). In this manner, a rare insertion event originally independent of homology can spread throughout a population, especially in naturally competent bacteria.

The CREEs are abundant in the meningococci, constituting approximately 2% of the genome (119, 183), but are not as numerous in *N. gonorrhoeae* or *N. lactamica* (42, 101), indicating that changes in these elements followed the evolutionary divergence of the three species. Although not thought to encode a protein product, the CREE contains an IHF (integration host factor) binding site and has features commonly associated with transposons (such as inverted terminal repeats) (21, 42). Inverted Correia elements can potentially act as transcription terminators (53), and, interestingly, the CREEs also contain promoter elements (150, 156, 160). The possibility exists that CREEs could influence the transcription of adjacent genes, but this was not found to be the case for a CREE located adjacent to genes encoding minor pilin proteins (98). The CREEs have also been located at sites of chromosomal inversions between different *N. gonorrhoeae* strains (163), suggesting they provide either regions of homology or an active function for genomic translocations.

The gonococcal genetic island. The gonococcal genetic island (GGI) is an approximately 57-kb DNA element present in 80% of *N. gonorrhoeae* isolates and 17% of *N. meningitidis* strains but is not normally found in commensal *Neisseria* (45, 200). The GGI has a G+C content of 43% compared to the average 52% for *N. gonorrhoeae* and fewer DUSs than average for the *Neisseria* genome, suggesting horizontal acquisition (45, 63, 157). The GGI is integrated at the *dif* site near the terminus of replication, causing a duplication of the *dif* gene into a functional *difA* and a divergent *difB* (46, 157). The action of the XerCD site-specific recombinase can excise the island at low frequencies (63).

The GGI encodes a type IV secretion system (T4SS), containing transfer and secretion genes related to the F factor (45). The T4SS actively secretes single-stranded DNA (ssDNA) into the extracellular environment, (45, 64), providing an efficient substrate for DNA transformation.

There is considerable variability in the GGI, with some T4SS versions of the GGI encoding the AtlA peptidoglycan hydrolase, whereas others contain the peptidoglycan endopeptidase *eppA* gene, and some contain a serum-resistant allele of *traG* (called *sac-4*) (45). The presence of both *atlA* and *sac-4* occurs more often in DGI isolates than in other clinical isolates (45). In *N. gonorrhoeae*, the island also contains genes encoding two DNA methylases, a topoisomerase, a helicase, orthologs to plasmid partitioning proteins, and an ssDNA binding protein (63, 77). The GGIs in *N. meningitidis* are more varied and contain deletions, which may indicate this element is being lost from this species (200). Although structural proteins of the T4SS play a role in TonB-independent iron acquisition of *N. gonorrhoeae*, aiding survival in human epithelial cells (207), the presence of the T4SS does not increase the adhesion and invasiveness of meningococci to human epithelial cells (200). Whether the GGI has other role(s) in neisserial virulence in addition to its role in genetic transfer remains to be determined.

Bacteriophages

Only a handful of double-stranded and M13-like filamentous phages and prophages have been identified in the *Neisseria*, and the lack of lytic or transducing phages precludes the use of bacteriophages for genetic manipulation. However, the ease of transformation has made it unnecessary to use phages to generate knockouts and insertions or to cleanly backcross mutations. Although research on neisserial phages has been slow to yield genetic tools, recently the *N. gonorrhoeae* Ngoϕ6 has been cloned as a phagemid and can function productively in a wide range of Gram-negative species, such as *Escherichia coli*, *Haemophilus influenzae*, *N. sicca*, and *Pseudomonas* species (122).

The first reports of neisserial bacteriophages were in nonpathogenic species, such as *N. flavescens* and *N. perflava* (120, 169, 175). Phage heads and plaques were later detected in culture supernatants of *N. meningitidis* (26), and although plaques were observed in lawns of *N. gonorrhoeae*, no phage particles were identified (24). Genome sequence analyses showed that many phage-related genes are present in neisserial genomes arranged as apparent prophages. Some phage gene clusters in *N. gonorrhoeae* and *N. meningitidis* are bordered by sites for a recombinase called Piv or Irg (85, 152) and others are integrated into the sites of the repeat sequence dRS3 (16). Prophage sequences present at the rearrangement break points suggest a possible active or passive role in recombination (48, 140, 163). However, there still remains little evidence that these prophages are competent to produce bacteriophage particles.

Of the identified phages, there are several examples of Mu-like prophages in the *N. meningitidis* genome. Phage Mu is a transposable element encoding its own transposase as well as more traditional phage genes like those for head, tail, and lytic proteins. Analysis of genomes revealed the Mu-like PMN1 and PMN2 phages from Z2491 (serogroup A) (119), the characterized MuMenB, which encodes membrane-associated antigens, from MC58 (serogroup B) (104), and a phage found in FAM18 (serogroup C) (48). Although Mu-like phages were not found in *N. gonorrhoeae* genomic sequences (104), five different double-stranded DNA (dsDNA) lysogenic phages were identified in strain FA1090 (Ngoϕ1-5), and their repressor genes were shown to be functional in *E. coli* (121). A repressor from Ngoϕ4 has been suggested to affect *N. gonorrhoeae* pathogenesis by regulating the ability of gonococci to adhere and invade human cervical cells (38). Although the dsDNA phages were not able to form plaques in commensal *Neisseria*, lambda-like particles from *N. gonorrhoeae* culture supernatants, presumed to be from the integrated prophages, were visualized by electron microscopy (121).

Both *N. meningitidis* and *N. gonorrhoeae* contain integrated filamentous prophages in their genomes. An 8-kb filamentous phage, known as Nf1 (85), is present in multiple *N. meningitidis* serotypes, some with several copies per strain. This phage contains an MDA (meningococcal

disease-associated) island whose presence is correlated with hyperinvasive phylogenetic groups in adults (15, 16), but a mechanistic basis for this correlation has not been elucidated. A circular double-stranded replicative form and a single-stranded mature form of the DNA were detected at low levels by polymerase chain reaction (PCR), but phage particles have not been demonstrated. Several plasmids isolated from *N. lactamica* and *N. meningitidis* had genes and organization similar to the MDA phage (193), suggesting horizontal gene transfer of MDA between the commensals and pathogenic species. Filamentous phages with similar organization have been reported in *N. gonorrhoeae* (123), but their roles in pathogenicity remain to be determined.

MECHANISMS OF HORIZONTAL GENE TRANSFER

DNA Transformation

DNA transformation is the dedicated process of taking up DNA from the environment and incorporating it, either by recombining it with homologous sequences or, in the case of plasmids, by sometimes establishing a new episome. Bacteria that are capable of transformation are said to be naturally competent, whereas methods to introduce DNA into bacteria like *E. coli* are called artificial transformation. Whereas most bacterial competence is transient and regulated, the *Neisseria* are constitutively able to uptake and incorporate DNA during all growth phases (17, 162), although not all cells in a population readily take up DNA (54a). Natural transformation has many possible benefits for the bacterial cell, such as a means to spread antibiotic-resistance determinants and other beneficial alleles or as a source of nutrients. It has also been suggested that uptake of homologous DNA can provide a template for use in DNA repair (161), although it is surprising that this has never been directly demonstrated.

Because the *Neisseria* are human-specific organisms, homologous DNA would come from other bacteria that colonize humans, with the highest probability being uptake of DNA from the commensal *Neisseria* that inhabit the nasopharynx or from other *N. gonorrhoeae* in the genital tract. *N. gonorrhoeae* may donate DNA to the environment by direct secretion or through cell lysis (62), and both ssDNA and dsDNA can be taken up from the environment (29, 165). The amount of homology between the transforming DNA and the chromosome influences the efficiency of transformation. A minimum of 73 nucleotides of flanking homology are required for inefficient transformation of *N. meningitidis* (5a, 134), but at least 500 nucleotides of flanking homology are required for efficient transformation frequencies (44).

DNA uptake sequence–stimulated transformation. As mentioned above, *Neisseria* genomes contain a nonpalindromic 10-bp DUS (56), which occurs at a frequency of approximately one per kilobase pair. Islands of DNA thought to have been acquired via horizontal gene transfer have fewer DUSs, and DUSs found within coding sequences of genome maintenance genes are over-represented relative to other coding regions (39). In *N. gonorrhoeae*, 76% of the 10-mer DUSs contain an additional two nucleotides, which provide slightly increased transformation efficiency (6). The DUS may also have a regulatory role in transcription, as half of 12-mer DUSs are arranged as inverted repeats at the ends of genes. It is likely that these inverted repeat DUSs function as Rho-independent transcriptional terminators (6, 56, 153), although this remains to be experimentally confirmed.

Although the DUS itself is not the target of a known restriction system, the *Neisseria* show preferential selectivity for the incorporation of DUS-containing DNA. *N. gonorrhoeae* can be inefficiently transformed with non-DUS-containing DNA (19); however, the efficiency of transformation is increased by orders of magnitude when the incoming DNA contains a DUS

(47, 165). Different *Neisseria* species show DUS variations (dialects) that affect the degree to which the DNA is transformed (28, 54); there is also variation in the transformation efficiency of the same piece of DNA between strains (47), and the location and number of DUS, but not orientation, also have mild effects on the transformation efficiency (5a).

Plasmids

Naturally occurring plasmids are found in *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* (193). There is a small 4.2-kb stably maintained plasmid called pJD1 found in 96% of all gonococci (128). Because the plasmid does not contain antibiotic resistance or identifiable virulence genes, it is also known as the cryptic plasmid. Other neisserial species contain cryptic plasmids of different sizes that hybridize to part of pJD1 from *N. gonorrhoeae* (76). The pJD1-encoded *cppB* (cryptic plasmid protein B) gene was used as a PCR target for *N. gonorrhoeae* diagnostics, although other nucleic acid amplification targets proved more specific for diagnosis (181, 188). pJD1 also carries a virulence-associated *vapDX* toxin-antitoxin system (91), of which the VapX antitoxin neutralizes the VapD ribonuclease. Some other antibiotic-resistant conjugative plasmids contain the *vapD* gene without an associated *vapX* gene (116), limiting them to exist only within pJD1-containing *N. gonorrhoeae*. The presence of the *vapDX* toxin-antitoxin system likely provides the selective pressure to maintain pJD1 within *N. gonorrhoeae* populations.

Plasmid-mediated antibiotic resistance. The commensal *Neisseria* are thought to be a reservoir of genetic material for the pathogenic strains, and plasmid transfer has been shown to occur between the pathogenic and commensal species (130, 192, 193). Plasmids are one way to exchange antibiotic-resistance elements and have contributed to β-lactamase (49) and tetracycline resistance in *N. gonorrhoeae* (112), as well as sulfonamide resistance in *N. meningitidis* (51, 129). Tetracycline resistance is mediated by the ubiquitous *tetM* gene and is usually carried on a conjugative plasmid (116) whose backbone can also mobilize the transfer of other plasmids containing other antibiotic-resistance markers (116, 129). Although much of the current resistance of *N. gonorrhoeae* to fluoroquinolones and cephalosporins is carried on the chromosome and not on plasmids, it is likely that resistance to future antibiotics may be acquired and rapidly spread via plasmids. Interestingly, there are fewer plasmid-encoded determinants of antibiotic resistance in the *Neisseria* as compared to enteric bacteria. The reasons for this are unclear, but it is likely that the selection pressures for gut microbes and their accessibility to other bacteria with antibiotic-resistance plasmids differ from those of bacteria that reside in the nasopharynx or genital tract.

Plasmids as genetic tools. Despite the presence of natural plasmids and the ease of transformation, plasmids have not been commonly used as tools for genetic complementation or protein expression in *Neisseria*. Unless there is homology in the *Neisseria* genome, the plasmid is lost following transformation (57). Instead, plasmids are frequently used as tools to construct gene knockouts and to place complementing genes on the *Neisseria* chromosome. The gene of interest is cloned into a vector flanked by regions of chromosomal homology, manipulated in vitro, and then transformed into *Neisseria*. Through double crossover events, the region between the flanking homologous DNA is replaced on the chromosome and the remaining vector is degraded (44). The result is a stable copy of the locus integrated into the chromosome. Thus, this strategy makes it so that constant selective pressure is not required for maintenance. There are some plasmids that have been constructed for laboratory use with neisserial plasmids as backbones: These include shuttle vectors between *N. gonorrhoeae* and either *Escherichia coli* (168) or *Haemophilus* species (117) and the Hermes system for complementation via a conjugative plasmid (92), as well as a vector for

the constitutive expression of green fluorescence protein (GFP) (164). Although not used as often as in *E. coli*, plasmids do have a role in the genetic manipulation of *Neisseria* in the laboratory.

Barriers to Genetic Transfer

Despite the *Neisseria* being naturally competent and having a marked preference for DUS-containing DNA, several barriers exist that reduce the transformation efficiency of DNA from other neisserial species and strains. The DUS dialect from one species corresponds to the ComP receptor of that species, and slight variations in sequence can reduce transformation efficiency (13). Additionally, each neisserial strain has multiple restriction-modification (R-M) systems to prevent entry of incorrectly modified DNA, and it was recently found that *N. meningitidis* has a CRISPR locus that functions against specific spacers.

Restriction-modification. R-M systems cleave foreign DNA that is not protected at a sequence-specific site. There are four main types of R-M systems in bacteria based on the types of subunits, sequence specificity, cleavage position, and cofactor requirements. Type I R-M consists of a three-subunit enzyme, generally encoded by the *hsdM*, *hsdS*, and *hsdR* genes, that recognizes asymmetric bipartite sequences. Type II R-M systems are the simplest and most common. They consist of separate methylation and restriction enzymes that both recognize the same 4–8-bp sequence, usually palindromic. Frequently, these are the enzymes used in laboratory cloning. The Type III R-M systems have a two-subunit enzyme consisting of modification and restriction subunits, and Type IV is a diverse family consisting only of methyl-dependent restriction enzymes (100). Types I–III have all been found in the *Neisseria* (167). Because chromosomal DNA is primarily converted to ssDNA as it enters the cell, it is usually not restricted; however, plasmids enter neisserial cells predominantly as dsDNA (18), making them a target for restriction (5a, 166). Consequently, chromosomal DNA has higher transformation efficiencies than plasmid DNA (62).

Some *N. meningitidis* isolates carry a *dam* gene whose product methylates the adenine of GATC sequences, such as in *E. coli*; however, the *N. meningitidis* Dam activity plays no role in mismatch correction (MMC), origin sequestration, or other regulatory functions attributed to the *E. coli* Dam. *N. meningitidis* strains without the *dam* gene carry an alternative gene called *drg* (for *dam*-replacing gene) at the same genomic locus. *drg* was originally reported to be associated with invasive strains, but a correlation was not observed when a larger sample size was subsequently examined (81). Drg is notable in that it cleaves GATC sequences with methyl groups, an activity opposite to that of Dam. The function(s) of Drg and Dam is not understood nor is the reason why different strains have these opposing allelic gene replacements.

Whereas in most bacterial strains, one or two R-M systems are sufficient, the pathogenic *Neisseria* can have at least fourteen different systems per strain (131, 167). For a genus that is generally receptive to its own DNA, it is unclear why there are so many redundant, strain-specific barriers to genetic transfer. The acquisition and spread of R-M systems have been used to determine phylogenetic clades of *N. meningitidis* (20), suggesting that the *Neisseria* exploit these systems to increase the stability of different strains. Like insertion elements and transposons, R-M systems have been proposed to be selfish genetic elements replicating in genomes solely for their own propagation (89). This does not seem like a sufficient reason for the *Neisseria* to have so many relative to many other bacterial species. Another line of thought is that the R-M systems confer a benefit to the bacterial host, whether as a defense against foreign DNA or as a means to preserve clonal lineages. It may be that there is a middle ground where *Neisseria* easily acquire selfish DNA that is then adapted to benefit the host cell. It is notable that other naturally competent genera, such as *Bacillus*, *Helicobacter*, and *Haemophilus*, also have an abundance of R-M systems (131).

CRISPR. Clustered regularly interspaced short palindromic repeat (CRISPR) loci in bacteria and archaea offer adaptive, sequence-based immunity against incoming DNA such as that found in plasmids and phages (78). These consist of 24–48-bp repeat spacer sequences separated by unique spacers from foreign elements. The locus is flanked by CRISPR-associated proteins (Cas) that assist in the processing and maturation of CRISPR-RNA. Although no CRISPR systems have been identified in *N. gonorrhoeae*, they have been found in *N. lactamica* and six strains of *N. meningitidis* (58, 206). The type II-C CRISPR system of *N. meningitidis* is unique in that each CRISPR repeat carries its own promoter, streamlining the CRISPR RNA maturation pathway by bypassing the requirement for pre-crRNA processing. The NmCas9 protein from the *N. meningitidis* system has recently been adapted for use in RNA-directed genome engineering of human pluripotent stem cells (73), showing promise as a genetic tool.

Given that natural transformation is the major means of horizontal gene transfer in the *Neisseria*, it is interesting to note that the CRISPR system does interfere with transformation of chromosomal DNA (206), in effect limiting the amount of genomic exchange that occurs in this usually receptive organism. It is likely that this CRISPR-mediated restriction of transformation occurs after ssDNA is integrated into a homologous DNA duplex in the cell. Self-targeting spacers, i.e., a spacer sequence that matches chromosomal DNA, are usually found with degenerate CRISPR elements (172); however, sequences that are identical to closely related organisms act to limit horizontal gene transfer. Spacer sequences in *N. meningitidis* match chromosomal sequences found in other *Neisseria* species (206). Perhaps this is a mechanism to maintain the clonality of *N. meningitidis* and other commensal inhabitants of the nasopharynx. *N. gonorrhoeae*, generally isolated in the genitourinary tract, does not often come in contact with other neisserial DNA and is not known to form clonal lineages. Therefore, it is likely that *N. gonorrhoeae* has no need for a CRISPR system to maintain clonality.

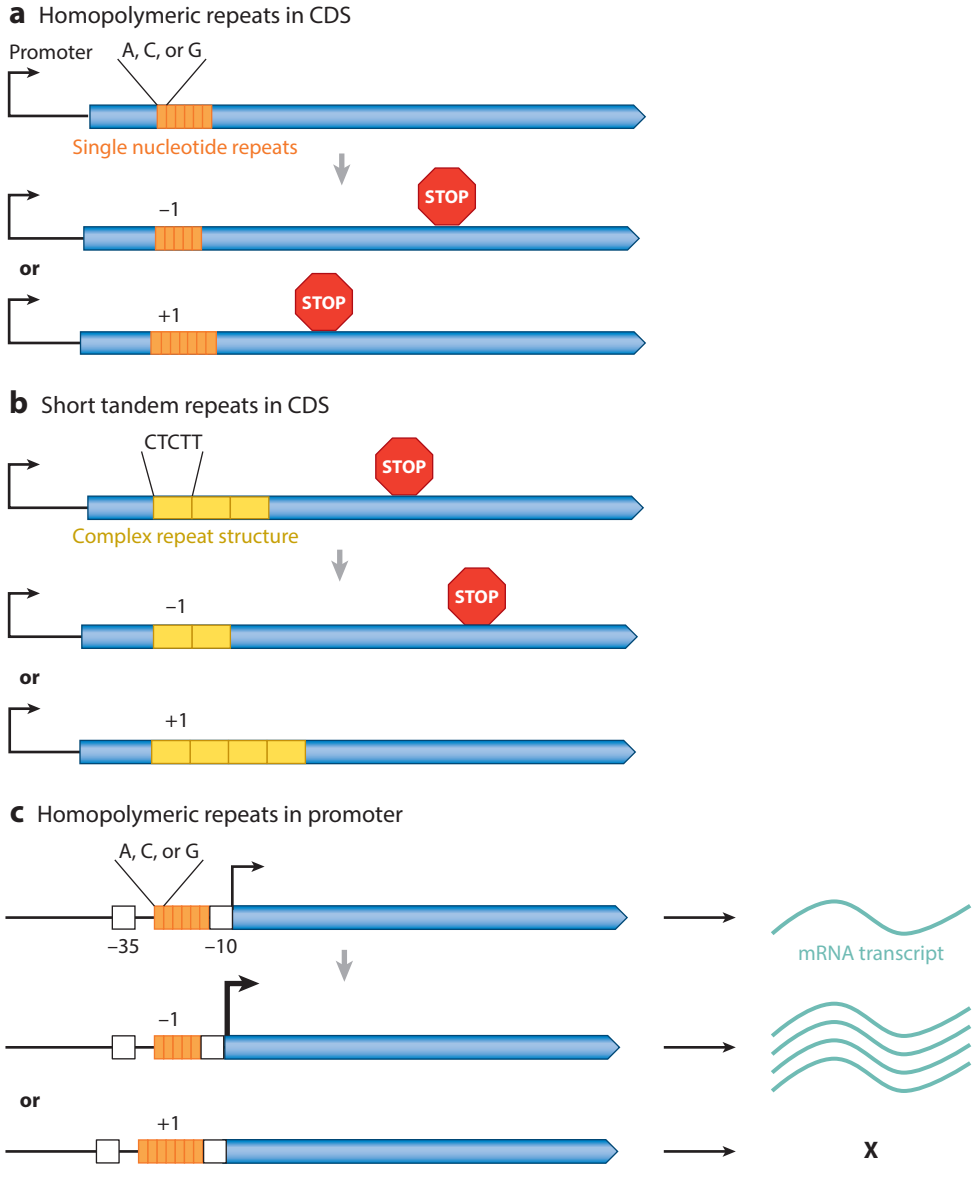
MECHANISMS OF DIVERSITY GENERATION

Antigenic variation is one of the more effective strategies used by pathogens to evade immune surveillance. The variability in surface-exposed structures allows the organisms to always have a subpopulation with divergent antigenic types. By changing the outer surface components, the *Neisseria* can avoid recognition by the adaptive immune system, prolonging a current infection and enabling reinfection to occur. Moreover, these antigenic variation systems can also provide functional variants that alter specific interactions with virulence factors and host molecules. The two major types of antigenic variation in the *Neisseria* are the reversible phase variation of hundreds of genes and the nonreciprocal gene conversion at the *pilE* locus. The variability of the Type IV pili, the lipooligosaccharide (LOS) antigen, and the outer membrane Opa proteins are major advantages to *N. meningitidis* and *N. gonorrhoeae*, facilitating their success in pathogenesis.

Phase Variation

Phase variation is the reversible change between defined expression states of genes, often a switch between an expressed (ON) and unexpressed (OFF) state or a switch between two forms of a gene product (113). Phase variation in bacteria can be mediated by invertible DNA segments, differential methylation, and changes in stretches of homopolymeric nucleotides or short tandem repeats (microsatellites) that alter the repeat number. If the repeat is in the coding region, the change causes a shift in reading frame, leading to the switch between ON and OFF states (**Figure 1*a*,*b***). Most phase variation in the *Neisseria* is mediated by polynucleotide repeat expansion or contraction during replication in a process called slipped-strand mispairing, in which one repeat is paired

with an adjacent repeat on the opposite strand (97), independent of homologous recombination functions (114). Depending on the gene measured, phase variation occurs at a rate between 10^{-2}–10^{-6}/cell/generation (105, 126), and there is a correlation between increased frequency of phase variation and a higher number of repeats (41, 114, 127). Neisserial genomes contain a large number of phase-variable genes; it is estimated that there are more than 100 phase-regulated genes altogether in the pathogenic *Neisseria*, with approximately 80 per strain (84, 137, 155). Many genes identified as virulence factors are phase variable, containing homopolymeric tracts or short tandem repeats. The combination of products from phase-variable genes contributes to the continual change in the expressed repertoire of surface antigens, facilitating immune evasion. Evidence for immune avoidance by phase variation includes the in vitro resistance of *N. meningitidis*



to bactericidal antibodies against the LgtG LOS modifier (10) and against the outer membrane porin PorA (182). However, it should be noted that not all phase-variable genes express surface antigens and they are not all directly involved in pathogenesis.

Phase variation through homopolymeric repeats. Genes with homopolymeric repeats typically contain runs of A, C, or G on the coding strand (**Figure 1*a***; **Table 1**), and there can be up to 17 repeats (127). Examples of genes containing homopolymeric repeats within the reading frame are those that encode for the glycosylation of pilin and LOS (*pgl* and *lgt* genes), the pilus accessory protein *pilC*, and the capsule polysialyltransferase *siaA* (**Table 1**). Polynucleotide repeats can also occur in promoter regions (**Figure 1*c***), where changes in repeat number alter the required spacing of the −10 and −35 sequences to affect transcription efficiency. Examples of this sort of phase variation occur in the *porA* and *opc* genes encoding outer membrane proteins and in the siderophore receptor encoding gene *fetA* (**Table 1**). Some variable genes, such as *pilC*, have been modified in the laboratory to replace every third nucleotide of the homopolymeric tract so as not to be variable (30, 111).

Phase variation of lipooligosaccharides. The LOS moiety is an endotoxin composed of a lipid A anchor in the outer membrane and an oligosaccharide core that is differentially glycosylated. The LOS is immunologically reactive, and there are 12 recognized LOS immunotypes in *Neisseria* (142), which have different sensitivities to human serum. The *lgt* genes of *N. meningitidis* and *N. gonorrhoeae* encode different LOS biosynthesis genes that transfer sugars to the LOS core, and a subset of the *lgt* genes contain homopolymeric repeats in their 5′ ends (**Table 1**). Phase variation allows combinations of possible glycosyltransferases, which produce different LOS structures (79). LOS is one of the main variable antigens of the *Neisseria*, along with Opa proteins and the Type IV pili, which are described below.

Phase variation through polynucleotide repeats. In addition to homopolymeric repeats, there are several types of polynucleotide repeats that contribute to phase variation in the *Neisseria* by using more complex repeat structures of 3–5-bp elements (**Figure 1*b***). These tandem repeats of short nucleotide sequences are less common than homopolymeric tracts (84, 155). Changes in polynucleotide repeat number in a promoter region can alter the binding site for a regulatory protein, such as the binding of IHF to the meningococcal *nadA* gene altering adhesin expression

Figure 1

Types of phase variation. The blue arrow represents the coding region of the gene in the direction of translation. The small black arrow is the promoter. Orange rectangles represent a single nucleotide repeat, and yellow rectangles represent a more complex repeat structure. Wavy blue lines represent mRNA transcript. (*a*) Homopolymeric repeats. The addition or deletion of a single nucleotide changes the reading frame of the gene. In this example, a normally translated gene is shifted out of frame and truncates early. (*b*) Short tandem repeats. The addition or deletion of a short DNA tract changes the reading frame of the gene if the number of nucleotides is not a multiple of three. In this example, the gene is out of frame. Deletion of one pentanucleotide repeat shifts the reading frame but not to the correct one, whereas addition of the repeat restores the proper frame. (*c*) Homopolymeric repeats in the promoter region alter the distance between the −10 and −35 polymerase recognition sites, affecting the degree of binding. In this example, changes to the promoter length alter transcription levels. Addition of one nucleotide causes more efficient transcription, whereas removal of one nucleotide leads to loss of transcription, denoted by X. Abbreviation: CDS, coding sequence.

Table 1 Examples of phase-variable regulated genes in *Neisseria*

Gene	Function	Repeat	Location of repeat	Organism	Reference
pglB	Pilin glycosylation	poly-A	5′ coding region	Nm	(124)
pglG,H	Pilin glycosylation	poly-C	5′ coding region	Nm	(124)
pglA/pgtA	Pilin glycosylation	poly-G	5′ coding region	Nm/Ng	(9, 80)
lgtA,C,D,G	Lipooligosaccharide biosynthesis	poly-G	5′ coding region	Ng, Nm	(79, 204)
pilC	Pilin associated	poly-G	5′ coding region	Ng, Nm	(82)
siaA	Capsule polysialylase	poly-C	5′ coding region	Nm	(65)
porA	Porin	poly-G	Promoter	Nm	(190, 191)
opc	Outer membrane protein	poly-C	Promoter	Nm	(136)
fetA	Siderophore receptor	poly-C	Promoter	Ng	(25)
nadA	Adhesin/invasin	TAAA	Upstream promoter binding	Nm	(102, 108)
opa	Opacity protein	CTCTT	5′ coding region	Ng, Nm, Nl	(173)
lip	Lipoprotein	15 bp	5′ coding region	Ng, Nm	(7, 201)
pilQ	Secretin	24 bp	5′ coding region	Ng, Nm	(187)
dcaC	Cell wall division	108 bp	Coding region	Ng, Nm, Nl	(160)
frpAC	Iron-regulated RTX family protein	27 bp	3′ coding region	Nm	(184)
modA	Type III DNA methylase	AGCC	5′ coding region	Ng, Nm	(164)
modB	Type III DNA methylase	CCCAA	5′ coding region	Ng, Nm	(164)

Abbreviations: Ng, *Neisseria gonorrhoeae*; Nl, *Neisseria lactamica*; Nm, *Neisseria meningitidis*.

(102, 108). One of the well-characterized tandem nucleotide repeats occurs in the *opa* genes that encode the opacity proteins on the surface of the cell (see below).

Neisseria also encode tandem repeats that have multiples of three nucleotides, such as the *lip*, *pilQ*, *dcaC*, and *frpAC* genes (**Table 1**). Although the mechanism of slipped-strand mispairing alters the repeat number, these genes are not subjected to reading-frame shifts. The structural effect on the resulting proteins can be considered another means of diversity generation (83). This mechanism is more similar to the well-known triplet repeat expansion genes common in neurological disorders (110).

Phase and antigenic variation of *opa* genes. The *opa* genes encode 25–30-kDa outer membrane proteins [some of which provide an opaque colony morphology on agar plates (177)], which bind to and signal through a variety of host cell receptors (reviewed in 135). In *N. meningitidis*, there are four *opa* genes (119, 183), whereas *N. gonorrhoeae* can encode up to 11 different *opa*s (14, 43). The *opa* genes each contain adjacent CTCTT repeats in the DNA encoding the signal sequence (**Table 1**) (171). There are from 2 to 20 CTCTT repeats in each *opa* (8, 171), and each gene can independently phase vary ON and OFF using slipped-strand mispairing. Thus, each individual Opa ON/OFF variation is mediated by a phase variation event (which results in the expression of the 11 different variants) and therefore represents an antigenic variation process mediated through phase variation. Additionally, there is a low frequency of recombination between *opa* gene copies that can contribute to strain diversification (3). The promoter strength has been mapped for three *opa*s in *N. gonorrhoeae* and correlated with the frequency of repeat changes (11). However, there is no correlation between orientation or distance of these three *opa* genes from the origin of replication and their expression levels. Strains containing deletions in all *opa* genes have

been created in both *N. meningitidis* (134) and *N. gonorrhoeae* (8, 95), and strains constitutively expressing specific *opa* genes have been constructed (32, 93) to facilitate studies examining the contribution of specific proteins to pathogenesis without the issue of phase variation confounding the results.

Phase-variable regulatory proteins. Phase variation can also have a regulatory function, as seen in the phasevarion. First described in *H. influenzae*, the phasevarion uses a phase-variable DNA methyltransferase to control the expression of other unlinked genes through differential methylation of promoter sequences. In the pathogenic *Neisseria*, the *mod* genes encode a Type III R-M DNA methyltransferase and are phase variable by containing polynucleotide repeat tracts (**Table 1**). Differential methylation by ModA and ModB of promoter regions directly affects expression of dozens of genes, including those that encode virulence factors, such as lactoferrin binding proteins in *N. meningitidis* and genes that mediate oxidative stress resistance in *N. gonorrhoeae* (164).

Impact of mismatch correction on phase variation. Although the genes of the MMC system are not phase regulated themselves, loss-of-function mutations to *mutL* or *mutS* can result in a 10–1,000-fold increase in phase variation (61, 96). Because MutS only recognizes stem-loop structures smaller than 4 bp (118), MMC mutations have an effect on homopolymeric tracts and short tandem repeats but not on the pentamer repeats of the *opa* gene family (103). Disruptions of the MMC pathway also result in a general mutator phenotype because mutations that arise from replication errors are not corrected (141). Although an increase in mutation rate can have a fitness cost for an organism, in the case of the pathogenic *Neisseria*, the benefit of an increase in phase variation may provide other selective advantages in the host (113). In support of this idea, an analysis of 95 serogroup A meningococci demonstrated that 54 had elevated mutation rates, of which 21 were due to defective *mutS* or *mutL* genes (127), a high percentage compared with other pathogenic bacteria (61). It is unclear whether these mutator strains form stable lineages that can transmit from host-to-host or exist only to provide a short term advantage within a host.

Pilin Antigenic Variation

Pilin Av is a representative type of diversity generation that some pathogens use to avoid immune surveillance (194). Although antigenic variation can be mediated by independent phase variation of multiple related genes, such as the LOS biosynthesis genes and the *opa* loci, pilin Av differs from phase variation in that the system promotes changes to a gene that alter the peptide sequence rather than the expression level, resulting in multiple forms of the antigen. The genetic information for producing the different variants is present within the cell, but only one variant is usually expressed at a time. Pilin Av is mediated by a gene conversion process, which is a nonreciprocal transfer of DNA from a donor homolog to a recipient locus without the donor locus being changed in the process. Gene conversion does not define a mechanism but instead refers to nonreciprocal events that occur during a recombination process. Some examples of directed gene conversion systems include the mating-type switch between MATa and MATα cells of the haploid yeast *Saccharomyces cerevisiae*, which incorporates one of two types of MAT genes into an expressed locus (60); the Ig gene diversity in chicken B cells, in which the single light-chain-combined V_λ and J_λ segments generate diversity with donor DNA from 25 nearby pseudo-V_λ genes (125); and the *vls* locus in the Lyme disease bacterium *Borrelia burgdorferi*, which incorporates 1 of 15 silent cassettes into the *vlsE* gene expressing the VlsE surface-exposed lipoprotein (205).

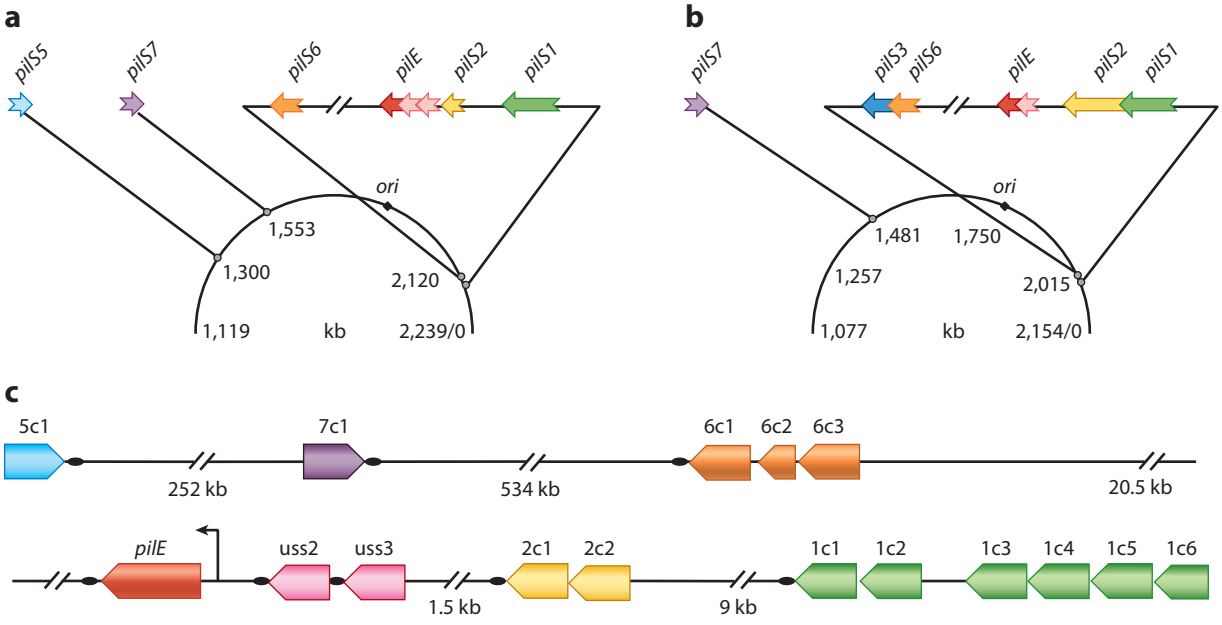


Figure 2

Chromosomal organization of pilin genes in *Neisseria gonorrhoeae*. A representative map of *pilE* and *pilS* loci arranged according to chromosomal position. Colored shapes indicate *pilE* and *pilS* gene copies, with the pointed end showing direction of translation. The coding sequence of *pilS* copies ranges from 300 to 400 bp in length, and the *pilE* coding region is 500 bp in length. The small black arrow in front of *pilE* indicates a functional promoter. The black oval represents the conserved *Sma/Cla* repeat. *ori* is the predicted origin of replication, experimentally determined for FA1090 (185). Numbers indicate chromosomal position in kilobase pairs. (*a*) Position and orientation of pilin loci with respect to the replication origin in strain MS11. (*b*) Position of pilin loci in strain FA1090. The presence and orientation are similar between the strains, except FA1090 does not have *pilS5* but does have a *pilS3* locus, more silent copies within *pilS2*, and only one upstream silent copy (uss). (*c*) Graphic representation of silent copies within each locus and the relative distances between them for strain MS11. The diagrams for MS11 and FA1090 are based on sequence data from GenBank (Accession numbers CP003909.1 and AE004969.1, respectively).

Pilin Av in *Neisseria* is one of the best-studied systems of gene diversification. The type IV pili of *Neisseria* are long filamentous surface structures involved in DNA transformation, twitching motility, and adherence to epithelial cells (162, 176, 199). Pilin (or PilE), the major component of the type IV pilus, is encoded by the chromosomal locus *pilE*. In the gonococci and other *Neisseria*, there are clusters of promoterless, truncated pilin genes scattered around the chromosome in different loci (**Figure 2**). These silent *pilS* copies share significant sequence similarity to the *pilE* gene and act as reservoirs of variant genetic information. *N. gonorrhoeae* has as many as 19 silent copies in 4 or 5 silent loci (66), whereas most meningococci have 4–6 silent copies in 1 locus. Given the number of silent gene copies in *N. gonorrhoeae*, it is surprising that direct recombination between them does not occur more frequently. Although some evidence does point to gross chromosomal rearrangements at *pilS* loci between gonococcal strains (163), the silent copies remain fairly stable within a strain.

Transfer of DNA sequences from a *pilS* copy into *pilE* results in changes to variable regions of the *pilE* gene, without reciprocal changes occurring in the *pilS* copy (59). The variable regions of *pilE* encode exposed regions on the pilus surface that are recognized by antibodies (52). The type of pilin expressed in *N. gonorrhoeae* is called class I, whereas some meningococci and all commensal *Neisseria* express a related class II pilin (4). The class II pilins lack the hypervariable region and they

do not undergo pilin Av (2, 69), although *N. meningitidis* expressing class II pili can still colonize and cause invasive disease. Differences in the exposed part of meningococcal pilin can affect cell signaling in endothelial and epithelial cell types (109).

Measuring pilin antigenic variation. The piliation state of *N. gonorrhoeae* was first described in the middle of the twentieth century as different colony phenotypes that correlated to the ability to infect people (86). Piliated cells form small domed colonies due to pilus-pilus interactions between cells, whereas nonpiliated cells grow faster and spread out to form large flat colonies (180). As colonies are allowed to grow over extended periods of time, a subset of bacteria convert to a nonpiliated phenotype, leading to irregular borders due to differences in growth rate and appearance (145). Serial passage in the laboratory can lead to the conversion of piliated colonies to nonpiliated and back to piliated again (179). In the course of human infection, gonococci continuously change pilin sequences (66, 146).

Factors that affect the frequency of pilin Av can be determined by measuring the appearance of nonpiliated colonies, a subset of the total possible variants, over time. These can be observed as blebs on the edge of a colony or as the percent of nonpiliated cells in a population. A reduction in the number of nonpiliated cells usually corresponds to reduced frequencies of pilin Av. However, nonpiliated variants can also be produced by phase variation of the *pilC* genes (82) or by the nonreversible deletion of the *pilE* gene (178), leading to situations in which the appearance of nonpiliated variants is not an exact measure of pilin Av. Moreover, nonpiliated variants are only produced from a subset of *pilS* copies, which can also result in misleading results. Pilin Av has also been assayed by a variety of Southern blot, PCR, and qRT-PCR approaches (71, 132, 147, 196), but the best way to measure the frequency is through assessing changes in *pilE* sequences via traditional or next-generation sequencing (35, 40). *pilE* sequencing provides an exact frequency (approximately 10% of variation) and the rate of antigenic variation can be calculated if the number of generations is known ($\sim 6.8 \times 10^{-3}$ recombination events per CFU per generation for *N. gonorrhoeae* strain FA1090, variant 1-81-S2) (35). This is one of the highest rates of change reported for a prokaryotic diversity generation system. The median rate reported for *N. gonorrhoeae* strain MS11 (1.7×10^{-3} events/CFU/generation), and *N. meningitidis* MC58 (1.6×10^{-3} events/CFU/generation), is lower but still results in a significant subpopulation of variants ($\sim$5%) (69). These rates are in the same range as the variation systems expressed by eukaryotic pathogens *Trypanosoma brucei* (189) and *Plasmodium falciparum* (72).

Factors required for pilin Av. There are general and specific factors known to be involved with recombination at the *pilE* locus that change the *pilE* sequence while keeping the silent copies intact. General factors include members of the classic set of proteins used in bacterial homologous recombination, including, but not limited to, RecA, RecX, RecO, RecR, RecJ, RecQ, RecG, and RuvABC (71, 90, 107, 144, 145, 151, 174). RecA is the master protein for recombination, catalyzing strand invasion, and annealing, which is enhanced by RecX in *N. gonorrhoeae*. RecO, RecR, RecJ, and RecQ are part of the RecF-like pathway in *Neisseria* (there is no RecF ortholog). The RecF pathway, characterized in *E. coli*, utilizes gapped DNA homologous recombination, and also assists RecA, whereas RecG and RuvABC catalyze resolution of the Holliday junctions created by the recombination events (91a). Inactivation of any of these proteins reduces the frequency of pilin Av, but not all mutants have the same magnitude of pilin Av reduction.

Although most changes to laboratory conditions, such as temperature, oxygen, or carbon source, do not significantly affect Av, the loss of iron in the medium boosted all recombination, including Av (148), suggesting that the iron-starved conditions of the human body activate pilin Av. The molecular basis of this stimulation by iron starvation is unknown.

Sequences required for pilin Av. At the end of each locus is a conserved 65-bp sequence known as the *Sma/Cla* repeat (flanked by the *Sma*I and *Cla*I restriction sites in *N. gonorrhoeae*) (**Figure 2*c***), which may be involved in pilin Av (196). Deletions of this repeat lower the frequency of pilin Av measured by a PCR-based assay, and the repeat has been shown to bind an unknown protein (195). It is presently unclear whether this repeat has any role in pilin Av in addition to providing extended regions of homology.

A genetic screen suggested that a *cis*-acting site was present in the region immediately upstream of *pilE* (87, 145), and subsequent directed mutational analysis identified a 16-nucleotide G-rich sequence in the region that forms a G4 (guanine tetraplex or quadruplex) structure in vitro (22). Individual point mutations at any of the G-C base pairs in this sequence disrupt pilin Av, whereas mutation of the T-A base pairs has no effect. These mutations altered the formation of the G4 structure in vitro, and treatment with *N*-methyl mesoporphyrin IX (NMM), which specifically interacts with G4 structures and not other forms of nucleic acids, inhibited pilin Av. Mutations that disrupted the G4 structure or NMM treatment also inhibited the appearance of nicks at the G4 sequence, whereas mutations in *recJ* and *recQ* enhanced the detection of nicks in a wild-type cell but not in a G4 point mutant (22). The G4 structure is the first genetic element shown to be specific for pilin Av and has led to the suggestion that alternative DNA structures may be involved in other recombination-based diversity generation systems.

Interestingly, there is a small RNA (sRNA) that overlaps with the *pilE* G4 motif that is required for the G4 structure to function during pilin Av (23). Mutations that block transcription of the G4 sRNA also block pilin Av, but expression of the G4 sRNA in an ectopic locus does not complement these promoter mutations. Altering the direction of transcription of the G4 sRNA or the strand it is expressed from still allows expression of the *pilE* gene, but prevents pilin Av from occurring, suggesting that this sRNA, and possibly the G4 structure, can act in only one orientation. It will be interesting to learn how the G4 and sRNA affect pilin genes in other neisserial species compared to the system found in *N. gonorrhoeae*.

Models for pilin Av. Several models have been proposed to explain the unidirectional recombination reactions that lead to pilin Av (reviewed in 70, 88). All models start with a break in the *pilE* sequence and alignment with a *pilS* copy (**Figure 3**). Unwinding at the break allows a 3′ ssDNA end of *pilE* to invade *pilS* at a region of microhomology. In the unequal crossing-over model (**Figure 3*a***), the displaced *pilS* strand becomes a template for the repair at the broken *pilE*. In the successive half-crossing-over model (**Figure 3*c***), one of the single-stranded *pilE* ends forms a direct link (a half-crossover) to the *pilS* in a RecF-like-dependent manner, which the second *pilE* end invades a short distance away for a second half-crossover, destroying the donor DNA.

The hybrid intermediate model (**Figure 3*b***) invokes a several-step recombination process occurring between two sister chromosomes and may be the reason for the diploid nature of the pathogenic *Neisseria*. This model was formulated to explain the detection of a putative recombination intermediate that was detected when pilin Av was blocked by a promoterless *cat* gene inserted into a silent copy (75). Pilin Av is initiated when a single crossover event occurs between a *pilS* copy and *pilE* at a region of microhomology to form a hybrid intermediate (**Figure 3*b***). Because the *pilS* and *pilE* loci are 5–450 kb apart, and deletions between these loci are not normally observed, this event must lead to destruction of the remainder of the donor chromosome, but it has not been directly tested. The creation of the hybrid intermediate is independent of (or requires little) RecA protein (74). The hybrid intermediate was proposed to be targeted for recombination with *pilE* on a different chromosome, as it recombined with a higher efficiency at *pilE* than at the originating *pilS* copy, and this targeting was RecA dependent. The recombination of the hybrid intermediate with *pilE* requires a crossover exchange at an extended region of homology and a

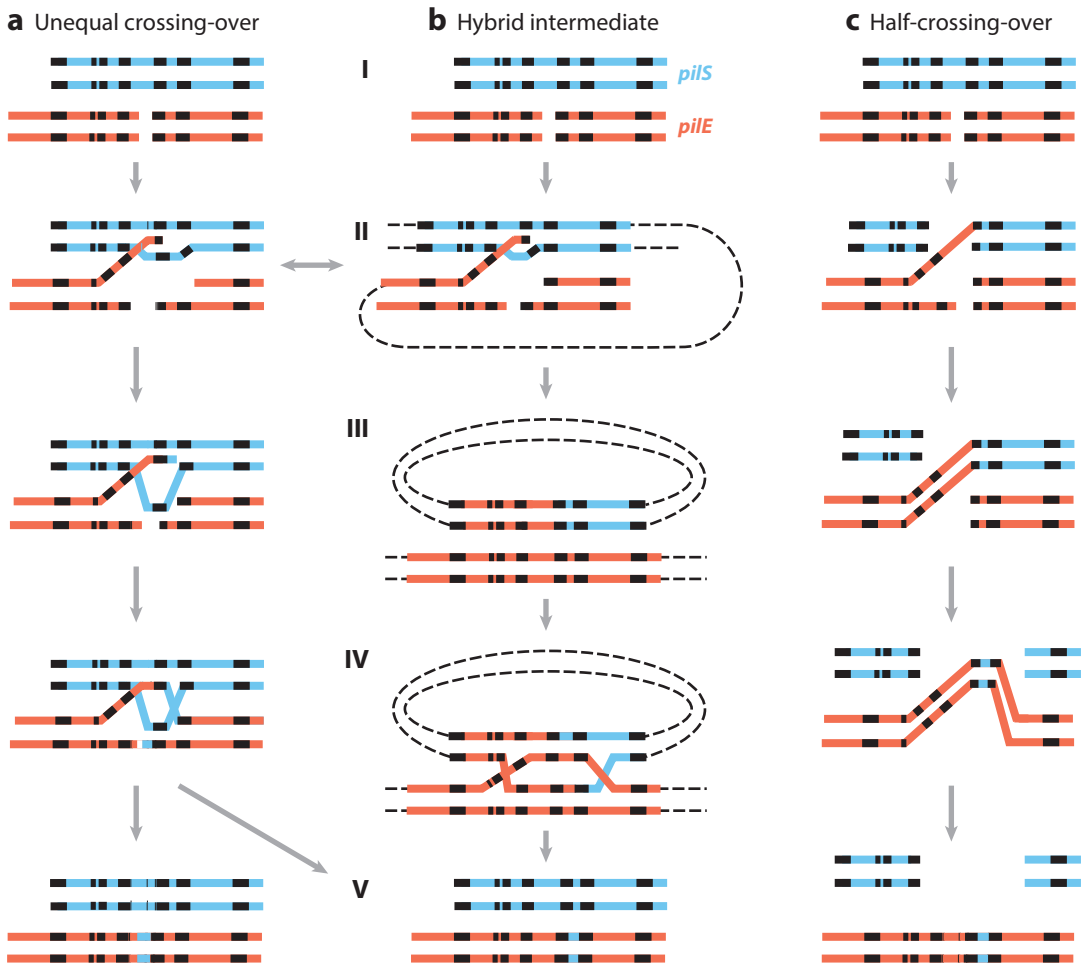


Figure 3

Models of pilin antigenic variation. Each pair of lines represents duplex DNA. Black lines represent regions of homology between *pilE* and *pilS*, and blue and red lines represent sequences unique to *pilS* and *pilE*, respectively. (*a*) The unequal crossing-over model depicts the displaced *pilS* strand acting as a template for repair at the broken *pilE*. After branch migration, the Holliday junctions are resolved into two molecules, with an unchanged pilS and a variant pilE. (*b*) The hybrid intermediate model involves first a recombination event to create a hybrid and two to resolve it. I. The first recombination event takes place intrachromosomally between *pilE* and any of the *pilS* copies within a locus. This could be initiated by a double-stranded break (*shown*) or a single-strand nick that produces a gap (*not shown*). II. A single strand of *pilE* invades the *pilS* locus at one of the regions of microhomology. III. A hybrid intermediate is formed with a crossover at the region of microhomology, with the remainder of the chromosome lost. The size of the intermediate depends on the distance between *pilE* and the *pilS* locus. IV. Two recombination events occur between the hybrid intermediate and the chromosomal copy of *pilE*: one at an extended homology that is RecA dependent and the second at a region of microhomology, which results in complete exchange between the two. Although depicted as two RecA-dependent events, the second recombination event at the microhomology is likely similar to the crossover formed in step III. V. The hybrid is lost, and the chromosome contains an intact *pilS* locus and a variant *pilE* sequence. (*c*) The half-crossing-over model consists of two successive half crossovers with little or no DNA synthesis. The second crossover occurs a short distance away from the first and results in replacement of the *pilS* donor sequence into *pilE* and destruction of the chromosome containing *pilS*. Modified from Reference 88.

second recombination at a region of microhomology (**Figure 3*b***), similar to the initiating event, and produces a variant *pilE* sequence with the original *pilS* locus apparently unchanged (**Figure 3*b***). How recombination at regions of microhomology is achieved is presently unknown but could be mediated by a resection and annealing process. It will be interesting to see how pilin Av compares to mechanisms of antigenic variation in other pathogenic organisms.

SUMMARY POINTS

1. *N. gonorrhoeae* and *N. meningitidis* are the two pathogenic members of an otherwise commensal genus of bacteria. *N. gonorrhoeae* is the sole agent of the sexually transmitted infection gonorrhea, and *N. meningitidis* can cause bacterial meningitis and septicemia.
2. The *Neisseria* genomes contain repeat elements, insertion sequences, and bacteriophages that contribute to genomic rearrangements and differences between species. The genomes also have multiple R-M systems and some species contain a CRISPR system.
3. The *Neisseria* are naturally competent during all phases of growth. DNA containing a 10-bp neisserial DUS is preferentially transformed by *Neisseria*. The DUS is common in neisserial genomes, occurring approximately once every kilobase.
4. Phase variation, or the random switching between the ON and OFF state of genes, occurs by slipped-strand synthesis at polynucleotide tracts and short tandem repeats. More than 100 genes are said to be phase regulated in the *Neisseria*, and many virulence factors are phase variable. There are two major surface antigens, LOS and Opa, that undergo antigenic variation through a multigene phase variation process.
5. Pilin Av is the unidirectional recombination of silent gene copies into the expressed *pilE* locus encoding the surface pili. The high rate of pilin antigenic variation is a means the *Neisseria* use to avoid immune surveillance.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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