Mobile genetic elements in *Neisseria gonorrhoeae*: movement for change

Review

Ana Cehovin[1*] and Steven B Lewis[1]

[1]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

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[*] Dr. Ana Cehovin
Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
Tel.: +44(0)1865275585
Email: ana.cehovin@path.ox.ac.uk

ABSTRACT

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhoeae, possesses several mobile genetic elements (MGEs). The MGEs such as transposable elements mediate intra-chromosomal rearrangements, while plasmids and the gonococcal genetic island are involved in inter-chromosomal gene transfer. Additionally, gonococcal MGEs serve as hotspots for recombination and integration of other genetic elements such as bacteriophages, contribute to gene regulation or spread genes through gonococcal populations by horizontal gene transfer. In this review we summarize the literature on the structure and biology of MGEs and discuss how these genetic elements may play a role in the pathogenesis and spread of antimicrobial resistance in *N. gonorrhoeae*. Although an abundance of information about gonococcal MGEs exists (mainly from whole genome sequencing and bioinformatic analysis), there are still many open questions on how MGEs influence the biology of *N. gonorrhoeae*.

INTRODUCTION

Mobile genetic elements are segments of DNA that can be transferred within and between bacterial genomes (Figure 1). The transfer of DNA between genomes is known as horizontal gene transfer (HGT) and can occur by transformation, conjugation and transduction. HGT is the main driver of evolution and diversification of many species, and can lead to spread of non-core, accessory genes which are not essential for survival and replication but encode small molecules conferring resistance to antibiotics and toxins, allowing bacteria to survive in specific niches (Rankin *et al.*, 2011).

Neisseria gonorrhoeae, or the gonococcus, is the causative agent of the sexually transmitted infection gonorrhoeae. It is closely related to *Neisseria meningitidis*, the meningococcus, which colonises the human nasopharynx and can cause septicemia and meningitis in susceptible individuals (Virji, 2009). The gonococcus and meningococcus are the only pathogenic species of the genus *Nesseria*, which also includes several commensals (Liu *et al.*, 2015). *N. gonorrhoeae* is acquired through sexual contact and establishes infection on the urogenital epithelia, although it can infect other parts of the body such as the conjunctiva, pharynx and rectal mucosa (Edwards & Apicella, 2004). Many virulence factors have been identified which allow the gonococcus to adapt to various host niches (reviewed in (Schielke *et al.*, 2010). Gonorrhoeae is a major public health concern and complications arising from gonococcal disease include infertility, pelvic inflammatory disease, ectopic pregnancy

and neonatal conjunctivitis, which can result in blindness (Unemo *et al.*, 2016). Early detection and treatment of gonorrhoeae is critical to prevent its transmission and long-term complications. However, treatment has become increasingly difficult due to the spread of antimicrobial resistance among gonococci (Unemo *et al.*, 2016), a trait which can be introduced and spread through the transfer of mobile genetic elements or genomic loci (Ochman *et al.*, 2000).

N. gonorrhoeae is naturally competent for DNA uptake and can acquire and incorporate DNA from related (Spratt *et al.*, 1992) and divergent species (Kroll *et al.*, 1998) in a process which is dependent on Type IV pili (Chen & Dubnau, 2004). Uptake of DNA by natural transformation is an important mechanism for transfer of chromosomal loci in the gonococcus, as evidenced, for example, by mosaicism in *penA* (Ameyama *et al.*, 2002) and *por* (Fudyk *et al.*, 1999). These events in *N. gonorrhoeae* have been extensively reviewed recently (Rotman & Seifert, 2014, Obergfell & Seifert, 2015) and will not be covered in this review.

Instead, this review focuses on genetic elements mediating intra- and inter-chromosomal rearrangements. The intra-chromosomal MGEs, such as insertion sequences (IS), Correia repeat enclosed elements (CREEs), minimal mobile elements (MMEs), Neisserial intergenomic mosaic elements (NIMEs) and transposons are sites of major inversion events, influence gene transcription, are hotspots for recombination and, in the case of transposons, carry antimicrobial resistance cassettes. Furthermore, we describe the biology of inter-chromosomal MGEs, including the gonococcal genetic island (GGI), bacteriophages and plasmids, and discuss how these elements can play a role in pathogenesis and the spread of antimicrobial resistance in the gonococcus. Although some of these topics have been covered in other reviews (Snyder *et al.*, 2005, Rotman & Seifert, 2014, Obergfell & Seifert, 2015), none of them has exclusively focused on *N. gonorrhoeae* which is the focus of the present review. The list of MGEs in the gonococcus is increasing steadily with the accumulation of whole genome sequences. However, how these elements impact the biology of *N. gonorrhoeae* is still uncertain and requires further investigation.

Transposable elements

Transposable elements (TEs) are small DNA segments that are capable of ‘jumping’ from one site in the genome to another (McClintock, 1950). TEs can be divided into two main groups, transposons and insertion sequence (IS) elements (Figure 1). Other elements such as Correia repeat enclosed elements (CREE), minimal mobile

elements (MME), the neisserial intergenomic mosaic elements (NIME) and Spencer-Smith repeat enclosed elements (SSREE) are not thought to be mobile but can be transferred between genomes through homologous recombination and can serve as hotspots for recombination. Transposons consist of terminal inverted repeats and an internal region containing genes necessary for transposition (encoding transposases, resolvases) and 'cargo genes' that are not essential for transposition but often confer a selective advantage for the host organism (Siguier *et al.*, 2014). ISs, on the other hand, contain only transposition-related genes within their terminal repeats and do not usually carry cargo genes. However, ISs can mobilise adjacent genetic elements by excising adjacent sequences (Skaar *et al.*, 2005, Jamet *et al.*, 2015). In addition, two identical ISs in close proximity can form a 'composite transposon', capable of excising and reintegrating the entire region between the two IS copies (Gyles & Boerlin, 2014, Siguier *et al.*, 2014). Multiple IS copies within a genome also lead to mobilisation through homologous recombination (Gyles & Boerlin, 2014).
Traditionally, TE insertion into a DNA molecule is regarded as a non-specific process but there are several examples of TEs that undergo site-specific integration (Siguier *et al.*, 2014). Although transposition is generally an intra-genomic phenomenon, TEs can be transferred across genomes indirectly on plasmids or bacteriophages (Siguier *et al.*, 2014). In the case of naturally competent bacteria such as *Neisseria*, inter-genomic transfer of TEs can also occur by transformation (Hamilton & Dillard, 2006). Excision of TEs can result in addition or removal of genes from an organism's repertoire, and insertion of TEs into coding or regulatory regions modulate local gene expression (Casacuberta & Gonzalez, 2013, Siguier *et al.*, 2014). Thus, TEs play a substantial role in shaping the evolution of host genomes.

Transposons

Several antibiotic resistance determinants in *N. gonorrhoeae*, such as bla_{TEM}, which confers resistance to penicillin, and *tetM*, which confers resistance to tetracycline, are resident on transposons on gonococcal plasmids (Figure 1). bla_{TEM} is found within the closely-related transposons Tn*1*, Tn*2* and Tn*3* (Bailey *et al.*, 2011). These transposons are integrated into the gonococcal β-lactamase plasmids and encode the *tnpA* transposase and *tnpR* resolvase (as well as bla_{TEM}) between two 38 bp inverted repeats (Partridge & Hall, 2005). Tn*1-3* share 99% nucleotide identity over most of their sequence but differ significantly (83-88% identity) at the resolvase-binding site (*res*), which is thought to have occurred through the acquisition of short regions from related transposons in a two-step recombination event (Partridge & Hall, 2005). The *tetM* determinant, found on the gonococcal conjugative plasmid, is

located on a 3.2 kb truncated form of Tn*916* (Swartley *et al.*, 1993, Pachulec & van der Does, 2010). This transposon is a member of the family of conjugative transposons or integrative conjugative elements (ICEs), large genetic elements of >16 kb that are capable of self-mobilisation (Scott & Churchward, 1995, Bellanger *et al.*, 2014). Using Tn*916* as a mutagenesis tool to examine its integration, Swartley *et al*. demonstrated that the transposon was capable of inserting into the chromosome of *N. meningitidis* either intact (class I insertion) or with deletions (class II form) (Swartley *et al.*, 1993). Class II insertions resulted in deletions of Tn*916* and integration of a preserved *tetM* determinant, so the authors concluded that *tetM* was most likely introduced into *N. gonorrhoeae* by a class II insertion of Tn*916* into the conjugative plasmid (Swartley *et al.*, 1993).

Insertion sequences

The gonococcal genome contains at least 16 different ISs (Marri *et al.*, 2010). Of these, IS*1016* is the most abundant, with approximately 23 copies present (Davies, 2010, Spencer-Smith *et al.*, 2012). IS*1016* elements can be found at both ends of DNA fragments that appear to have inverted in some strains, suggesting that these ISs are accompanied by large scale chromosomal rearrangements (Spencer-Smith *et al.*, 2012). In addition, truncated copies of IS*1016* are present around the recently described *maf* genomic islands (MGIs). MGIs are horizontally-acquired islands that encode a polymorphic toxin and an immunity protein that protects the host from the effects of the toxin (Jamet *et al.*, 2015). Of the five MGIs that were identified in *N. gonorrhoeae*, three (MGI 3–5) contained IS*1016* elements in one or both of their flanking regions, indicating that these elements are likely responsible for MGI integration into the genome.

Two additional *N. gonorrhoeae* ISs were discovered by *in silico* identification of eight genes related to members of the Piv/MooV family of site-specific recombinases and IS*110*-like transposases (Skaar *et al.*, 2005). These invertase-related genes (*irg*) all contain the catalytic residues DED, which is analogous to the DDE motif found in canonical transposases (Skaar *et al.*, 2005, Siguier *et al.*, 2014). Seven of the *irg* genes (*irg2-6* and *irg8*) are almost identical, whilst the eighth, *irg7*, is distinct but has homology with two genes in the *N. meningitidis* genome (*pivNM-1A* and *pivNM-1B*). Furthermore, the flanking regions of *irg2-6* and *irg8* are almost identical to regions surrounding *irg7*, *pivNM-1A* and *pivNM-1B*. No terminal inverted repeats have been identified around the *irg* genes, which is unusual for IS elements in general but is a common feature of the IS*110* family (Skaar *et al.*, 2005). Thus, these two groups of

homologous loci have been designated as new neisserial IS*110*-like elements, termed IS*Ngo2* (loci with *irg1-6* and *irg8*) and IS*Ngo3* (loci with irg7*, pivNM-1A* and *pivNM-1B*). No Irg transposase homologues have been identified in the commensal *Neisseria* species, indicating that IS*Ngo*s are likely to be specific to pathogenic *Neisseria* spp. (Skaar *et al.*, 2005). Both IS*Ngo*2 and IS*Ngo3* are invariably found at the 3' end of Nf prophages and it has been suggested that these phages were integrated by a unique mechanism involving Irg transposases (Kawai *et al.*, 2005, Skaar *et al.*, 2005). Alignment of all Nf1 copies (containing IS*Ngo3*) found within three *N. meningitidis* and one *N. gonorrhoeae* genomes revealed that the loci are located in the middle of a 20 bp consensus repeat sequence known as dRS3 (Kawai *et al.*, 2005). This suggests that the IS*Ngo3* transposases target dRS3 sequences for integration. However, no discernible consensus sequence or repeat region was found either side of Nf4 (containing IS*Ngo2*), suggesting that IS*Ngo2* transposases may function by a different mechanism (Kawai *et al.*, 2005). IS*Ngo2* has also been found in close proximity to an inverted chromosomal fragment in some gonococcal strains, indicating its potential involvement in chromosomal rearrangements (Skaar *et al.*, 2005, Spencer-Smith *et al.*, 2012).

Correia repeat enclosed elements

The Correia repeat enclosed element (CREE), also known as the *Neisseria* miniature insertion sequence (NEMIS), is a 50 to150 bp sequence consisting of a variable internal fragment flanked by inverted repeats of 26 bp known as the Correia repeat (CR) (Correia *et al.*, 1986, Buisine *et al.*, 2002). The CR is present approximately 250 times in the gonococcal chromosome, of which 122 are associated with CREEs and 132 are present as a single repeat (Marri *et al.*, 2010). Although the structure of the CREE resembles that of an IS, the internal region is non-coding, meaning that the element lacks the machinery for transposition (Buisine *et al.*, 2002). Although active CREE mobilization has never been demonstrated, CREE inversions have been found by repeated passaging of *N. gonorrhoeae* (Elbeyioglu *et al.*, 2017). It has been suggested that CREEs are remnants of ancient TEs that have lost their ability to mobilise, or represent a 'non-autonomous mobile element' that moves through the action of other transposases (De Gregorio *et al.*, 2003, Schoen *et al.*, 2009). There is sequence identity between CREEs and the N-terminus of the IS*1106* transposase gene, indicating that this transposase may be capable of mobilising CREE (Schoen *et al.*, 2009), which contain an integration host factor (IHF) binding site that may facilitate synapse formation during excision (Buisine *et al.*, 2002). Like other IS elements described above, CREEs have been located around sites of chromosomal

inversions (Spencer-Smith *et al.*, 2012). CREEs may also be involved in gene regulation. For example transcription of the operon encoding the MtrCDE efflux pump in *N. meningitidis* can be upregulated through the binding of IHF at the centre of a CREE positioned upstream of the promoter (Rouquette-Loughlin *et al.*, 2004). Gene regulation by CREE also occurs by the presence of consensus promotor sequences associated with CREE positioned 5' of a gene, which may influence the regulation of expression of these genes (Buisine *et al.*, 2002, Snyder *et al.*, 2009, Siddique *et al.*, 2011).

Minimal mobile elements

Minimal mobile elements (MMEs) are DNA segments consisting of two conserved genes flanking a variable region that contains different genes in different strains (Saunders & Snyder, 2002, Snyder *et al.*, 2004). A typical example is the region between *trpE* and *purK.* In *N. gonorrhoeae*, this region contains genes for a restriction modification system, whilst the corresponding region in *N. meningitidis* contains a gene encoding a hypothetical protein, and *N. polysaccharea* contains no ORF at this site (Zhu *et al.*, 1999, Snyder *et al.*, 2007, Davies, 2010). MMEs do not contain transposase-encoding genes or terminal inverted repeats, so are not expected to be mobile. Instead, these elements are likely to be transferred from genome to genome through homologous recombination with conserved flanking genes, with genes initially inserted by a rare insertion event (Snyder *et al.*, 2007). As different genes are present at the same site, these regions may be hot spots for recombination, although the reason for this is unclear (Davies, 2010). Comparative genomics have revealed 39 candidate MME sites in *Neisseria*. Together, these MMEs harbour more than 100 internal cassettes, some of which appear to be non-neisserial in origin (Snyder *et al.*, 2007).

Neisserial intergenomic mosaic elements

The neisserial intergenomic mosaic elements (NIMEs) are loci consisting of terminal dRS3 repeats separated by 19 to 214 bp of repetitive sequences known as repeat sequence (RS) elements (Parkhill *et al.*, 2000, Bentley *et al.*, 2007). NIMEs themselves are often clustered together in large intergenic 'NIME repeat arrays', ranging in size from 200 - 2700 bp (Parkhill *et al.*, 2000). No NIME-specific transposases have been detected within these elements, with RS fragments being ostensibly non-coding. Thus, these loci are likely to be non-mobile but rather serve as hotspots for recombination by virtue of their highly repetitive nature or the presence of specific recombinase target sequences (*e.g.* dRS3, see insertion

sequences section) (Parkhill *et al.*, 2000). Indeed, NIME repeat arrays sometimes contain other mobile elements such as CREEs and ISs (Bentley *et al.*, 2007). Importantly, NIME repeat arrays are often found either side of genes encoding variable cell surface proteins, such as TbpB and LbpB, leading to the hypothesis that these arrays serve to promote antigenic variation of immunogenic proteins by providing the sites necessary for recombination (Bentley *et al.*, 2007).

Spencer-Smith repeat enclosed elements

In a study comparing the genomes of *N. gonorrhoeae* strains, two copies of a new ~650-bp element were found either side of a large chromosomal fragment that had undergone inversion (Spencer-Smith *et al.*, 2012). These so-called Spencer-Smith repeat enclosed elements (SSREE) contain 19 bp terminal inverted repeats and a nearly identical ~610 bp non-coding internal segment. The internal segment contains one or two predicted ORFs, but these do not show homology to transposase genes. Furthermore, the location of SSREE elements does not differ between genomes. Due to their almost identical nucleotide composition, SSREE elements may have facilitated an inversion event in strain FA1090 by serving as a template for homologous recombination (Spencer-Smith *et al.*, 2012). Alternatively, they could act as a recognition sites for IS*1016* elements, two of which are located 30 kb and 100 kb from the 5' and 3' ends of the inverted fragment, respectively (Bentley *et al.*, 2007).

In summary, the advancement of whole genome sequencing and bioinformatics analysis has identified new repeat genetic elements that illustrate the flexibility of *N. gonorrhoeae* genome. Transposable elements provide hotspots for recombination, inversion events and other chromosomal rearrangements. While the diversity of genome organization has been revealed by bioinformatics analyses, the consequences of chromosomal rearrangements on host-pathogen interaction still needs to be understood.

Bacteriophages

Bacteriophages are the most abundant life forms on the planet. Many bacterial genomes contain viral genomes, or phages, which integrate into the chromosome at tRNA genes, intergenic regions and open reading frames (ORFs) (Canchaya *et al.*, 2004). Infecting phages either propagate vigorously and kill their host by a process known as lysis, or integrate into the genome in a quiescent state called lysogeny and become prophages (Fortier & Sekulovic, 2013). Under certain circumstances, the

prophage does not integrate into the bacterial chromosome but rather exists as an episome, a linear or circular plasmid, without inducing the lytic cycle (Los & Wegrzyn, 2012). Phages are classified into two major groups: filamentous ssDNA bacteriophages, and tailed bacteriophages, which package dsDNA (Salmond & Fineran, 2015). Due to their antibacterial activity and the ability to encapsulate DNA in phage particles, phages have been exploited for use in molecular biology and, more recently, in other biotechnological areas such as targeted gene delivery, vaccine administration, peptide expression and phage therapy (Haq *et al.*, 2012). Recently, NgoΦ6, a gonococcal broad-host-range filamentous phage has been cloned as a phagemid, a hybrid cloning vector containing DNA from the filamentous phage and a plasmid, for use in genetic manipulation, such as site-directed mutagenesis and phage display (Greenstein & Brent, 2001, Piekarowicz *et al.*, 2014).

By examining the genome sequence of *N. gonorrhoeae* FA1090, nine genetic islands have been identified which harbor five dsDNA tailed bacteriophages (NgoΦ1 – 5) and four filamentous phages (Piekarowicz *et al.*, 2007). The filamentous phages in the gonococcus share high homology with *N. meningitidis* phage islands that are integrated into a transposase of a small insertion sequence (IS) found on the neisserial chromosome (see section “Transposable elements”) (Bille *et al.*, 2005, Kawai *et al.*, 2005). One of the subtypes of neisserial filamentous phages (Nf1, also called meningococcal disease associated (MDA) island), is present in a truncated form in *N. gonorrhoeae* (Kawai *et al.*, 2005). Compared with meningococcal Nf1, the gonococcal Nf1 lacks genes involved in the production of circular phage DNA, adsorption to and infection of target cells and capsid production (Kawai *et al.*, 2005, Meyer *et al.*, 2016) (Figure 2A). These genes, which are important for the production of replicative form of the phage, its packaging and entry of the phage into a target bacterium (Meyer *et al.*, 2016), are present in other filamentous phages in the gonococcus, such as Nf4 (Figure 2A). Meningococcal Nf1 has been associated with meningococcal invasive disease in young adults (Bille et al., 2008). In contrast, filamentous phages are present in many strains of *N. gonorrhoeae* isolated from different disease manifestations, and no association has been found between gonococcal filamentous phages and the likelihood of causing disseminated gonococcal infection (DGI) (Piekarowicz et al., 2006).

At least five large phage clusters of the tailed bacteriophage islands are found in *N. gonorrhoeae* FA1090 and MS11 (Remmele *et al.*, 2014). Genes of gonococcal tailed phages are modularly organized, with distinct regions encoding structural and

assembly proteins, toxin-antitoxin systems (TAs), and factors involved in phage replication (Piekarowicz *et al.*, 2007). Although the tailed bacteriophages were not able to form plaques in commensal *Neisseria* species, its repressor and holin genes are active in *E. coli*. The phage repressor maintains phage lysogeny by down-regulating the expression of most phage genes while the holin mediates host cell lysis to liberate phage particles. Autoplaquing in *N. gonorrhoeae* has been observed (Campbell *et al.*, 1985) and phage particles have been detected in gonococcal supernatants (Piekarowicz *et al.*, 2007) suggesting that the prophage can be excised from the gonococcal chromosome under certain circumstances and form particles.

Excision of phages from the bacterial chromosome can contribute to horizontal gene transfer between bacteria due to phages accidentally packaging bacterial DNA and delivering it to the neighboring cells, in processes known as generalized or specialized transduction (Salmond & Fineran, 2015). Although any bacterial gene can be transferred by phages, in recent years phages have received much attention because they may contain genes involved in antimicrobial resistance (AMR), including the bla_{TEM} genes, which confer resistance to penicillin (see below) (Modi *et al.*, 2013, Balcazar, 2014, Quiros *et al.*, 2014). The spread of genes involved in AMR can occur either through transduction or by inducing bacterial lysis and release of bacterial DNA, which is then acquired by a neighboring bacterium by transformation (Salmond & Fineran, 2015). Whether phages promote the spread of AMR amongst *N. gonorrhoeae* remains to be determined.

Pathogenic bacteria often contain multiple prophages which encode molecules altering their virulence, such as multiple virulence factors in *Salmonella enterica* serovar Typhi and the Shiga-like toxins in *Escherichia coli* O157 (Canchaya *et al.*, 2004). Environmental stimuli and exogenous agents which oxidize DNA or cause DNA strand breaks, such as reactive oxygen species and antibiotics, can activate prophage genes involved in the lytic cycle (Fortier & Sekulovic, 2013). Additionally, phages may alter bacterial virulence properties by encoding genes involved in adhesion, colonization and invasion into host cells and by increasing resistance to components of the immune system (Wagner & Waldor, 2002). Most prophage genes in the gonococcus are transcriptionally silent under laboratory conditions with the exception of those encoding phage repressor proteins that actively maintain phage lysogeny (Remmele *et al.*, 2014). Prophage genes are differentially regulated in gonococcus under specific conditions (Stohl *et al.*, 2005, Jackson *et al.*, 2010, Isabella & Clark, 2011, Yu *et al.*, 2016). For example, the phage repressor protein

Npr (NGO1013), part of the NgoΦ4 prophage, is iron repressed and regulated by Fur (Ducey *et al.*, 2005, Jackson *et al.*, 2010). Npr regulates the expression of gonococcal genes required for adherence to cervical cells and mucosal colonization (Daou *et al.*, 2013). In anaerobic conditions, 47 gonococcal genes belonging to NgoΦ1, 2, 3 and 5 phage islands are induced and may be involved in biofilm maintenance, such as in *Pseudomonas aeruginosa* (Isabella & Clark, 2011). Exposure of gonococci to oxidative stress, which occurs during gonococcal infection, also upregulates phage genes (Stohl *et al.*, 2005). Accordingly, prophage genes are highly expressed in gonococci from cervical lavage samples from women infected with *N. gonorrhoeae* suggesting that phage proteins are differentially regulated during gonococcal infection (McClure *et al.*, 2015). Many of the phage genes in gonococcus encode proteins with unknown functions and it is not known whether any of these proteins are involved in gonococcal pathogenesis. A recent study has shown that a gene (ORF9), belonging to the gonococcal filamentous phage and with similarity to a toxin from *Vibrio cholerae*, is expressed on the surface of *N. gonorrhoeae* and may be involved in human tissue damage during infection (Piekarowicz *et al.*, 2016); however, this has not been experimentally confirmed.

Although extensive bioinformatic analyses of gonococcal genomes have identified and characterized integrated filamentous and tailed bacteriophages, no evidence exists about the role of phage components in the gonococcal lifestyle or pathogenesis; the function of many phage proteins in the gonococcus remains elusive.

Plasmids

Plasmids are vehicles for the horizontal transfer of genetic material, including antibiotic resistance markers, and can rapidly spread through bacterial populations. In gonococci, plasmids have contributed to the emergence of high-level tetracycline and penicillin resistance (Roberts, 1989). Most plasmids found in gonococci are 4 - 9kb in size, with the exception of the conjugative plasmid which is larger, ranging from 39 to 42 kb.

Non-conjugative plasmids

The first plasmid to be identified in gonococcus was cryptic plasmid pJD1 with no phenotypic traits in the gonococcus (Engelkirk & Schoenhard, 1973). Cryptic plasmid is a 4.2 kb plasmid, which is present in 96% of gonococcal strains (Roberts *et al.*, 1979, Korch *et al.*, 1985) (Figure 2B). In some gonococcal strains, a stable 12.6 kb

plasmid was also found, composed of three directly repeated copies of 4.2 kb cryptic plasmid (Johnson *et al.*, 1983, van Passel *et al.*, 2006). It is possible that the cryptic plasmid could integrate into the chromosome as both the plasmid and the chromosome share repetitive sequences, however, these findings were never confirmed (Sarandopoulos & Davies, 1993).

The cryptic plasmid contains open reading frames encoding a replicase, a toxin-antitoxin (TA) system VapD/VapX, mobilisation proteins, a replicase and four hypothetical proteins, one of which is a DNA-binding protein with a helix-turn-helix (HTH) domain (Figure 2B) (Korch *et al.*, 1985, Sarandopoulos & Davies, 1993, Daines *et al.*, 2004). The VapD/VapX TA system is found in other bacteria, such as *Haemophilus influenzae* (Daines *et al.*, 2004), *Dichelobacter nodosus* (Katz *et al.*, 1994) and *Helicobacter pylori* (Kwon *et al.*, 2012). In *H. influenzae*, four *vap* operons have been identified, all of which show characteristic features of type II TA modules where the toxin activity is inhibited post-translationally by an unstable anti-toxin protein (Daines *et al.*, 2004). VapD functions as a potent ribonuclease, cleaving mRNA and enhancing the survival of *H. influenzae in vivo* (Daines *et al.*, 2004, Ren *et al.*, 2012). Whether VapD functions in a similar manner in the gonococcus remains to be determined.

Cryptic plasmids do not appear to confer a phenotype or virulence in gonococcus despite being widespread (Roberts, 1989). The three promoters identified in the cryptic plasmid can direct transcription in *E. coli* but are not active in *N. gonorrhoeae* under laboratory conditions (Sarandopoulos & Davies, 1993). However, certain conditions may upregulate genes on pJD1, suggesting that the gene products may be important for gonococcus under specific conditions, including iron excess and anaerobic conditions (Sarandopoulos & Davies, 1993, Isabella & Clark, 2011).

The β-lactamase plasmids were first isolated from *N. gonorrhoeae* in 1976 when the gonococcus became increasingly resistant to the then drug of choice penicillin (Dillon & Yeung, 1989). In penicillinase-producing *N. gonorrhoeae* (PPNG) strains, several types of structurally-related β-lactamase plasmids have been identified: Asia/pJD4 (7.4 kb) (Figure 2B), Africa/pJD5 (5.6 kb), Nimes (6.8 kb), New Zealand (9.3 kb), Rio/Toronto (5.1 kb), Johannesburg (4.8 kb) and Australia (3.2 kb) (Pagotto *et al.*, 2000, Muller *et al.*, 2011, Trembizki *et al.*, 2014). All gonococcal β-lactamase plasmids are derivatives of the prototypical Asia plasmid, in which DNA rearrangements have occurred due to the presence of inverted repeats and insertion

sequences (Pagotto *et al.*, 2000). The Asia/pJD4 plasmid has a broad host range and is also found in *E. coli*, *H. influenza* and *Salmonella minnesota* (Dillon & Yeung, 1989). The plasmid contains three distinct origins of replication (*ori1, ori2* and *ori3*), which are thought to be functional in different hosts (Jain & Srivastava, 2013), and two genes encoding replication initiation proteins, which are necessary for either *ori1*, or *ori2* and *ori3*, respectively. The Asia/pJD4 plasmid belongs to the incompatibility (Inc) group W but it also has a silent IncFII replicon which is active solely in the presence of *ori1*. The Africa/pJD5 plasmid, a derivative of Asia-type plasmid, contains only *ori1* which belongs to the IncFII group (Pagotto & Dillon, 2001).

Plasmid-encoded antibiotic resistance is conferred by the bla_{TEM} gene, which encodes an enzyme that hydrolyzes the cyclic amide bond in the β-lactam ring and renders the gonococcus resistant to benzyl penicillin, ampicillin and cephaloridine (Roberts, 1989). The first β-lactamase gene to be identified in gonococcus was $bla_{\text{TEM-1.}}$ Since then more than 220 bla_{TEM} alleles have been identified (NCBI, 2016). One of these alleles, the $bla_{TEM\text{-}135}$, is carried predominantly by the Rio/Toronto plasmid (Muhammad *et al.*, 2014) and it differs from $bla_{TEM\text{-}1}$ by only one nucleotide, resulting in the amino acid substitution, M182T. This mutation increases enzyme stability and further changes to key amino acids could lead to the emergence of stable, extended-spectrum β-lactamase (Orencia *et al.*, 2001) which would render *N. gonorrhoeae* resistant to all cephalosporins (including ceftriaxone).

The gonococcal β-lactamase plasmids do not contain genes required for conjugation but can be transferred to other cells by larger conjugative plasmids in a process called mobilisation. Experimentally, the β-lactamase plasmids have been transferred to other *Neisseria* spp., where they are stable in the absence of antibiotic pressure (Roberts, 1989). The broad host range pJD4 plasmid can be mobilised by a number of conjugative plasmids, including IncP, IncFIV, IncFII and Inclα plasmids from *E. coli* (Dillon & Yeung, 1989). In the gonococcus, mobilisation of smaller plasmids is promoted by the large conjugative plasmids (Figure 2C) (Flett *et al.*, 1981, Roberts & Knapp, 1988). It has been shown that mobilization of pJD4 requires the mobilization protein MobA, enabling the transfer of DNA during conjugation, and origin of transfer *oriT*, which MobA binds to (Rodriguez-Bonano & Torres-Bauza, 2004). In Rio/Toronto β-lactamase plasmids, this mobilization region is missing and their mobilization occurs by co-integration with the conjugative plasmid (Scharbaai-Vazquez *et al.*, 2007).

Conjugative plasmids

The gonococcal conjugative plasmids belong to the IncP1 group (Pachulec & van der Does, 2010), a diverse family of broad host range plasmids which can harbour a variety of antibiotic resistance genes (Popowska & Krawczyk-Balska, 2013). The backbone of gonococcal conjugative plasmids is similar to other IncP1 plasmids and they contain genes for the replication initiation (*ssb* and *trfA*), conjugative transfer (*tra*), mating pair formation (*trb*), and plasmid inheritance and control genes (*kor*, *kle*, *inc* and *kfr*). The plasmid structure is modular with accessory gene elements incorporated in the plasmid backbone between *tra* and *trb* regions. Differences between neisserial conjugative plasmids and other IncP1 plasmids occur in the: i) *traJ*/*nic* region, to which the TraJ protein binds, ii) *trbK* gene encoding the entry exclusion protein which mediates incompatibility (Haase *et al.*, 1996) and iii) the inheritance and control region, which is smaller than in other IncP1 plasmids (Pachulec & van der Does, 2010).

One of the mechanisms for maintaining a plasmid in a bacterial population is post-segregational killing (PSK). PSK is a system based on toxin/antitoxin (TA) loci. Of note, the gonococcal conjugative plasmid lacks characteristic PSK genes typically found in other IncP plasmids (Schluter *et al.*, 2007, Pachulec & van der Does, 2010). Instead, in the neisserial conjugative plasmid, two types of Zeta/Epsilon TAs have been identified which belong to type II TAs (Pachulec & van der Does, 2010) (Figure 2C). Zeta toxin disrupts peptidoglycan synthesis (Mutschler & Meinhart, 2013). Plasmid-encoded Zeta/Epsilon TAs are usually found in Gram positive bacteria and have only recently been identified in Gram negatives. The gonococcal Zeta/Epsilon TA loci lack the transcriptional regulator typically associated with Zeta/Epsilon TAs and therefore the mechanism of their regulation is unknown (Mutschler & Meinhart, 2013).

In addition to the Zeta/Epsilon loci, a third toxin, VapD, is also present in the conjugative plasmid (Figure 2C). However, the associated antitoxin VapX is not present. VapD on conjugative plasmid shares only 30% similarity with VapD on pJD1. It is thought that VapX on the gonococcal cryptic plasmid could function as antitoxin to VapD on the conjugative plasmid (Daines *et al.*, 2004, Pachulec & van der Does, 2010), which could contribute to the maintenance of cryptic plasmid in gonococcal population and determine the spread of conjugative plasmids, including the plasmid harboring the *tetM* resistance marker (see below), in *Neisseria*.

Three types of conjugative plasmid have so far been identified in *N. gonorrhoeae*: pLE2451 (the markerless 39 kb plasmid) and two types of 42 kb plasmids carrying *tetM* determinants. The 42 kb plasmids were first found in 1982 in US clinical isolates with high-level tetracycline resistance (Knapp *et al.*, 1987). The *tetM* determinants are present in a number of species, such as *Streptococcus* spp. (Clewell & Gawron-Burke, 1986), *Clostridium difficile* (Hachler *et al.*, 1987), *Ureaplasma urealyticum* (Roberts & Kenny, 1986), *Neisseria* spp. and other members of Neisseriaceae family (Knapp *et al.*, 1988). Based on restriction enzyme mapping, two types of *tetM*-carrying 42 kb plasmid have been identified: Dutch and American. The two types differ by an insertion sequence which is related to Tn*916* and is located upstream of *tetM* (Turner et al., 1999, Pachulec & van der Does, 2010). Although it has been suggested that both plasmids arose by divergent evolution (Gascoyne *et al.*, 1991), sequence comparisons of large regions of both plasmid types indicate that they evolved from a markerless conjugative plasmid by *tetM* transposition (Pachulec & van der Does, 2010).

Most research on the biology of the gonococcal plasmids was conducted in the 1970s and 1980s. Recently, gonococcal plasmids have gained attention due to epidemiological studies of plasmid-harboring *N. gonorrhoeae* strains and the spread of AMR (Unemo & Shafer, 2014, Unemo *et al.*, 2016). Up to date research into the biology of gonococcal plasmids and their contribution to the pathogenesis of *N. gonorrhoeae* is lacking. A number of genes on gonococcal plasmids encode proteins of unknown function that may be important in the biology of the gonococcus. For example, as discussed, specific conditions may upregulate genes on gonococcal cryptic plasmid, which also encodes genes such as *vapD* that in other bacteria can enhance *in vivo* survival. More research into the role of cryptic plasmid in the biology of *N. gonorrhoeae* is warranted. Similarly, the conjugative plasmid encodes several TA systems whose function is unknown and may contribute to the pathogenesis or maintenance of this plasmid in *N. gonorrhoeae*.

Gonococcal Genetic Island

The gonococcal genetic island (GGI) is a 57 kb genomic island found in approximately 80% of *N. gonorrhoeae* isolates and appears to function as a mobile element (Figure 1). There is clear evidence that the GGI has been horizontally acquired, possessing a lower GC content (44%) compared with the rest of the genome (51%), and short direct repeats in its flanking regions (Dobrindt *et al.*, 2004,

Hamilton *et al.*, 2005). The island is also capable of mobilising in and out of genomes using a host site-specific recombination system and a self-encoded DNA secretion system (Hamilton *et al.*, 2005, Dominguez *et al.*, 2011). Since the discovery of the GGI in *N. gonorrhoeae*, various degenerate forms of the island have been identified in ~20% of *N. meningitidis* strains (Woodhams *et al.*, 2012). In contrast, the island is rarely found in the genomes of commensal *Neisseria* spp. (Dillard & Seifert, 2001, Woodhams *et al.*, 2012), with the exception of one *N. bacilliformis* isolate (Pachulec *et al.*, 2014).

The GGI is always inserted in the same location in the neisserial chromosome, near the *ung* gene at the replication terminus (Hamilton *et al.*, 2005, Snyder *et al.*, 2005). The precise site of insertion is a 28 bp consensus sequence known as a *dif* site, which acts as a recognition sequence for the XerC and XerD recombinases (Hamilton *et al.*, 2005). *In E. coli*, XerCD recombinases resolve chromosomal and plasmid dimers that occur during DNA replication (Carnoy & Roten, 2009). GGI-positive isolates harbour two *dif* sites either side of the island, a consensus site (*difA*) and a second 'degenerate' site (*difB*) that contains four nucleotide mismatches in their XerD binding region, whilst GGI-negative isolates harbor the *difA* sequence only (Hamilton *et al.*, 2005). The *difA* site can mediate insertion of exogenous genetic material into the chromosome, since exposure of a GGI-negative *N. gonorrhoeae* strain to cloned DNA containing a *dif* site and 90 bp of the GGI results in insertion at the *difA* site (Hamilton *et al.*, 2005). This has led to the hypothesis that the GGI utilizes the XerCD site-specific recombination system to integrate into genomes by virtue of *dif*-like sequence (Hamilton *et al.*, 2005, Ramsey *et al.*, 2011). The XerCD system is also exploited by the CTXΦ bacteriophage for insertion into the *Vibrio cholerae* chromosome (Huber & Waldor, 2002). The GGI can also excise from the chromosome at low frequencies in wild-type gonococci in a *xerD*-dependent manner, forming a non-replicative plasmid that can be transferred (Dominguez *et al.*, 2011). Excision may provide a mechanism by which the GGI can be lost from the host genome and which could explain why 20% of gonococcal isolates do not harbour the island (Dominguez *et al.*, 2011). The rate of excision is enhanced by mutating the degenerate *difB* to the consensus *difA* sequence, indicating that mutation of the *difB* stabilizes the GGI within the neisserial chromosome (Dominguez *et al.*, 2011, Ramsey *et al.*, 2011).

The GGI contains at least 63 ORFs, with around 18 genes encoding a type IV secretion system (T4SS) (Hamilton *et al.*, 2005, Pachulec *et al.*, 2014). T4SSs are

structurally related but functionally diverse group and include conjugation systems as well as systems that secrete proteins into eukaryotic cells to facilitate intracellular survival or induce pathogenicity (Wallden *et al.*, 2010). T4SSs can be classified into three subgroups based on similarity to the prototypical conjugative plasmids, IncF (F plasmid), IncP (plasmid RP4), and IncI (plasmid R64) (Lawley *et al.*, 2003). The T4SS locus on the GGI shows similar gene organisation to the transfer region of the IncF plasmid and contains homologues of genes only found in F-like T4SSs (Lawley *et al.*, 2003, Hamilton *et al.*, 2005).

The GGI T4SS contributes to the transfer of DNA from its host to other gonococci. A co-culture assay demonstrated movement of a chromosomal antibiotic resistance marker between two *N. gonorrhoeae* strains (Hamilton *et al.*, 2005). As the marker transferred by the GGI T4SS was located outside of the island, T4SS is capable of secreting non-GGI DNA. Indeed, it is possible that the entire chromosome can be secreted together with the GGI in a single-stranded form when integrated forms of the island are exported through the T4SS (Ramsey *et al.*, 2011). This ability to secrete chromosomal DNA is likely a significant driver of gene transfer in the gonococcus. DNA transfer events appear to involve transformation, since addition of DNase reduced transfer (Hamilton *et al.*, 2005). It should be noted that the MS11 strain used in these secretion studies contains a truncated variant of the gene encoding TraA, the primary constituent of the T4SS pilus (Ramsey *et al.*, 2011), and it has been reported that expression of full-length TraA abolishes secretion into the environment (Llosa *et al.*, 2009). The vast majority of *N. gonorrhoeae* strains contain an intact *traA* gene (Llosa *et al.*, 2009; personal observations).

Since the T4SS locus comprises less than half the sequence of the GGI, the genes located within the remaining region might confer additional roles. The remaining 30 kb region contains 36 predicted ORFs, most of which encode hypothetical proteins (Hamilton *et al.*, 2005). Notable exceptions include ORFs showing homology to DNA methylases (*ydg* and *ydhA*), a helicase (*yea*), partitioning proteins (*parA* and *parB*), a topoisomerase (*topB*), and a single-stranded DNA binding protein (*ssb*) (Hamilton *et al.*, 2005). The genes encoding the two putative partitioning proteins, the topoisomerase and the single-stranded DNA (ssDNA) binding protein form part of a cluster of eight genes that is present at the boundary of several genomic islands in different proteobacteria (Jain *et al.*, 2012). These genomic islands are often integrated conjugative elements containing a T4SS (Jain *et al.*, 2012). The *parA* and *parB* genes from the cluster on the GGI are required for type IV secretion of DNA in

N. gonorrhoeae (Pachulec *et al.*, 2014) and the protein product of *ssb* (SsbB) enhances topoisomerase activity, possibly stabilizing the circular form of the GGI following excision from the chromosome (Jain *et al.*, 2012). Thus, it has been suggested that the conserved cluster may play a wider role in transport and/or stability of genomic islands (Jain *et al.*, 2012).

In addition to its role in DNA transfer, the GGI appears to enhance bacterial survival and pathogenicity. For example, type IV secretion-mediated secretion of ssDNA contributes to the initial stages of biofilm formation (Zweig *et al.*, 2014), which likely protects the gonococcus against antibiotics and the host immune system. In addition, recent analysis of 289 gonococcal isolates revealed an association between the presence of the GGI and multi-drug resistance, suggesting that GGI may promote antibiotic resistance (Harrison *et al.*, 2016). This could be mediated by enhanced spread of resistance determinants by the T4SS (Dillard & Seifert, 2001, Harrison *et al.*, 2016), or by an as yet unidentified mechanism.
Interestingly, the GGI appears to play a role in iron acquisition by gonococci. During investigations into the Ton iron acquisition complex, which is essential for intracellular survival in strains lacking the GGI, it was found that a GGI-positive strain could survive within human epithelial cells following inactivation of the Ton complex, which transfers energy to outer membrane iron transporters for iron uptake (Zola *et al.*, 2010). In contrast, inactivation of the Ton complex in a GGI-negative strain completely abolished intracellular survival (Hagen & Cornelissen, 2006). Ton-independent survival in GGI-positive bacteria relied on the T4SS machinery. It has been suggested that the T4SS may act as a conduit through which iron can enter the cell, or may secrete a factor that stimulates release of sequestered iron (Zola *et al.*, 2010, Ramsey *et al.*, 2011). Finally, a survey of 115 low-passage isolates revealed that certain loci within the GGI are associated with cases of disseminated gonococcal infection (DGI) (Dillard & Seifert, 2001). These loci were the peptidoglycanase-encoding gene *atlA* and a specific allele of the gene for structural T4SS protein TraG, known as the *sac4* allele. The *sac4* allele of *traG* confers serum resistance when introduced into a serum-sensitive strain, although the mechanism is unknown (McShan *et al.*, 1987, Dillard & Seifert, 2001).

The GGI is associated with several functions in the gonococcus, such as transfer of genetic material, enhancement of bacterial survival under certain conditions, such as the presence of antibiotics, and iron sequestration. Although DNA donation involving

661 the T4SS locus on the GGI is well defined, the roles of GGI in neisserial virulence still
662 need to be determined.
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665 **CONCLUSIONS**
666 In this review we present a comprehensive catalogue of MGEs in *N. gonorrhoeae*
667 and discuss their involvement in chromosomal rearrangements, gene regulation,
668 horizontal gene transfer and antibiotic resistance. With the accumulation of data from
669 gonococcal whole genome sequences and advances in bioinformatics, the
670 identification of such genetic elements is becoming more straightforward. However,
671 the key question of understanding how MGEs impact the biology and pathogenesis
672 of the gonococcus is much more challenging. MGEs such as transposable elements,
673 filamentous phages and the GGI have received a lot of attention recently and
674 important discoveries have been made: the finding that the ISs and SSREEs
675 (Spencer-Smith *et al.*, 2012) are involved in chromosomal rearrangements, the
676 discovery of a gonococcal broad-host-range filamentous phage, which encodes
677 proteins expressed on the gonococcal surface (Piekarowicz *et al.*, 2014, Piekarowicz
678 *et al.*, 2016) and the association of the GGI in biofilm formation (Zweig *et al.*, 2014)
679 and antibiotic resistance (Harrison *et al.*, 2016). Additionally, there has been limited
680 recent work on gonococcal plasmids apart from epidemiological studies of
681 gonococcal strains harbouring β-lactamase and *tetM*-containing conjugative plasmids
682 because of their capacity for spreading antimicrobial resistance elements (Fayemiwo
683 *et al.*, 2011, Muhammad *et al.*, 2014, Trembizki *et al.*, 2014, Unemo *et al.*, 2016). In
684 addition, several plasmid gene annotations, in particular for cryptic and β-lactamase
685 plasmids, are largely incomplete. While the structure of the conjugative plasmid has
686 been extensively analysed recently (Pachulec & van der Does, 2010), there are
687 several genes present on this plasmid, including three TA systems, which encode
688 proteins with unknown functions. Analysis of these and other elements should shed
689 light on the adaptation of the gonococcus to its human host, and its evolution into a
690 multi-drug resistant pathogen.
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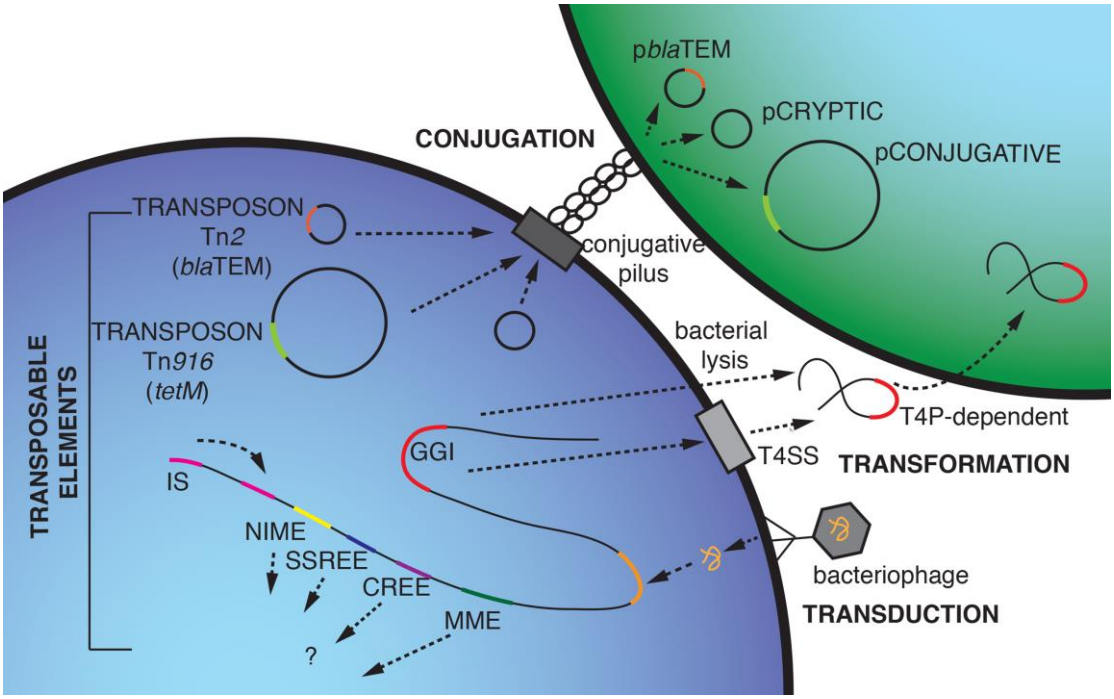
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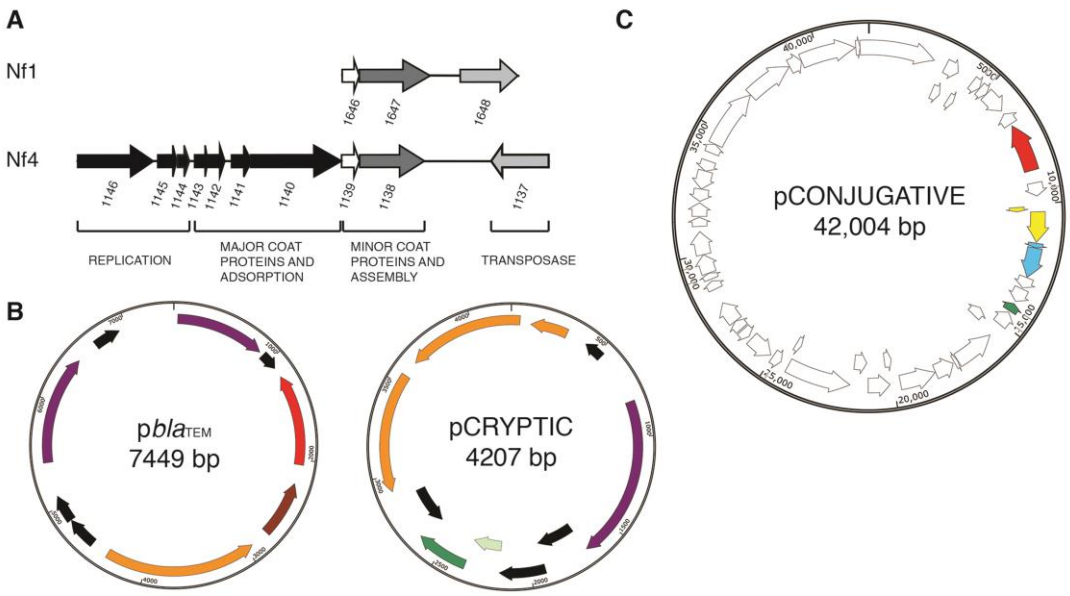
Figure 1.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MINOR COAT PROTEINS AND ASSEMBLY
TRANSPOSASE
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Figure 2.