

Methods in Molecular Biology Introductory Overview

Overview of *Neisseria meningitidis*

Neisseria meningitidis, or the meningococcus, is a Gram negative, non-motile diplococcus that can cause septicaemia and meningitis in susceptible individuals. It is closely related to *N. gonorrhoeae*, or the gonococcus, which is the causative agent of the sexually transmitted infection gonorrhoea. *N. meningitidis* and *N. gonorrhoeae* are the only pathogenic members of the genus *Neisseria*, which includes several commensal species¹. Both *N. meningitidis* and *N. gonorrhoeae* are obligate human pathogens. *N. meningitidis* is acquired through person-to-person contact via aerosols and oral or nasal secretions and is a member of the normal nasopharyngeal microbiome in healthy individuals. Once acquired, the meningococcus may be carried transiently or for a period of several months before carriage is lost. Carriage studies have shown *N. meningitidis* resides in 3-35% of the population depending on geographic location, climate and local disease status².

N. meningitidis is considered an accidental pathogen, as the bacterium only rarely crosses into the bloodstream causing life-threatening diseases such as septicaemia and meningitis. Meningococcal disease occurs endemically as sporadic cases in a community, or in epidemics, such as those observed in the African meningitis belt. The most susceptible individuals are infants under 1 year of age, teenagers and young adults^{3, 4}. Others at risk from meningococcal disease are those with deficiencies in their complement system and asplenia⁴. The onset of disease is rapid and case fatality rates range between 10-20%, despite the availability of antibiotic treatment. Of the survivors, 10-20% develop long-term sequelae, including hearing loss, loss of limbs, skin scarring and neuro-developmental deficits, and up to 36% develop deficits in physical, cognitive or psychological functioning^{5, 6}.

Typing of the meningococcus

Several schemes have been used to type *N. meningitidis*. Meningococcal isolates are typed according to differences in their polysaccharide capsule, lipooligosaccharide and the outer membrane proteins PorB and PorA, defining an isolate's serogroup, immunotype, serotype and serosubtype, respectively. There are thirteen meningococcal serogroups, each with a chemically distinct capsular polysaccharide; of these, six serogroups, A, B, C, W, X and Y, cause disease. The capsules of serogroups B, C, W and Y are composed of polysialic acid or sialic acid linked to glucose or galactose⁷, while the capsules of serogroups A and X are composed of N-acetyl mannosamine-1-phosphate⁸ and N-acetylglucosamine-1-phosphate⁹ respectively.

Sequencing based typing methods for *N. meningitidis* include, multi-locus sequence typing (MLST), ribosomal MLST and core genome MLST. MLST was the first nucleotide sequence based approach for characterising the sequence type of bacterial isolates. This technique uses seven gene fragments to reliably and reproducibly identify major clones, or clonal complexes, within *N. meningitidis* populations¹⁰. The unique sequences for each locus generate an allelic profile, known as a sequence type (ST), which enables the grouping of isolates into clonal complexes. The establishment of clonal complexes has furthered our understanding of *N. meningitidis* epidemiology, by revealing that most disease is caused by temporally stable hyperinvasive lineages that correspond to specific clonal complexes^{11, 12}. Since whole genome sequencing data became quick and relatively inexpensive to collect, MLST has been replaced by core genome MLST. This approach groups isolates into lineages

based on a core set of 1605 genes. Core genome MLST provides higher resolution of an isolates genetic composition in comparison to MLST and has shown that disease isolates are a diverse recombining population from which invasive lineages have independently emerged on several occasions^{13, 14}.

Meningococcal adhesion

N. meningitidis possesses a number of virulence factors that enable adherence to host cells. These adherence molecules can be considered as major or minor adhesins. The major adhesins are Type IV pili (Tfp) the Opa and the Opc proteins¹⁵. The minor adhesins include NadA (*Neisseria* adhesin A)¹⁶, NspA (*Neisserial* surface protein A)¹⁷ and the autotransporters MspA (meningococcal serine protease)¹⁸ and Msf (meningococcal surface trimeric autotransporter fibril)¹⁹.

The human nasopharynx is lined with a columnar epithelium that forms a cellular barrier, which excludes mucosal pathogens. The majority of the cells in the epithelial layer are ciliated, although there are areas of non-ciliated cells, to which *N. meningitidis* preferentially adheres²⁰. Initial adhesion is mediated by Tfp and is followed up by a more intimate adhesion facilitated by proteins such as Opa and Opc²¹. After attachment, *N. meningitidis* can form microcolonies on the surface of the epithelial cells and induce reorganisation of the cell surface. This leads to elongation of the microvilli, which grow to surround the microcolonies, and can ultimately result in engulfment of *N. meningitidis* into endocytic vacuoles²⁰. Penetration of the epithelial layer by the meningococcus occurs via a transcellular route, which is Tfp dependent and does not disrupt the integrity of the epithelium^{20, 22}.

Type IV pili

Tfp are functionally versatile nanomachines that participate in an array of seemingly unrelated functions such as adhesion, motility, protein secretion and DNA uptake²³. Tfp form filamentous hair-like projections that extend out from the surface of the bacterium and mediate the initial contact of meningococci to epithelial and endothelial cells. Assembly of functional pili on the bacterial surface requires a complex machinery composed of over 20 proteins²⁴. The major pilin subunit, PilE, forms a conserved "lollipop" structure composed of an α 1N-helix "stick" and a globular "head". PilE subunits are arranged in a helical conformation to form a coil-like structure, in which the globular domains face outwards and are connected by polar and hydrophobic interactions²⁵. In the latest model of *N. meningitidis* Tfp, the α 1N-helix was observed to form a partially melted structure in the assembled pilus, which accounts for the flexibility and elasticity of the pilus and the ability of Tfp to stretch under force²⁵.

In *Neisseria* spp. Tfp are grouped into two classes based on their reaction (class I) or lack of reaction (class II) with the monoclonal antibody SM1²⁶. The *pilE* subunits of class I and class II pili are located in distinct genomic contexts²⁷. Located downstream of class I *pilE* are several promoterless *pilS* cassettes that can recombine with the 3' end of the *pilE* gene in a process known as gene conversion²⁸. This results in *pilE* genes encoding a conserved N-termini, which is ultimately buried within the pilus filament, and highly variable C-termini, whose globular head groups are exposed on the pilus surface. Conversely, class II pili are conserved and do not undergo antigenic variation, rather class II PilE subunits have up to five glycosylation sites, which are located on the pilus

surface²⁹. These glycans are proposed to enable immune evasion by decreasing antibody access to the pilin polypeptide chain and by undergoing glycan variation, rather than protein variation²⁹.

Tfp are essential for the natural competence of *N. meningitidis* and enable cells to uptake exogenous DNA. Imported DNA can be used as a template for repairing damaged DNA, as a source of nutrition or to generate genetic variation through homologous recombination³⁰. The latter of these accounts for the high level of horizontal gene transfer observed in *Neisseria* spp., which enables the generation of extensive genetic diversity³¹. *Neisseria* spp. typically uptake homotypic DNA, facilitated by the minor pilin component ComP, which selectively recognises and binds genus-specific DNA uptake sequence motifs^{32, 33}.

Opa proteins

Opa (opacity associated) proteins are a family of closely related proteins expressed in the outer membranes of the meningococcus and gonococcus³⁴. Meningococci typically express four Opa proteins whose loci are dispersed throughout the genome and subject to phase variation³⁵. Opa proteins bind CEACAM (carcinoembryonic antigen-related cell adhesion molecule) receptors 1, 3, 5 and 6 via the non-glycosylated face of the Ig N-domain³⁶. Opa proteins have four extracellular loops that link together eight antiparallel β -strands, which form a β -barrel structure, situated in the outer membrane³⁷. Three of these loops contain variable regions and sequence diversity within these regions confers specificity for host receptors³⁶. The recent NMR derived structure of Opa₆₀ shows that these extracellular loops are highly dynamic with little evident secondary structure, which would maximise the number of conformations made by these highly variable regions, enabling them to engage with a variety of host receptors³⁸.

Autotransporters

Autotransporters are a class of proteins secreted by Gram-negative bacteria. They are composed of an N-terminal signal sequence, a central functional passenger domain(s) and a C-terminal translocator domain. The N- and C-terminal domains enable translocation across the inner membrane through the Sec machinery and passage across the outer membrane via the Bam complex, respectively (reviewed in ³⁹). *N. meningitidis* is known to have eight autotransporter proteins, IgA1 protease, MspA, App, Msf, AutA, AutB, NadA and NalP^{18, 40, 41}. Three of these (IgA1, MspA and App) are type Va autotransporters that harbour an S6-family serine endopeptidase domain⁴². NalP has subtilisin-like serine protease activity⁴¹, while Msf and NadA are trimeric autotransporters, whose passenger domains remain attached to the bacterial cell surface, where they act as adhesins⁴³. Msf was previously called *Neisseria* hia homologue A (NhhA) or *Haemophilus* surface fibril (Hsf)¹⁹.

IgA1 protease specifically cleaves the hinge region of human immunoglobulin A, releasing the Fc domains⁴⁴. The central passenger domain of IgA1 protease is composed of three domains, serine-protease, γ -peptide and α -peptide, the latter of which contains a NLS (nuclear localisation sequence), and can bind heparin and DNA^{45, 46}. Autocatalytic cleavage of the passenger domain releases the serine-protease domain⁴⁷, whilst NalP mediated cleavage releases a larger unit composed of the serine-protease and α -peptide domains⁴⁸. However, the size and composition

of the IgA passenger domain released by cleavage can vary between meningococcal strains⁴⁵. In hyperinvasive ST-11 isolates, the NLS in the α -peptide allowed efficient transport of the secreted IgA protease into the nucleus of infected cells, where it cleaved the p65/RelA component of the NF- κ B complex⁴⁹.

Autotransporters App and MspA are both immunogenic and can mediate bacterial–host cell adhesion^{18, 40, 50}. The passenger domains of both proteins are released from the cell surface by auto-catalytic cleavage or NalP protease^{18, 50}. Upon release, these domains specifically bind mannose or transferrin-1 receptors, thereby mediating endocytosis⁴¹. Evidence suggests that once inside the cell, App and MspA passenger domains are trafficked into the nucleus, where they bind core histone proteins and can proteolytically cleave histone H3 in a dose-dependent manner⁵¹. Furthermore, the App and Msp passenger domains can induce cell death via a caspase dependant pathway⁵¹.

AutA and AutB are structurally similar and their expression is often disrupted by various genetic factors in *Neisseria* spp. AutA is found in pathogenic and commensal *Neisseria* spp., whilst AutB is only found in the meningococcus and gonococcus^{52, 53}. AutA and AutB are both subject to phase variation and AutB is predominantly in the phase-OFF state. AutA and AutB are both secreted and exposed on the cell surface, where AutA induces auto-aggregation, potentially influencing the formation of biofilms⁵³ and AutB promotes biofilm formation and impedes the transit of the meningococcus through epithelial cell layers⁵².

NalP contains a C-terminal lipobox at the end of the autotransporter signal sequence. This lipid moiety retards the release of the NalP central passenger domain, temporarily retaining the protein at the cell surface⁴⁸. This enables NalP to cleave targets on the surface of *N. meningitidis* including IgA protease⁴⁷, App⁴⁷, AusI (autotransporter serine protease)⁵⁴, NHBA⁵⁵ and LbpB (lactoferrin binding protein B)⁴⁷. In addition to bacterial targets, NalP has been reported to cleave human complement protein C3 into C3a-like and C3b-like fragments⁵⁶, which potentially promotes the survival of *N. meningitidis* in human blood⁵⁷.

The trimeric autotransporters Msf and NadA were both identified in a genome-based approach aimed at identifying surface-exposed meningococcal proteins able to induce protective immunity against the meningococcus⁵⁸. Both Msf and NadA produce bactericidal antibodies and are recognised by sera from patients convalescing after meningococcal disease⁵⁹. Msf interacts with the extracellular matrix components heparin sulphate, laminin and vitronectin, and is essential for the colonisation of nasal mucosa in a mouse model of meningococcal disease^{19, 43}. Exposure of macrophages to Msf results in an increased rate of caspase dependent apoptosis⁶⁰. Furthermore, Msf promotes macrophage differentiation towards a CD200R^{hi} immune homeostatic phenotype, which *in vivo* can alleviate inflammatory responses stimulated by meningococci, enhancing bacterial colonisation⁶¹. Msf is present in the majority of meningococcal isolates⁶², whilst NadA is present in only ~30% of *N. meningitidis* isolates, is subject to both phase and antigenic variation and its level of expression varies significantly among isolates^{63–65}. The structure of the NadA5 ectodomain, the part of NadA5 that extends into the extracellular space,

revealed it forms a trimer composed of a novel coiled-coil fold topped by an unusual N-terminal head domain, formed from three protruding wing-like structures⁶⁶. Transmission electron microscopy imaging of the NadA3 variant in Bexsero revealed how the coiled-coil stalk could undergo bending, which could confer functional advantages⁶⁶.

Phase variation

Phase variation is the ON and OFF switching of gene expression, typically mediated by mutations in simple tandem repeats or homopolymeric nucleotide tracts situated upstream or in the open reading frame of genes. Mutations in repeat regions are commonly mediated by slipped-strand mispairing during DNA replication, resulting in the deletion or inclusion of more sequence repeats⁶⁷. Ultimately mutations in intergenic regions can influence transcription by altering the spacing of the promoter elements, while mutations in open reading frames can result in frame shift mutations. *N. meningitidis* disease isolate MC58 contains 82 candidate phase variable genes, each of which can switch on or off to creating a pool of genetically indistinguishable individuals that express a mosaic of phenotypes⁶⁸. The commonly held hypothesis is that phase variation allows *N. meningitidis* populations to survive changes in the host micro-environment, as switching between different expression states could aid immune evasion or promote association with specific host tissues. *N. meningitidis* genes that are phase variable include the Opa proteins, the haemoglobin receptor HmbR, O-linked protein glycosylation enzymes, DNA methyltransferases and the outer-membrane porin PorA^{35, 54, 69-72}.

Free iron is rarely available the human host, as it is sequestered by iron binding proteins such as transferrin, ferritin, lactoferrin, haemoglobin and haptoglobin-haemoglobin complexes. The meningococcus acquires iron from host iron-binding proteins during host colonisation and pathogenesis. HmbR and HpuAB are *Neisserial* TonB dependent iron acquisition systems, which bind haemoglobin and both haemoglobin and haptoglobin-haemoglobin, respectively^{73, 74}. HmbR and HpuA are both subject to phase variation and contain homopolymeric G tracts within their open reading frames⁷⁰. Generally HmbR is over-represented and HpuAB is under-represented in disease isolates, with the latter often lost by complete deletion or replacement with an insertion element. Analysis of phase variation status revealed one or both receptors were in an ON state in 91% of disease and 71% of carriage isolates⁷². A separate study that compared six isolates undistinguishable by conventional typing found HmbR was in an ON state in the invasive disease isolates, but in an OFF state in the carriage isolates⁷⁵. These data suggest that HmbR is important for systemic spread of the meningococcus and their surface-expression and association with disease suggest that these receptors could be potential vaccine targets⁷⁶. However, a recent study that tested polyclonal sera raised against HmbR, HpuA and HpuB in the serum bactericidal assay found none of the sera was bactericidal, suggesting immune responses against these receptors may not be protective⁷⁷.

Neisseria spp. encode a variety of glycosyltransferases and can synthesise more than thirty different glycoforms⁷⁸. Glycan tags are covalently attached via an O-link to several surface exposed and periplasmic proteins. In *N. meningitidis*, five protein glycosylation genes contain simple sequence repeat regions, four of which encode glycosyltransferases and one of which encodes an acetyltransferase⁷⁸. Whole genome sequencing of paired carriage isolates taken 6-9 weeks apart from 50 asymptomatic individuals revealed extensive phase variation in one or more glycosylation

locus^{78, 79}. Differential expression of these glycosyltransferase genes resulted in high levels of glycan microheterogeneity, in which the same proteins were decorated with different glycans⁷⁸.

Type III restriction modification systems are two subunit enzymes consisting of a DNA-methyltransferase, encoded by a *mod* gene, and a restriction endonuclease, encoded by a *res* gene⁷¹. *N. meningitidis* contains three phase variable DNA methyltransferases, ModA, ModB and ModD, which contain repetitive DNA tracts within their open reading frames^{80, 81}. Phase variation of *mod* genes, leads to differential expression of the methyltransferases resulting in bacterial populations with distinct patterns of DNA methylation, which in turn leads to altered gene expression driven by epigenetic regulation. In the case of *modA11* switching to an OFF state leads to a four-fold reduced susceptibility to the antibiotics ceftazidime and ciprofloxacin⁸². Phase variation resulting from *mod* gene ON and OFF switching is referred to as the phasevarion⁷¹. Single-molecule real-time (SMRT) DNA sequencing⁸³ has been employed to identify the consensus methylation sequences of three meningococcal DNA-methyltransferases, *modA11*, *modA12* and *modD1*, which methylate adenine residues at CGY^{m6}AG, AC^{m6}ACC and CC^{m6}AGC respectively⁸⁴.

Meningococcal vaccines

Back in 1907 Davis made the first attempt to generate a meningococcal vaccine, which was prepared from heat killed *N. meningitidis* cultures isolated from the patients own cerebrospinal fluid⁸⁵. It was unclear if these first vaccination attempts were successful, as one patient died, whilst the other survived. Almost thirty years later during the 1931 meningococcal outbreak in Northern Sudan, Riding and Corkill prepared whole cell vaccines obtained from the lysates of 9 different isolates. However, this whole cell vaccine had no discernible protective effect⁸⁶.

The late 1960s saw the introduction of polysaccharide vaccines by Gotschlich and colleagues, which elicited bactericidal antibodies in human volunteers, but produced a short lived, T-cell independent response^{87, 88}. Plain capsular polysaccharide vaccines were improved upon in the 1980s with the advent of glycoconjugate vaccines. These coupled the immunogenic polysaccharide capsule to carrier proteins such as the tetanus and diphtheria toxoids. The resulting glycoconjugates activate a longer lived T-cell dependent response, which generates plasma and memory B cells^{89, 90}. In November 1999, the UK became the first country to introduce a meningococcal serogroup C national immunisation program⁹¹. Although the serogroup C vaccine had a short lived effect in infants (less than one year), this was offset by herd immunity, which reduced serogroup C carriage and disease incidence⁹². Between 1999 and 2001, serogroup C cases in the UK decreased by 86.7%⁹¹. Now glycoconjugate vaccines are available for five (A, C, W, X and Y) of the six disease causing serogroups.

Unlike the other disease causing serogroups, the polysaccharide capsule of serogroup B is poorly immunogenic⁹³. Therefore, alternative vaccination strategies were sought for protection against serogroup B disease. In the 1970's Gotschlich and colleagues found outer membrane vesicles (OMV) could elicit protective antibodies⁹⁴, which led to the era of OMV vaccines. OMVs are continually formed by Gram-negative bacteria and are essentially blebs from the bacterial outer-membrane with a lumen that contains various periplasmic constituents. OMVs were prepared in response to strain specific serogroup B outbreaks in Cuba⁹⁵, Norway⁹⁶ and New Zealand^{97, 98}. The dominant antigen in OMVs is the outer membrane porin PorA, which is antigenically variable. Thus the major drawback of OMV vaccines is that they provide limited cross-protection and are largely PorA variant specific. To expand the coverage of serogroup B OMVs HexaMen and NonaMen were developed⁹⁹.

¹⁰⁰. These are prepared from two or three *N. meningitidis* strains each engineered to express three different PorA variants. At the time NonaMen was predicted to provide coverage for over 75% of global serogroup B strains⁹⁹.

The early genomic era saw the advent of reverse vaccinology, which identified serogroup B vaccine candidates from a whole genome sequence⁵⁸. This approach identified 570 open reading frames predicted to express surface exposed or exported proteins, 350 of these were successfully expressed and these candidates were whittled down to NadA, NBHA and fHbp (factor H binding protein), the three protein components in the licensed meningococcal vaccine Bexsero¹⁰¹. Concurrently, using a classical biochemical fractionation approach, fHbp was identified as a key vaccine antigen and is now a component of the licensed meningococcal vaccine Trumenba^{102, 103}. Today, Bexsero and Trumenba are the only two commercial subunit vaccines that offer a degree of protection against serogroup B meningococcal disease. Both vaccines contain fHbp; Trumenba solely consists of two lipidated fHbp peptides, V1.55 (B01) and V3.45 (A05)¹⁰⁴ while Bexsero is composed of the fusion proteins GNA2091-fHbp (V1.1, B24) and NHBA-GNA1030, a NadA trimer and an OMV component derived from *N. meningitidis* serogroup B strain NZ98/254¹⁰¹. Bexsero was approved by the European Medicines Agency in January 2013. Today, Bexsero is approved for persons 10-25 years of age in the United States and for persons over 2 months of age in the European Union, whilst Trumenba is approved for persons 10-25 years of age in the United States and Canada and for persons >10 years of age in the European Union and Australia.

Next generation vaccines for improved protection against serogroup B disease have also been developed. These include chimeric vaccine antigens, which are composed of an fHbp scaffold onto which an immunogenic PorA moiety is grafted¹⁰⁵; OMVs with over-expressed fHbp proteins¹⁰⁶; a subunit opa vaccine, composed of 14 different opa variants¹⁰⁷ and an NMB0315 DNA vaccine, which expresses the outer-membrane protein NMB0315 inside host cells¹⁰⁸.

Meningococcal disease in Africa

The unique geography and climate of Africa, has led to the emergence of the African meningitis belt; a region that extends from Senegal in the west to Ethiopia in the east, where large epidemics of meningococcal disease occur every eight to twelve years during the dry season. Traditionally, these epidemics were caused by serogroup A, which accounted for over 90% of cases¹⁰⁹. This prompted the development of an affordable serogroup A vaccine for Africa. A partnership between the World Health Organisation and Program for Appropriate Technology in Health funded by the Bill and Melinda Gates Foundation led to the development of MenAfriVac, a serogroup A conjugate vaccine. Between 2010-2017 over 270 million doses of MenAfriVac¹¹⁰ were received by local populations in the meningitis belt. Following immunisation, cases of serogroup A meningococcal disease declined dramatically, driven by the direct effects of vaccination and herd protection generated by eliminating of serogroup A carriage^{109, 110}. By 2016-2017 serogroup A was responsible for only 0.8% of invasive meningococcal disease cases in immunised regions¹¹¹. Carriage studies in Burkina Faso and Chad performed following immunisations with MenAfriVac found serogroup A carriage dropped from 0.39% to undetectable levels, and from 0.75% to 0.02%, respectively¹¹².

Following the successful reduction of serogroup A, other disease causing serogroups have emerged. Cases caused by serogroups C, W and X were reported in Nigeria, Niger and Burkina Faso. In Niger, the serogroup C epidemic in 2015 had over 8,000 reported cases, which then spread to Nigeria

causing the 2017 epidemic in which there were over 10,000 cases. In Burkina Faso, serogroup X cases have occurred persistently since 2011 and serogroup W still causes endemic invasive meningococcal disease and occasional epidemics^{110, 113}. In Morocco, 95% of invasive disease isolates collected between 2011-2016 belonged to serogroup B¹¹⁴. The incidence of other meningococcal serogroups in Africa highlights the importance of developing and introducing a six serogroup vaccine.

A pentavalent ACWXY conjugate vaccine, developed by the Serum Institute of India, has successfully undergone Phase I clinical trials. Phase II trials are currently underway to evaluate its safety and immunogenicity in children between 12-16 months of age¹¹⁵. Although this vaccine will cover the five disease causing serogroups in the African meningitis belt, the prevalence of serogroup B in North Africa suggests that future vaccination strategies should also include protection against this serogroup.

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