Neisseria meningitidis: using genomics to understand diversity, evolution and pathogenesis

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Abstract | Meningococcal disease remains an important cause of morbidity and death worldwide despite the development and increasing implementation of effective vaccines. Elimination of the disease is hampered by the enormous diversity and antigenic variability of the causative agent, *Neisseria meningitidis*, one of the most variable bacteria in nature. These features are attained mainly through high rates of horizontal gene transfer and alteration of protein expression through phase variation. The recent availability of whole-genome sequencing (WGS) of large-scale collections of *N. meningitidis* isolates from various origins, databases to facilitate storage and sharing of WGS data and the concomitant development of effective bioinformatics tools have led to a much more thorough understanding of the diversity of the species, its evolution and population structure and how virulent traits may emerge. Implementation of WGS is already contributing to enhanced epidemiological surveillance and is essential to ascertain the impact of vaccination strategies. This Review summarizes the recent advances provided by WGS studies in our understanding of the biology of *N. meningitidis* and the epidemiology of meningococcal disease.

Meninges
The membranes surrounding the brain and spinal cord.

Fulminant
Coming on suddenly and with great severity.

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Neisseria meningitidis (the meningococcus) is a Gram-negative, extracellular bacterium that asymptomatically colonizes the mucosal surface of the oropharynx of ~10% of the human population[1] and is transmitted between individuals through inhalation of respiratory secretions and saliva during close contact with a carrier (FIG. 1). Invasive meningococcal disease (IMD) is a relatively rare event that occurs when the bacterium traverses the mucosal epithelium and invades the bloodstream. As observed for *Haemophilus influenzae* and *Streptococcus pneumoniae*, the meningococcus may cross the blood–brain barrier and disseminate to the meninges. Systemic infection usually results in potentially life-threatening meningitis and/or septicaemia, but other forms of disease, such as pneumonia, arthritis, urethritis and conjunctivitis, may also develop[2]. In spite of more than five decades of effort to develop effective meningococcal vaccines[3,4] (BOX 1), IMD continues to be a major global public health challenge, with an estimated burden of disease of approximately one million cases annually worldwide[5] and overall mortality ranging from 4% to 20%, depending on the infecting strain and the age of the individual[6]. The incidence of meningococcal disease is generally highest in infants younger than 1 year, with half of the cases occurring in children younger than 5 years. Another peak of IMD can be seen in adolescents and young adults — the age groups with the highest prevalence of oropharyngeal carriage[1,5]. The course of the disease can be fulminant; early recognition of symptoms is crucial, and immediate treatment, including the administration of antibiotic therapy, is the only effective measure when IMD has developed[7]. In addition, a substantial proportion of survivors experience severe sequelae, such as deafness, mental impairment and amputations[8].

N. meningitidis is naturally competent for genetic transformation and readily undergoes homologous recombination[9]. Consequently, the genomic diversity of meningococcal lineages is extensive, and recombination, rather than mutation[10], is the predominant source of new genetic information and the essential driving force behind the evolution of the bacterium, permitting rapid adaptation to fluctuating environmental conditions. With whole-genome sequencing (WGS) becoming relatively inexpensive, several thousand meningococcal genomes, which are each ~2.2 million nucleotides in length, are currently available for in-depth analyses. This source of data, although still underutilized, is starting to provide new insights into the plasticity of the genome of this human commensal with occasional pathogenic potential. In this Review, we discuss how high-throughput sequencing approaches have advanced

Core genome
The set of genes that are present in all (or nearly all) strains of a species or population.

our understanding of the diversity and evolution of *N. meningitidis* and the pathogenesis of *N. meningitidis* infection and are contributing to explaining the epidemiology of meningococcal disease.

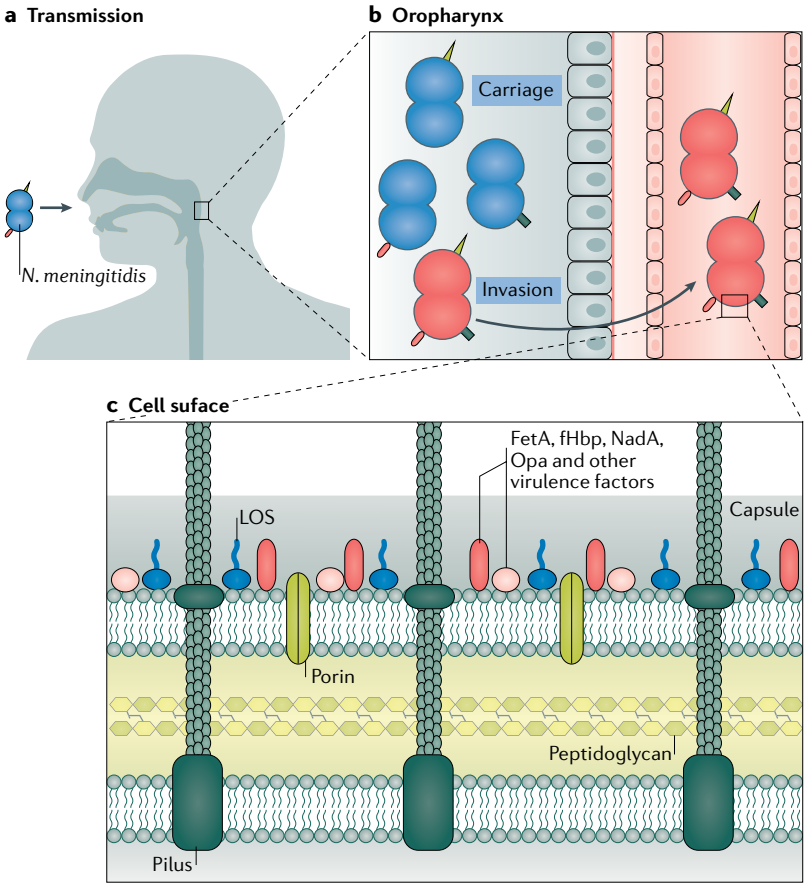

Fig. 1 | **Overview of *Neisseria meningitidis* transmission, carriage state, invasion and virulence factors of the meningococcal outer membrane. a** | Transmission occurs through contact with respiratory droplets or secretions that enter through the mouth or nose, with subsequent colonization and proliferation of bacteria in the oropharynx or nasopharynx. **b** | In the proliferation phase, a wide range of phenotypic variation is stochastically created by genetic reassortment through various mechanisms, especially phase variation as a result of the expansion or contraction of repeat elements[122], but also intragenomic and intergenomic recombination and post-translational modification of surface proteins[146]. Most *N. meningitidis* infections never result in clinical disease, and the bacteria remain in a carriage state (illustrated in blue). However, for genetically primed strains, a potentially invasive phenotype may eventually emerge in the proliferation and reassortment phase (illustrated in red). Invasive phenotypes penetrate the mucosal epithelium and gain access to the bloodstream. **c** | An invasive phenotype displays a number of immunogenic molecules on its cell surface. They can be categorized broadly into adhesins, invasins and iron acquisition systems. The outer membrane contains type IV pili and the surface-bound proteins NadA and Opa, all of which function to allow attachment to host cell surfaces and invasion through the mucosa. The membrane also contains immune modulators such as factor H-binding protein (fHbp) and NspA (which also binds complement factor H) and, in nearly all hypervirulent strains encountered to date, the bacterium is surrounded by a polysaccharide capsule that protects against complement-mediated phagocytosis. Additionally, the outer membrane of the bacterium contains lipooligosaccharides (LOS; endotoxin), which have both adhesion and immune evasion properties. Proteins such as ferric enterobactin transport protein (FetA) and HmbR enable meningococci to acquire from the human host iron, a crucial growth factor during disease. Meningococcal porins, class 1 porin (PorA) and PorB, are sometimes also classified as virulence factors since they interact with the immune system of the host[147]. Some fragments of inflammation-promoting peptidoglycan from the cell wall may also be released during growth[148]. Parts **a** and **b** adapted with permission from REF.[122], Elsevier.

The genus *Neisseria*

The genus *Neisseria* comprises at least 25 species, most of them colonizing the mucosal and dental surfaces of warm-blooded animals, usually as harmless commensals[11,12]. Of the 11 species colonizing humans, most are non-pathogenic, although some may cause disease in immunocompromised hosts. However, the two genetically closely related species *N. meningitidis* and *Neisseria gonorrhoeae* (the gonococcus) are globally important pathogens. The commensal species, as well as the gonococcus, are a reservoir of genes that can be acquired by the meningococcus through horizontal genetic transfer. DNA fragments several kilobases long can be imported into *N. meningitidis* from related organisms co-colonizing the mucosa[13]. A recent study using WGS identified a core genome of 1,111 gene families conserved among *Neisseria* species[14]. In particular, there is a high level of sequence similarity (~96%) between the genomes of the two human-specific pathogens *N. meningitidis* and *N. gonorrhoeae*. Phylogenetic analyses based on sequencing of the 53 ribosomal genes have shown that *N. meningitidis* and *N. gonorrhoeae* evolved recently from a common ancestor[15,16], but separated as a result of colonization of distinct ecological niches (FIG. 2). Whereas *N. gonorrhoeae* has been documented in dental calculus of Neanderthals[17], the origin of meningococcal disease is probably only a few centuries old[18]. Genomic analyses showed that an ancestral gonococcal strain acquired a number of genetic factors allowing it to colonize the oral mucosal surface instead of the urogenital environment[19]. One of the main differences between *N. meningitidis* and *N. gonorrhoeae* is the lack of a capsule in the gonococcus, whereas disease-causing meningococci in immunocompetent individuals are nearly always encapsulated. On the basis of sequence similarity, it has been hypothesized that the genes for capsule synthesis and other virulence factors in meningococci were probably acquired through horizontal gene transfer from members of the family Pasteurellaceae after the phylogenetic split[20]. Newer analyses that identified various genes of the capsule operon in non-pathogenic *Neisseria* species suggested multiple acquisition and loss events during the evolution of the genus *Neisseria*[21].

Epidemiology

Of the three main bacterial pathogens associated with meningitis (*H. influenzae*, *S. pneumoniae* and *N. meningitidis*), *N. meningitidis* is the only one (with the exception of serotype 1 *S. pneumoniae*) that can cause large outbreaks. Although meningococcal disease frequently occurs endemically, with scattered and apparently unrelated cases, large, devastating and unpredictable epidemics may develop in some parts of the world, sometimes encompassing several continents (that is, a pandemic situation). A zone south of the Sahara, called the 'African meningitis belt'[22], which stretches from Senegal in the West to Ethiopia in the East (encompassing parts or the whole of 26 countries), has the highest incidence of meningococcal disease in the world[23]. Historically, incidence rates in that region have exceeded 800 cases per 100,000 population per year during serogroup A meningitis epidemics[24].

Dental calculus
A form of hardened dental plaque that is caused by precipitation of minerals from saliva and gingival fluid on the teeth.

Multilocus enzyme electrophoresis
A method for characterizing organisms by the relative mobilities under electrophoresis of a large number of intracellular enzymes.

Multilocus sequence typing
A procedure to characterize microbial isolates using the DNA sequences of internal fragments of multiple housekeeping genes.

Twelve serogroups are defined on the basis of the structure of the capsular polysaccharide, and six of them (serogroups A, B, C, W, X and Y) are responsible for nearly all cases of disease worldwide[6]. Genomic analysis of the capsule locus has been used to support the serogroup nomenclature[25]. The incidence of disease caused by the different serogroups is changing constantly, both temporally and geographically, possibly as a result of changes in the human population immune status[26]. Large epidemics have traditionally been caused by serogroup A meningococci. However, in high-income countries these epidemics stopped in the 1970s, whereas they continued in sub-Saharan Africa until the successful introduction of a monovalent conjugate vaccine against serogroup A disease in large-scale mass vaccination campaigns, starting in 2010 (REF.[27]). In much of the developed world, including North America, South America, western Europe, Australia and New Zealand, serogroup B was the cause of endemic and hyperendemic disease in the last part of the twentieth century, with serogroup C causing sporadic outbreaks and epidemics[7]. Following the introduction of effective conjugate vaccines in national vaccination programmes, the incidence of serogroup C disease has decreased in the past decade in many high-income countries[28]. In recent years, there have been increases in the number of cases of IMD caused by serogroup Y (first in the USA and then in western Europe)[29,30], as well as by serogroup W, mainly in Africa, South America and Europe[31–34].

Epidemiological studies using the large genetic variability of the meningococcus to identify specific lineages or clonal complexes within the species have demonstrated substantial differences in the invasive potential of strains. The first studies of genetic diversity and population structure of *N. meningitidis* used variation in a small number of housekeeping genes, first elucidated through multilocus enzyme electrophoresis[35,36] and subsequently through multilocus sequence typing[37]. The molecular epidemiological studies revealed the existence of particularly virulent lineages: sequence types (STs) highly associated with outbreaks and epidemics and only rarely identified in samples from healthy carriers[38]. With the increased availability and affordability of WGS, a new era has been opened to tackle many issues regarding the epidemiology of IMD, linked to the evolution of this pathogen[39,40]. Clonal complexes first defined on the basis of multilocus enzyme electrophoresis and confirmed by multilocus sequence typing are now scrutinized using WGS[41,42]. The Neisseria PubMLST database currently includes more than 20,000 WGS-complete genomes (genome size greater than 2 Mb) of *Neisseria* species isolates, including more than 15,000 genomes of *N. meningitidis* (accessed 14 June 2019). In spite of the extensive recombination potential of the species, there is strong congruence of the various classification schemes. A phylogeny of a randomly sampled subset of the meningococcal isolates in the database (~5% of all isolates in the database) and their associated lineages is shown in FIG. 3.

Among the hypervirulent lineages identified in *N. meningitidis*, lineage 11 is one of the most ancient, identified in 1917. An interesting feature of lineage 11 is its association with the four capsular serogroups containing polysialic acid (that is, serogroups B, C, W and Y). Most of the lineage 11 isolates from the 1960s and 1970s expressed the serogroup B capsule[40], whereas in the 1990s a new variant of ST-11 (designated as ST-11/ET-15) gave rise to serogroup C outbreaks in North America before spreading to Europe[43]. As a consequence, monovalent serogroup C conjugate vaccines were developed[44]. Although serogroup W lineage 11 isolates already existed in the 1970s, a severe epidemiological situation occurred in 2000 when such isolates were introduced in Saudi Arabia during the hajj pilgrimage, causing a large outbreak. Following the hajj in 2000, the strain spread worldwide, and ever since serogroup W lineage 11 has been a problem, first in sub-Saharan Africa, then in South America, Europe and Australia[31–34,45–47]. WGS has been essential in elucidating the existence of different clades of serogroup W lineage 11, providing an understanding of their epidemiological importance. With use of a gene-by-gene approach encompassing 1,546 core loci of 750 lineage 11 isolates, two sublineages were differentiated; lineage 11.1 included the strain introduced during the hajj pilgrimage, whereas lineage 11.2 included the South American strain and the so-called original UK strain that emerged in the UK in 2009 (REF.[40]). Another new variant was identified in the UK in 2013 that was associated with a severe increase in serogroup W disease and has since spread to other European countries[34,48–50]. Three putative recombinational events and four point mutations distinguished the UK 2013 strain from the original UK strain, including genes encoding antigens, such as the haemoglobin–haptoglobin receptor complex HpuAB, the genetic regulator MtrR and a number of metabolic genes[50]. This novel and rapidly expanding UK 2013 strain, which also possesses the *Neisseria* adhesion A (NadA) antigen, has been associated with unusual clinical features, including

Box 1 | **Genomics and meningococcal vaccines**

There are effective protein-conjugate polysaccharide vaccines for four of the six current disease-causing serogroups of *Neisseria meningitidis*[126,127], and a low-cost conjugate vaccine also including serogroup X is in a clinical phase II trial[128]. Such conjugate vaccines are effective in infants, elicit longer-lasting immune responses and may induce herd protection by affecting transmission of the bacterium in the human population. Polysaccharide-based vaccines against serogroup B have not been pursued owing to poor immunogenicity of the serogroup B capsule and self-antigen concerns[129]. In the search for potential subcapsular antigens for a vaccine against serogroup B disease, genomics has been essential. With use of the first available *N. meningitidis* complete whole genome and an approach called 'reverse vaccinology'[130], novel protein vaccine candidates were identified and recombinant DNA technology was used to produce and test these antigens for suitability. The new targets, including a neisserial adhesin NadA, the neisserial heparin-binding protein and factor H-binding protein (fHbp) were combined with the outer membrane vesicle from a New Zealand vaccine strain[126] to produce Bexsero[131]. Given the known sequence diversity of fHbp[132], another protein-based vaccine, Trumenba, was developed to cover the two subfamilies of fHpb and thus potentially target all of *N. meningitidis* serogroup B, and other serogroups as well[133]. Large sequencing projects were undertaken to estimate the coverage of these vaccines based on the diversity of the antigens among meningococcal populations circulating in various geographical areas[134,135]. With whole-genome sequencing, any set of antigen-encoding genes within a large collection of isolates can be efficiently characterized and thus the potential coverage of different vaccines can be compared. Whole-genome sequencing is also valuable in determining the changing prevalence of the vaccine antigens following vaccine introduction and identifying escape mutants.

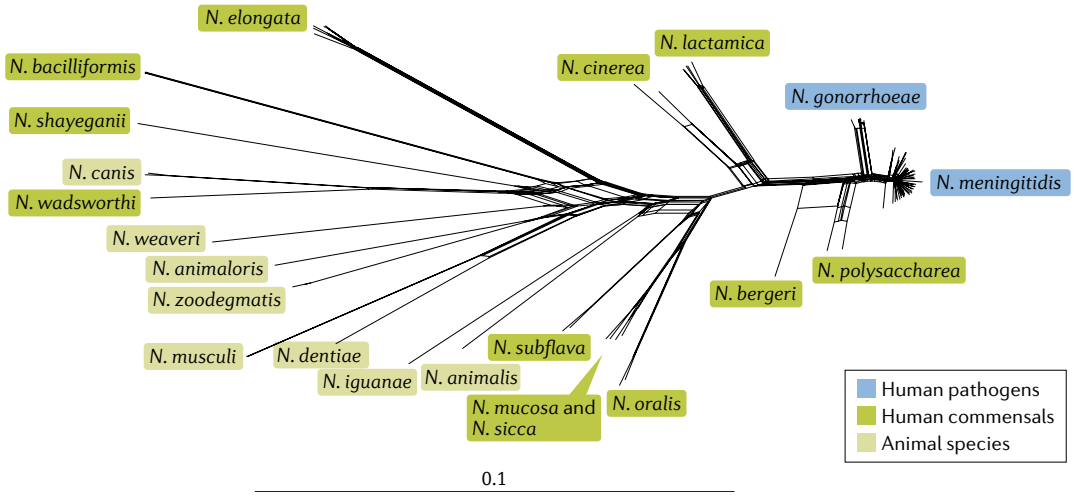

Fig. 2 | **Phylogenetic network of the relationships between species in the genus *Neisseria*.** Most species are distantly related to each other and exhibit a variable degree of clonality. Intraspecies variation is large in *Neisseria lactamica*, *Neisseria oralis*, *Neisseria subflava*, *Neisseria cinerea*, *Neisseria polysaccharea* and *Neisseria elongata*, with the latter having multiple defined subspecies (*Neisseria elongata* subsp. *elongata*, *Neisseria elongata* subsp. *glycolytica* and *Neisseria elongata* subsp. *nitroreducens*). At the other end of the spectrum, *Neisseria gonorrhoeae* is a particularly clonal species, with all isolates clustering tightly together. The major pathogens, *Neisseria meningitidis* and *N. gonorrhoeae*, are more closely related than other species within the genus. Species with overlapping ecology are more closely related than those that inhabit distinct niches. For example, *N. oralis*, *Neisseria mucosa*, *Neisseria sicca* and *N. subflava* are all non-pathogenic species that inhabit the human pharynx and are situated on the same branch. *N. lactamica*, *N. cinerea*, *N. polysaccharea* and *Neisseria bergeri* are also non-pathogenic inhabitants of the human nasopharynx, although they are each located on distinct branches of the phylogenetic tree. To date, there are no publicly available sequences for *Neisseria flava*, and it is therefore not displayed in the figure. Note that *N. sicca* and *N. mucosa* did not form monophyletic groups and were not distinguishable with this method, and could therefore possibly represent a single species or, alternatively, more than two separate species. On the other hand, *N. elongata*, which is also found in the human pharynx, is distantly related to other commensal species. The left side of the network is occupied by different distinct animal strains. The figure was created as follows: 93 isolates were manually selected from the 21,615 isolates openly available on the *Neisseria* PubMLST platform as of May 2019. For rare species, a minimum of two isolates were manually selected. For *N. meningitidis*, one representative was selected from each defined clonal complex. For *N. gonorrhoeae* and *N. lactamica*, representatives from the major sequence types were selected. In all cases, the selection procedure gave preference to isolates that maximized geospatial and temporal variation. The network was created by concatenating the sequences of all 53 loci included in the *Neisseria* ribosomal multilocus sequence typing scheme[149], as defined in PubMLST. The concatenated sequences were aligned with MAFFT[150], and the Hamming distances from the resultant distance matrix were fed to the Neighbor-Net algorithm[151] as implemented in SplitsTree4 (REF. [152]). The scale bar corresponds to a relative distance of 10% across the length of the alignment.

severe gastrointestinal symptoms[51]. Another variant of lineage 11 has become adapted to colonization of the urogenital tract, being transmitted mainly within the population of men who have sex with men[52–54] (BOX 2).

Lineage 10 (previously designated as the ST-5 clonal complex/lineage III)[41], one of the three predominant serogroup A lineages, has been responsible for major epidemics of meningitis worldwide, but principally in the meningitis belt of sub-Saharan Africa after its introduction in the continent in 1988–1989 (REFS[55,56]). Three successive waves of epidemics have occurred in the meningitis belt associated with three distinct clones of lineage 10, namely ST-5, ST-7 and ST-2859. As for lineage 11, the principal antigens that invoke effective immunity (for example, the class 1 porin (PorA), the ferric enterobactin transport protein (FetA) and factor H-binding protein (fHbp))[57,58] have remained unchanged in these successive clones. Genome comparisons of the emerging ST-2859 strains versus the ancestral ST-7 strains identified 13 recombination blocks, allowing replacement of ST-7 by ST-2859 as a main cause of disease shortly after ST-7 outbreaks. In 20% of the recombination events the acquired DNA came from other species. The *pgl* locus, which determines the glycosylation patterns of major protein antigens, genes involved in the regulation of pilus expression and genes involved in the synthesis of *maf3* adhesins were affected by the recombination events[58]. Emergence and expansion of the ST-2859 clone was explained by these changes in cell surface structures, enabling the new clone to multiply in human populations having developed mucosal immunity against ST-7. WGS analysis of 153 lineage 10 isolates covering the successive epidemic waves provided conflicting results. Although the genomes were highly uniform within epidemic waves, with an average of 67.2% of all loci exhibiting identical alleles, the emergence of the successive epidemic clones was linked to 11 genetic events, primarily involving core genes encoding metabolic processes[59]. The acquired DNA was found to be identical to that in unrelated, hyperinvasive meningococci, suggesting that the epidemic clones emerged through the acquisition of pre-existing metabolic gene variants rather

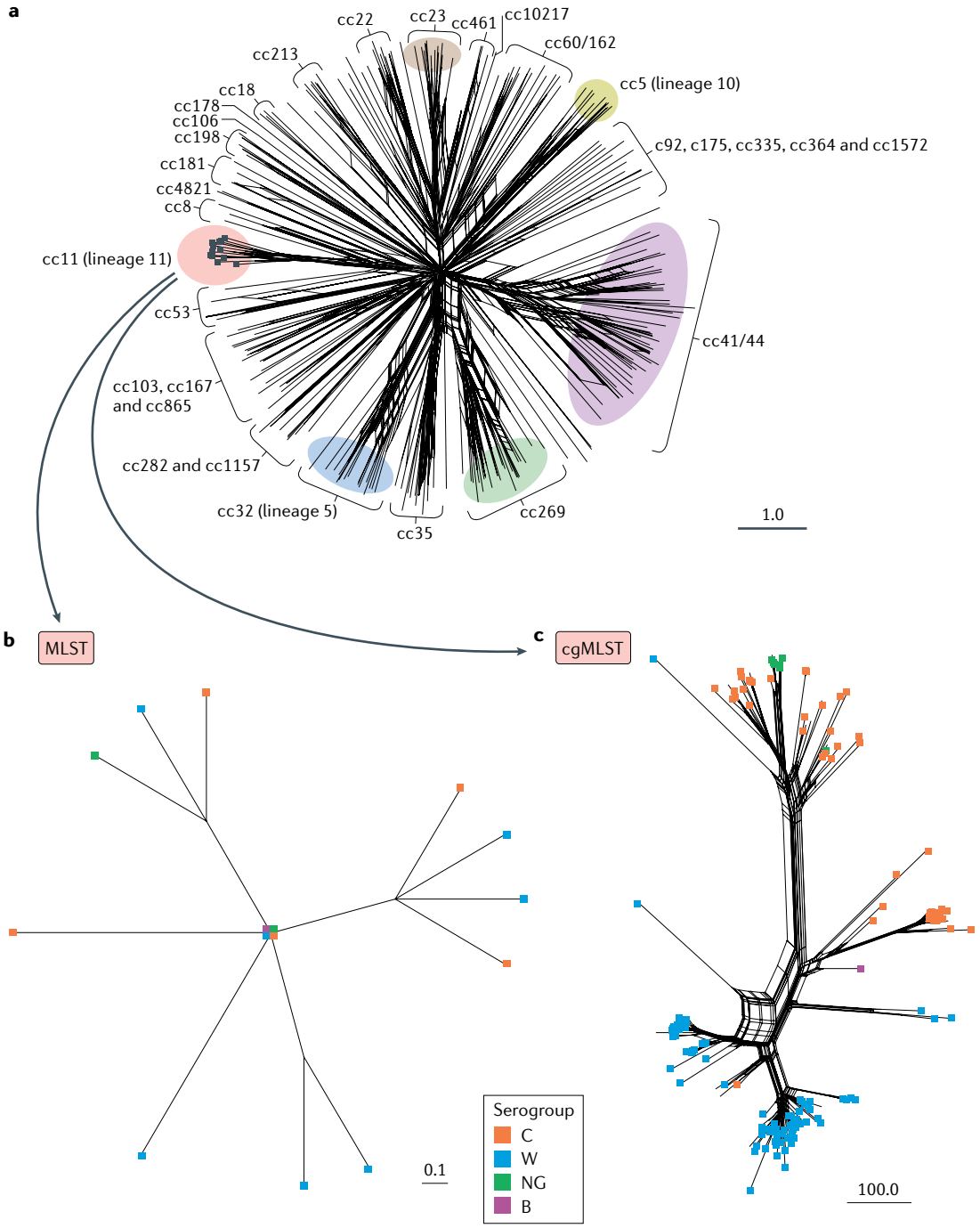

Fig. 3 | **Phylogenetic networks of *Neisseria meningitidis* at different resolutions. a** | Relationships between the major clonal complexes (cc) defined in *N. meningitidis* using multilocus sequence typing (MLST). This network shows a proportional representation of all *N. meningitidis* genomes submitted to PubMLST as of May 2019. The largest clonal complexes are circled. Alternative lineage terminology is used for cc11 (lineage 11), cc5 (lineage 10) and cc32 (lineage 5). Individual sequence types are not shown. From the total of 15,711 *N. meningitidis* genomes available from PubMLST as of May 2019, each isolate was randomly chosen with a probability of 0.05, resulting in a total of 762 genomes. Allelic distance profiles were loaded into SplitsTree4 (REF.[152]), and the Neighbor-Net algorithm[151] was applied. The scale bar corresponds to an allelic distance of 1.0. The maximum distance between any two isolates in an MLST comparison is seven. Unique lineage 11 sequence types are indicated by boxes. **b** | Neighbor-Net network of lineage 11 obtained with MLST data. MLST can resolve phylogenetic relationships to the clonal complex level, but the lack of discriminatory power becomes apparent when one is looking within a single clonal complex. Although this network consists of 193 isolates, only 12 distinct genotypes were found. One hundred and eight-two of the isolates have an identical genotype and are located on the central node. Isolates are annotated by serogroup. **c** | Neighbor-Net network of lineage 11 obtained by core genome MLST (cgMLST). Whole-genome sequencing drastically increases the resolution of the phylogenetic network. This figure was made with the 1,605 loci defined in the PubMLST core genome MLST scheme. In this scheme, all 193 strains have unique genotypes, and it becomes evident that isolates cluster by serogroup.

Box 2 | ***Neisseria meningitidis* as a cause of urethritis**

N. meningitidis and *Neisseria gonorrhoeae* conventionally colonize distinct ecological niches — the mucosa of the oronasopharynx and that of the genital tract, respectively; however, there is overlap. The first report of isolation of *N. meningitidis* from the urogenital tract dates from 1933 (REF.[136]), and isolation of the meningococcus from the cervix, urethra or anal canal started to increase in the 1970s[137]. Sexual practices of men who have sex with men were hypothesized as an important factor for transmission. Orogenital transmission was first confirmed when the same C:2a:P1.5 strain was isolated from a patient's urethral exudates and from his sexual partner's pharynx using pulsed-field gel electrophoresis for genotyping[138]. In the past 5 years, there have been increasing reports of *N. meningitidis* as the exclusive cause of symptomatic urethritis. A case caused by a serogroup W of the sequence type 11 clonal complex was reported in Japan in 2013 from an HIV-positive man[139]. During 2015, outbreaks of *N. meningitidis* urethritis emerged in several cities in the USA. In all cases, isolates were non-encapsulated and typed as sequence type 11 (REFS[52,140,141]). Surprisingly, the clone emerged primarily among heterosexual men and represented a new clade of lineage 11.2. The strain had adapted to the urogenital environment by deletion of the capsule through insertion of *IS1301* in the capsule operon, enhancing mucosal adherence, and by acquisition of the gonococcal denitrification pathway, promoting anaerobic growth[141].

The strain evolved from a common ancestor that likely existed in 2011 (REF.[54]). One study showed that a wide range of meningococcal genotypes have the ability to cause urethritis[142]. Further, urethritis isolates of the same clade of clonal complex 11 were recovered from men who have sex with men in Germany and France[143]. The gene encoding factor H-binding protein (fHbp), which binds human factor H, a negative complement regulator leading to enhanced survival in blood, had a premature stop codon in all urethritis isolates, similarly to the non-functional homologue of fHbp found in *N. gonorrhoeae*.

N. meningitidis urethritis raises major public health issues as urogenital diagnostic methods usually do not encompass the meningococcus. Because nucleic acid amplification tests are most frequently used for rapid diagnosis of gonorrhoea, such meningococcal urogenital cases may also be unreported if further investigatory tests are not performed[144]. Potential acquisition of antimicrobial resistance from the gonococcus by horizontal gene transfer is an additional concern[145].

than virulence-associated or antigen-encoding genes; thus, evasion of host immune responses is unlikely to explain the emergence of the epidemic waves in Africa[59]. Whether changes in metabolic genes leading to small differences in transmission fitness have a major role in explaining changes in epidemic clones needs to be confirmed for other hypervirulent lineages.

Lineage 5 (previously designated as the ST-32/ET-5 clonal complex) has been responsible for serogroup B outbreaks globally for more than 40 years. Major epidemics were recorded in Norway, Cuba, Chile and Brazil in the 1980s and 1990s, and prolonged outbreaks were recorded in the Pacific Northwest of the USA and in Normandy, France[35,60,61]. In contrast to lineages 11 and 3, similar or closely related clones of lineage 5 may express very different major antigenic proteins. Analysis of the core genome of a small but global collection of lineage 5 isolates revealed three distinct sublineages — the 'Asian group' (sublineage 5.1), the 'north European–Norwegian group', which contained isolates with the PorA type P1.7, 16 (sublineage 5.2), and a 'Latin American group' with PorA type P1.19, 15 (sublineage 5.3) — as well as several isolates which did not fall into any of these groups. The most variable genes were those encoding surface-exposed lipoproteins associated with iron acquisition[62].

As generation and analysis of WGS data is now rapid and relatively inexpensive, WGS offers powerful opportunities for enhanced molecular surveillance and is being established as a routine technique for epidemiological studies of large meningococcal strain collections. The Meningitis Research Foundation Meningococcus Genome Library (MRF-MGL) was the first genome-based initiative to make sequence data from strains collected in the UK readily available[63]. Starting in 2009, the MRF-MGL now includes more than 4,000 sequenced isolates, and the isolate information is deposited in the Neisseria PubMLST database. A representative European meningococcal strain collection of 799 IMD isolates from the epidemiological year from July 2011 to June 2012 was also put together and whole genome sequenced[64]. In addition to allowing tracking of the pathogen and assessment of virulence and antimicrobial resistance, such genome libraries are especially valuable to document potential vaccine coverage and to demonstrate the impact of non-capsular vaccines[65] (BOX 1).

Carriage versus disease

Hyperinvasive lineages can be isolated from the oropharynx of healthy carriers. Their prevalence in carriage studies differs substantially, probably as a result of different virulence potential and duration of colonization[66,67]. Except for very rare cases, usually in immunocompromised individuals, disease-causing meningococci are encapsulated, whereas isolates recovered from culture of oropharyngeal swabs often do not harbour, or express only at low levels, a polysaccharide capsule[66–68]. Absence or decreased expression of capsule is seen in meningococci of identical genotypes recovered from the nasopharynx compared with the invasive isolate in individual patients[69]. The loss of capsule, which may result from downregulation of capsule gene expression, phase variation in the capsule synthesis genes (see later) or inactivation of genes in the capsule gene cluster (*cps*), is crucial in meningococcal biology, as intimate adhesion on human mucosal surfaces and formation of microcolonies can then be mediated by type IV pili[69].

cps has been identified as a spontaneous point mutation hotspot, and many carriage isolates do not possess a complete capsule operon[70,71]. In some instances, the genetic inability of strains to produce a capsule is a consequence of a lack of the *cps* operon. In capsule-null strains, the *cps* operon is replaced by a non-coding region (*cnl* region)[69,71]. *cnl*-carrying meningococci have been described in various lineages[71–73], but with rare exception[74] disease caused by such strains occurs only in immunocompromised individuals. Comparative genome sequencing has been used in an attempt to discover other potential differences between carried and invasive isolates. A study looking at genetic differences between serogroup A isolates from carriers and patients with IMD during an epidemic in Chad provided no evidence of consistent differences between the carried and invasive isolates[75]. A study performed in the course of the serogroup B epidemic in New Zealand compared the whole genomes of 12 throat isolates recovered from household contacts of seven patients with the genome of the index strain[76]. Within a household, isolates of the same ST differed from the index strain by between 9 and 210 single-locus polymorphisms after exclusion of

pilS antigenic variation and predicted tandem repeats. Of 94 variants found to cause amino acid substitutions, insertions or deletions, only five were predicted to alter protein function. These were in *lgtC* which encodes a glycosyltransferase, a gene that encodes a chloride transporter, *hmbR* which encodes a TonB-dependent haemoglobin receptor, *tbpA* which encodes transferrin-binding protein A and a gene encoding a citrate transporter family protein[76]. On a similar note, the genomes of capsulated invasive isolates and both capsulated and non-capsulated isolates from asymptomatic carriers from the lineage 5 outbreak in Normandy, France, were compared using a gene-by-gene analysis[77]. Genes involved in iron acquisition differed between the capsulated invasive and carrier isolates, in particular the *hmbR* gene, was switched off in capsulated carriage isolates[77].

Population structure

Transformation and homologous recombination have a major role in the genome plasticity and virulence of meningococci[78]. The meningococcus relies on sequence diversification to produce a surplus of variants that might provide increased fitness and survival in a changing environment, allowing survival of a bacterial subpopulation that is able to avoid the host immune defences. Sequence diversity accumulates rapidly, largely as a consequence of recombination between different lineages of the species during the carriage state or with other related species colonizing the oral mucosa. In experimental transformation, imported sequences were found to be longer in the recipient if the genomic DNA is derived from an intraspecies donor versus an interspecies donor[79]. Uptake of foreign DNA from other meningococcal strains and closely related species (that is, the gonococcus and *Neisseria lactamica*) is facilitated by the presence of ~2,000 copies of DNA uptake sequences throughout the meningococcal genome[80], whereas distinct variants of DNA uptake sequences found in other members of the Neisseriaceae are efficient barriers to interspecies recombination[81].

Although the meningococcus has a highly dynamic population structure as a result of horizontal gene transfer, isolates can still be readily grouped into clonal complexes or lineages owing to their similarity to a central allelic profile. The diversity is limited by purification events resulting from bottlenecks during transmission of the bacteria to new geographical areas. This is suggested by the strong geographical structuring observed in the genomic diversity of lineages 5 and 11 (REFS[40,62]). Collapse of the genomic diversity within ST-2859 of lineage 10 was demonstrated in a systematic longitudinal study of meningococcal carriage and disease isolates collected over a period of more than 10 years in northern Ghana[82]. After a gap of 2 years, when the clone was displaced by a serogroup W strain, ST-2859 meningococci reemerged, both as a colonizer and as a meningitis-causing agent. A profound reduction in genomic diversity among isolates of the second wave was observed, with the expanding new clone differing in only one single-nucleotide polymorphism in a gene encoding the conserved hypothetical integral membrane protein NMAA_141 from some isolates of the original ST-2859 population, a striking example of how genomic diversity of an epidemic clone can collapse when passing through a population bottleneck[82]. Models of the population structure for this highly recombining species have been debated since 1993, when an epidemic population model was proposed[83] in which occasional clonal propagation of highly fit organisms occurs in a predominantly recombining population structure. More recently, a model of predominantly clonal evolution, with restrained recombination, has been suggested to better fit the newer genetic data[84]. Bottlenecks[82] and founder events resulting from the spread of a few organisms to new geographical areas[85,86] have the most substantial role in the periodic decreased diversity of the species.

Phase variation

Phase variation is a feature of certain host-adapted pathogens, such as *N. meningitidis*, regulating a high-frequency stochastic, reversible switching of gene expression. Phase variation allows rapid changes in the bacterium when it encounters a hostile host environment. The *N. meningitidis* pan-genome harbours more than 100 genes that undergo phase variation by expansion or contraction of simple sequence repeats, allowing the switching of gene expression between on and off states[87]. Each meningococcal isolate carries on average more than 4,000 simple sequence repeats in its genome, with each repeat unit usually being between one and ten nucleotides in length[87]. Changes in the number of repeats within a coding sequence can alter translation by introducing frameshifts in the reading frame, and in the proximity of a promoter, it can modulate transcription. Rates of phase variation are often several orders of magnitude greater than basal mutation rates, and the frequency of switching is, in part, determined by the number of repeats in the repeat tract[88]. In an analysis using 78 complete or partial genome sequences of *N. meningitidis*, a mean of 47 phase-variable genes per genome was calculated[89]. Phase-variable genes include virulence factors such as those involved in capsule biosynthesis, restriction-modification systems, metabolic proteins, bacteriocins and surface-expressed proteins, such as pili glycosylation or modulation proteins, adhesins and lipopolysaccharides[87–92]. Iron acquisition systems that enable the meningococcus to acquire iron from various iron-binding proteins within the human host are particularly prone to phase variation[93,94]. The TonB-dependent surface receptors HmbR and HpuAB both undergo phase variation owing to poly(G) tracts in the open reading frame of the genes[95]. Phase variation frequencies are controlled by a number of genetic factors, including genes of the mismatch excision repair and other DNA-repair pathways[78,96,97].

The importance of phase variation in relation to disease occurrence was recently illustrated in a study which assessed phase variation in 737 UK serogroup W invasive and carriage isolates of lineage 11 analysed by WGS[98]. A selection of phase-variable genes encoding outer membrane proteins were compared between the original UK strain and the UK 2013 strain[40]. Statistically significant increases in repeat number were detected in the UK 2013 strain in genes encoding PorA, NadA and

DNA uptake sequences
Small repeated sequences that are required for DNA binding or uptake in natural transformation in members of the genus *Neisseria*.

Pan-genome
The sum of genes that are found in at least one strain of a species or population. In addition to the core genome, this includes the accessory genome, which contains dispensable genes present in a subset of the strains.

Simple sequence repeats
DNA tracts in which a short base pair motif is repeated several times, which can be found within the open reading frame or within the promoter region of a gene.

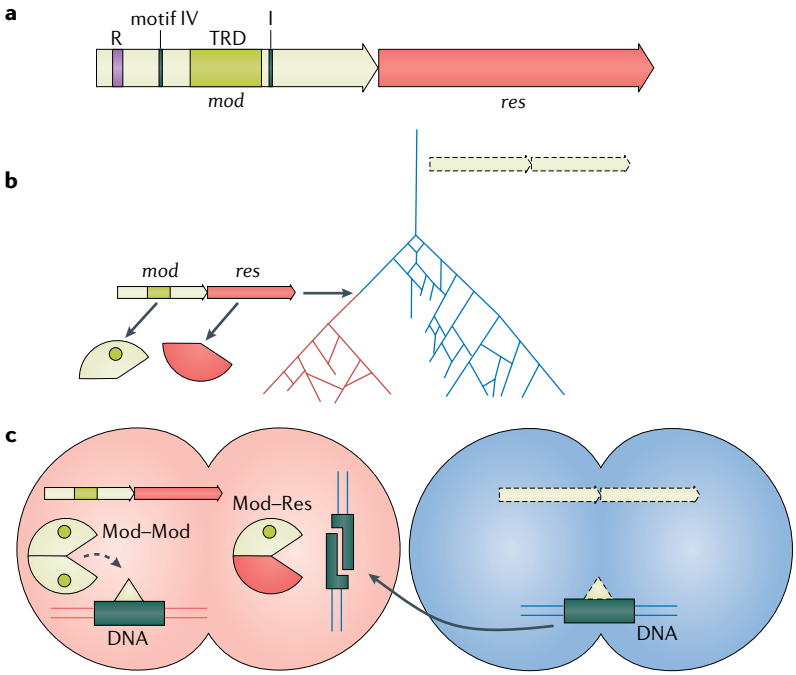

Fig. 4 | **Restriction-modification systems in *Neisseria meningitidis*.** Briefly, restriction-modification systems work to protect the cell against foreign DNA by cleavage at specific motifs, as mediated by restriction endonuclease genes, with protection of self through the methylation of these same motifs mediated by methyltransferase genes. **a** | Overview of type III restriction-modification systems in *N. meningitidis*. The system consists of two genes; the methyltransferase (modification) gene (*mod*) and the restriction endonuclease gene (*res*). Three different *mod* genes have been described in *N. meningitidis*: *modA*, *modB* and *modD*[105]. The three genes occupy different loci and do not display substantial sequence similarity in their functional domains. The *modA* gene is found in all *N. meningitidis* isolates sequenced to date, *modB* is found in 78% and *modD* is found in 25%[104]. A single *mod* gene contains an amino-terminal tetranucleotide (*modA*) or pentanucleotide (*modB* and *modD*) repeat that is subject to phase variation (R). Towards the centre of the gene, a variable target recognition domain (TRD) is located, and this region determines the site of recognition and methylation. The TRD is flanked on each side by conserved active site sequences DPPY (motif IV) and FxGxG (also called motif I). **b** | Evolution of *N. meningitidis* with ample recombination, interrupted by the invasion of a novel restriction-modification system, which prevents further recombination and drives the evolution of a new, red lineage. The restriction-modification system is absent in the blue lineage, as indicated by the dotted boxes. The *mod* and *res* genes produce Mod and Res proteins, subunits of the functional enzyme complexes that perform the methyltransferase and restriction endonuclease tasks. **c** | In the DNA of the red bacterium, the specific recognition site (dark green) for the TRD has been methylated (light green triangle) by an enzyme complex of two Mod subunits (light green circle sector), protecting it from self-cleavage. In the blue bacterium, the equivalent recognition site is not methylated (the dotted triangle representing absent methylation) since it has not acquired this particular restriction-modification system. The blue and red bacteria can both freely recombine internally with other members of their respective lineages. However, the blue bacterium cannot donate DNA containing the specific recognition site to the red bacterium because unmethylated recognition sites are recognized and subsequently cleaved in the red bacterium by an enzyme complex consisting of a Mod subunit (light green circle sector) and a Res subunit (red circle sector). Recognition of the specific recognition site for both methylation and restriction is determined by the TRD in the Mod subunit (light green circle sector). Part **a** adapted with permission from REF.[101] Seib, K. L., Jen, F. E., Scott, A. L., Tan, A. & Jennings, M. P. Phase variation of DNA methyltransferases and the regulation of virulence and immune evasion in the pathogenic *Neisseria*. *Pathog. Dis.* (2017) **75** ftx080, by permission of Oxford University Press. Parts **b** and **c** adapted with permission from REF.[153], Proceedings of the National Academy of Sciences USA.

two opacity proteins, OpaD and OpaJ. Invasive and carriage isolates exhibited similar repeat numbers but the absence of *pilC* expression was frequently associated with disease. It was speculated that the rapid expansion of the UK 2013 strain was due to a higher phase variation rate, allowing it to avoid the immune response of the host during transmission and consequently resulting in an increased number of disease cases[98].

Restriction modification and epigenetics

Restriction-modification activity consists of methylation of a specific DNA sequence by a methyltransferase and cleavage of unmethylated DNA by a restriction endonuclease (FIG. 4). Such mechanisms are found in all bacteria, but are particularly prevalent in naturally competent organisms such as *N. meningitidis*[99,100]. These systems are assumed to protect the bacterium against infections by foreign DNA and may result in sexual isolation[99]. All *N. meningitidis* strains possess type III restriction-modification systems, with modification enzymes encoded by *mod* genes[101]. The protein Mod catalyses the methylation of a single strand of DNA at a specific 4–6-bp region recognized by its DNA recognition domain. Three phase-variable DNA methyltransferases (ModA, ModB and ModD), which mediate epigenetic regulation of distinct phase-variable regulons (phasevarions), have been identified in *N. meningitidis*[102]. Variable expression of methyltransferases leads to variable genome-wide methylation differences and altered expression of multiple genes through epigenetic mechanisms[103]. An investigation of the distribution of *mod* genes and alleles in a collection of 1,689 meningococcal genomes showed that *modA* was present in all isolates, whereas *modB* and *modD* were present in 78% and 25% of the isolates, respectively[104]. Each *mod* gene has distinct alleles, defined by their DNA recognition domain. *modA* alleles A12 and A11 predominated (identified in 70% and 27.5% of the isolates, respectively), whereas *modB2* and *modB1* were the most common *modB* alleles (found in 49% and 42% of the *modB*-positive isolates, respectively) and 75% of the positive *modD* isolates had the allele *modD1* (REF.[104]). A strong association with distinct meningococcal lineages was observed, although the dominant alleles were found in multiple lineages. The *modD1* phasevarion, which alters resistance to oxidative stress[101], was found to be associated with hypervirulent lineages[104].

These different alleles of the *mod* genes target and methylate different DNA sequences, thereby regulating distinct gene sets. Phase variation of *modA11* produced moderate (an average of twofold) alterations in the expression of 285 genes, including those encoding immunogenic outer membrane proteins such as the lactoferrin-binding proteins, and also modulates DNA repair and antibiotic sensitivity, while *modA12* differentially regulated 26 genes, some of them also involved in iron acquisition[105–107]. Thus, with up to three independently switching on or off *mod* genes, *N. meningitidis* has a powerful epigenetic mechanism available to stochastically vary gene expression and permit adaptation in response to the highly selective immune pressure of the host environment.

Glycosylation

Glycosylation of proteins is a post-translational modification associated with crucial biological processes implicated in host–pathogen interactions[108]. *N. meningitidis* exhibits a general *O*-linked protein glycosylation (*pgl*) system in which several surface-exposed and periplasmic proteins are glycosylated[109]. Protein glycosylation is important for protein function and interactions with other microorganisms and host cells, and affects the pathogenicity and virulence of the bacterium. The *pgl* core locus encodes three enzymes (PglB, PglC and PglD) involved in the synthesis of an undecaprenyl diphosphate monosaccharide and three glycosyltranferases (PglA, PglE and PglH) that can modify the monosaccharide by addition of sugars. The disaccharide and trisaccharide forms can be further modified through O-acetylation mediated by PglI. PglF is responsible for translocation of the glycan to the periplasmic side of the inner membrane, and the PglO/PglL oligotransferase adds the sugar chain onto the protein[109,110]. The *pglA*, *pglE*, *pglH*, and *pglI* genes are phase variable, which results in the formation and expression of multiple glycoforms. Although a variety of glycosylated proteins has been identified in *Neisseria* species, the PilE subunit of the pilin and the nitrate reductase AniA are the best characterized neisserial glycoproteins[111]. WGS has also revealed extensive polymorphism in the *pgl* core locus, with a variable presence of *pglG* (a putative glycosyltransferase), *pglH* and *pglI*[109,112,113]. Homologous recombination in the *pgl* loci was suggested to have a substantial role in the replacement of ST-7 by ST-2859 in the meningitis belt of Africa. Compared with the ST-7 strains, ST-2859 strains acquired a recombination block encompassing the *pglD*, *pglC*, *pglB* and *pglH* genes, whereas four other independent recombination events affected this locus in individual ST-2859 isolates[58].

Within-host variation

WGS provides new means to investigate the genomic evolution of bacteria during colonization and infection, and in particular to study mechanisms of within-host adaptation. Applying WGS to multiple isolates collected from the same host is increasing our understanding of the processes occurring during within-host evolution[114]. To assess within-host genetic changes in isolates from blood and the throat of four individuals with IMD, ultradeep WGS (average coverage of 1,500-fold) was performed on isolates recovered from both sites within 24 hours[115]. Eleven mutational events affecting eight different loci (average of three events per isolate pair) genetically separated the blood from the throat isolates in each individual. These comprised eight slipped-strand mispairing events and three recombinational events due to gene conversion. The slipped-strand mispairing events were located in *pilC1* (three events), *modA12* (two events), *pglI*, a phage-tail encoding gene and the promoter of *fetA* (one event each), whereas the three recombinational events included a Mu-like prophage, an haemagglutinin-like adhesin and the major subunit of the pilin protein, revealing that genes involved in type IV pilus biogenesis were predominantly affected[115]. A comparison of paired blood and cerebrospinal isolates from 195 individuals with IMD showed that most pairs had at least one variant locus between the two samples, again with genes related to pilus biosynthesis being the most frequently mutated[116]. Following infection with a laboratory strain, several modifications of phase-variable genes were detected after human passage, with *porA*, *lgtA*, *lgtC* and *hpuA* being among the most affected[117]. During long-term carriage, phase variation may reduce the expression of genes that encode surface proteins to avoid detection by the host immune system[118]. WGS analyses of paired isolates from 50 individual meningococcal carriers collected 2 months apart revealed changes in genes belonging to the pilin family and the restriction-modification systems and genes involved in glycosylation[119]. Substantial changes in the *pgl* genotype and/or glycan phenotype were identified in 48 of the 50 paired isolates[113].

Virulence

The factors that determine whether invasive disease will develop or not when a strain encounters a new host are yet not completely understood. The variation in the rates of IMD caused by different lineages suggests that the properties of the bacterium are essential, but the genetic basis for the observed differences in virulence is still a matter of investigation. Except for the genes involved in capsule biosynthesis and a functional filamentous prophage (designated MDAΦ for 'meningococcal disease-associated island') that has been associated with increased invasiveness[120,121], most potential virulence genes identified in meningococci are also present in other non-pathogenic *Neisseria* species[19] (FIG. 5). Thus, it has been hypothesized that the propensity to cause disease is a multifactorial property, depending on a combination of genetic elements that are commonly found in non-pathogenic species[122]. When compared with the genomes of isolates from individuals with IMD, similar isolates identified in the throat of close contacts of the individuals were found to harbour many differences, especially in genes known to be phase variable[76]. Following transmission of one or a few bacterial cells to a new host, the founder cells will start to proliferate, and the stochastic assortment of expressed virulence factors will produce a variant capable of crossing the nasopharyngeal mucosa and invading the bloodstream[122]. It has been suggested that hypervirulent lineages differ not in their colonization ability but rather in their ability to modulate the expression of genes, for example those involved in the oxidative stress response that allow dissemination within the host[123]. However, acquisition by horizontal gene transfer of a functional capsule locus and the MDAΦ prophage, which is known to encode the immunoglobin-binding protein TspB[124], appears to be sufficient to transform an asymptomatic carriage strain into a hypervirulent clone with potential for causing large epidemics[125].

Conclusions

WGS has made possible the exploration of the genome of the highly variable species *N. meningitidis* in unprecedented detail. As a consequence, many new insights have been gained during the past few years regarding its origin, evolution, adaptation to the host and virulence properties. Although there is compelling evidence

that *N. meningitidis* separated rather recently from *N. gonorrhoeae*, the ecological separation between the two species is becoming less clear. Increased contact between the species, as a result of changing sexual attitudes and practices, might have important public health consequences, with concerns for diagnosis methods, antimicrobial resistance development and treatment. WGS has already become an invaluable tool for researchers studying *Neisseria* species, replacing other molecular methods for surveillance and outbreak investigations.

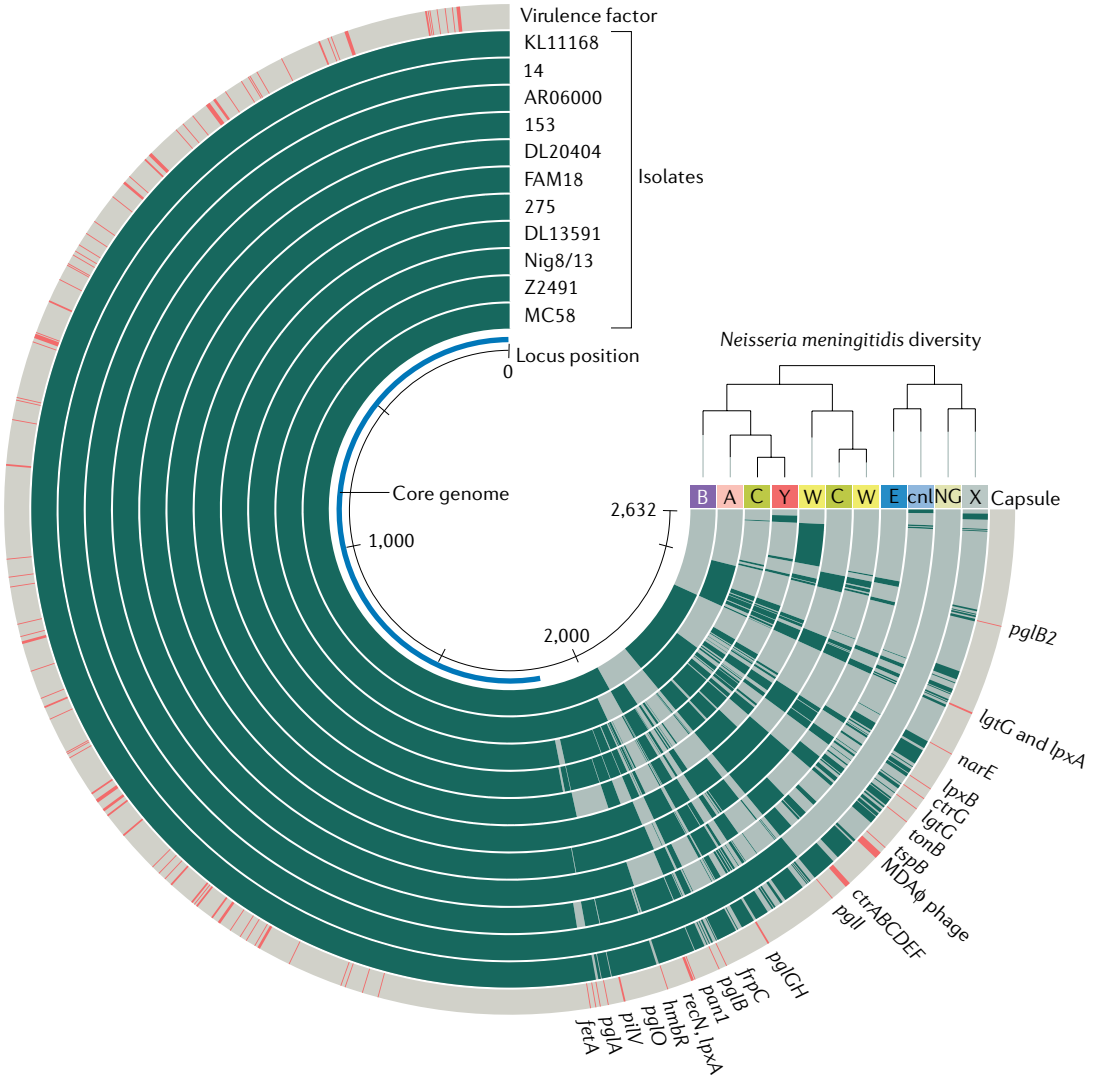

Fig. 5 | **Overview of the pan-genome content of 11 closed genomes of *Neisseria meningitidis*.** The presence (dark green) and absence (light green) of genes in 11 publicly available *N. meningitidis* genomes with different capsule phenotypes (coloured boxes on the right side) is shown. Above the capsule annotations, a phylogenetic tree shows the relation (as determined from the locus presence or absence patterns) between the isolates. In the outermost circle, known virulence factors are annotated with red ticks. 'Virulence factor' here refers to any genetic element known to be central to colonization, host invasion, immune system evasion or survival in adverse conditions such as in the presence of antibiotic compounds. In the accessory genome, the names of these virulence factors are shown. Virulence factors in the core genome are not annotated. They include a wide range of iron acquisition systems, invasins, efflux pumps, stress response proteins, pilins, glycosylation systems, other adhesins and catalase. As for virulence factors in the accessory genome, they include the capsule (including the capsule translocation system), the MDAΦ phage, *hmbR*- and *fetA*-mediated iron acquisition, the *frpC* and *narE* toxin genes, lipooligosaccharide-related genes such as *lgtG* and *lpxAB*, as well as different pilin glycosylation (*pgl* family) genes. The invasive isolates in this figure are FAM18, Z2491, MC58 and Nig8/13. There are no single known virulence factors that clearly differentiate these invasive isolates from the non-invasive ones. The isolates and the associated information (*Neisseria* PubMLST ID, serogroup, invasive or non-invasive infection and location) are as follows: KL11168 (84412, serogroup X, non-invasive, Burkina Faso), 14 (30, non-groupable, non-invasive, Germany)[154], AR06000 (83698, serogroup null, non-invasive, Ethiopia), 153 (2077, serogroup E, non-invasive, Germany)[154], DL20404 (84413, serogroup W, non-invasive, Burkina Faso), FAM18 (698, serogroup C, invasive, USA)[155], 275 (9756, serogroup W, non-invasive, Germany)[19], DL13591 (84387, serogroup Y, non-invasive, Burkina Faso), Nig8/13 (21589, serogroup C, invasive, Nigeria)[125], Z2491 (613, serogroup A, invasive, The Gambia)[156], and MC58 (240, serogroup B, invasive, UK)[157]. These isolates were selected from publicly available closed genomes to represent a diverse range of serogroups.

The availability of large genome projects such as the MRF-MGL and open-access Web-based databases such as PubMLST allows in real time the detection and characterization of outbreaks and refines our understanding of global meningococcal epidemiology, as has been illustrated by the analysis of lineage 11. The diversity of the species is restricted by population bottlenecks as well as by restriction-modification systems and DNA uptake sequences, which might limit horizontal gene transfer between lineages and species. Although a few hypervirulent lineages have been responsible for most of the IMD cases worldwide, the genetic properties that are required to cause disease are still not fully established. The capsule is a prerequisite to IMD development in almost all immunocompetent individuals due to its antiphagocytic properties and by providing resistance against complement-mediated killing. The MDAΦ phage also appears to be important for disease development, although its role and importance are still subject to speculation. Many of the studies comparing the genetic properties of isolates collected from different types of clinical samples or with different disease status identified the same genetic elements apparently under selection pressure (that is, type IV pili, glycosylation proteins, iron acquisition proteins, adhesins and evasins) through an on/off regulation of expression by simple sequence repeats. Further large-scale WGS studies are clearly warranted to resolve conflicting results on which virulence factors are essential for disease development and how stochastic events on the mucosal membrane can trigger pathogenicity. Improved and new technologies permitting analyses of bacterial genetic material directly from clinical samples without the cultivation step will also be essential. Stochastic variation affecting expression of the large array of phase-variable genes may be an important factor affecting invasion potential, but further studies using deep sequencing are needed to better assess which genetic factors or combinations of factors have to be expressed for disease development. Most currently generated WGS data are based on the short-read Illumina sequencing technology. With WGS used in combination with long-read sequencing, such as Nanopore sequencing, which allows researchers to better tackle sequence variation in the numerous repetitive elements present in *N. meningitidis*, questions regarding the evolution and epidemiology can be further elucidated. WGS has proved valuable in the development of vaccines against serogroup B disease (BOX 1) and is now the most efficient way to determine potential coverage of these protein-based vaccines, as well as to assess the changing prevalence of vaccine antigens in the meningococcal populations following vaccine introduction.

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