Molecular mechanisms leading to diversity in *Neisseria meningitidis*

Abstract

Neisseria meningitidis **causes devastating disease around the globe. Yet, key questions concerning colonization, emergent disease and diversity remain unanswered. Only a subset of individuals colonized with *N. meningitidis* contracts devastating invasive disease. The factors determining the establishment and progression of disease remain largely unknown. *N. meningitidis* exhibits a huge genetic and phenotype diversity. Extensive research on this variation will extend fundamental knowledge needed for the development of an efficient vaccine. Crucial for handling this unpredictable pathogen in the future, are insights about how rapid phenotypic switching and allelic diversity is mediated at molecular level. The present review highlights the molecular events that drive *N. meningitidis* genetic diversity which potentially affects the phenotype, as well as the issues to be resolved.**

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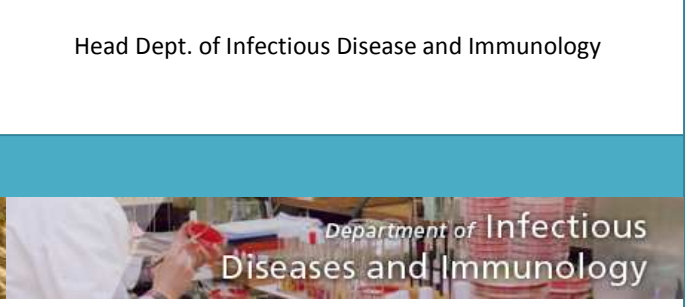

Introduction

Neisseria meningitidis, commonly named meningococcus, is a Gram-negative aerobic-bacterium that exclusively colonizes humans. The first descriptions of symptoms, later known to correlate with *N. meningitidis* infections, appeared at the beginning of the 19th century. At the end of that century the bacterium was isolated for the first time and linked to endemics of several clinical manifestations including meningitis (1). Next to meningitis, the pathogen can cause septicemia, pneumonia and many other manifestations (2). The various manifestations of *N. meningitidis* infections potentially complicate the diagnosis. Despite that approximately 10-35% of healthy adults are asymptomatic carriers of *N. meningitidis*, development of invasive disease is relatively rare. Incidences vary across the globe and range from up to 1/1000 in African regions to 0.2/100,000 in Europe (3-5). More than 10% of meningococcal disease is lethal and up to 19% of survivors suffer from long-term sequelae, like neurological disorders or loss of limbs (6). Young children are most at risk to develop invasive disease, followed by adolescents and young adults (3).

Structure and classification

Most isolates of *N. meningitidis* have a diplococcal shape, (figure 1) (7). The bacteria can be either capsulated or non-capsulated. Non-capsulated variants only rarely cause invasive disease in contrast to these being capsulated. Isolates obtained from blood or cerebrospinal fluid (CSF) rely on capsular expression to modulate opsonic and non-opsonic phagocytosis and avoid killing by the complement system. The composition of the polysaccharide capsule varies between strains and this has enabled classification of *N. meningitidis* into different serogroups. At this time 13 serogroups are distinguished of which 6 are known to cause invasive disease, namely strains A, B, C, W-135, X and Y (3). Expression of the capsule is encoded by the *cps* locus, which encompasses all information necessary for biosynthesis and translocation of the capsular polysaccharide (8). Most serogroups (B, C, W-135

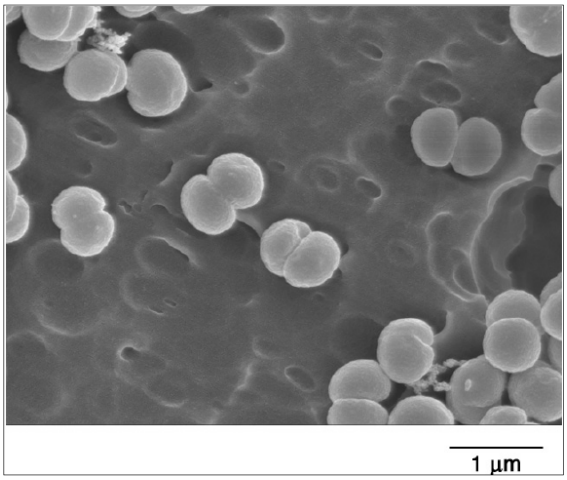

Figure 1. Electron microscopic detection of a diplococcal shaped *N. meningitidis* strain, here non-capsulated. Adapted from Talà et al. 2008.

and Y) carry a sialic acid capsule, while serogroup A contains a linked mannosamine (4). Sialic acid is associated with immune evasion properties of *N. meningitidis*, which is also true for the major outer membrane component Lipopolysaccharides (LPS). LPS is subject to multiple variations and takes a major part in modulating *N. meningitidis* antigenicity and influences its interaction capacity with host cells (9). *N. meningitidis* LPS is composed of an inner and outer oligosaccharide core attached to lipid A. The outer core has a variable composition and is linked to the HepI part of the diheptose of the inner core. The core oligosaccharide is attached to the lipid A that anchors the molecule in the membrane (9, 10).

Since about a decade, *N. meningitidis* strains are classified by multi-locus sequence typing (MLST). During MLST sequence types are distinguished on the basis of genetic differences in specific regions of seven housekeeping genes (11). Several hypervirulent lineages have been correlated to the emergence of invasive disease. It seems that the genetic constitution of hypervirulent strains remains surprisingly stable, when considering that *N. meningitidis* is one of the most versatile bacteria known (12). Nevertheless, clear criteria reliably predicting the outbreak of invasive disease are still a matter of investigation.

Genome composition

The *N. meningitidis* genome consists of a single circular chromosome. On average, the genome has a length of approximately 2.2 Mb depending on the strain. About 2000 genes are encoded in the genome. Compared to other species, it is remarkable that 20% of the entire chromosome consists of repeating sequences. This causes genetic instability and variability leading to antigenic variation and phase variation (13, 14). The *N. meningitidis* genome is about 90% homologous to the genomes of *Neisseria gonorrhoeae* and *Neisseria lactamica*. *N. gonorrhoeae* infects the uro-genital tract and can lead to local inflammation, but is not associated with invasive disease in contrast to *N. meningitidis*. *N.lactamica* is considered a harmless commensal bacterium that mainly colonizes children (15, 16).

Transmission and emergence of disease

Transmission of *N. meningitidis* occurs from person to person. Asymptomatic carriers can pass the bacterium via droplets out of the upper respiratory tract or saliva (6). Most individuals that encounter *N. meningitidis* will become carriers of the pathogen and will not develop invasive disease. In carriers, the pathogen typically colonizes the upper respiratory tract and the pharynx.

The carriage is usually asymptomatic and can persist for days to several months. When the bacteria gain access to the bloodstream, invasive disease may develop, which can lead to severe septic manifestations or other conditions like meningitis or pneumonia (1). Progression to sever illness is usually observed within the first two weeks after contact with the bacterium (17). To enter the bloodstream, the pathogen has to pass mucosal surfaces. Up to date, the "Trojan horse theory" is believed to adequately explain how penetration into the bloodstream occurs. It states, that *N. meningitidis* can penetrate and/or transcytose through epithelial or endothelial cells, and is carried across the cellular barriers (1, 4). Once inside the bloodstream, replication rates increase drastically and a strong inflammatory response is induced, mainly by LPS. Also the blood brain barrier can be passed and multiplication may occur in the CSF. Many factors have been determined to propagate invasive disease development, but it has not be fully revealed, which mechanisms account for the switch from the carriage state to progression of disease during *N. meningitidis* infections. Several virulence factors and the ability to rapidly switch between distinct phenotypes are important. The presence of the capsule is the only condition believed to be required for development of systemic disease, although capsular expression alone is not sufficient to evoke invasive disease (18, 19). Further factors correlated to disease development include host susceptibility, genetic polymorphisms, smoking, co-infections and young age (4, 20, 21). Especially variation in regulators of the complement system appear to be pivotal in determining disease progression (22). Overall, the low incidence of invasive disease compared to the carriage rates enhances the idea that *N. meningitidis* is a pathogen by accident and has no benefit from evoking severe conditions. Interestingly, certain hypervirulent lineages have been shown to persist for decades and to emerge during outbreaks. When considering that invasive disease is not advantageous for the spread of *N. meningitidis*, invasive strains might be expected to die out. The persistence of hypervirulent strains may indicate that the pathogenic potential is linked to increased carriage efficiency (23).

Initial colonization

To colonize its host, adhesion to the human mucosa is essential for *N. meningitidis.* Therefore the pathogen expresses redundant adhesins with binding capacities to various receptors like CD46, CD66, integrins and many more on epithelial or endothelial cells (24). Initially during infection, type IV pili confer adherence to eukaryotic cells and propagate dispersal. At later phases of

infection the pili retract and bacterial and host cell membranes undergo close interactions leading to engulfment of the pathogen. Primary adhesins among others involved in these processes include Opc, Opa proteins, PilC, PilQ and Factor H binding protein. Once internalized, *N. meningitidis* replicates inside host cells, often without causing serious harm, and thus masking itself from recognition by immune mediators (25).

Epidemiology

The emergence of different *N. meningitidis* serogroups is restricted geographically. Highest incidence rates for invasive disease are observed in periodic waves in regions of sub-Saharan Africa known as the "Meningitis Belt", evoked by serogroup A. Up to 1000 disease cases in a population of 100,000 have been reported during these outbreaks, with case-fatality rates of 75% in patients under the age of 15 (6). Further, serogroups A, C, Y and W-135 are responsible for carriage and disease in Africa and Asia. Serogroups B and C are predominantly detected in Europe. Disease rates vary between countries from 0.2 to 14 cases per 100,000. Invasive disease is mainly evoked by serogroup B in European countries which introduced serogroup C vaccines (5, 26). In the United States serogroups B, C and Y are most prevalent. All serogroups account comparable for disease, but frequencies change during periods relatively to each other. The overall disease rates in the United States are positioned around one case in 100,000. Globally, the highest rates of disease are observed in children below the age of 4 and in adolescents (3, 6).

Vaccines

Effective vaccines against *N. meningitidis* have been developed during the past decades. However, not all serogroups can be targeted yet and a universal vaccine protecting against all serogroups is desirable. First vaccines against *N. meningitidis* have been developed in the 1960s, based on targeting capsular polysaccharides of serogroups A and C. Due to poor immunogenicity, especially in children below the age of 2, conjugated vaccines have been introduced since the end of the 1990s. These conjugated vaccines improved the immunization effectiveness, but still significant loss of memory was a major drawback. Further conjugative vaccines, also targeting other serogroups, have been introduced or are currently tested in clinical trials. For example, a huge campaign has being initiated, having the immunization of 250 million people in 25 African countries as goal, by distributing a novel serogroup A conjugated vaccine (27). Despite all ongoing progress, an effective vaccine directed against multiple serogroups could not be implemented yet. Ditto, no vaccine protecting against all serogroup B strains is available (1, 26).

Thus replacement of non targeted serogroups remains a threat demanding surveillance, as learned from vaccine campaigns against *Streptococcus pneumoniae* (1). Applied reverse vaccinology issuing the identification of conserved antigens has led to promising approaches for upcoming vaccines. Clinical trials are ongoing to evaluate safety and effectiveness of novel serogroup B vaccines. However, development of non-strain specific vaccines will be a tough task to fulfill for future developers. Variants can emerge spontaneously and may make vaccines ineffective (27).

Diversity in *N. meningitidis*

N. meningitidis appears to be one of the most versatile bacteria known. Different mechanisms aid immune evasion and adaption to changing environments. Rapid on/off switch of gene expression allows *N. meningitidis* to appear in markedly different phenotypes within a single strain (28). Frequent allelic and antigenic variation of key surface components drives *N. meningitidis* immune escape (1). The mosaic like structure of the *N. meningitidis* genome witnesses an evolutionary history of recombination between mixed DNA (23). DNA uptake mechanism are regarded to be central in providing foreign DNA to subsequently enlarge or alter the genomic content by recombination (1). The three classical routes for lateral DNA exchange in bacteria, namely transformation, transduction and conjugation, have been described in *N. meningitidis* (29-31). Next to these processes, phase variation devoted lots of attention concerning phenotype switching in *N. meningitidis.* During phase variation genes, e.g. coding for outer membrane proteins or involved in capsular expression, are shut on and off in a high frequency fashion. The ability to regulate expression of components which might be advantageous in only specific colonization conditions makes *N. meningitidis* a highly adaptive pathogen (28). Also environmental regulation of gene expression is a common adaptive mechanism which alters the *N. meningitidis* phenotype. Various environmental stress conditions regulate *N. meningitidis* gene expression. Microarray analysis of the *N. meningitidis* genome revealed extensive gene regulation in changing environments which are summarized elsewhere (32).

Despite solid approaches characterizing mechanisms responsible for *N. meningitidis* diversity, unanswered questions concerning the development of diversity and regarding the population structure remain. For example, individuals are typically colonized with only one strain. In the time course of carriage, allelic diversity of adhesins develops. According to current understanding development of suchlike diversity demands lateral DNA exchange between

heterologous strains bearing genes coding for variants of the respective adhesins. No such co-colonization has been observed by today. It is thus poorly understood how extensive allelic variation develops from initial colonization with only one *N. meningitidis* strain (1). Unexpectedly, evolutionary successful lineages which expand clonally during outbreaks, show only diminished genetic and antigenic variation. In order to effectively adapt to changing environments during spread, adaptive behavior by genetic variation would be more obvious (12). Another remarkable fact is, that despite being antigenically and genetically extremely diverse, disease evoking variants are only presented by a small subset (23).

The purpose of this review is to describe the mechanisms responsible for emergent genetic diversity which are revealed and supposed yet, and their potential contribution in the context of *N. meningitidis* as a potent immune evading unpredictable occasionally, as well as fascinating from a microbiologists view, pathogen.

DNA uptake mechanisms in *N. meningitidis*

Natural competence

N. meningitidis is natural competent during its entire life-cycle, which is true for the entire *Neisseria* genus. Intra- as well as interspecies lateral genetic exchange within the *Neisseria* genus contribute to genetic diversity (29). Uptake of DNA involves four stages, passage of the outer membrane, transport across the periplasm, shuttling through the inner membrane and often incorporation of imported DNA into the chromosome via homologous recombination (33). *N. meningitidis* DNA uptake is restricted to the presence of DNA uptake sequences (DUS) within the donor DNA. DUS are repeated DNA sequences between 9-10 or 12 base pairs in length that can be detected all over the genome (29). DUS are mainly found as inverted repeats. Due to that orientation DUS are speculated to also act as intrinsic transcriptional terminators, by forming hairpins through their orientation within the genome when getting transcribed (34). For *Neisseria* genomes the number of DUS approximates about 1900, which is relatively high compared to other human-colonizing bacteria in which similar sequences have been found (16, 35). An over-expression of DUS within *Neisseria* has been reported, indicating that lateral DNA exchange within that genus is a standard mechanism favoring fitness rather than an extraordinary evolutionary event. Frequent recombination of mixed DNA between *Neisseria* facilitates antigenic variation. However, *Neisseria* species remain in their ecological niche. It is believed

that effective DUS mediated inter-genomic exchange must be restricted to a certain degree between *Neisseria*. Otherwise, mergence of individual *Neisseria* species to one species would be likely (36). Until recently the DUS were thought to share the common 10-mer 5‘GCCGTCTGAA3‘. The common sequence was updated to a 12-mer, as it was evidenced that a T at -1 of the 10-mer and an A at -2 are widespread in the *N. meningitidis* genome. Transformation efficiency assays revealed preference of the 12-mer DUS to the 10mer DUS. Consequently the 12-mer DUS is recommended to consider in future studies (29). Interestingly, DUS seem to be specifically concentrated within genes contributing to DNA repair, recombination, restriction modification and replication. Next to a possible contribution for establishing diversity, it is thus speculated that DUS may play a role in the recovery of the entire population during genotoxic stress by acquiring undamaged copies of the essential DUS containing genes (35).

Because *N. meningitidis* is Gram-negative, DNA has to pass the outer membrane during the uptake process. The type IV pili apparently play an essential role in fulfilling this task. They are found on the bacterial cell surface as filamentous appendages, (figure 2) (4). Different pili are characterized by their morphology and assembly mechanisms. Type IV pili are the most commonly found pili in Gram-negative bacteria and they are multifunctional. Next to DNA uptake, type IV pili are important colonization factors, conferring motility, aggregation, cell adhesion and further functions, making these hair-like structures potent virulence factors (37). To get insights into the fine tuning of the type IV pilus function, an assessment to identify the function determinants of the type IV pilus has been done. By evaluating the impact on competence of genes composing type IV pili that are dispensable for the biogenesis, pilin ComP, which is only found in low abundance, and PilT, associated with twitching motility, have been identified as essential for competence in *N. meningitidis*. Another low abundance pilin, PilV, could be characterized as competence antagonist (38, 39). However, the molecular mechanisms of

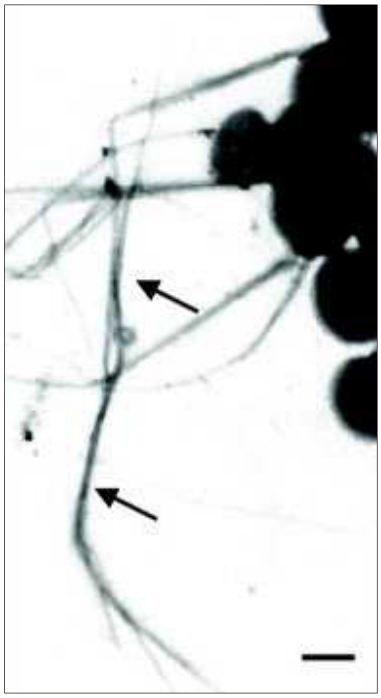

Figure 2. Electron micrograph of a specific *N. meningitidis* strain, with pili attached indicated by arrows. The bar scales 0.5 µm. Adapted from Hill et al. 2010.

assembly or structural information remain poorly characterized. As the type IV pilus is essential for competence, it is not surprising that DNA uptake is equally dependent on the expression of compounds needed for pilus biogenesis. Secretins are responsible for translocating individual compartments through the outer membrane during pilus biogenesis. PilQ proteins present a subset of these secretins. They confer extrusion and contraction capabilities to the type IV pilus. *N. meningitidis* mutants lacking these proteins lose natural competence. Also various mutations affecting type IV pili expression strongly reduce competence (33). It remains to be investigated if this is due to the lack of composing an entire functional pilus, or if specific interactions between DNA and pilus assembly components take a major role in conferring competence. PilE is the major pilin protein and composes polymeric fibers which establish the pilus. In vitro assembly studies of PilQ and type IV pili indicate that a described PilQ complex could be the channel shuttling PilE across the outer membrane to the bacterial surface (40). Which mediators take control for DUS selection is not described. PilQ has affinity for DNA and DNA binding was stronger than for the complexed type IV pilus (33). The affinity of PilQ for DNA however was not restricted to DNA containing DUS, indicating that specific selection for DNA take up happens at another stage. Furthermore binding to ssDNA was more effective compared to dsDNA binding. Based on these observations, a potential mechanism for DNA presentation to *N. meningitidis* as an initial event to happen during transformation was hypothesized. It has been suggested that the binding of DNA to PilQ is linked to retraction of the type IV pilus, as the positively charged PilQ channel gets exposed to the environment then and potentially attracts DNA. Structural analysis of the PilQ complex, defining it as central chamber which gets polymerized and can form a channel, support this theory (41). Next, other mediators capable of binding DNA possibly execute selection for DUS and further DNA processing. Preference of PilQ for ssDNA binding opens room for speculation. It has been argued that one strand of dsDNA might be degraded, ssDNA is encountered more frequently in the *N. meningitidis* natural environment, or other DNA uptake mechanisms next to transformation account for this finding (33). More interaction partners that bind DNA non-specifically have been discovered, but mediators keeping the responsibility for the DUS selection remain undefined (42).

The actual persistence of DNA uptake restriction by DUS may be considered unfavorable from an evolutionary perspective. Acquiring useful foreign DNA sustaining adaption and immune evasion not only between *Neisseria* could outweigh negative aspects of DNA uptake. It might

thus be expected that DUS gets negatively selected in favor of fitness, rather than accumulating in high numbers as it is observed. In the molecular drive model, it is hypothesized that DUS probably overcome such like selection in a "selfish" manner, and possibly do not contribute to fitness in *N. meningitidis* at all, thus being actually adverse. On the other hand, DUS dependent DNA uptake restriction contingently act as surveillance system to protect the bacterium against harmful DNA like transposable elements or material derived from phages. But because it was evidenced that DUS are under-expressed in novel laterally acquired genes, and there is no omnipresent distribution of DUS in DNA which potentially could be taken up, it was suggested that DUS thwart diversity, rather than assisting diversity emergence (43). Thus, it can be argued that horizontal gene transfer might be more significantly impacted by other means than transformation, and processes like transduction or conjugation deserve deepen attention. However, two different theories concerning the role of transformation come into conflict here (43, 44). Whether there is no or only a small apparent role for transformation in laterally acquired diversity, or if transformation is a mediator facilitating antigenic diversification in spite of the reported contrary findings, will demand further investigations.

Phages and diversity

Several sequences in *N. meningitidis* have been identified that originate from phages. The presence of prophages is a commonly observed phenomenon in bacterial genomes. Up to 20% of a bacterial genome may consist of prophage derived sequences that often provide functional genes. Functional genes from prophages that are beneficial to the host frequently withstand negative selection. Remnant sequences of the prophage get lost, leaving the selected genes in the genome (45). The presence of Mu-like phages was discovered in *N. meningitidis.* Mu-like phages found were often present as mosaic DNA, suggesting emergence from different related phages by horizontal DNA transfer. Computational predictive analysis revealed a subset of these sequences to encode membrane associated genes. It was thus suggested that these genes contribute to antigenicity and might have an influence on virulence and pathogenicity. These findings hint that transduction may influence membrane protein composition. However, it is essential to note that in this case the phage infected its host bacterium in an initial evolutionary event. After selection of phage derived sequences, no functional prophage is left. Specific phage derived genes acquire mobility themselves, rather than being transmitted via transduction subsequently. Following, transduction seems to happen initially, but the mediation of lateral DNA transfer might be taken

over by transformation in subsequent generations (31). Further, a whole genome comparison of *N. meningitidis* isolates was done to disclose genes associated with invasive disease. A prophage of the filamentous bacteriophage family could be detected in isolates correlated to invasive disease. It was further found that this phage behaves like a mobile genetic element, as the sequence was detected in form of a circular nuclease resistant element within the supernatant of bacterial cell cultures (46). The genetic element was unraveled to be actively secreted by the bacterial cell and was suggested to encode genes propagating transition from a commensal state to invasive disease. PilQ could be identified as essential for active secretion of the element. Interestingly, the mechanism behind seemed to be independent on pilus extrusion. How the mechanism is mediated is not revealed, but these findings, again, suppose a central role of PilQ for the active lateral exchange of DNA (33, 46). A subsequent approach was carried out to identify, whether the relation of this particular phage to invasive disease is truly causal, rather than due to clonal expansion. The effect of the phage on virulence of separate clonal complexes was assessed for this purpose. Interestingly, it could be shown that up to 45% of hypervirulent strains tested in this study had obtained their disease evoking potential by that particular phage. No conclusive homologies to any known virulence factors have been found. In conclusion, the phage was characterized to bear genes acting as virulence factor, which have a strong impact on the pathogenic potential of *N. meningitidis* (47). Another phage investigated, named "neisserial filamentous phage", revealed a novel integration mechanism in *N. meningitidis.* The repeat sequence dRS3 is part of a family of rapid sequences with a 20 base pair consensus found in *N. meningitidis.* dRS3 could be identified as target site for integration of the neisserial filamentous phage, which was assumed to use its own specific transposase to integrate (48). Subsequently it was suggested that this integration complex of the neisserial filamentous phage could be responsible for recombination events of any dRS3 repeat within the *N. meningitidis* genome, thus resulting in genome reconstitution (13).

The mentioned observations hint to a crucial role for phages in acquiring diversity in *N. meningitidis.* Nevertheless, more insights are needed to draw a clear picture of the relevance of phages for diversity and for the development of invasive meningococcal disease. It should be noted that it is rather difficult to distinguish transduction and transformation considering that phage sequences can acquire mobility independently from a functional phage. Thus transformation may also play a pivotal role.

Conjugation and diversity

Lateral exchange of conjugative plasmids has been observed in *N. meningitidis*. Plasmids recognized as being transferred between *N. meningitidis* confer various resistances to antibiotics. However, these plasmids only minor contribute to diversity, as plasmids capable of mobilizing chromosomal elements have thus far not be identified in *N. meningitidis* (42). Nevertheless conjugation may play a role in DNA exchange, especially within the *Neisseria* family. *N. meningitidis* is capable of receiving plasmids containing genes coding for beta-lactamases from *N. gonorrhoeae* via conjugation (30). Similar observations involving other resistance carrying plasmids have been reported including inter- and intra- species conjugation (49). There is only limited data questioning which basic underlying molecular mechanisms are involved in *N. meningitidis* conjugation, although Type IV pili are known to be essential (25). PilQ could be shown to bind effectively DNA in a DUS independent manner, but a possible link of this observation to conjugation has only been hypothesized (33). Thus a defined correlation between conjugation and the emergence of diversity could not be drawn so far.

In conclusion, the three classical routes of lateral DNA transfer, conjugation, transduction and transformation, occur naturally in *N. meningitidis.* Natural transformation is frequently regarded as the most important route for DNA uptake. As mentioned, recently doubts about this position have been raised, and the importance of other routes, especially of transduction, might be underestimated. The classical view awarding a central setting of competence to diversity development appears to be highly contentious. There are numerous findings hinting to molecular mechanisms responsible for lateral DNA transfer, but mediators and routes are due to be identified to obtain an explicit picture. In how far intergenomic recombination in *N. meningitidis* contributes to diversity is difficult to estimate clearly. Homologous recombination between intergenomic elements has been considered one of the major forces facilitating diversity in bacteria including *N. meningitidis.* Co-colonization with any pathogenic or non-pathogenic bacterial species has been supposed to provoke novel variants (50). For instance, high frequencies of recombination of certain alleles between *N. meningitidis* and *N.lactamica* have been described (51). However, it has to be kept in mind that recombination is not necessarily the primary mechanism for acquiring genetic diversity and enhancing adaptation. Numerous studies indicate, that intergenomic recombination mainly aids DNA repair, occasionally resulting in adaptive diversity. The actual presence of DUS and a lack of multiple DNA repair genes support this

hypothesis (16). Yet, these findings do not thwart the importance of recombination for the development of diversity. For the generation of novel genomes in *N. meningitidis*, intergenomic recombination has been estimated to have 10 times higher impact than spontaneous mutations (23). Because DUS selects for the uptake of *Neisserial* DNA, ingested DNA likely shares homology with the genome. Also, homologous recombination happens efficiently in *N. meningitidis*. It is thus not surprising that recombination occurs at high frequencies. Most housekeeping genes have a mosaic structure, indicating that recombination is a major determinant of the genome make up (52). Whether transduction might be more significant for lateral DNA exchange than has been argued so far and if natural competence plays a key role in genome maintenance awaits future research.

Intragenomic events involved in *N. meningitidis* variability

N. meningitidis is remarkably specialized in switching its structural and antigenic appearance to adapt to environmental changes and to avoid immune detection. Various genes have been reported to be present in multiple copies within the *N. meningitidis* chromosome, strongly favoring intragenomic recombination (4). Recombination of distinct loci which share regions of homology facilitates antigenic variation (53). Phase variation is another major mechanism contributing to bacterial phenotype diversity. Through mispairing of repeat sequences during DNA repair or replication, the transcription and translation of genes is controlled (54). The ability of independently switching off and on the expression of different gene products makes *N. meningitidis* a successful colonizing organism by rapidly adapting to distinct environments (4). Under ongoing investigation are mechanisms, which putatively mediate epigenetic gene regulation in *N. meningitidis*. Phase variation of genes coding for methyltransferases may be responsible for rapid switches between distinct expression patterns of multiple genes (55). Genes which are sequence variable and of which the expression is controlled are commonly involved in establishing cell surface compartments. For instance, the expression of gene products essential for the composition of surface glycans, LPS, lactoferrin, transferrin binding proteins and the capsular are controlled by phase variation. Either expression levels can be modulated, or the expression of a gene product can be shut on or off completely during phase variation. For instance, capsular expression is observed to be variable, either by switching to distinct serotypes, as well by switching to a non-capsulated phenotype (4, 13). The major constituent of intragenomic variation is the presence of repeat DNA sequences. Next to DUS, further families

of DNA repeats have been described, facilitating the events of phase variation as well as recombination. Appearance of DNA repeats in *N. meningitidis* ranges from repeats spanning a few base pairs, like it is the case for simple sequence repeats (SSRs) sized between one and seven nucleotides involved in phase variation, to 26 base pair repeats conferring mobility to genetic elements of a few hundred base pairs in size (13, 56).

Phase variation

Phase variation is crucial for *N. meningitidis* adaptation to changing environments. By reversible high frequency on/off switching of surface compartments, *N. meningitidis* can rapidly change between distinct phenotypes and thus alter its actual life style. Different niches can be occupied if advantageous for survival, like switching between intracellular and extracellular appearance. Possibly many of these switching events are involved in converting *N. meningitidis* to an invasive pathogen. More than 80 genes are assumed to undergo phase variation, which results in a enormous number of possible phenotypes the bacterium is capable of presenting itself (28, 56). Phase variability of surface antigens has been correlated with emergence of invasive *N. meningitidis* cells (57). It is thus of crucial interest and significant clinical relevance to understand the underlying mechanisms of phase variation.

Phase variation is mainly mediated by slipped strand mispairing. During slipped strand mispairing, the number of repeats within SSRs increases or decreases. This occurs while DNA replication or DNA repair takes place. Due to misalignment during complementary base pairing between the parent strand and the daughter strand, the synthesized DNA can decrease or increase the number of the SSRs. If this occurs within a sequence having an effect on the expression of a protein, phase variable gene expression might be mediated. Transcription is affected, if the repeats are positioned within the spacer between the -10 region and the -35 region of a transcriptional promoter. In this way, efficient binding of the sigma factor which directs the RNA polymerase to promoter sites to initiate transcription, is abolished (58, 59). Spacing of 17 base pairs between both element gains most efficient transcription. Alteration in length of the 17 base pairs spacing on a single nucleotide results in significant loss of transcription efficiency, while alteration of a few base pairs commonly disrupts transcription (60). A phase variable effect can also be achieved when the repeats are located outside the promoter region, by affecting the stability of mRNA or altering the binding affinity of regulatory proteins. When the repeats are positioned within a protein coding sequence, translation might be affected. If the misalignment

results in a frame shift that is not a multiple of three, these mutations will disrupt the function of the synthesized protein (61). The introduction of a stop codon will lead to premature translational arrest and thus to phase variation (62). Phase variation rates considered up to now occur at relatively constant rates, specific for individual sequences (63).

Sequences involved in N. meningitidis translational phase variation

In *N. meningitidis* the expression of numerous genes has been revealed to be controlled by translational phase variation. As mentioned previously, capsular expression is strongly linked to invasive disease, as *N. meningitidis* cannot survive within blood or CSF in a non-capsulated stage. However, the role for capsular expression during colonization and dissemination is still not clear. It has been proposed that reversible capsular expression shut off during colonization of *N. meningitidis* might favor progression to invasive disease. Isolates obtained from patients during an outbreak of meningococcal disease were shown to contain either an insertion or deletion within a DNA residue normally containing a $(dC)_7$ box within the *siaD* gene. This particular gene codes for the alpha-2,8 polysialyltransferase responsible for the biosynthesis of the poly-sialic acid capsule. The insertion of an additional cytosine residue frame shifts downstream sequences leading to translation termination shortly after the $(dC)_7$ box. On the other hand, deletion of one cytosine residue generates a stop codon 30 base pairs downstream of the $(dC)_7$ box. Insertion or deletion most likely occurs via slipped strand mispairing and yields an un-functional protein, thus no capsular biosynthesis can be achieved in that state. The process described is reversible and thus could play a crucial role in facilitating rapid adaption, as capsular re-expression was estimated to occur at rates of 10^{-3} (62). Consequently, these observations considered together with similar data derived by other studies established a scenario in which *N. meningitidis* needs to shut off capsular expression during passage through mucosal epithelium. Upon entering the circulation, capsular expression is needed again, in order to evade immunity (64).

Essential for *N. meningitidis* pilus formation and adhesion to epithelial cells is the pilus associated protein PilC. The expression of PilC is controlled by translational phase variation. PilC is constituted by conserved and variable regions. Commonly, *N. meningitidis* strains carry two homologous variants of PilC, PilC1 and PilC2 respectively. PilC1 is usually detected in isolated strains while PilC2 appears to be present only in a subset of isolates (65). In *N. gonorrhoeae* alterations in a guanine repeat were found to be responsible for inducing slipped strand mispairing and thus translational control of PilC (66). In *N. meningitidis* a similar mechanism

was identified by the presence of a $(dG)_9$ box at the 5' end of the *pilC* gene (67). Frame shifting of the coding sequence results in translational control and thus on/off switch of PilC. Switching frequencies for PilC of up to 10^{-4} have been reported. On and off switch of PilC likely provides advantages for survival in different environments and passing barriers between these (65).

Variation of the *lgtA* gene of *N. meningitidis* results in high frequency on/off switch of the entire *lgtABE* locus. This locus encompasses three genes coding for glycosyltransferases which are essential for the synthesis of the terminal LPS structure lacto-N-neotetraose (9). By utilizing a homologous hybridization probe targeting a known gene involved in LPS phase variation in *Haemophilus influenzae*, the *lgtABE* locus could be identified in *N. meningitidis*. Phase variation mediated by slipped strand mispairing was evidenced to occur in the *lgtA* gene by a set of $(dG)_{14}$ positioned at the 5' end of the gene. Either insertion or deletion of guanine residues during DNA repair or replication resulting in a different number of guanine repeats frame-shifts the upstream start codon and thus prevents translation of the entire locus. Via this regulation the LPS structure can be switched to two distinct immunotypes (68).

Opa proteins are major adhesion and invasion factors, which determine the tissue tropism to a significant extend. Most abundant, four to five loci coding for Opa proteins are found in *N. meningitidis*. All characterized *opa* genes contain a CTCTT tandem repeat at the genes 5' end (25). In *N. gonorrhoeae*, an alteration of the number of repeats of this pentamer by slipped strand mispairing could be related to a frame-shift of a start codon found in *opa* genes. The striking sequences could equally be detected in *N. meningitidis*, supposing the same mechanism in both species. Thus, translation of Opa proteins is individually effectively controlled. This raises a vast number of combinations of distinct Opa proteins, determining the phenotype of *N. meningitidis* and influencing tropism and host immunity (69).

In order to acquire essential iron, *N. meningitidis* has evolved expression of the two component receptor HpuAB and the receptor HmbR. Both receptors are responsible for haemoglobin binding and the expression of both receptors is individually controlled by on/off phase variation. Most likely, expression of two phase variable receptors fulfilling a similar task facilitates immune evasion, while ensuring uptake of iron needed for growth of the pathogen. Within the *HmbR* locus either a $(dG)_9$ box or a $(dG)_{10}$ box is present, of which a nine guanine repeat yields an functional protein eventually. Slipped strand mispairing leads to a frame shift and incorporation of an additional guanine terminates translation after 396 amino acids. Interestingly this putative inactive translate would present a 44 kDa protein. This putative protein could not be detected so

far, but possibly has an unknown function, as usually translation termination takes place more close to the 5' end of a gene yielding significantly smaller translates. During phase variation of the HpuAB receptor only 49 amino acids are translated before premature translation arrest happens. Here as well, variants positive for HpuAB were found to have a $(dG)_{10}$ box within the *hpuA* locus, while HpuAB negative variants had an additional guanine residue inserted at this spot. Further, translational arrest within the *hpuA* locus affected the *hpuB* locus equally, thus resulted in shutting off the expression of the entire HpuAB receptor (70).

Approximately 70% of the tested *N. meningitidis* isolates contain the gene coding for the autotransporter AusI. That protein belongs to the serine protease family, which is unique to *N. meningitidis* (71). Serine proteases in *N. meningitidis* have been speculated to play a role in colonization and invasion by exploiting host antibodies (72). However, the function of AusI can only be speculated based on sequence comparison analysis. Experimental evidence for any function is lacking. Anyways, phase variation of AusI could be indicated. Isolates positive for AusI were disclosed to contain either a $(dC)_6$ box or a $(dC)_9$ box within the *ausI* gene. AusI negative variants contain a $(dC)_{10\text{-}11}$ box, shifting the *ausI* gene out of frame, which yields an un-functional protein (71).

Sequences involved in N. meningitidis transcriptional phase variation

Transcriptional phase variation presents another major control mechanism of phase variable gene expression. The *N. meningitidis porA* gene codes for pore forming class one outer membrane proteins. Here, a $(dG)_{11}$ box located within the spacer between the -10 an -35 domain of the *porA* promoter is a hot spot for slipped strand mispairing. Deletion of one guanine residue results in less efficient transcription, while the deletion of two guanine residues prevents transcription entirely. Frequencies of decrease or shut down of *porA* expression are estimated at 10^{-3} in clinical samples. It is supposed that slipped strand mispairing during replication accounts for the variable phenotypic appearance (73). Phase variation of class one outer membrane proteins facilitates host immune evasion (74).

Another event of phase variation of which the molecular mechanism could be suggested, concerns the Opc protein. Opc is expressed in most *N. meningitidis* strains and was characterized as adhesin (25, 57). A cytosine repeat regulates transcription efficiency of the *opc* gene by slipped strand mispairing. Start of *opc* transcription was supposed to take place 13 base pairs downstream of a -10 region. Upstream of this region a $(dC)_{12\text{-}13}$ box was identified in Opc

positive samples, extending through the -35 region. Either a $(dC)_{11}$ or $(dC)_{14}$ box has been detected to down regulate transcription. Other repeat numbers of cytosine residues observed where correlated to Opc protein shut off. 5-10 fold reduction of *opc* derived mRNA was present in Opc down regulated samples, whereas no mRNA could be detected in Opc negative *N. meningitidis* having an altered cytosine number in the identified spot. Thus, the cytosine repeat stretch is either located in a binding site essential for *opc* promoter function, or interferes with transcription by being positioned between the putative binding site and the -10 region (75).

The ferric enterobactin receptor FetA, involved in iron uptake via ferric enterobactin, was identified in *N. gonorrhoeae* and *N. meningitidis*. In *N. gonorrhoeae* FetA could be characterized as highly phase variable with switching frequencies observed to occur up to $1.3*10^{-2}$. As FetA is a major outer membrane protein, its phase variable regulation strongly sustains immune escape. Because FetA is present in both species, *N. gonorrhoeae* and *N. meningitidis*, it may descend of a common gene pool in the *Neisseria* species. The molecular mechanisms responsible for FetA phase variation in *N. gonorrhoeae* likely resemble molecular events occurring in *N. meningitidis* (76, 77). Spacing between the -10 and -35 region of the FetA promoter can be regulated by slipped strand mispairing in *N. gonorrhoeae*. A $(dC)_{12}$ box was correlated with high expression of FetA and the spacing of both promoter elements was found to be 17 base pairs long. Changes in cytosine repeats leading to spacing lower than 17 base pairs or higher than 18 base pairs were correlated with low expression of FetA. Interestingly 18 base pairs spacing was correlated to high expression either, while 17 base pairs would be the optimal spacing length as mentioned previously. The *FetA* promoter was deduced to be extraordinary powerful, probably due to the high amount of cytosine present within the spacer region. Thus 18 base pairs of spacing seemed not to affect promoter binding to an extend as previously expected (76).

Another approach implicated phase variation of the adhesin NadA which is commonly expressed in *N. meningitidis*. Upstream of the -35 region of the promoter, a tetranucleotide repeated tract (TAAA) was discovered. Variations in the number of this SSR between 8 and 13 were tested. A clearly phase variable effect leading to NadA down regulation could be proven for repeat numbers of 9 and 12 of the SSR. As the remaining repeat numbers tested were correlated to high NadA expression, no periodic pattern correlating the repeat numbers to NadA expression was evident. Further investigations considered more varying numbers of repeats, as well as a mutant lacking the SSR entirely, which showed intermediate expression of NadA. Only a pattern of quasiperiodicity could be reasoned (58). However, a phase variable effect was evident, and it was

supposed that this SSR from *N. meningitidis* could act as an “upstream element”. The presence of “upstream elements” has been evidenced in *Escherichia coli* (54). In *E.coli*, a sequence, rich in adenine and thymidine residues, positioned upstream of the promoters -35 region, was previously shown to strongly stimulate transcription. The observed enhancement is achieved by binding of the RNA polymerase α-subunit to the upstream element and thereby enhancing binding of the RNA polymerase to the promoter (54, 78). A similar mechanism responsible for phase variation of the *N. meningitidis* NadA adhesin was supposed, due to apparent genetic similarities concerning adenine and thymidine richness, as well, due to a likewise position of the SSR respectively the promoter region. (54). Probably by slipped strand mispairing the efficiency of that mechanism is lowered and thus down regulates NadA expression. Subsequently, it was revealed, that regulation upon altering the number of SSRs occurs via adjusting the affinity of the transcription factor integration host factor (IHF) to the *nadA* promoter. It was argued, that this effect was either achieved by deviating binding efficiency of IHF to the specific SSR, or indirect via binding to a spot that is frame shifted due to mispairing. Phase variation of NadA likely is crucial for switching between a phenotype mainly advantageous for transmission, to an appearance more suitable for carriage (79).

Further sequences not experimentally evidenced to undergo phase variation, are speculated to be controlled by phase variation based on sequence comparison in genetically differing variants. The expression of thirty-three genes was thus identified as putatively phase variable (54). Overall when considering all proteins under the control of phase variation characterized thus far, it is obvious that a large number of variations between all these proteins makes *N. meningitidis* able to present itself in a vast number of distinct phenotypes. This not only enables *N. meningitidis* to compete with other species for niches to be occupied, but will also challenge the host immune system to recognize the bacterium. For future approaches, it is worth to consider other factors influencing phase variation. For instance, the impact of the environment, or the influence of the bacterium’s physiological state on phase variation, could have a controlling effect on this fascinating mechanism, opening further questions to the stance of phase variation in a fundamental, as well in a clinical perspective (63).

The N. meningitidis phasevarion

Restriction modification systems (RMS) are commonly found in bacteria. It is thought that these systems protect bacteria against foreign DNA, e.g. of invading phages, by sequence specific cleavage of non-self DNA. RMS are divided into three groups based on characterization of cleavage recognition sequences, subunits incorporated and cofactor dependency. For high frequency variation in gene expression, the type III RMS is considered relevant by today. Type III RMS are composed of a complex consisting of two methyltransferase subunits and two restriction endonucleases subunits. The methyltransferase, encoded by *mod* genes, methylates DNA specifically, while the restriction endonucleases, encoded by *res* genes, cleaves sequence specific unmethylated double stranded DNA. The restriction endonuclease is dependent on dimerization with the respective methyltransferase, whereas the methyltransferase can execute its function independently (80). Recent findings indicate that type III RMS are under the control of phase variation and hence influence expression of multiple genes. This machinery has been described as "phase-variable regulon" or "phasevarion" (81). In *N. meningitidis* several *mod* alleles have been identified, of which *modA11* and *modA12* are most predominant. A tandem repeat $(AGCC)_n$ within *modA11* and *modA12* was identified to mediate frame-shift mutations of a distal start codon resulting in on/off phase variation. Strikingly, phase variation of both *mod* alleles apparently regulates the expression of multiple genes, by either down-or up regulating them, when tested under conditions mimicking a physiological relevant state. *modA11* influenced the expression of almost 300 genes and *mod12* affects 36 further genes, including outer membrane proteins, evidenced in an experimental setting. As further *mod* alleles were found in *N. meningitidis*, probably each of them is able to regulate a distinct set of genes. It is not elucidated yet, which part in the phasevarion is taken by active restriction endonucleases. For other species it has been observed, and preliminary findings hint to that model equally in *N. meningitidis*, that the restriction endonuclease needs to be inactivated in order to obtain gene regulation by methyltransferases (82). Despite not proven experimentally yet in *N. meningitidis*, epigenetic gene regulation mediated by differing methylation patterns of genes among bacteria is not a novel finding (83). Because inactivated restriction endonucleases have been identified in bacterial cells in which multiple genes were controlled by methyltransferases, an epigenetic regulation via methylation seems obvious. Potentially, *mod* phase variation would allow rapid switch between markedly different cell types by altering methylation patterns of multiple genes (84). Either, virulence factors could be controlled and randomized according to this model. This would lead to

tremendous variations of pathogenic properties within individual cells in a colonizing population (55). Further functions for phase variation of type III RMS have been proposed. Via altering the methylation pattern of specific genes, DNA uptake could be enhanced temporally, e.g. by loosening the selection for DUS. This would allow uptake of potentially useable genes. The mosaic structure of the *N. meningitidis* genome sustains such a model. Also the actual emergence of the mosaic genome structure despite the presence of strict DNA uptake control mediated by DUS could be explained that way. Another related approach states that type III RMS might induce degradation of self DNA. Suchlike mediated "bacterial suicide" of a population part would facilitate release of DNA into the environment. Extracellular DNA could be taken up by the residual population and serve for DNA repair or recombination, thus enhancing diversity (55). However, data sustaining the latter two models is needed to further argument into these directions.

Recombination mediated antigenic variation

A mechanism for antigenic variation mediated by recombination is well characterized in *N. gonorrhoeae* and suggested to occur similarly in *N. meningitidis* (53). Here, the PilE expression locus, *pilE,* recombines with at least one of several silent *pil* loci (*pilS*) in a unidirectional fashion in frequencies of up to $4*10^{-3}$/cell/generation, and thus mediates phenotypic alterations. *pil* genes in general are constructed of conserved and variable regions. However, *pilS* loci do not contain promoter regions and can consequently not be expressed (85). During the recombination event, the part of the *pilE* sequence which undergoes recombination with *pilS* gets lost and is replaced by the corresponding *pilS* sequence. The *pilS* sequence remains unchanged. Hence, the result of this recombination event is considered to be comparable to resultant gene conversion observed during recombinational repair in other species (63, 85, 86). Recombination mediated pilus antigenic variation is strictly dependent on the RecA protein (63). Based on characterization in *E.coli*, RecA was identified as a crucial part of the RecABCD pathway mediating DNA double strand brake repair via recombination. RecA was also shown to significantly prolong survival in *N. meningitidis* upon induced DNA damage (87). The molecular background of how RecA mediates unidirectional transfer of genetic material remains largely unknown. Different molecular models have been proposed, including various theoretical explanations for inter – and intragenomic recombination possibly accounting for recombination between *pilE* and *pilS* (63). As mentioned previously, the role of intergenomic recombination for the development of

diversity, and especially, antigenic variation has become controversial. Data obtained from distinct studies support the idea that intergenomic recombination does not contribute significantly to pilus antigenic variation. One model strongly sustaining this view states, that the flanking homology around distinct loci within a chromosome and between taken up DNA would strongly favor homologous recombination of these regions. Therefore, relatively more efficient recombination between *pilE*/*pilE* and between *pilS*/*pilS* would not allow the recombination observed between *pilE* and *pilS* (85). The model widely accepted to probably describe the actual mechanism most closely, involves insertion of "mini cassettes" from *pilS* into *PilE*. This is proposed to occur via recombination of "mini cassettes" within p*ilS* encompassing variable *pil* sequences, embedded between conserved sequences found equally in *pilS* and *pilE*, finally resulting in gene conversion (88). Still, the working mechanism of RecA for producing gene conversion remains speculation. For sure, the mechanism behind has to enable the occurrence of a recombinational event despite the presence of considerable non-homology between "mini cassettes" and corresponding *pilE* sequences (85). Assuming that RecA works similar to a synaptase probably adequately approaches the actual mechanism (63). Synaptases are involved in fusion of homologous sequences and introducing homologous single stranded sequences to double stranded DNA (89). Further models, including exposition of respective sequences to DNAse and thus introducing double strand brakes initiating RecA dependent unidirectional DNA transfer, as well as involvement of other DNA repair mechanisms, have been speculated and contribute to obtain a clear picture of the underlying mechanisms (85). However, as it is difficult to catch this event while actually occurring due to its stochastic appearance, only the resulting gene conversion has been scrutinized so far. Hence pilus antigenic variation still is a challenging field of investigation. Further studies, especially considering *N. meningitidis* pilus antigenic variation, would clarify the overall picture of this phenomenon.

Concluding remarks

N. meningitidis remains a poorly understood pathogen in many respects. Understanding of factors determining disease progression, as well the development of a non-strain specific effective vaccine are still major challenges. Thanks to novel approaches, vaccine development forges ahead. Promising trials are ongoing for serogroup B vaccines. Huge campaigns around the globe issuing implementation of serogroup A and C vaccines have led to successful immunization of

large populations. However, *N. meningitidis* genetically and functionally is one of the most diverse bacteria found perhaps due to being an exclusive human pathogen. Both, allelic diversity and rapid phenotypic changes mediated by phase variation, makes this pathogen a potent immune evader and colonizer of different anatomical niches. Rapid switching between distinct *N. meningitidis* surface antigen compositions complicates valid estimations for vaccine efficiency. Once a vaccine is implemented, replacement by different serogroups is a threat seriously awarding consideration. The probably best way out of this misery would be the formulation of a universal vaccine composed of antigens that are conserved among all strains. Equally central are insights about the settings in which a colonizing state alters to conditions of invasive disease. *N. meningitidis* needs to journey through various compartments and different environments to enter the blood stream and CSF. It is obvious that this only hardly would occur without adaption to allow rapid changes between different phenotypes that can escape host immune mediators and enable entry into host cells. It is thus of major importance to understand the molecular mechanisms conferring diversity in *N. meningitidis* and to lay open weak points of the pathogen.

Phase variation is a key feature of *N. meningitidis* allowing rapidly switching between distinct phenotypes. Slipped strand mispairing is one of the driving mechanisms behind phase variation. The mechanisms of phase variation of several proteins have been unraveled, all contributing to randomly switch between vast numbers of distinct phenotypes. Especially phase variation of *mod* genes, the phasevarion, probably confers rapid switching between two different cell types and randomization of virulence factors. Future research putting light on the importance of the phasevarion in disease and adaption likely will tell interesting gene regulatory mechanism. Additional sequences are predicted to undergo phase variation. Precise characterization of the phase variation mechanisms in these sequences and which proteins are influenced will help to better understand *N. meningitidis* virulence and immune escape capabilities. It is still an ongoing debate, to what extend natural competence is a contributor to genome composition. Phage transduction probably accounts for the mosaic like structure of the *N. meningitidis* genome more than it is assumed in most publications. Confirming data of assumed mechanisms behind recombination mediated pilus antigenic variation is due to be presented. Conceiving the exact processes occurring would broaden our knowledge, not only for specifically dealing with pathogens, but also in fundamental genetics.

Based on the evident variability and adaptability of the fascinating bacterium *N. meningitidis*, it surely will further challenge future vaccine developers. Ditto, all factors responsible for disease

progression on either sites, the host and *N. meningitidis*, remain to be uncovered. Any therapeutic intervening with invasive meningococcal disease will be a huge step forward. Fundamental understanding of how *N. meningitidis* manages diversity emergence is crucial to answer these questions in the future. Not only characterizations in a single organism, but also matching diversity mechanisms found within other *Neisseria* species, will aid the prevention of *N. meningitidis* evoked invasive disease in the future.

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