Genetic variation in Neisseria meningitidis
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General discussion
In the previous chapters, the data were discussed separately. Still, this leaves a number of observations for discussion. Also, some data in different chapters should be combined in order to clarify some of the questions that arose in chapter 1. In the following paragraphs, four issues are addressed. First, the genetic diversity of serogroup A strains is reviewed with respect to the population structure of *N. meningitidis*. Some of the conclusions that are valid for serogroup A, may be applicable to other serogroups as well. In turn, this raises questions on the role of natural transformation in meningococci. What could be the main Darwinian evolutionary advantage of this phenomenon? Next, the data from chapters 5 through 7 are discussed. The proposed hypothesis and the used approach, which followed from chapter 4, are briefly reviewed. The search for the determinants of the hyperendemic character of lineage III strains yielded a restriction modification system, *NmeSI*. The possible link between *NmeSI* and the hyperendemic character of lineage III is discussed. Finally, the possible role of restriction modification systems in meningococci in general is discussed.
In chapters 2 and 3, the genetic diversity of a collection of serogroup A strains was studied. One of the main conclusions is, that the data suggest a clonal, rather than an epidemic population structure as suggested for meningococci in general by Maynard Smith et al. (17). This has been inferred for the serogroup A strains from previous MEE data as well (30). However, the significance of the data presented in chapter 3 is, that they are obtained by the combination of three independent methods, of which the independent markers are shown to display considerable linkage.

It was noted before, that serogroup A shows two main clades (9), but it is possible that more clades will be identified when other geographic areas are sampled. Spratt et al (30) have argued, that the apparent limitation of recombination can arise from biological (lack of sexual mechanisms), ecological (different bacteria rarely meet each other) or epidemiological (the rate of spread is sufficiently high to reduce the effects of recombination) reasons. They suggested that the latter two possibilities might form the principal reason for the lack of recombination in serogroup A. However, these arguments are not completely satisfactory.

Firstly, though carriernesship in African countries may be low, in China it is high (1), and serogroup A strains of both clades have apparently been able to reside in the Chinese population in the 1970s between the pandemic waves. Also, members of the two clades have been isolated from Dutch patients in the same year (1989 and 1990). This means that these bacteria coexisted in the Dutch population, thus having access to each other’s gene pool as well as the vast pool of genes supplied by strains of the other serogroups. It is unlikely, that the Dutch patient isolates represent the repeated introduction of these organisms from putative isolated serogroup A populations. Theoretically, ecological exclusion could be due to different habitats of each of the serogroup A clades. However, there are no indications that the serogroup A meningococci occupy other niches in the nasopharynx and oropharynx than meningococci from other serogroups. Secondly, the epidemiological explanation remains unsatisfactory, for similar reasons. The two clades also include isolates from carriers, and rapid spread, preventing the effects of recombination to become apparent, contrasts with the long periods between epidemic waves. Moreover, this rapid spread did not occur in several industrialized countries after introduction of the subgroup III isolates following the Haj pilgrimage, even though subgroup III isolates have been found in e.g. the Netherlands afterwards. In addition, a hit-and-run lifestyle of serogroup A strains should not interfere with the possibilities to undergo recombination: in a country with high carriage rates and a diverse meningococcal population, the net effect is similar for the resident meningococcus that encounters a certain number of acquired meningococci per time unit compared to the newly acquired meningococcus encountering the same number of resident meningococci per time unit.

The above arguments suggest that there is a biological reason for the limited recombination for serogroup A strains. It is unlikely that the physical properties of the serogroup A capsule
are the cause of this phenomenon. Most isolates from the throat are not encapsulated, thus an
effect for instance of a difference in surface charge of the serogroup A capsule as compared
to other capsular polysaccharides is unlikely to be the reason for the limited horizontal gene
transfer. Perhaps the clonality should be seen in a different light: clonality implies linkage,
thus also the genetic information comprising the unknown reasons for the clonality of the
serogroup A strains is linked to the genes necessary for the biosynthesis of the serogroup A
capsule.
The absence of panmixia in serogroup A strains is in accordance with a lower ratio between
recombination and mutation, as suggested by the studies of Morelli et al. (21) and recent data
of Feil et al (10), who observed a lower recombination to mutation ratio in serogroup A
meningococci than in other N. meningitidis serogroups. Is clonality confined to meningococci
with the serogroup A capsule? The answer may very well be negative: some of the pandemic
serogroup B/C strains also have been relatively stable for prolonged periods of time. This has
been interpreted before as reflecting clonality (3). The apparent diversification observed for
some of the hyperendemic B/C isolates may very well be the consequence of a higher
sampling efficacy in countries as Norway and the Netherlands, detecting rare recombination
events. In addition, founder effects (comparable to sequential bottlenecks (1), as observed for
the ET-5 cluster of serogroup B meningococci, may also contribute to population divergence
once such a clone spreads to a different geographical entity. In this respect, the different
clones within for instance the ET-5 cluster would be comparable to the subgroups within the
serogroup A clades.
The argument for the epidemic model is that members from certain highly successful clones
become overrepresented in the strain collections. The comparison of carrier and disease
isolates shows that the strains that are the principal cause of disease, are represented in the
carrier population at a lower level (3, 5). Thus, the successfulness of meningococci of
overrepresented clones is not in the competition for a certain niche, but in causing disease.
Again, this implies linkage of the typing characteristics with the capability to cause disease,
meaning clonality, in meningococci of certain clones associated with increased incidence of
disease.
Together, the arguments above suggest that the role of recombination in diversifying
Neisserial populations may be very limited, possibly as its effects are diminished by the (non
sexual) proliferation of the bacteria. This of course does not underestimate its importance in
the spread of antibiotic resistance (28, 29) or new surface antigens (16).
POSSIBLE ROLE FOR NATURAL TRANSFORMATION

In view of the thoughts outlined above, it may be of importance to address the role of natural transformation in *Neisseria* species. One of the characteristics of this process in these bacteria is its genus specificity, mediated by the *Neisseria* DNA uptake sequence (DUS). If the evolutionary advantage of the natural transformation machinery was found in the uptake of an energy rich substrate, one would not expect this specificity. There is no point in being dainty in the choice of one's food. An advantage in the occasional uptake of different alleles from unrelated meningococci is unlikely, as this will only rarely be the case. Most of the DNA that will be encountered by a given meningococcal cell will be a remnant of a sister cell. Moreover, in this scenario, one would expect the DUSs to be confined during evolution to the close proximity of genes that encode surface antigens, for which variation is beneficial. In contrast, the (incomplete) genome sequences suggest that the DUSs are evenly spread over the genome, similar to *Haemophilus influenzae* (27).

This leaves another possibility, being the uptake of DNA for the reparation of (detrimental) mutations in the DNA. Phase-variation of surface structures seems to be an important aspect in the interaction of Neisseria (and similar mucosal pathogens) with the host (19). For a number of proteins, the phase variation was shown to be due to variation in the length of stretches of repeated nucleotide motifs in the genes encoding these proteins. Such variation may lead to an altered promoter strength, and therefore lower transcription, or to an out of frame mutation, and therefore to incorrect translation of the transcript of a gene. The variation in length of these stretches is thought to be caused by slipped strand mispairing during replication. Recently, it was suggested that these mechanisms of phase variation are influenced by mismatch repair (7). Maybe, the *Neisseria* have a slightly elevated mutation rate (or lower repair efficiency), which would be beneficial for high frequency phase variation by the aforementioned mechanisms. The disadvantages of a higher mutation rate could be counterbalanced by the uptake of neighboring DNA as a template for repair. In fact, a high recombination rate within a population and a relatively low recombination rate between populations implies a purifying (diversity purging) effect of recombination within a population, maintaining the clonality of this population (6). This may also explain why only few one basepair mutations are found within a cluster of genetically related strains by MLST (10).

HYPERVIRULENCE-ASSOCIATED GENOMIC SEQUENCES

Having discussed possible reasons for clonality, still leaves the question why certain clones cause more disease. It was hypothesized that meningococci belonging to these clones carry genetic elements on their chromosomes being informative for their more virulent character, which are not present in meningococci rarely causing disease. Representational Difference
Analysis (RDA) was used in an attempt to identify such sequences in meningococci belonging to the lineage III clone. First, the assumptions on which this analysis was based will be shortly reviewed. Lineage III, characterized by the P1.4 serosubtype, seems responsible for the increase in cases of meningococcal disease in the Netherlands (4, 25, 26). As described in chapter 4, different clones expressed this serosubtype in the 1960s, without causing an increase in meningococcal disease. In addition, lineage III strains with different serosubtypes were isolated. Therefore, the results described in chapter 4 suggest that the P1.4 epitope is not the only characteristic that is responsible for the spread of this clone. Since certain P1.4 strains were not associated with increased incidence of disease, such strains were used in representational difference analysis. The driver strains were deliberately chosen from serogroup B, as the aim of the study was not the comparison between strains from different serogroups, but comparison of serogroup B strains with different epidemiological behavior. Finally, the driver strains belonged to a lineage (lineage IV) that was more related to lineage III than other lineages (4).

A major conclusion of the representational difference analysis, is that no pathogenicity island was found in lineage III, as in pathogenic strains of *Helicobacter, Salmonella, Escherichia coli,* and *Yersinia* (12). Although it could be argued that this is due to a limitation of the chosen method, this is unlikely since for the specific 1.8 kb locus that was identified, three RDA amplicons were found, containing more than half of the locus. Should there be a pathogenicity island present (often more than 30 kb), it is expected that a number of amplicons of that putative pathogenicity island were detected. The specific fragments that were identified, encode a restriction modification (RM) system with a regulatory protein. As discussed also in chapter 6, a different RM system could be involved in the divergence from the commensal relationship with the host towards the more pathogenic relationship observed for lineage III, with detrimental effects for both host and bacterium. Different methylation patterns as well as the action of the regulatory protein can interfere with (a) regulatory mechanism(s), resulting in subtle changes in the timing of certain processes, skewing the balance towards disease. When the RM system has replaced another RM system, differential methylation could be either the presence of the newly acquired methylation pattern or the absence of the existing (“non-hyperendemic”) methylation pattern. Loss of methylation or new methylation of a regulatory site, or the binding of a newly introduced regulatory protein, could potentially influence the “normal” pattern of transcriptional regulation. Part of this speculation can be addressed relatively simple: the site that is methylated by NmeSIM was identified (chapter 6), and a binding site of NmeIC was proposed (Chapter 7). Identification of these sites in the lineage III genome should not prove to be too difficult. However, only indirect evidence will be obtained by analysis of the location of these sequences, as we do not know what phenotype should be looked for. A model for assessing hyperendemicity is lacking.
STRAIN-SPECIFIC RESTRICTION MODIFICATION SYSTEMS MAY AFFECT CLONALITY

The restriction modification system \textit{NmeSI}, of which the identification was described in chapters 5, 6 and 7, is specific for lineage III strains. It is not surprising that all lineage III strains tested contain this system: RM systems can behave as selfish genes, killing the cells that loose expression of the methylase gene by the action of the restriction enzyme, and preventing invasion of a different RM system, a process that has been called apoptotic mutual exclusion (22). A few non-lineage III strains were found that were positive for the \textit{nmeSIM} PCR. These strains probably represent strains in which the \textit{nmeSIM} gene was obtained by recombination after uptake via transformation, either from lineage III strains or from the unknown organism that served as the donor to lineage III.

Inter-strain differences caused by restriction modification systems exist in many bacterial species. The traditional phage typing method is based on differences in RM systems, RM systems have been called "bastions of polymorphism" (20). Notably, in the preliminary sequence of the genome of the serogroup A strain that is being sequenced, a different RM system seems to be located at a position identical to the site of integration of \textit{NmeSI} in lineage III strains. This does not render the arguments, put forward in the previous paragraph on possible connections between \textit{NmeSI} and the hyperendemic character of lineage III, worthless. The serogroup A strain belongs to a clone that has caused epidemics, which suggests that the aforementioned putative effect of differential methylation is primarily caused by the absence of a previously existing ("non-hyperendemic") methylation pattern rather than the introduction of a new methylation pattern.

Alternatively, a different RM system can very well affect clonality by influencing the rate of successful recombination. Although usually RM systems are thought to decrease successful recombination, it may very well have the opposite effect. The observations, from which a negative influence is concluded, are usually confined to one specific locus, or on a very long stretch of DNA. If one performs a transformation experiment, selecting for recombination of a certain locus containing a cleavage site recognized by a resident restriction endonuclease of the acceptor strain, one is bound to observe lower recombination rates. Indeed, this has been observed in \textit{Neisseria}, though only to a small extent (11). However, experiments in \textit{E. coli} suggest a role of RM systems in creating mosaic genes after recombination, possibly by providing recombinogenic ends (18). It was proposed earlier, that RM systems could serve to accelerate evolution by providing highly recombinogenic ends (8, 23). As the \textit{NmeSI} recognition site AGTACT seems highly underrepresented in the \textit{Neisserial} genome, only a limited positive effect on recombination would be expected in this respect. In general, a strain carrying a RM system with a less frequent recognition site should display a lower rate of recombination than a strain carrying a RM system with a more frequent recognition site, all other parameters \textit{(e.g. other RM systems)} being equal. This may explain why lineage III is
relatively clonal, having a RM system with infrequent recognition sites as compared to other meningococci containing RM systems with more frequent recognition sites.

The polymorphisms in RM loci in the pathogenic Neisseria, known from literature (31) and from the comparison of the locus downstream of the helicase in the serogroup A genome and the NmeSI locus, suggest some evolutionary advantage of maintaining RM systems and of maintaining diversity of RM systems. Although RM systems may act as "selfish genes", resisting loss, this does not explain why a cell that acquires a RM system should outgrow a sister cell that did not acquire the RM system. A feasible explanation may be that RM systems are protective against bacteriophages. Previously, no bacteriophages were found for meningococci, but the preliminary data from one of the genome projects revealed sequences of a prophage (13, 24). For E. coli, it was suggested that bacteriophages might impose frequency-dependent selection, favoring rare restriction modification types (14). Bacteriophages could play a role in maintaining diverse RM systems in N. meningitidis as well. Still, the exact nature of the difference in virulence potential between lineage III strains and endemic strains remains to be explained.

REFERENCES

24. Sanger Centre, personal communication


