Extensive Genomic Variation within Clonal Complexes of Neisseria meningitidis

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Accepted: 9 November 2011

Data deposition: Seven draft genomes are presented in this article and have been submitted to GenBank: AGBN00000000, AGB00000000, AGBP00000000, AGBQ00000000, AGBR00000000, AGBS00000000, and AGBT00000000.

Abstract

Meningococcal disease is a widely distributed complex disease affecting all age categories. It can cause severe meningitis and septicemia, especially in unvaccinated infants and young children. The causative agent, Neisseria meningitidis (Nm), can be phenotypically and genetically differentiated into serogroups and sequence types (STs) and has a highly dynamic population structure. To obtain a deeper understanding of the epidemiology of Nm, we sequenced seven Nm genomes. Large-scale genomic analysis was conducted with these 7 Nm genomes, 27 additional Nm genomes from GenBank, and 4 other Neisseria genomes. We observed extensive homologous recombination in all gene functional categories among different Nm genomes. Homologous recombination is so frequent that it has resulted in numerous chimeric open reading frames, including genes in the capsule biosynthesis cluster and loci targeted by commercial vaccines. Our results reveal that, despite widespread use, evolutionary relationships inferred from the standard seven-gene multilocus sequence typing (MLST) method could not predict virulence gene content or strain phenotype. In fact, up to 28% of the virulence-associated genes could differ between strains of identical STs. Consistent with previous studies, we found that allelic recombination is also associated with alterations in antibiotic susceptibility. Overall, these findings emphasize the extensive genomic plasticity of Nm and the limitations of standard molecular methods to quantify this genotypic and phenotypic diversity.

Key words: homologous recombination, horizontal gene transfer, genome evolution, multilocus sequence typing, virulence gene.

Introduction

Neisseria meningitidis (Nm) is an encapsulated Gram-negative bacterium. Although it is a common commensal with a carriage prevalence of about 20% in human populations (Claus et al. 2005), Nm can cause severe meningitis and septicemia (Stephens et al. 2007). The genetic basis for the remarkable differences in meningococcal virulence is still a matter of investigation (Schoen et al. 2008). Some efforts have been made to identify virulence factors that are associated with the invasive disease (Dunning Hotopp et al. 2006). However, many designated virulence genes in Nm were also found to be present in nonpathogenic species such as Neisseria lactamica (Snyder and Saunders 2006; Bennett et al. 2010). Similarly, commensal and nonpathogenic species serve as reservoirs of virulence alleles and they engage extensively in the exchange of genes and gene pieces, largely via homologous recombination (Nakamura et al. 2004; Marri et al. 2010).

The high frequency of recombination results in a highly dynamic population structure (Holmes et al. 1999). Multilocus sequence typing (MLST) groups sequence types (STs) into clonal complexes (CCs) by their similarity to a central allelic profile (genotype) (Maiden et al. 1998). Since its initial introduction (Maiden et al. 1998), MLST has been embraced for characterizing bacterial variation and facilitating epidemiological investigations (Maiden 2006). Despite the increasing
popularity of MLST, it has been recognized that MLST cannot be used to infer phenotypic characteristics. For instance, MLST cannot be used to predict vaccine coverage due to the poor correlation between MLST and antigenic variability (Bambini et al. 2009) and the isolates of identical ST, but distinct serogroup have been described (Yazdankhah et al. 2004). Evolutionary relationships of Nm may be better resolved by using more loci (Didelot et al. 2009) or whole genome approaches. A recent analysis using genomic data has suggested that the Nm species population has evolved into distinct phylogenetic clades larger than the lineages identified by MLST (Budroni et al. 2011).

Despite the wide recognition of homologous recombination in the Nm species, the extent of recombination still demands a systematic investigation. In this study, we sequenced 7 Nm isolates, 6 of which form 3 pairs with identical serotypes and serosubtypes. We then conducted a large-scale comparative analysis on these seven genomes together with additional 31 Neisseria genomes. We attempted to assess the extent of homologous recombination, especially among strains with identical STs and CCs, and address potential functional implications of these recombination events through analysis of genes involved in antibiotic resistance, capsule biosynthesis, and vaccine efficacy.

### Materials and Methods

**Genomes Sequenced and Analyzed**

The seven clinical Nm strains were all blood isolates from cases of invasive disease. Six of them represent three matched pairs (table 1). Paired strains are of identical serotype and serosubtype and were isolated from elderly patients from Ontario, Canada. However, they are temporally distinct and were obtained 4–6 years apart. The seventh isolate, Nm1140, is an unusual invasive strain that could not be serogrouped by serogroup-specific capsular antigen antisera (National Microbiology Laboratory, Public Health Agency of Canada) or real-time PCR (Public Health Laboratories, Public Health Ontario; Taha 2000; Corless et al. 2001; Mothershed et al. 2004). Genome sequences for the seven Nm strains were generated via GS-FLX (Roche/454 Life Sciences, Branford, CT) pyrosequencing. Draft genomes were assembled using the gsAssembler (Roche) and CAP3 (Huang and Madan 1999), and further edited and visualized using the Phred/Phrap/Consed software package (Gordon et al. 2001). The seven draft genomes have been submitted to GenBank and the accession numbers are AGBN00000000 (Nm8187), AGBG00000000 (Nm8663), and AGBR00000000 (Nm2732), AGBP00000000 (Nm6938), AGBN00000000 (Nm6756), AGBS00000000 (Nm6140), and AGBT00000000 (Nm1140). Genome sequences for additional 27 Nm, 1 N. lactamica (Nlact), and 3 Neisseria gonorrhoea (Ng) strains were obtained from GenBank (supplementary table S1, Supplementary Material online).

**Virulence Genes and Homolog Identification**

A set of 177 putative virulence genes in the Neisseria genus was originally identified in Marri et al. (2010) by searching published literature and available databases. Out of these 177 genes, 164 genes are present in the Nm species, 153 of them are present in the MC58 genome (Tettelin et al. 2000), and the remaining 11 are absent from MC58 but present in the Z2491 genome (Parkhill et al. 2000). Sequences of these 153 MC58 genes and 11 Z2491 genes were extracted from the reannotated genomes in the NeMeSys database (Rusniok et al. 2000). Sequences of these 153 MC58 genes and 11 Z2491 genes were used as queries in a BlastN search (Camacho et al. 2009) and used as the query sequences to identify virulence genes present in other genomes (for a complete list of these virulence genes, see supplementary table S2, Supplementary Material online). The nucleotide sequences of the examined genes were used as queries in a BlastN search (Camacho et al. 2009). Homologues of these genes were identified using BlastN with an E-value <10−28 and match length >85%. To identify orthologues, we required genes present as single-copy per genome.

**Multilocus Sequence Typing**

A standard seven-locus scheme (Maiden et al. 1998) was used to determine multilocus ST. The determination of STs was conducted on the Neisseria MLST website (http://pubmlst.org/neisseria/) developed by Jolley and Maiden (2010) and based at the University of Oxford, UK.

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**Table 1**

Nm Isolates Sequenced in the Study

| Isolates | Serogroup | Serosubtype | ST  | CC  | Year | Patient Age/Gender | Penicillin MIC (μg/ml) |
|----------|-----------|-------------|-----|-----|------|--------------------|-----------------------|
| Nm8187   | Y         | NT/P1.5     | 3,923 | 167 | 2002 | 77/M               | 0.12                  |
| Nm3127   | Y         | NT/P1.5     | 3,980 | 167 | 2006 | 73/F               | 0.50                  |
| Nm6938   | W135      | NT/P1.6     | 22    | 22  | 2001 | 72/M               | ≤0.06                 |
| Nm2732   | W135      | NT/P1.6     | 22    | 22  | 2007 | 96/F               | 0.12                  |
| Nm8663   | Y         | 14/P1.-     | 23    | 23  | 2002 | 80/M               | ≤0.06                 |
| Nm6756   | Y         | 14/P1.-     | 23    | 23  | 2008 | 73/M               | ≤0.06                 |
| Nm1140   | NG-cnl    | —           | 1,136 | 1,136 | 2009 | 10/M               | ≤0.06                 |

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*NG-cnl, capsule null locus.*
Determination of Nonvertically Acquired Genes between Identical-ST Genomes

We assumed that, if the strains from the same ST are truly clonal, all nucleotide differences within the same ST should be solely due to point mutations. Given the identical seven MLST loci, we can estimate an upper bound of nucleotide divergence for these genome pairs. If these mutations are independent random events, under a binomial distribution, the $P$ value to observe no change in a 3288-nucleotide sequence (the total concatenated length of the seven MLST sequences) is 0.001 when the divergence is set to be 0.0021 (and is 0.05 when the divergence is 0.00091).

We assumed that the divergence is 0.0021 between any two genomes from the same ST, and genes that had more than the expected number of nucleotide changes at a significance level of 0.001 were deemed as nonvertically acquired genes. All statistical tests were conducted using the R package (R Development Core Team 2009). It is important to note that the significance level of 0.001 was chosen to obtain a more conservative estimate for the identification of nonvertical genes, and the use of a relaxed significance level of 0.05 would have resulted in the identification of more nonvertical genes.

Simulations were performed to assess the robustness of the nonvertical genes determination. In brief, the MC58 whole genome sequence was used as a reference genome and substitutions were randomly introduced as in the Jukes–Cantor model (Jukes and Cantor 1969) to generate "derived" genomes and, as done in Hao and Golding (2008), no indels were allowed. Nucleotide sequences of the MC58 single-copy genes (1822 in total) were used as query sequences in a BlastN search (Camacho et al. 2009) against the derived genome sequences, and the best Blast hits were collected as orthologues for further analysis.

Phylogenetic Analyses

Homologous sequences were aligned using MUSCLE (Edgar 2004). DNA distance among sequences was measured using dnadist of the PHYLIP package (Felsenstein 1989) version 3.6. The taxon with the shortest phylogenetic distance was identified as the nearest neighbor (Koski and Golding 2001). Nucleotide diversity (Tajima’s $\pi$), which is the average number of nucleotide differences per site between any two DNA sequences from the sample population (Tajima 1983), was calculated using the BioPerl Perlymorphism module (Stajich and Hahn 2005). Phylogenetic trees were constructed using a maximum likelihood method via the RAxML program (Stamatakis 2006) under a GTR $+\Gamma+\I$ substitution model.

There were 1072 single-copy genes universally present in all 38 examined Neisseria genomes (for a complete list of the 1,072 genes, see supplementary table S3, Supplementary Material online). The phylogeny of the concatenated sequences of the 1,072 genes (992,796 characters) was constructed using the RAxML program (fig. 1). Five hundred bootstrap replicates were generated. Furthermore, a maximum likelihood tree was constructed for each individual gene using the

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**FIG. 1.**—Maximum likelihood tree constructed using the concatenated sequences of 1,072 universally present genes. The tree was rooted using N. lactamica as the outgroup (not shown). Five hundred bootstrap replicates were generated, and only the bootstrap values $<99\%$ are shown. A maximum likelihood tree was also constructed for each individual gene and the proportion of genes (out of 1,072) that support each branch was calculated and shown in parentheses only for the relatively well-supported branches (with a support $\geq0.1$). CCs are red.
of minimum inhibitory concentrations (MICs). Isolates were tested for susceptibility to penicillin (0.004 to 8.0 μg/ml) at the concentrations noted (table 1), and interpretive criteria were used according to the CLSI document M100-S21, 2011 (CLSI 2011). Testing was repeated on a different day and yielded identical results.

Results and Discussion

Population Structure and Phylogenetic Incongruence

On the concatenated 1072-gene tree, branches leading to the individual CCs are all supported by a 100% bootstrap value, whereas most branches representing deeper relationship among the CCs are poorly resolved (fig. 1). Phylogenetic networks based on the concatenated sequences revealed a similar picture such that the strains from the same CC are closely related and form monophyletic groups, yet the overall topological structure is star-like, suggesting poorly resolved deep phylogenetic relationships among the CCs (supplementary fig. S1, Supplementary Material online). This supports the view that Nm population has experienced selective sweeps, rapid population growth, and very high levels of recombination, all of which remove phylogenetic signals and result in a star-like phylogeny (Smith et al. 1993). Unlike the bootstrap support, the support by individual gene trees was universally low except for a value of 0.96 on the branch leading to the N. lactamica species (fig. 1). This suggests that most individual gene trees are topologically different from the concatenated gene tree. For instance, the branch leading to the N. lactamica species is supported by only 37% of individual gene trees. In other words, about 63% of genes support a closer relationship between a fraction of the N. lactamica strains and either N. gonorrhoeae or N. lactamica rather than a monophyletic origin of N. lactamica. The support observed for individual CCs ranges from 0.12 (CC269) to 0.53 (CC167). The AU test was applied on each universal gene against the concatenated 1072-gene tree (supplementary table S4, Supplementary Material online). One thousand forty-one genes (97.11%) showed significant incongruence ($P < 0.01$) with the concatenated 1072-gene tree. Furthermore, a significant amount of universally present genes were involved in intragenic recombination. The detected proportion of genes involved in intragenic recombination is 48.51% by RDP, 66.51% by Phi, and 79.48% by OnePop (table 2). All together, it suggests extensive homologous recombination at both the gene and intragenic levels among the N. meningitidis genomes.

Similarly, a high frequency of recombination was observed within the N. gonorrhoeae species. Out of the 1,072 genes, there are 436 genes supporting the clustering pattern ([TCDC-NG08107, NCCP11945], FA1090), 150 genes supporting ([NCCP11945, FA1090], TCDC-NG08107), 140 genes supporting ([TCDC-NG08107, FA1090], NCCP11945), and 346 genes that are not phylogenetically informative. That is, among the phylogenetically informative genes, 60.06%, 20.06%, and 19.28% of genes support the three possible phylogenetic relationships,
Among the 61 universally present virulence genes, 1,830 possible gene pairs were examined by the AU test (fig. 2), of which 1,773 (96.88%) pairs showed significant phylogenetic incongruence ($P < 0.01$) supported by both gene nucleotide sequences, and all pairs showed significant incongruence supported by at least one gene. Similar results were observed when analyzing translated protein sequences. One thousand thirteen (93.61%) protein pairs showed significant incongruence supported by both sequences and 1,828 (99.89%) protein pairs showed significant incongruence supported by at least one sequence (supplementary fig. S2, Supplementary Material online). The slightly decreased number of significant protein pairs could be explained by fewer informative sites due to synonymous substitutions as well as the reduced length of amino acid sequences relative to gene sequences. It is also notable that several genes show marginally significant or nonsignificant $P$ values against many other gene topologies (seen as vertical blocks of marginally significant or nonsignificant $P$ values in fig. 2 and supplementary fig. S2, Supplementary Material online). The less than significant $P$ values are due to the lack of informative signals in these particular gene sequences (e.g., low sequence diversity), and the data illustrating these sequence features are not shown. Importantly, when the AU test was performed on the randomly concatenated genes, all pairs showed significant incongruence supported by at least one gene, and the number of pairs that showed significant incongruence supported by both genes are 1,805 (98.63%) for concatenation of 3 genes, 1,811 (98.96%) for concatenation of 6 genes, and 1,811 (98.96%) for concatenation of 6 genes, and 1,811 (98.96%) for concatenation of 3 genes, 1,811 (98.96%) for concatenation of 3 genes.

Extensive Recombination in Virulence Genes

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### Table 2

| Genes* | Total | RDPb | Phi* | OnePopb |
|--------|-------|------|------|---------|
| Universal | 1,072 | 520(48.51%) | 713(66.51%) | 852(79.48%) |
| Information storage and processing | 206 | 103(50.00%) | 131(63.59%) | 152(73.79%) |
| Cellular processes and signaling | 194 | 100(51.55%) | 140(72.16%) | 160(82.47%) |
| Metabolism | 382 | 215(56.28%) | 288(75.39%) | 338(88.48%) |
| Poorly characterized | 194 | 79(40.72%) | 123(63.40%) | 150(77.32%) |
| Not in COG | 123 | 35(28.46%) | 56(45.53%) | 78(63.41%) |
| Virulence | 164 | 82(50.00%) | 95(57.93%) | 117(71.34%) |
| Universally present | 61 | 32(52.46%) | 37(60.66%) | 52(85.25%) |

*a Universal genes were grouped according to their Clusters of Orthologous Groups of proteins (COG) functional categories. Information storage and processing includes COG categories I, A, K, L, and B; cellular processes and signaling includes COG categories D, Y, T, M, N, Z, W, U, and Q; Metabolism includes C, G, E, F, H, I, P, and Q; Poorly characterized includes R and S. Because some genes have been annotated into multiple COG categories, the sum of individual groups is slightly larger than the total number of single-copy genes.

*b After Bonferroni correction for multiple tests at the 0.05 level.

**Significance was determined when $P < 0.05$ was observed in all three tests (NSS, Max $\chi^2$, and Phi) in the Phi package.
processes and signaling to 7.76 for genes involved in information storage and processing (supplementary fig. S4, Supplementary Material online). Furthermore, a significant proportion of genes from all functional categories were observed to undergo intragenic recombination (table 2). All together, this suggests that not only virulence genes but also genes in all functional categories are involved in extensive recombination.

How Clonal Is a CC in Nm?

Given the high frequency of recombination at both the gene and intragenic levels, it is important to examine the extent of recombination within a CC. As such, we sought to address how many virulence genes were involved in recombination within the same ST. Unfortunately, it has been well demonstrated that existing recombination detection programs do not effectively identify recombination among low-diversity sequences (Drouin et al. 1999; Posada and Crandal 2001; Wiuf et al. 2001; Posada 2002). In this study, we took a novel approach by estimating the maximum possible nucleotide differences caused by mutation and identifying genes that exceed such estimates. A significant percentage of nonvertically acquired virulence genes were found in 10 identical-ST genome pairs, ranging from 5.8% in Nm6756-Nm8663 to 27.8% in FAM18-ES14902 (fig. 3). In other words, the strains in the same ST are not strictly clonal. Differences were observed in up to 27.8% of the virulence genes and some of the differences impact protein sequences and could result in functional consequences.

To further assess the robustness of the detection of nonvertically acquired genes, we conducted a series of simulations. As shown in supplementary fig. S5, Supplementary Material online.
the relationships for individual genes in the analysis. Similarly, however, the relationships of the strains may not represent still possible to obtain a relatively well-supported relationship. A 100% bootstrap value in figure 1. In other words, despite studies, all major CCs were found to be supported by consistent with the explanation that extensive recombination examined genome pairs than in the simulated genomes. This is Furthermore, DNA distance increased much faster in the five genome-wide sequence divergence of 0.0021 should be considered conservative. genomes, whereas there are 63.5% identical genes between the most diverse genome pair within an ST (FAM18-S0108). To have a similar fraction of genes identical with at least 1 outgroup genome. It is most likely that these genes resulted from recent horizontal transfer (via homologous recombination) from different CCs. Because this analysis considered identical genes and not intragenic recombination, the true number of transfers should be much higher than the estimates in figure 4. In table 3, the nearest neighbors of the 1,072 universal genes in M13399 were identified. Even though M13399 belongs to CC269, only 47.39% of its genes have their nearest neighbors in CC269. Of the genes, 11.29% have their nearest neighbors in CC32, whereas 28.45% of the genes have their nearest neighbors in a CC other than CC269 and CC32. It is noteworthy that the extensive recombination between CCs is not unique to the strain M13399. A significant number of horizontal transfer events were observed in several other strains (fig. 4). Recently, Budroni et al. (2011) reported that the Nm strains contain clade-associated restriction modification systems and suggested that these systems could serve as a barrier to DNA exchange between different populations. Our results, however, suggest that even with the proposed barrier generated by clade-associated restriction modification systems, a significant number of genes could still recombine between different CCs. Our results are consistent with the findings from several previous studies that indicate restriction modification systems may not only provide defense against foreign DNA but may also play a significant role in genetic exchange process. By providing recombinogenic ends for exonucleases or helicases to further act on (Dubose et al. 1988) and limiting the size of transferred fragments, such systems could facilitate small and multiple sequence replacements (McKane and Milkman 1995). The complete functional consequences of clade-associated restriction modification systems, therefore, demand further thorough investigation in future studies.

Detectable Functional Consequences of Recombination Events

Recombination and Mosaic Capsule Gene Cluster

The capsule gene cluster is located downstream of the dnaJ and cfr genes. The galE-rfbBAC operon (for lipooligosaccharide biosynthesis) and the tex gene (putative regulator) are universally present in all Nm strains, including the x14 and

![Figure 3](image-url)
Nm1140 that are incapable of capsule production (Claus et al. 2002). All serogroupable strains (A, B, C, Y, and W135) also contain the **ctrABCD** operon (capsule transport genes) and the methyltransferase operon **hphIAB**. The two A strains contain the **sacABCD** operon (for capsule biosynthesis), whereas B, C, Y, and W135 strains contain the **siaABCD** operon (for capsule biosynthesis). The **siaD** gene in serogroups Y and W135 shares no homology with other serogroups and is also called synF/G (Claus et al. 2009). The B and C strains also contain the **ctrG** gene downstream of the **siaABCD** operon, and specifically in C strains, **oatC** that encodes polysialic acid-specific O-acetyltransferase is adjacent to **ctrG** (supplementary fig. S6, Supplementary Material online). Furthermore, the **lipAB** operon is often found downstream of the duplicated **galE-rfbBAC** operon (Beddek et al. 2009). The **lipAB** operon was not included in the supplementary figure S1, Supplementary Material online because the operon was absent from the seven genomes sequenced in this study.

It is notable that the distribution of different serogroups is not associated with the evolutionary relationship of the strains (supplementary fig. S6, Supplementary Material online) and different serogroups were even found in the same ST (e.g., B and C in ST11; supplementary table S1, Supplementary Material online). In other words, the entire capsule gene cluster could be involved in recombination, which is known as capsule switching (Swartley et al. 1997; Beddek et al. 2009). Gene movement is also evident within the capsule gene cluster (supplementary fig. S6, Supplementary Material online). Gene cluster inversion seemed to have taken place multiple times. For instance, the operons **sacABCD**, **ctrABCD**, and **tex** are in different orientations between the two A strains (Z2491 and WUE_2594), and different operon orientations were also found in serogroup B strains. A number of transposable elements (or IS elements) were observed at various locations in several capsule gene clusters. They are **IS4** inserted between **ctrA** and **siaA** in M01-240194 and NZ-05/33; **IS101** between **ctrA** and **siaD** in G2136; **IS1016** flanking **synF/G** in Nm3127 and Nm8187; **IS1016** flanking **synF/G** in Nm6756; and **IS1016** (between **ctrA** and **siaA**) and **IS1655** (between **rfbA** and **rfbC**) in M04-240196 (supplementary fig. S6, Supplementary Material online). The

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### Table 3

**Distribution of the Nearest Neighbors of the 1,072 Universally Present Genes in M13399**

| ST/CC | ST/CC | Genes | Percentage | Genes | Percentage |
|-------|-------|-------|------------|-------|------------|
| CC269a | 508 | 47.39% | 50,050 | 46.69 |
| CC32 | 121 | 11.29% | 12,019 | 11.21 |
| CC41/44 | 57 | 5.32% | 4,935 | 4.60 |
| CC23 | 37 | 3.45% | 3,527 | 3.29 |
| CC8 | 35 | 3.26% | 3,124 | 2.91 |
| ST5 | 28 | 2.61% | 2,582 | 2.36 |
| ST751 | 25 | 2.33% | 2,317 | 2.16 |
| ST177 | 18 | 1.68% | 2,131 | 1.99 |
| CC11 | 17 | 1.59% | 1,682 | 1.57 |
| ST23 | 14 | 1.31% | 1,339 | 1.25 |
| CC22 | 13 | 0.93% | 1,072 | 1.00 |
| ST731 | 10 | 0.84% | 1,173 | 1.09 |
| ST1136 | 8 | 0.75% | 829 | 0.77 |
| ST4821 | 7 | 0.65% | 695 | 0.65 |
| Nlact | 1 | 0.09% | 160 | 0.15 |
| Ng | — | — | 15 | 0.01 |
| No signal | 138 | 12.87% | 16,272 | 15.18 |
| Total | 1,072 | — | 107,200 | — |

*It is important to note that CC was assigned whenever the nearest neighbor was one of the genomes from a CC even through some genomes in a CC might be substantially different from the rest of the CC. For this reason, the percentage supporting M13399 to be CC269 here (47.39%) is much higher than the phylogenetic support (0.12) from individual gene trees in figure 1. Similar criteria also apply for other CCs.*

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**Fig. 4.**—A conservative estimate on genes horizontally transferred between CCs. Lineages A and B are required to belong to the same CC. Genes in A that are different from all members of B and C but identical to at least one outgroup genome were counted.
recent occurrence of IS elements is consistent with the fast turnover of IS elements in the bacterial genomes (Wagner 2006).

Furthermore, genes within the capsule gene cluster could undergo intragenic recombination beyond both CCs and serogroups. The 5’ end (1-360 nt) of ctrG in a serogroup B strain M04-240196 is identical with two C strains (FAM18 and NEM8013), whereas its 3’ end is virtually identical with other B strains. The third C strain 053442, however, has a 5’ end identical to most B strains and shares a very similar 3’ end (with only 1 nucleotide different) with FAM18 and NEM8013 (fig. 5A). The ctrD gene in a serogroup B strain H44/76 differs 57 nucleotides from another B strain MC58. It is likely that position 1-285 nt in H44/76 is a recent transfer from a fairly divergent genome. Such a divergent sequence is also present in a W135 strain Nm6938 and a Y strain Nm2756, but the breakpoints are different (fig. 5B). The two A strains, namely Z2491 and WUE_2594, contain a very divergent ctrC sequence compared with other strains. The 3’ end (685-786 nt) of this divergent-type sequence was also found in a710 and Nm2732. There is a third sequence type in position 579-639 nt. Position 579-639 in a CC41/44 strain a710 is different from the other two CC41/44 strains, and the same region in a CC32 strain H44/76 is also different from the other CC32 strain MC58 (fig. 5C). Similarly, mosaic structure was also found in the tex gene in multiple strains. The supplementary figure S7, Supplementary Material online shows origins of different regions based on breakpoints specific to the a710 strain. Position 1177-2271 in a710 is different by only 1 nucleotide, otherwise identical with other CC41/44 strains (M01-240149 and NZ-05/33), whereas position 1-1176 in a710 differs 67 nucleotides from both M01-240149 and NZ-05/33 and positions 1-148, 149-1176 in a710 yield two different origins (supplementary fig. S7, Supplementary Material online). Such frequent recombination in capsule gene cluster at both gene cluster level and intragenic level could potentially lead to the escape of vaccine-induced or natural protective immunity.

Nonvertical Evolution and Antibiotic Resistance

The seven sequenced strains showed different levels of antibiotic susceptibility (table 1). Among the three closely related genome pairs, two pairs have different penicillin MIC values. That is, Nm3127 (MIC = 0.50) has lower susceptibility to penicillin than Nm8187 (MIC = 0.12), and Nm2723 (MIC = 0.12) has lower susceptibility to penicillin than Nm6938 (MIC ≤ 0.06). It is important to note that antibiotic susceptibility played no role in choosing isolates to be sequenced. In fact, antibiotic susceptibility results were reviewed after the whole genome sequencing data were analyzed. The observed decrease of susceptibility in recent isolates could result from bias in small sample size and/or genuinely decreased susceptibility to penicillin in Ontario, Canada (Brown et al. 2001). Different penA sequences were found to be associated with the difference in susceptibility within these two pairs. There are 10 amino acids different between Nm3127 and Nm8187, and 16 amino acids different between Nm2723 and Nm6938 (fig. 6). Nine of these amino acid sites have been previously linked to the decreased susceptibility to beta-lactam

**Fig. 5.**—Chimeric genes (A, ctrG; B, ctrD; and C, ctrC) in the capsule gene cluster. Only nucleotide changes are shown in the alignment. The suggested topology based on the concatenated 1,072 genes (fig. 1) is shown along with each alignment. Branches involved in the same CC are colored in green, when the members of the CC don’t share the same type of sequence. Serogroups are shown as one letter in parentheses (W for W135). Spaces in the ctrG alignment signify gene truncation in G2136 and MC58.
antibiotics, such as penicillin and cephalosporins (Thulin et al. 2006; Taha et al. 2007; du Plessis et al. 2008). Furthermore, mutations in some other genes (e.g., mtrR, porB, and ponA) also contribute to changes in antibiotic susceptibility in Neisseria species, and the degree of antibiotic susceptibility is determined by the combination of all these genes (Orus and Vinas 2001; Lindberg et al. 2007; Lee et al. 2010). Such an association was also found in this study. Nm8187 exhibits intermediate resistance (MIC 5 0.12), whereas Nm8663 and Nm6756 are penicillin susceptible (MIC /C20 0.06), yet all have identical penA sequences. The decreased susceptibility to penicillin in Nm8187 could be explained by differences in the mtrR and porB genes. Similarly, the highly susceptible Nm1140 shares 8 nonsynonymous penA changes with the less susceptible Nm3127 and Nm2732 strains. Although changes in penA do not correlate with the low MIC observed in Nm1140, analysis reveals mtrR and porB alleles that are highly similar to sequences from the penicillin-susceptible strains Nm8663 and Nm6756. The three closely related genome pairs all have nearly identical mtrR and porB genes (supplementary fig. S8, Supplementary Material online). Only 1 nonsynonymous difference was observed in porB between Nm8187 and Nm3127, and only two differences in ponA were observed between Nm6938 and Nm2732. This supports the conclusion that differences between the penA genes are most likely responsible for the different penicillin susceptibility profiles of Nm3127 and Nm8187 and between Nm2732 and Nm6938.

Intragenic Recombination of Vaccine Target Gene NHBA (GNA2132)

Currently, a new universal vaccine (4CMenB) against the serogroup B meningococcal disease is being tested (Toneatto et al. 2011). The new vaccine is based on surface protein antigens rather than capsular polysaccharide antigens and is composed of five targets, GNA1870 (fHbp), GNA1994 (NadA), GNA2132 (NHBA), GNA1030, and GNA2091. Among the five genes, we found significant phylogenetic incongruence between the NHBA gene tree and the concatenated gene tree. Recombination has been observed in CC11, CC41/44, and CC269 (fig. 7). Strikingly, the NHBA in OX99-30304 (serogroup B) yields 99 amino acids different from the other three CC41/44 strains (M01-240149, NZ-05133, and M0579) but is identical with the NHBA in a serogroup C and CC11 strain FAM18 and serogroup A strain Z2491 (fig. 7 and supplementary fig. S9, Supplementary Material online). It is noteworthy that the NHBA sequences in the 23 strains are of significantly shorter length than those in the remaining 15 strains. Furthermore, after applying the recombination detection programs, we found strong evidence of intragenic recombination in the NHBA gene supported by various tests (\( P_{NSS} = 0, P_{Max x^2} = 0, P_{phi} = 0, P_{RDP} = 2.69 \times 10^{-16}, P_{OnePop} = 0 \)). The occurrence of intragenic recombination in the NHBA gene has introduced multiple amino acid substitutions. These could potentially have functional consequences, such as vaccine escape, because existing vaccines are less likely to be effective against distant variants of the antigenic
proteins (Brunelli et al. 2011) and extensive recombination and gene conversion have been suggested to be associated with evasion of the host immune response (Andrews and Gojobori 2004; Helm and Seifert 2010). On the other hand, given the high frequency of recombination between the different serogroups, and even between species, the antigens recognized by the new 4CMenB vaccine are unlikely to be serogroup B specific. Thus, strains from other serogroups than B, or maybe even from Ng, could potentially be preventable by the 4CMenB vaccine, if they contain 4CMenB-recognized antigen genes.

Conclusion

We have found extensive recombination during the genome evolution of Nm. More than 92% of all possible gene pairs display significant phylogenetic incongruence and more than 48% of genes exhibit significant signals for intragenic recombination. These findings confirm that, within a CC determined by the 7 MLST loci, many genes do not evolve in a clonal manner, and in the strains of the same ST, up to 28% of genes may be nonvertical. A significant amount of recombination between CCs has been observed, even though the Nm strains contain restriction modification systems that have been suggested to generate barriers to recombination between clades (Budroni et al. 2011). Extensive recombination, especially changes in virulence genes and capsule genes, could lead to important functional consequences in strains that appear identical by standard classification methods, such as MLST. In this study, we found that recombination events altered penicillin susceptibility in 2 of our 3 closely matched genome pairs. Furthermore, we discovered intragenic recombination in the vaccine target NHBA, which raises the concern that recombination events could impair vaccine efficacy and lead to vaccine escape of Nm.

Supplementary Material

Supplementary figures S1–S9 and tables S1–S4 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

We would like to acknowledge P. Rawte, S. Lo, and H. Siebert for their assistance with the Nm strains and susceptibility testing. This work was supported by funds from Sanofi-Pasteur to D.E.L. and a Natural Sciences and Engineering Research Council of Canada (NSERC) fellowship to W.H.
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Associate editor: Takashi Gojobori