Conservation of Meningococcal Antigens in the Genus *Neisseria*

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ABSTRACT ***Neisseria meningitidis*, one of the major causes of bacterial meningitis and sepsis, is a member of the genus *Neisseria*, which includes species that colonize the mucosae of many animals. Three meningococcal proteins, factor H-binding protein (fHbp), neisserial heparin-binding antigen (NHBA), and *N. meningitidis* adhesin A (NadA), have been described as antigens protective against *N. meningitidis* of serogroup B, and they have been employed as vaccine components in preclinical and clinical studies. In the vaccine formulation, fHbp and NHBA were fused to the GNA2091 and GNA1030 proteins, respectively, to enhance protein stability and immunogenicity. To determine the possible impact of vaccination on commensal neisseriae, we determined the presence, distribution, and conservation of these antigens in the available genome sequences of the genus *Neisseria*, finding that fHbp, NHBA, and NadA were conserved only in species colonizing humans, while GNA1030 and GNA2091 were conserved in many human and nonhuman neisseriae. Sequence analysis showed that homologous recombination contributed to shape the evolution and distribution of both NHBA and fHbp, three major variants of which have been defined. fHbp variant 3 was probably the ancestral form of meningococcal fHbp, while fHbp variant 1 from *N. cinerea* was introduced into *N. meningitidis* by a recombination event. fHbp variant 2 was the result of a recombination event inserting a stretch of 483 bp from variant 1 into the variant 3 background. These data indicate that a high rate of exchange of genetic material between neisseriae that colonize the human upper respiratory tract exists.**

IMPORTANCE The upper respiratory tract of healthy individuals is a complex ecosystem colonized by many bacterial species. Among these, there are representatives of the genus *Neisseria*, including *Neisseria meningitidis*, a major cause of bacterial meningitis and sepsis. Given the close relationship between commensal and pathogenic species, a protein-based vaccine against *N. meningitidis* has the potential to impact the other commensal species of *Neisseria*. For this reason, we have studied the distribution and evolutionary history of the antigen components of a recombinant vaccine, 4CMenB, that recently received approval in Europe under the commercial name of Bexsero®. We found that fHbp, NHBA, and NadA can be found in some of the human commensal species and that the evolution of these antigens has been essentially shaped by the high rate of genetic exchange that occurs between strains of neisseriae that cocolonize the same environment.

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The genus *Neisseria* is a large group of Gram-negative bacteria. Besides several human species that are only rarely associated with disease, this genus also contains two major human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The latter generally colonizes humans asymptomatically but can cause systemic disease in a small percentage of cases.

The taxonomy of the genus *Neisseria* based on sequence analysis is problematic. Studies based on three genes, *argF*, *recA*, and *rho*, and on the 16S rRNA have shown that five phylogenetic groups can be identified, each including more than one named species (1). However, probably because of the high level of recombination between these organisms, the species within these groups are less distinct than what is normally accepted in molecular taxonomy, as in the case of *Neisseria cinerea*, which is often placed within the species *N. meningitidis*. Recently, the complete phylogeny of the neisseriae has been reconstructed by ribosomal multilocus sequence typing and an accurate interpretation of the relationships between different species has been proposed (2). Moreover, many of the genes that are associated with virulence in *N. meningitidis* are also present in commensal neisseriae, and it has been speculated that the latter might constitute a reservoir of virulence factors for the pathogenic species (3). In some cases, genetic exchange is less frequent because of the difference in ecology, such as that between *N. meningitidis* and *N. gonorrhoeae*, for instance (4).

Currently, capsular polysaccharide-based vaccines for the A, C, Y, and W-135 serogroups of *N. meningitidis* are available, while the development of a polysaccharide vaccine for serogroup B was not possible because of the similarity to a human carbohydrate that induces a weak immune response and carries the risk of autoimmunity. For this reason, a multicomponent protein-based vaccine known as 4CMenB has been proposed (5). 4CMenB was recently approved in Europe under the commercial name Bexsero®. 4CMenB includes three main antigens, the factor H (fH)-binding protein (fHbp), the neisserial heparin-binding antigen (NHBA), and *N. meningitidis* adhesin A (NadA). Two additional

proteins, GNA2091 and GNA1030, were incorporated into fusion proteins with fHbp (GNA2091-fHbp) and NHBA (NHBA-GNA1030), respectively, to enhance protein stability and increase immunogenicity (5). NadA was included as a single-protein antigen.

fHbp is a surface-exposed lipoprotein that binds fH, a key inhibitor of the complement alternative pathway, leading to evasion of killing by the innate immune system (6). The expression of fHbp by *N. meningitidis* strains is important for survival in human blood and human serum (7). With very few exceptions (8, 9), the gene coding for fHbp is present in serogroup B strains of *N. meningitidis* in three major genetic variants, i.e., 1, 2, and 3, and in *N. gonorrhoeae* in one of the three variants (10). The three variants are not fully cross-protective (11, 12); variant 1 does not show cross-protection activity against variants 2 and 3, and vice versa. NHBA, predicted to be a surface-exposed lipoprotein, was selected as a vaccine candidate with the name GNA2132 (i.e., genome-derived *Neisseria* antigen 2132) in a genome-wide vaccine target discovery program (13). GNA2132 was shown to be able to induce bactericidal antibodies in humans and to bind heparin, improving the survival of the meningococcus in human serum. For this reason, it was later renamed NHBA (14). NHBA is ubiquitous in meningococci of serogroup B (9, 15, 16). NadA is a meningococcal surface-anchored protein from the family of trimeric autotransporters (17) that are understood to export themselves to the bacterial surface with no external energy source or auxiliary proteins. Recent data suggest that other proteins might be involved in their secretion, and further investigation is ongoing (18). The gene coding for NadA is present in three out of the four hypervirulent lineages of *N. meningitidis* of serogroup B and is absent from pathogenic *N. gonorrhoeae* and the commensals *N. lactamica* and *N. cinerea* (19, 20). Although their functional activity is not known, GNA1030 and GNA2091 were shown to induce protective immunity in mice in serum bactericidal activity assays (5).

The *fHbp*, *nhba*, and *nadA* genes are also present in invasive strains of *N. meningitidis* belonging to the other serogroups (15, 16, 21, 22). The gene coding for fHbp in non-B epidemic strain isolates, in particular, of serogroups A, X, and W-135 from Africa, has been sequenced (22–25). These studies demonstrated the high clonality of the non-B isolates, with the exception of serogroup C, not only in terms of genetic diversity among strains but also in terms of molecular variability of the antigens.

To evaluate whether these antigens are present only in pathogenic neisseriae or are also present in other species belonging to this genus, we analyzed their presence, distribution, and conservation in the complete and draft genome sequences of 80 strains of pathogenic and commensal neisseriae that were available in public databases. We used the single nucleotide polymorphisms (SNPs) retrieved from the alignments of these sequences against the genome of *N. meningitidis* MC58 to resolve the relationships between the species closely related to *N. meningitidis* that could not be clearly distinguished by 16S rRNA sequence analysis. By comparing the phylogenetic relationships between the genes to the whole-genome phylogenetic tree and by analyzing the conservation of the genomic loci where the genes were inserted, we demonstrated the effect of the interspecies transfer of genetic material in their evolution.

RESULTS

Phylogeny of the genus *Neisseria*. A phylogenetic analysis of the genus *Neisseria* based on 48 16S rRNA sequences that are representative of the diversity of the genus resulted in a tree with generally low levels of bootstrap support (Fig. 1). However, we could distinguish one branch containing the two species that are pathogenic in humans, i.e., *N. meningitidis* and *N. gonorrhoeae*, together with the human commensals *N. cinerea* and *N. polysaccharea*. Within this branch, different strains of the same species did not form monophyletic groups. This inability to resolve the relationships among the different species was due both to the weak phylogenetic signal in 16S rRNA sequences and to the high level of homologous recombination within and between these species, which can be visualized by constructing a phylogenetic network (see Fig. S1 in the supplemental material) where it is almost impossible to clearly distinguish branches corresponding to named species. We estimated the relative contributions of recombination and mutation to the diversification of this set of sequences by measuring the population-scaled recombination rate ρ and the mutation rate θ. We obtained $\rho = 0.042$ and $\theta = 0.015$, yielding a ρ/θ ratio of 2.8. This value, computed across the different neisserial species, is only marginally smaller than the estimated ρ/θ ratio of 3.1 obtained from strains belonging only to *N. meningitidis* by using the sequences of fragments of seven housekeeping genes (40, 41). The fact that the value obtained by analyzing exclusively sequences of *N. meningitidis* is comparable to the value obtained by analyzing sequences of isolates belonging to different species of neisseriae suggests that homologous recombination plays a fundamental role not only in the diversification of lineages within the species *N. meningitidis* (41) but also in driving the speciation process within the genus *Neisseria* (42).

In Fig. 1, we also indicate the species for which at least one genome sequence was available (Table 1). Although the sample was biased toward the species that colonize humans, the available genomes allowed the sampling of all of the branches of the tree. In these genomes, we verified the conservation of the three major antigens contained in the 4CMenB vaccine, namely, *fHbp*, *nhba*, and *nadA*, and highlighted (in purple in Fig. 1) those sequences where none of the three antigens was present, while the other sequences are colored according to the species. With the exception of *N. flavescens*, all of the genomes where at least one antigen was present were concentrated in one branch of the tree, including *N. meningitidis*, *N. gonorrhoeae*, *N. polysaccharea*, *N. lactamica*, *N. cinerea*, *N. flava*, *M. cerebrosus*, *N. macacae*, and three unidentified neisseriae.

To resolve the ambiguities of the taxonomy of this branch, we performed a genomewide phylogenetic analysis of the available complete and draft genome sequences (Fig. 2). This analysis included all of the sequences where at least one of the three genes, *fHbp*, *nhba*, or *nadA*, was found. *N. flavescens* was excluded from this analysis because the available genome was too divergent from the other sequences to allow a reliable alignment. The different species were well separated, with no ambiguities, and all of the branches were supported by high levels of bootstrap support (for clarity, the branches containing more than one strain of the same species were grouped in gray areas; for the complete tree, see Fig. S2 in the supplemental material). Among these species, *N. gonorrhoeae* was the one most closely related to *N. meningitidis* and showing the smallest variability among the sequenced strains,

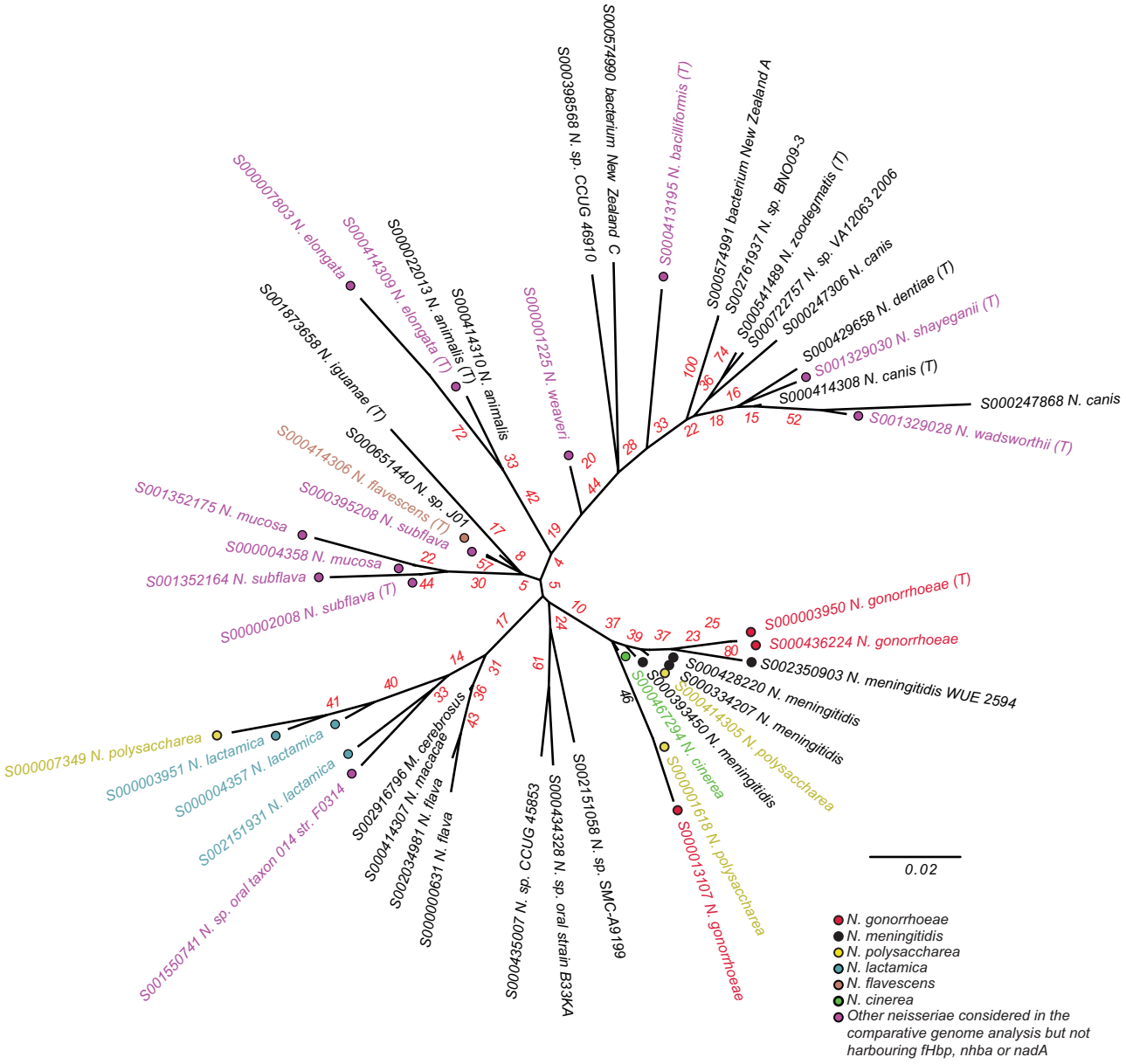

FIG 1 Maximum-likelihood phylogenetic tree obtained with 16S rRNA sequences. Bootstrap values are shown with red labels. In this tree, strains of the same species do not form monophyletic branches. A branch containing sequences from *N. meningitidis*, *N. gonorrhoeae*, *N. polysaccharea*, and *N. cinerea* can be identified. Colored circles and labels indicate species whose whole-genome sequences were available. In particular, purple indicates those sequences where none of the three antigens was conserved, while the other sequences are colored according to the species. A capital T indicates the type strain of the species.

suggesting that this species differentiated recently from an ancestor of contemporary *N. meningitidis*. In this reconstruction, the sequenced strains of *N. cinerea* were distantly related to *N. polysaccharea* and clearly distinct from *N. meningitidis*, confirming recent results based on the analysis of the concatenated sequences of core genes (3).

Conservation of the antigens. (i) fHbp. Among the sequenced genomes of the genus *Neisseria*, the *fHbp* gene was present only in species closely related to *N. meningitidis*, i.e., *N. gonorrhoeae*, *N. cinerea*, and *N. polysaccharea*, while it was absent from, among others, all of the *N. lactamica* strains (Table 1). Interestingly, in six-sevenths of the available strains of *N. polysaccharea*, the gene was frameshifted (FS) in different positions, suggesting that this gene does not confer a significant selective advantage on this species. In all of the strains of *N. gonorrhoeae* and *N. polysaccharea*, the *fHbp* gene could be attributed to variant 3, while in *N. cinerea*, the gene could be classified as variant 1 (Fig. 3; see Fig. S2 in the supplemental material). The major differences between the gonococcal and meningococcal *fHbp* genes were concentrated in the N-terminal portion of the molecule (see Fig. S3 in the supplemental material) where the insertion of a single base (G) at position 40 causes a frameshift in all gonococcal strains, which results in the loss of the lipobox motif.

When *fHbp* was present, the entire locus harboring the gene was conserved and it was located between the homologues of NMB1869 and NMB1871 (named according to strain MC58

TABLE 1 Summary of the levels of conservation of the *fHbp*, *nhba*, and *nadA* genes in 80 complete and draft neisserial genomes[a]

		fHbp			NHBA			NadA		
Species	No. of strains (closed genomes)	No. of strains harboring fHbp	Species avg % fHbp identity to fHbp-1.1	Note(s)	No. of strains harboring NHBA	Species avg % NHBA identity to peptide 2	Note(s)	No. of strains harboring NadA	Species avg % NadA identity to NadA-3.8	Note(s)
N. bacilliformis	1	0			0			0		
N. cinerea	5	5	93.9	Variant 1	0			2	54.4	1-200 aa at contig border
N. elongata subsp. *glycolytica*	1	0			0			0		
N. flavescens	2	0			1	61.2	FS	0		
N. gonorrhoeae	17 (3)	17	63.3	4 FS, variant 3	17	81.2	2 FS, 1 at contig edge	0		
N. lactamica	8 (1)	0		OPA-like protein replaces fHbp	8	83.8	2 at contig edge	0		
N. macacae	1	0			0			0		
N. meningitidis	27 (14)	27	83.7	Variants 1-3	27	82.6		12	89.2	2 FS, 2 at contig edge, 1 IS*4* insertion
N. mucosa	2	0			0			0		
Neisseria oral taxon	1	0			0			0		
N. polysaccharea	7	7	69.3	5 FS, 1 at contig edge, variant 3	7	84.3	2 at contig edge	0		
N. shayeganii	1	0			0			0		
N. sicca	3	0			0			0		
N. subflava	1	0			0			0		
N. wadsworthii	1	0			0			0		
N. weaveri	2	0			0			0		

[a] Percent identity was calculated with respect to the form of the same proteins that are included in the 4CMenB vaccine (5). Besides intact genes, we included the *fHbp* and *nhba* genes of *N. polysaccharea*, *N. lactamica*, *N. flavescens*, and *N. gonorrhoeae*, which are FS or interrupted by a contig edge; a 200-aa fragment of *nadA* in *N. cinerea* close to a contig edge; two FS *nadA* genes; and one interrupted by IS*4* in *N. meningitidis*.

[Fig. 4a and b]). As previously reported (43), the locus was also highly conserved in *N. lactamica*, where the *fHbp* gene was always replaced by a gene annotated as a "putative opacity protein" on the opposite strand (Fig. 4a). Finally, in most of the strains lacking a clear homologue of *fHbp*, we observed the presence of weak similarity hits (between 30 and 40% similarity) against proteins that were annotated mainly as "fHbp proteins" or weaker hits (approximately 20% similarity) against proteins annotated as "hypothetical lipoproteins." Despite a conserved lipoprotein motif, these hits, located in a different genomic region, could not be aligned with *fHbp* of *N. meningitidis*.

We then analyzed a multiple-sequence alignment of a region of 10 kbp, including the *fHbp* gene, to identify homologous recombination events that contributed to its evolution (Fig. 5). For clarity, a single representative of each group of strains with a similar pattern of recombination is shown, i.e., FA1090 for gonococci, CCUG27178A for *N. cinerea*, MC58 and M04-240196 for meningococcus *fHbp* variant 1, 8013 for variant 2, and M01-240355 for variant 3. For a complete diagram, including all of the events in a selection of 22 representative sequences, see Fig. S4 in the supplemental material. Two major events could be identified in Fig. 5. (i) *fHbp* variant 1 was imported into *N. meningitidis* from *N. cinerea* in an event also spanning the *cbbA* gene, which is located upstream of *fHbp*. (ii) *fHbp* variant 2 originated by the replacement of a portion of a variant 3 strain with the homologous sequence from a variant 1 strain. The latter event could be seen clearly in a multiple-sequence alignment of variant 2 and 3 *fHbp* sequences (see Fig. S5a in the supplemental material). The putative donor was identified by RDP3 (46) as *N. cinerea* strain CCUG27178A, but given the high similarity of the *N. cinerea* gene to the *N. meningitidis* variant 1 genes, we could not exclude the possibility that other undetected intermediate steps occurred. This complex pat-

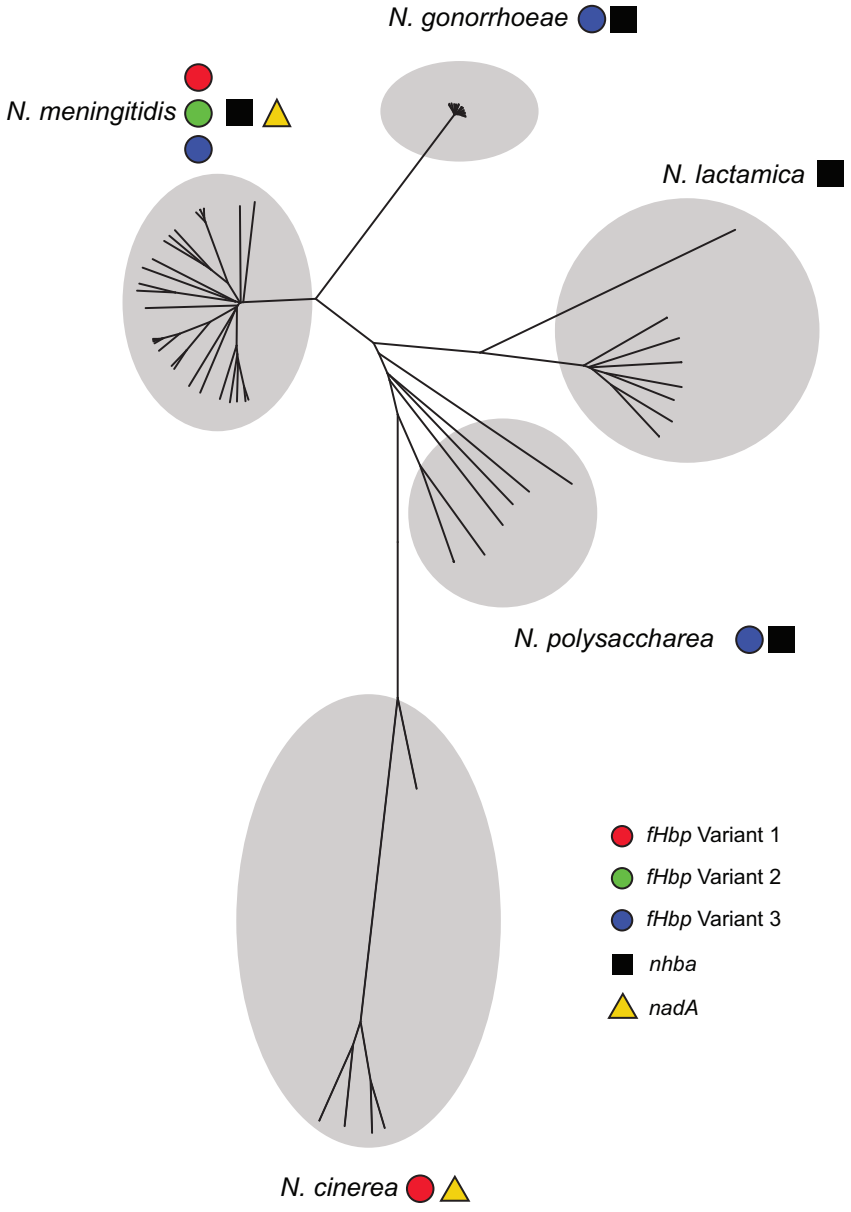

FIG 2 Neighbor-joining phylogenetic tree with evolutionary distances calculated by the maximum composite likelihood method obtained from the SNPs in the portion of the multiple-sequence alignment shared by all of the strains studied. The alignment includes 64 strains of *N. meningitidis*, *N. gonorrhoeae*, *N. polysaccharea*, *N. cinerea*, and *N. lactamica*. The presence of *fHbp*, *nhba*, and *nadA* is indicated. The colors indicate the major allelic variants of *fHbp*. One of the three major *fHbp* variants is present in all of the strains of *N. meningitidis*. *nadA* is present in *N. meningitidis* in 7 out of 27 strains with the correct translation frame. *nhba* is ubiquitous, with the exception of *N. cinerea*.

tern of recombination was probably what led to the observed modular organization of the *fHbp* gene (44).

The structuring of the splits in a phylogenetic network of the *fHbp* sequences (see Fig. S5b in the supplemental material) was also clearly related to the modularity of the gene. Five different contiguous portions of the molecule could be highlighted in the *fHbp* sequence alignment (see f1 to f5 in Fig. S5a in the supplemental material) that corresponded to the major splits in the network. f1 and f2 were the major contributors to the split between variants 2 and 3, while f3 and f5 determined the split between variant 1 and variants 2 and 3. All of the portions segregated with variant 1 or 3 molecules (except f4, which defined a portion of the molecule under a very different pattern of recombination), and variant 2 molecules could be described as a chimera of variant 1 and 3 molecules, as previously suggested (44) and supported by our analysis of the recombination events spanning this region.

(ii) NHBA. Besides being present in all strains of *N. meningitidis*, conserved homologs of the NHBA-encoding gene were also found in *N. lactamica*, *N. polysaccharea*, *N. gonorrhoeae*, and *N. flavescens* (Table 1). In all of the species where NHBA was present, the entire locus harboring the *nhba* gene was well conserved (Fig. 6). A highly conserved locus was also found in *N. flavescens* strain NRL30031 H210, but the NHBA coding gene was FS. In the only other available sequenced genome of *N. flavescens*, the gene was absent. However, since the latter sequence was not complete, definitive conclusions could not be drawn. In other species, where the *nhba* homolog was absent (Table 1), there was a very weak similarity hit, in any case inferior to 30% sequence identity.

While in most cases *nhba* sequences from different species did not segregate together in a phylogenetic tree (Fig. 7), all gonococcal *nhba* molecules clustered in a branch of the tree that was well supported by bootstrap testing (94%). In the rest of the tree, although it was possible to identify small groups of closely related sequences with high levels of statistical support, most inner branches had very low levels of bootstrap support. In addition, the conservation of the *nhba* sequences within *N. meningitidis* (9, 16, 38) was of the same order of magnitude as the conservation across the whole *Neisseria* genus. These results provided further support for the existence of a high rate of genetic exchange between the different species that homogenize the distribution of the NHBA molecules within the genus *Neisseria*, with the possible exception of gonococci.

A phylogenetic network analysis evidenced the influence of homologous recombination on the complex evolutionary history of this set of sequences (see Fig. S6 in the supplemental material), since it was impossible to separate phylogenetic clades corresponding to the species. The only exceptions were the gonococcal strains that were grouped in a separated branch, although we also found evidence of reticulate evolution between the gonococcal branch and the other neisseriae. We also analyzed the evidence of recombination events in the region, including 10 kbp upstream and downstream of the gene. In accordance with the network analysis of the gene sequences, the pattern of homologous recombination was very complex, and we found many dis-

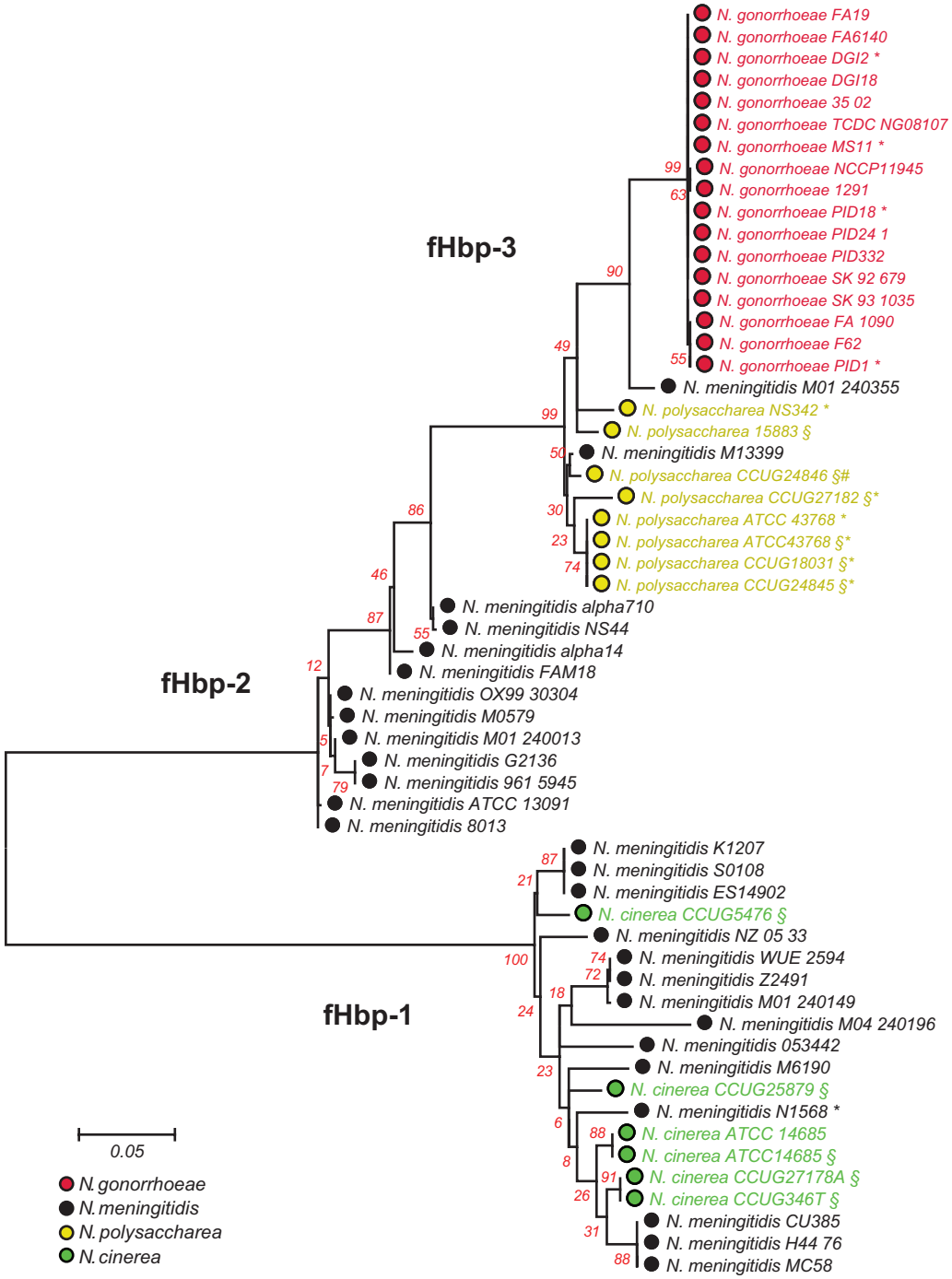

FIG 3 Maximum-likelihood phylogenetic tree of the *fHbp* gene. The tree was obtained with the Kimura two-parameter model with gamma correction. Bootstrap values are shown with red labels. The average gene variability (Pi) was 0.131 (SE, 0.011). Also, including sequences from other species, the tree showed the typical structuring in three branches, variants 1, 2, and 3, as already described in *N. meningitidis*. In particular, *N. gonorrhoeae* strains harbored variant 3-like genes, as did the two *N. polysaccharea* strains. *N. cinerea* harbored a variant 1 *fHbp* gene very similar to that of *N. meningitidis*. Colored circles and labels indicate the different *Neisseria* species. The § symbol indicates sequences that were downloaded from the BigsDB database; the others came from GenBank. The # symbol indicates genes that were at the border of a contig. Asterisks indicate genes that were FS.

tinct events overlapping the *nhba* gene (see Fig. S7 in the supplemental material).

The level of conservation of two important functional motifs described for NHBA, namely, the lipobox motif (LXXC) and the Arg-rich heparin-binding motif (RFRRSARSRRS), was checked. The lipobox motif was conserved in all of the strains. The Arg-rich heparin-binding motif was well conserved in the central part (RSARSR). Where present, the initial 3 amino acids (aa) of the motif (RFR) were less conserved. In all of the gonococcal strains, in one *N. lactamica* strain (Y92 1009), in *N. polysaccharea*, and in

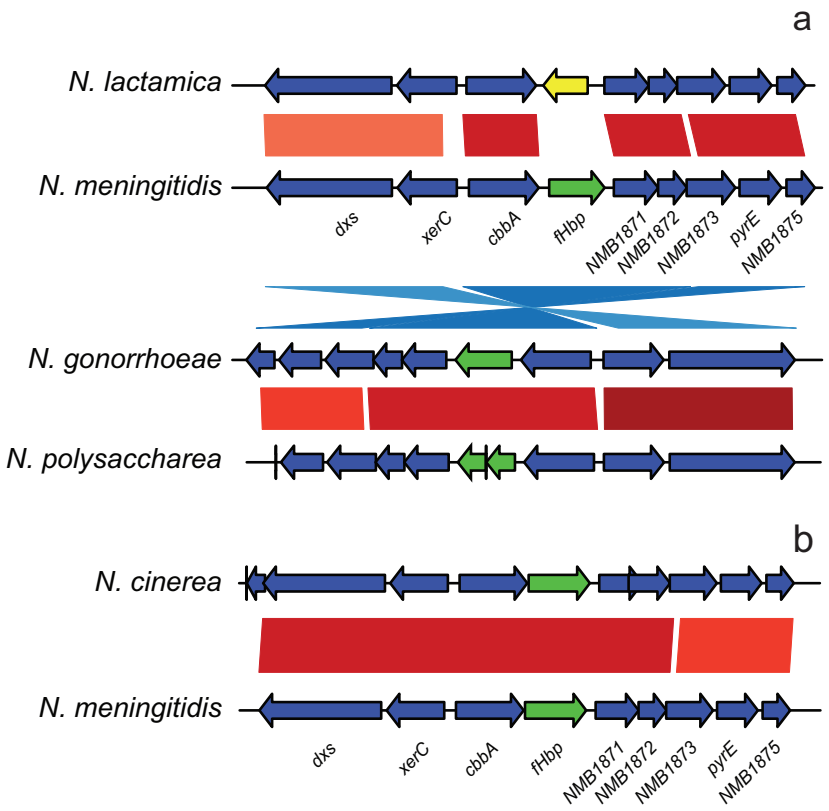

FIG 4 (a) Alignment of the *fHbp* locus in *N. lactamica* strain 020-06, *N. meningitidis* strain M01-240355, *N. gonorrhoeae* strain FA1090, and *N. polysaccharea* strain ATCC 43768. The *fHbp* gene is indicated in green. In three cases (*N. meningitidis* M01-240149, WUE_2594, and Z2491), the intergenic region between the homologues of *NMB1869* and the *fHbp* gene showed the insertion of a highly conserved, AT-rich DNA fragment of 186 or 187 bp (49) that corresponds to the direct-repeat portion of IS*1106* (GenBank accession no. Z11857.1) and is responsible for the separation of the *fHbp* promoter from its start codon. The entire locus is conserved in the reverse strand in all strains of *N. gonorrhoeae* and *N. polysaccharea*, where fHbp is always variant 3, while in both strains of *N. polysaccharea* the homologue of the *fHbp* gene is FS. The *fHbp* gene is absent from all strains of *N. lactamica*, where it is replaced by a protein annotated as a "putative opacity protein" on the opposite strand (in yellow). (b) *fHbp* loci in *N. meningitidis* MC58 and *N. cinerea* strain ATCC 1468. In both cases, the *fHbp* gene is variant 1.

three *N. meningitidis* strains (8013, alpha14, and N1568), a deletion of 7 or 8 aa (relative to the MC58 protein) was present that included the initial 3 aa (RFR) of the heparin-binding motif. This region, located upstream of the heparin-binding motif, is the cleavage site of the NalP protein (14) and was described as variable in gonococci (10).

(iii) NadA. The *nadA* gene was present in 12 of the sequenced strains of *N. meningitidis* (Table 1). In two strains, the coding sequence of the *nadA* homolog was interrupted by a premature stop codon introduced by a frameshift. In another strain (M6190), *nadA* was disrupted by insertion element IS*4*. In two cases, the coding sequence of *nadA* was interrupted by the border of a contig and the functionality of the gene could not be established. We found no homolog of the *nadA* gene in *N. lactamica* or *N. polysaccharea*, although in those species, as well as in the *N. meningitidis* strains that do not harbor the gene, the locus was well conserved (Fig. 8), suggesting that the *nadA* gene was the result of a recent insertion that left the neighboring genes unchanged (19). We found an intact homolog of *nadA* in one strain of *N. cinerea*, while in another strain we could reconstruct a *nadA* gene by joining two distinct contigs that contained, respectively, 850 bp of the N-terminal end and 600 bp of the C-terminal end of the molecule. However, the integrity of the gene could not be established. In two *N. cinerea* strains, fragments of *nadA* could be found in small contigs, but it was impossible to establish whether the gene was present. Finally, in one *N. cinerea* strain, the locus was conserved but *nadA* was replaced by a gene coding for a small hypothetical protein. When we used the concatenated pseudogene and the complete *nadA* gene from *N. cinerea* to build a phylogenetic tree (Fig. 9), we found that the *N. cinerea* sequences were related to the *nadA4* and *-5* forms from *N. meningitidis* (9). In all other species, the locus was not conserved. In *N. lactamica*, *N. mucosa*, and *N. sicca*, weak similarity hit (less than 45% sequence identity) was present, but the homology was limited to a C-terminal portion of the molecule of about 120 aa that is common to a family of YadA-like surface proteins (Table 1) and not specific of *nadA*.

The analysis of the presence of recombination revealed a limited number of events that related the formation of the meningococcal *nadA4* and *-5* molecules to a contribution from a donor similar to *N. cinerea nadA* (data not shown).

(iv) GNA1030. The gene for GNA1030 was present in all of the strains analyzed, with the exception of the draft genome of *N. wadsworthii* 9715. In *N. polysaccharea* ATCC 43768, the gene was interrupted at the border of a contig. In two gonococcal strains (PID332 and SK_93_1035), the poly(A) stretch following the ATG start codon introduced an adenine that was responsible for a frameshift of the gene. In the other species, the gene sequences were well conserved and the average gene variability of the whole *Neisseria* genus was reflected by a polymorphism index (Pi) of 0.095 (standard error [SE], 0.005). For a phylogenetic tree based on the aligned sequences, see Fig. S8a in the supplemental material.

(v) GNA2091. The gene for GNA2091, which is predicted to code for a hemolysin, was present in all of the species. In both *N. weaveri* LMG 5135 and ATCC 51223, the gene was interrupted at the border of a contig. The predicted start codon (ATG) was not always conserved but was in some cases replaced by a GTG (valine) or AAG (lysine) codon. The gene was always in frame and well conserved in the genus *Neisseria* (Pi = 0.128; SE, 0.005). For a phylogenetic reconstruction of the molecule, see Fig. S8b in the supplemental material.

DISCUSSION

The genus *Neisseria* includes a large group of bacteria, some of which are responsible for life-threatening diseases in humans, while others are mainly harmless colonizers. We have studied the distribution of *fHbp*, *nhba*, and *nadA*, the genes for three antigens included in the 4CMenB vaccine, in the available genome se-

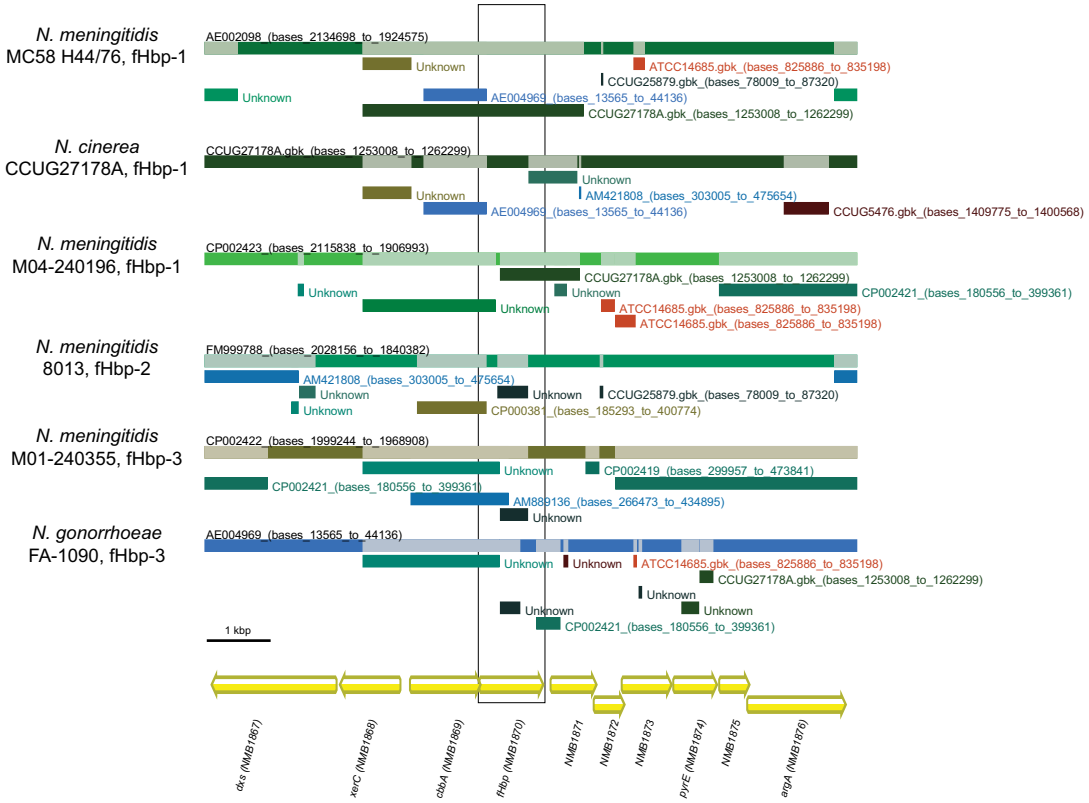

FIG 5 Schema of the recombination events spanning the *fHbp* gene. The positions of the genes in the MC58 sequence are shown. The black box indicates the position of the *fHbp* gene. A single representative of each group of sequences showing a similar pattern of recombination events is reported. Each horizontal bar represents the locus in the genome indicated on the left. Light boxes drawn within each bar represent putative segments transferred by recombination. The imported fragments are represented by specific boxes that are drawn below each bar. The name of a putative donor strain is indicated on the right. *fHbp* variant 1 (strain MC58) appears to have been imported into *N. meningitidis* by a recombination event also including the upstream *cbbA* gene, while *fHbp* variant 2 was the result of the recombination of a small fragment of a variant 1 sequence in a variant 3 background.

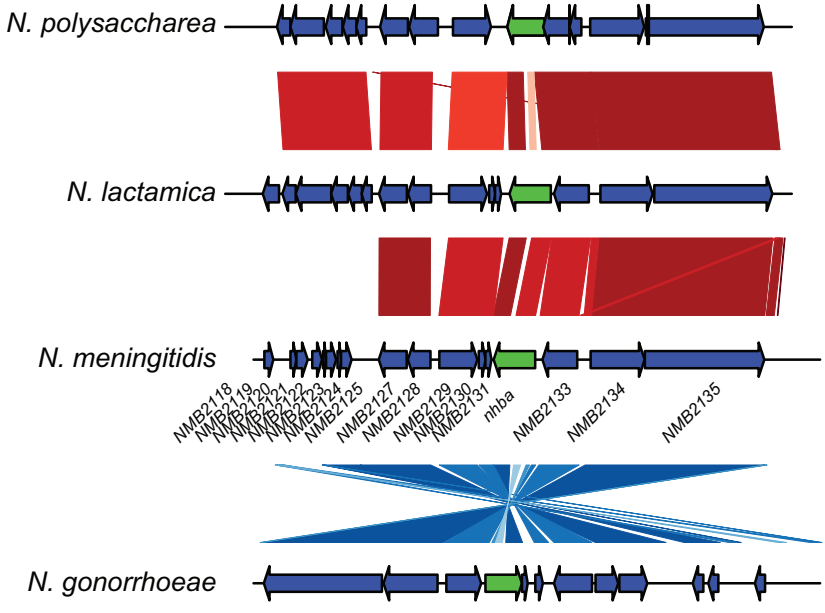

FIG 6 Alignment of the *nhba* loci of *N. lactamica* strain 020-06, *N. meningitidis* strain MC58, *N. gonorrhoeae* strain FA1090, and *N. polysaccharea* strain ATCC 43768. The *nhba* gene is indicated in green.

quences of the commensal and pathogenic neisseriae. We also studied the GNA2091 and GNA1030 proteins, which are included in the 4CMenB vaccine as fusion proteins with fHbp (GNA2091-fHbp) and NHBA (NHBA-GNA1030). *fHbp* was present in one of its three major variants in all strains of *N. meningitidis*, in *N. gonorrhoeae*, and in all of the commensal human neisseriae that have been sequenced to date, except *N. lactamica*. All of the *N. gonorrhoeae* and *N. polysaccharea* strains harbored *fHbp* variant 3, while strains of *N. cinerea* harbored close homologs of *N. meningitidis fHbp* variant 1. It was not possible to assess unambiguously in which species the protein evolved primarily, and all hypotheses involved at least one interspecies recombination event and possibly two. Given the relatively lower rate of recombination between *N. meningitidis* and *N. gonorrhoeae* (4) and the fact that an *fHbp* variant 3 gene also exists in *N. polysaccharea*, it is likely that an *fHbp* variant 3 gene was present in their most recent common ancestor. We also found evidence that the *fHbp* variant 1 gene evolved in *N. ci-*

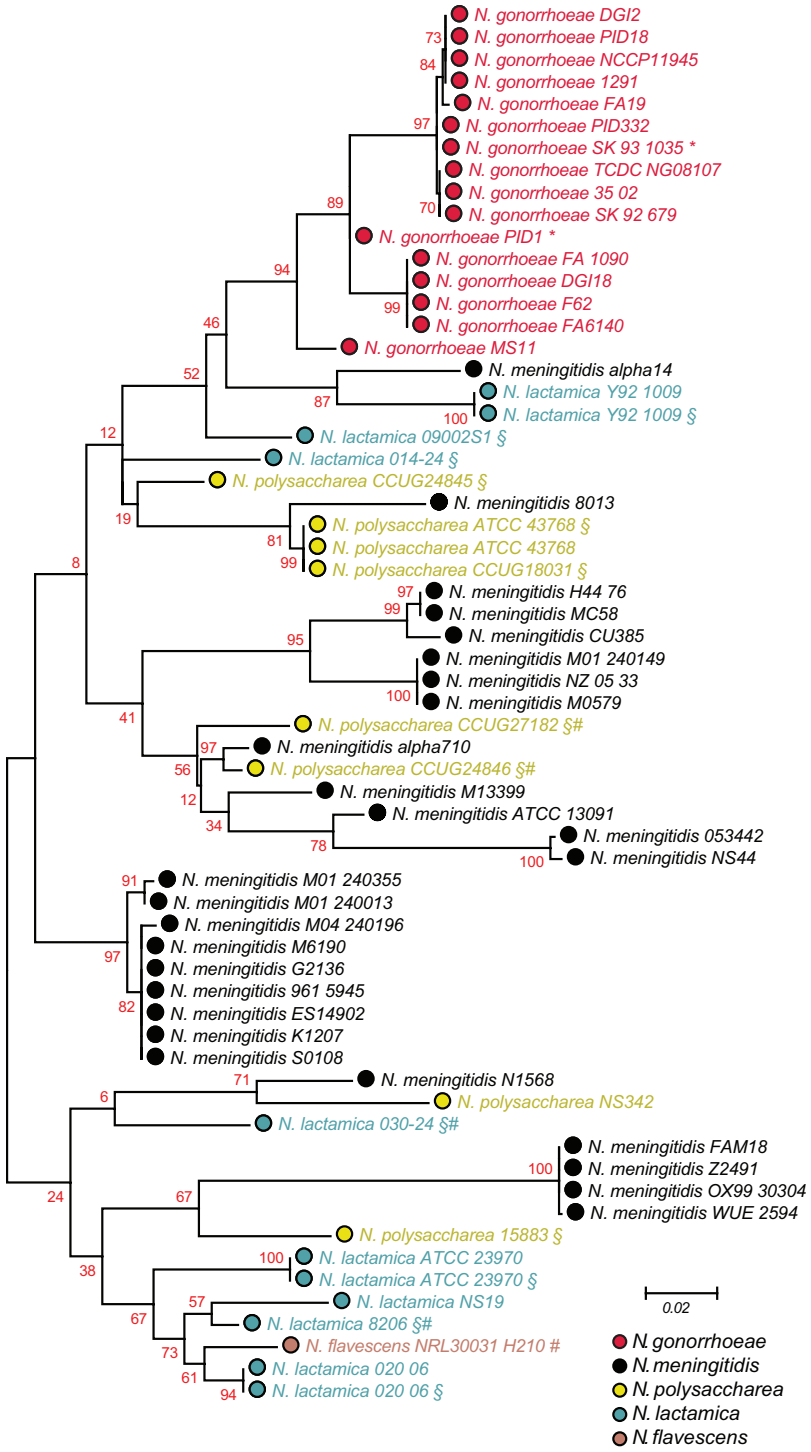

FIG 7 Maximum-likelihood phylogenetic tree of the *nhba* gene. The tree was obtained with the Kimura two-parameter model with gamma correction. Bootstrap values are shown with red lebels. The average gene variability (Pi) was 0.084 (SE, 0.004). Gonococci formed a monophyletic branch closely related to the rest of the isolates. The other species, *N. polysaccharea*, *N. lactamica*, and *N. flavescens*, were scattered throughout the entire tree. Colored circles and labels indicate the different *Neisseria* species. The § symbol indicates sequences that were downloaded from the BigsDB database; the others were from GenBank. The # symbol indicates genes that were at the border of a contig. Asterisks indicate genes that were FS.

nerea and was introduced into *N. meningitidis* by a recombination event also involving the neighboring *cbbA* gene. Finally, sequence analysis suggested that one more homologous recombination event generated *fHbp* variant 2 by inserting a variant 1 fragment into a variant 3 background.

A distribution similar to that of *fHbp* was found for *nhba*, which was absent from *N. cinerea*, while it was present in *N. lactamica*. Also, the gene was present in all strains of *N. gonorrhoeae*, and its sequences showed little variability within this species. This result was compatible with the overall low variability of the genomes of *N. gonorrhoeae* and suggested a recent diversification of the gonococci from *N. meningitidis*. The *nhba* gene showed low variability within *N. meningitidis* (9, 16, 38), and sera of mice vaccinated with one variant were shown to be bactericidal against heterologous strains (11). Interestingly, the heparin-binding motif was only partially conserved across all of the species where the gene was present. The site of cleavage of NHBA by NalP, situated upstream of the heparin-binding region, was not conserved, suggesting different posttranslation protein processing.

The gene coding for NadA is part of an independent genetic unit (19) that consists of a promoter region, the coding sequence, and a terminator region. *nadA* was present in only a portion of the *N. meningitidis* strains and was never found in any other commensal or pathogenic neisseriae, with the exception of two strains of *N. cinerea*. Analysis of the sequences immediately adjacent to this genetic unit showed that the segment was always flanked by direct repeats, indicating that a recombination mechanism could have determined its distribution among the meningococcal lineages. Finally, homologues of GNA1030 and GNA2091 were present and well conserved in most isolates of the genus *Neisseria*.

Taken together, these findings highlight the complex evolutionary history of the human neisseriae. With the exception of *N. gonorrhoeae*, the human neisseriae are carried asymptomatically in the nasopharynxes of healthy individuals. It has long been known that homologous recombination is the main process contributing to the genomic evolution of *N. meningitidis* and that some antigens can be exchanged between pathogenic and commensal species (45). We found that, when present, the sequences of *fHbp*, *nhba*, and *nadA* from other species of the genus *Neisseria* do not introduce radically new variants compared to the variability already present in *N. meningitidis*.

The distribution of the 4CMenB vaccine antigens in the commensal neisseriae suggests that vaccination could have an impact on the composition of the commensal flora. In particular, since the variability of *nhba* across the different neisseriae (*N. gonorrhoeae*, *N. lactamica*, and *N. polysaccharea*) was similar to the

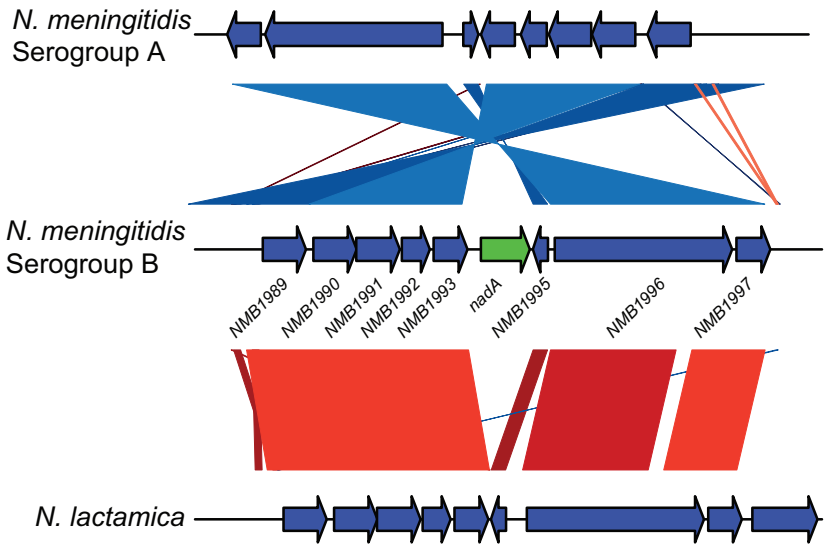

FIG 8 Alignment of the *nadA* loci of *N. meningitidis* serogroup A strain Z2491, *N. meningitidis* serogroup B strain MC58, and *N. lactamica* strain 020-06. The *nadA* gene is indicated in green. Although the locus is well conserved, the *nadA* gene is missing from both *N. meningitidis* serogroup A strain Z2491 and *N. lactamica* strain 020-06. In *N. cinerea* ATCC 14685, the *nadA* gene is interrupted by a contig edge; in *N. cinerea* CCUG346T, the gene is intact. In both strains, the gene is placed in the same locus as in *N. meningitidis* serogroup B strain MC58.

intraspecies variability within *N. meningitidis*, a recombinant vaccine based on NHBA could have an impact on the species harboring this protein. However, as recently shown for the 4CMenB vaccine (46), the ability of vaccine-induced antibodies to mediate killing depends on their ability to react with the variant/peptide present in the vaccine and on the amount of antigen expressed by a given strain. The latter might vary within and between species in response to as-yet-unknown environmental factors, as recently demonstrated for NadA (47, 48). Other factors, like surface accessibility, must also be taken into account (8). Therefore, genetic typing is not sufficient to predict strain coverage and further studies are needed to evaluate whether this vaccine will have any impact on commensal strains.

MATERIALS AND METHODS

Phylogenetic analysis of the genus *Neisseria*. We downloaded 1,080 aligned 16S rRNA sequences of the species belonging to the genus *Neisseria* (January 2012) from the RDP website (http://rdp.cme.msu.edu/index.jsp). The sequences were pruned by keeping only sequences that were more than 1% divergent, and a sample of 48 sequences that were representative of the diversity of the genus was obtained. From these, a maximum-likelihood phylogenetic tree based on the Tamura three-parameter model was computed (26). Site variability was modeled with a gamma distribution (five categories, $G = 0.1273$) plus invariants ($I = 66.74\%$ of the sites). The values of the parameters were optimized on the data. For each branch, bootstrap consensus was inferred from 500 replicates. The analysis was conducted with MEGA5 (27). Phylogenetic networks were computed with SplitsTree (28, 29).

Estimation of recombination and mutation rates. The population-scaled recombination rate ρ and the population-scaled mutation rate θ were estimated by the LDHat method (30) and with the Watterson estimator (31), respectively, with the software package RDP3 (32).

Genome sequences. The 18 complete and 49 draft genome sequences of 67 isolates belonging to the genus *Neisseria* were retrieved from GenBank (December 2011). To improve the representativeness of the data set,

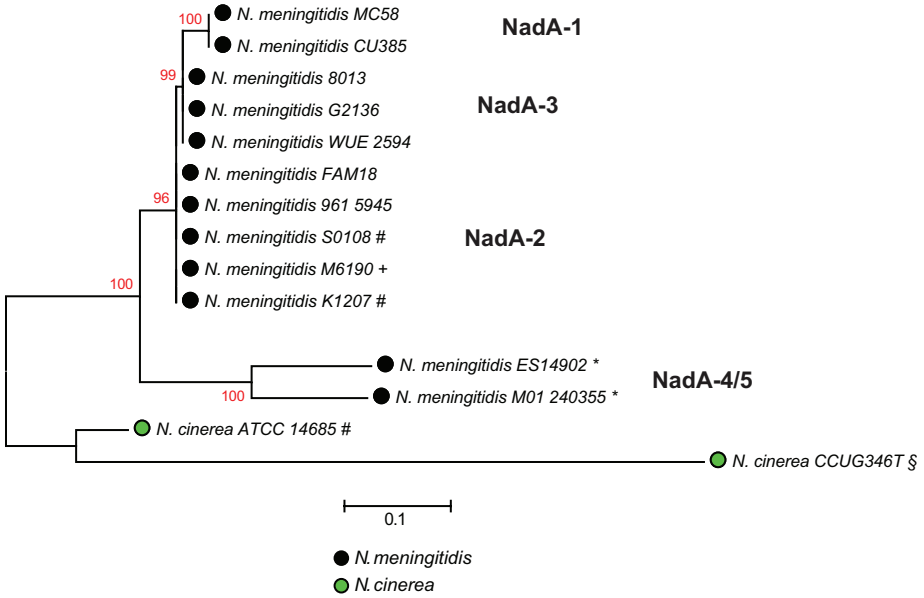

FIG 9 Maximum-likelihood phylogenetic tree of the *nadA* gene. The tree was obtained with the Kimura two-parameter model with gamma correction. Bootstrap values are shown with red labels. The sequence of the *nadA* gene from *N. cinerea* strain ATCC 14685 was obtained by joining two fragments at the border of two distinct contigs, and it was therefore not possible to assess the integrity of the gene. Colored circles and labels indicate the different *Neisseria* species. The § symbol indicates sequences that were downloaded from the BigsDB database; the others were from GenBank. The # symbol indicates genes that were at the border of a contig. The plus sign indicates genes that were interrupted by IS*4*. Asterisks indicate genes that were FS.

we downloaded (December 2012) 18 additional genome sequences of *N. cinerea* (five strains), *N. polysaccharea* (seven strains), and *N. lactamica* (six strains) from the *Neisseria* PubMLST database (http://pubmlst.org/neisseria/). Of these, five (one *N. cinerea* strain, three *N. lactamica* strains, and one *N. polysaccharea* strain) were resequencings of strains already present in the GenBank data set that we included to correct potential sequencing errors in the draft genomes. The draft genomes consisted of 23 to 574 contigs. For a complete list of the isolates, see Table S1 in the supplemental material. For the aligned gene sequences of *fHbp*, *nhba*, *nadA*, *gna1030*, and *gna2091* and the aligned locus sequences of *fHbp* and *nhba*, see the Text S1 file in the supplemental material. As indicated in Table S1, of the 27 strains of *N. meningitidis*, 2 were characterized as serogroup A, 17 were serogroup B, 5 were serogroup C, 1 was serogroup X, 1 was serogroup Y, and 1 was not typeable because of a capsule null locus.

Genome alignments and phylogenetic analysis of sequenced strains. Pairwise genome alignments of all sequences against the genome sequence of *N. meningitidis* MC58 were computed with Nucmer (33). From these, 223,369 SNPs were identified in the core genome, and the corresponding alleles in all of the strains were extracted. Phylogenetic analysis was conducted by the neighbor-joining method (34). Evolutionary distances were calculated by the maximum composite likelihood method (35). The analysis was conducted with MEGA5 (27). Phylogenetic networks were calculated with SplitsTree4 (28, 29).

Genome annotation. Annotation transfer for draft genomes was performed with RATT (36). In particular, for *N. polysaccharea* ATCC 43768 (accession number ADBE00000000), we used the annotation of *N. gonorrhoeae* FA1090 (accession number AE004969) as the template. For *N. cinerea* ATCC 14685 (accession number ACDY00000000), we used the annotation of *N. meningitidis* MC58 (accession number AE02098) as the template.

Criteria for gene presence or absence. The presence of the *fHbp*, *nhba*, and *nadA* genes was verified by nucleotide alignments against predicted coding genes and finally checked with TFASTY (37) to find possible pseudogenes or coding sequences not predicted in the genome annotation. fHbp query sequences were representative of the three major molecular variants (11). For NHBA, nucleotide sequences coding for peptides 1 to 21, representative of the diversity of the molecule in *N. meningitidis* (16, 38), were used as query sequences. For NadA, the query sequences were representative of the five major protein forms (38). To identify possible distant homologues, the BLAST *E* score cutoff was set to 10^{-5} and the sequence alignments were then manually checked to identify true homologues. Gene loci were also inspected by considering the whole-genome alignment performed with Mauve (39) and the level of conservation of each locally collinear block containing the genes.

Phylogenetic analysis of *fHbp*, *nhba*, and *nadA*. Maximum-likelihood phylogenetic trees based on the Kimura two-parameter model with gamma correction were built for each antigen. Branch stability was tested by bootstrap consensus of 500 replicates. The analysis was conducted with MEGA5 (27). Phylogenetic networks were built with SplitTree4 (28). Phylogenetic distances were computed with the Kimura two-parameter model with gamma correction by using the values of the parameters obtained with MEGA5.

Identification of recombination events. To test the presence of recombination, a multiple-sequence alignment of each locus was built by extending the *fHbp*, *nhba*, and *nadA* loci by 10 kbp upstream and downstream (or to the edge of the contig in the unfinished genomes if this was closer than 10 kb). The draft genomes that were fragmented in the neighborhood of the gene locus were not included in the alignment (see Text S1 in the supplemental material). Recombination events were inferred with RDP3 (32) by the RDP, Geneconv, MaxChi, Bootscan, chimaera, 3Seq, and Siscan methods.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00163-13/-/DCSupplemental.

Text S1, TXT file, 1.8 MB.
Figure S1, PDF file, 0.4 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.3 MB.
Figure S5, PDF file, 2.1 MB.
Figure S6, PDF file, 0.3 MB.
Figure S7, PDF file, 0.2 MB.
Figure S8, PDF file, 0.5 MB.
Table S1, XLS file, 0.1 MB.

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