NadA, a Novel Vaccine Candidate of *Neisseria meningitidis*

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Abstract

Neisseria meningitidis is a human pathogen, which, in spite of antibiotic therapy, is still a major cause of mortality due to sepsis and meningitis. Here we describe NadA, a novel surface antigen of *N. meningitidis* that is present in 52 out of 53 strains of hypervirulent lineages electrophoretic types (ET) ET37, ET5, and cluster A4. The gene is absent in the hypervirulent lineage III, in *N. gonorrhoeae* and in the commensal species *N. lactamica* and *N. cinerea*. The guanine/cytosine content, lower than the chromosome, suggests acquisition by horizontal gene transfer and subsequent limited evolution to generate three well-conserved alleles. NadA has a predicted molecular structure strikingly similar to a novel class of adhesins (YadA and UspA2), forms high molecular weight oligomers, and binds to epithelial cells in vitro supporting the hypothesis that NadA is important for host cell interaction. NadA induces strong bactericidal antibodies and is protective in the infant rat model suggesting that this protein may represent a novel antigen for a vaccine able to control meningococcal disease caused by three hypervirulent lineages.

Key words: *N. meningitidis* • meningococcus • adhesin • pathogenesis • vaccine

Introduction

Neisseria meningitidis is a capsulated gram-negative bacterium, which is a major cause of meningitis and sepsis, two devastating diseases that can kill children and young adults within hours despite the availability of effective antibiotics. The bacterium is found only in humans and is classified into 13 serogroups on the basis of the chemical composition of the capsular polysaccharides. Five serogroups (A, B, C, Y, and W-135) cause disease (1, 2). The strains, isolated from invasive meningococcal disease, have been classified by multilocus enzyme electrophoresis into a small number of hypervirulent lineages: electrophoretic types (ET)* ET37, ET5, cluster A4, lineage III, and subgroups I, III, and IV-1 (3, 4). Recently, a new sequence-based classification, multilocus sequence typing, has been introduced, which classifies the above strains into sequence types (ST) ST11, ST32, ST8, ST41, ST1, ST5, and ST4, respectively (5). Strains isolated from healthy carriers usually fall into many different ET and ST types.

The reported annual incidence of meningococcal disease varies from 0.5 to 10 per 100,000 persons; however, during epidemics the incidence can rise above 15 and up to 400 per 100,000. The case fatality rate ranges from 5 to 15%, and up to 25% of survivors are left with neurological sequelae. The first successful vaccines against meningococcus consisted of purified polysaccharides against four (A, C, Y, and W-135) of the five pathogenic serogroups (6–8). These vaccines are highly effective in adults but less efficacious in infants and young children, the age groups mostly exposed to disease. Second generation glycoconjugate vaccines have recently been introduced against serogroup C. meningococcus (9, 10). These vaccines have shown extremely high efficacy (>90%) in infants, children, and adolescents and are presently a valid measure to eliminate the disease caused

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**Abbreviations used in this paper:* ET, electrophoretic type; GC, gonococcus; MenB, serogroup B *N. meningitidis*; ORF, open reading frame; ST, sequence type.

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by serogroup C. Similar vaccines against serogroups A, Y, and W-135 are in the later phases of development (11). Currently, there are no licensed vaccines available in the U.S. and Europe for the prevention of serogroup B *N. meningitidis* (MenB) disease, which is responsible for 32% of all meningococcal disease in the United States and for 45–80% of the cases in Europe and for >50% of the cases in the rest of the world, with the exception of sub-Saharan Africa where serogroup A is responsible for 90% of the cases (12, 13). The use of capsular polysaccharide as the basis of a vaccine for prevention of MenB diseases has been problematic because the MenB capsular polysaccharide is identical to a widely distributed human carbohydrate ($\alpha[2\rightarrow8]$*N*-acetyl neuraminic acid or polysialic acid), which is a self-antigen, and therefore, is poorly immunogenic in humans. Furthermore, the use of this polysaccharide in a vaccine may elicit autoantibodies (14, 15). An alternative approach to vaccine development is based on surface-exposed proteins contained in outer membrane vesicles. These vaccines have been shown both to elicit serum bactericidal antibody responses and to protect against meningococcal disease in clinical trials (4, 16). Although outer membrane vesicle vaccines have been used for vaccination in Central and South America, they have limitations that prevented their use in other countries. Their main problem is that the major protein antigens show sequence and antigenic variability (17, 18) and, although they induce protective antibodies against the homologous strain, they fail to induce protection against heterologous strains (19).

Recently our laboratory published the use of the genomic sequence to discover novel antigens capable of inducing protection against MenB (20). During the screening of the genome, we found a protein, NMB1994, able to elicit bactericidal antibodies, which has the same carboxyl terminal region architecture as YadA and UspAs. Since UspAs are promising protective antigens against *M. catarrhalis* infection (21) and YadA has been implicated in virulence (22), we further investigated this protein. Here we describe NMB1994 (NadA) a novel surface-exposed oligomeric protein, which binds to host cells in vitro suggesting that it could belong to this novel class of adhesins. NadA is encoded by an open reading frame (ORF) that was found in the genome of the meningococcus B strain MC58 (23) and was absent in the genome of the meningococcus A strain Z2491 (24). We show that the gene is present in a subset of *N. meningitidis* strains but is overrepresented in three hypervirulent lineages and may represent a new antigen for vaccine development and a tool to dissect the evolution and pathogenic mechanisms of this bacterium.

Materials and Methods

Computer Analysis. Computer programs included in the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package (v10.0) and the Biosoft Sequence Processor GeneJockey II were used to analyze and compare the nucleotide sequences from different Neisseria strains.

Sequence homology searches, protein features, and possible functions were performed using the programs BLAST, SignalP (25), SIMPA96 (26), PAIRCOIL (27), ZZIP (28), and CLUSTALW.

Nucleotide Sequence Accession Nos. The complete genome sequences of *N. meningitidis* serogroup B strain MC58 (23) and *N. meningitidis* A strain Z2491 (24) are available under GenBank/EMBL/DDBJ accession nos. AE002098 and AL157959. The nucleotide sequences of *nadA* genomic region of each strain analyzed have been submitted to the GenBank database GenBank/EMBL/DDBJ accession nos. AF452465–AF452488.

Bacterial Strains, Culture Conditions, and Chromosomal DNA Isolation. 175 *N. meningitidis* strains were chosen for analysis. For a detailed list, see Online Supplemental Material.

Bacteria were grown overnight at 37°C in a humidified atmosphere of 5% CO_2 in air on gonococcus (GC) medium agar (Difco) supplemented with Kellogg's supplement solution (0.22 M D-glucose, 0.03 M L-glutamine, 0.001 M ferric nitrate, and 0.02 M cocarboxylase; Sigma-Aldrich) as described previously (29, 30). One loopful of meningococci was dissolved in 500 μl of PBS and chromosomal DNA was prepared as described previously (31).

PCR and Nucleotide Sequencing of Gene Fragments. PCR amplification of the *nadA* genes were performed on 10 ng of chromosomal DNA using primers, mapping 350 nt upstream and downstream the coding region (forward primer: GTCGACGTCCTCGATTACGAAG; reverse primer: CGAGGCGATTGTCAAACCGTTC), and Platinum Hifi Taq Polymerase (GIBCO BRL). PCR conditions were: 30 cycles of denaturation at 95°C for 30 s; annealing at 60°C for 30 s; and extension at 68°C for 1 min. PCR products were analyzed on 1% agarose gel and the sizes were determined using a molecular weight marker 1-Kb Plus DNA Ladder (GIBCO BRL). The amplified fragments were purified on a Qiaquick column (QIAGEN) and then automated cyclo-sequenced (model 377; Applied Biosystems) by primer walking on both strands of the amplified fragment.

Cloning, Expression, and Purification of rNadA. The *nadA* sequences (allele 1: aa 24–362; allele 2: aa 24–343; allele 3: aa 24–350), were amplified by PCR on chromosomal DNA and cloned into pET21b$^+$ vector (Novagen). The plasmids were transformed in *Escherichia coli* BL21 (DE3) to express the proteins as COOH-terminal Histidine fusions. Protein expression was induced at 30°C by adding 1 mM IPTG at OD_{600nm} 0.3 and growing the bacteria for an additional 3 h; expression was evaluated by SDS-PAGE. Recombinant fusion proteins were purified by affinity chromatography on Ni^{2+}-conjugated chelating fast-flow Sepharose 4B resin (Amersham Pharmacia Biotech).

Western Blot Analysis. Bacterial colonies were grown to stationary phase in GC broth supplemented with 0.3% glucose. Samples were taken at different times, pelleted by centrifugation at 3,000× *g* for 10 min, resuspended in PBS, and thawed/frozen up to bacterial lysis. Equal amounts of proteins were subjected to SDS-PAGE on 12.5% polyacrylamide gels and electrotransferred onto nitrocellulose membranes.

Dot Blotting. The presence of *nadA* gene in 50 strains out of the 175 strains was tested by dot-blot. We used as probe the whole *nadA* gene, as amplified from 2996 strain (allele 3) and labeled with digoxigenin using the Roche DIG High-Prime DNA Labeling and Detection kit. 10 μl aliquot of cell suspension of each strain were boiled for 10 min and spotted on nylon membrane (Boehringer Mannheim). The membranes underwent cross-linking of DNA by 2′ exposure to UV light and other stan-

dard procedures for preparation and signal detection as reported by the manufacturer.

Preparation of Polyclonal Anti-NadA Antisera. To prepare antisera, 20 μg of purified recombinant proteins were used to immunize 6-wk-old CD1 female mice (Charles River Laboratories). 4–6 mice per group were used. The proteins were given intraperitoneally, together with CFA for the first dose and incomplete Freund's adjuvant for the second (day 21) and third (day 35) booster doses. Bleed out samples were taken on day 49 and used for the serological analysis.

50 μg of purified rNadA from allele 3 were used to immunize guinea pigs. Two animals per group were used. The immunization was performed subcutaneously together CFA for the first dose and complete Freund's adjuvant for the further three doses (days 28, 56, and 84). Bleed out samples were taken on day 105 and used for the animal protection assay.

Binding of Antisera to the Surface of Meningococci. The ability of mouse polyclonal anti-rNadA antisera to bind to the surface of live meningococci was determined using FACScan™ flow cytometer of the encapsulated NMB and its isogenic nonencapsulated mutant M7 strains (32). We used R-phycoerythrin-conjugated goat $F(ab)_2$ antibody to mouse Ig (Jackson ImmunoResearch Laboratories) to detect antibody binding.

Complement-mediated Bactericidal Activity. Serum bactericidal activity against *N. meningitidis* strains was evaluated as described previously (20, 33), with pooled baby rabbit serum (CedarLane) used as complement source. Serum bactericidal titer, was defined as the serum dilution resulting in a 50% decrease in CFUs per ml after 60 min incubation of bacteria in the reaction mixture, compared with control CFU per ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60 min of incubation.

Animal Protection. The ability of anti-NadA Abs (guinea pigs antisera) to confer protection against *N. meningitidis* bacteremia was evaluated as previously described in infant rats challenged intraperitoneally (34). Briefly, 5–7-d-old pups from litters of outbred Wistar rats (Charles River Laboratories) were randomly redistributed to the nursing mothers. Two experiments were performed using two different MenB strains (8047 and 2996). Each strain has been serially passaged three times in infant rats. In experiment 1, groups of four were challenged intraperitoneally with 100 μl of a mix of bacteria from strain 8047 (7×10^3 CFU) and heat inactivated guinea pig antiserum or control mAb. In experiment 2, group of six animals were treated with the mAb or different dilutions of guinea pig antiserum at time 0. 2 h later, they were challenged with the bacteria from strain 2996. The anticapsular control mAb used was SEAM 3 (35). In both experiments, blood cultures were obtained 18 h after the challenge, by puncturing the heart with a syringe and needle containing ~25 U of heparin without preservative (Fuijisawa). Bacteria numbers in the blood cultures were obtained by plating out 1, 10, and 100 μl of blood onto chocolate agar overnight. For calculation of geometric mean CFU/ml, animals with sterile cultures were assigned a value of 1 CFU/ml.

Binding Assay. Chang epithelial cells (Wong-Kilbourne derivative, clone 1–5c-4, human conjunctiva) were maintained in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 15 mM L-glutamine, and antibiotics. Cells were nonenzymatically detached using cell dissociation solution (Sigma-Aldrich), harvested, and resuspended in RPMI 1640 medium supplemented with 1% FBS. 5×10^4 cells were mixed with rNadA (allele 3), rGNA2132 proteins, devoid of the COOH-terminal Histidine fusion or medium alone, and incubated 30 min at 4°C. Cells were incubated with antisera on ice for 1 h, then for 30 min on ice with R-phycoerythrin-conjugated goat $F(ab)_2$ antibody to mouse Ig. Cells were analyzed with a FACScan™ flow cytometer. The mean fluorescence intensity for each population was calculated.

Online Supplemental Material. Characteristics of the 175 *N. meningitidis* strains analyzed in this study.

Results

A Coiled-Coil Protein Anchored to the Outer Membrane. During the screening of the *N. meningitidis* genome for novel vaccine candidates (20), *nadA* (*nmb1994*) was found to be one of the ORFs encoding a protein able to elicit serum bactericidal antibodies in mice. This ORF encodes a predicted outer membrane protein with 32–34% identity to the ubiquitous surface protein A2 of *M. catarrhalis* (UspA2; reference 36) and to an adhesin-invasin expressed by enteropathogenic *Yersinia* species (YadA), respectively (37, 38).

NadA is a protein of 362 amino acids with a possible leader peptide of 23 amino acids. The mature protein has a predicted molecular weight of 35363.49 daltons and an isoelectric point of 4.50. Analysis of the predicted secondary structures revealed several features that are summarized in Fig. 1. The COOH-terminal region (aa 310–362) has a predicted amphipatic β-structure, and a terminal aromatic amino acid, which are typical features of outer membrane anchoring domains (39). The amino terminal region (aa 23–90) has not a defined secondary structure, whereas the rest of the protein has mainly an α-helix propensity (84.6%). Within this region, residues from 90–146 and 183–288 have high probability of forming coiled coils. In addition, residues 122–143 contain four leucine residues in the "a" positions of the heptad repeats (L-x[6]-L-x[6]-L-x[6]-L) that may form a leucine zipper domain. It is known that both coiled coils and leucine zipper sequences are involved in dimerization (40) and may mediate oligomerization of monomers via association of two or more α helices.

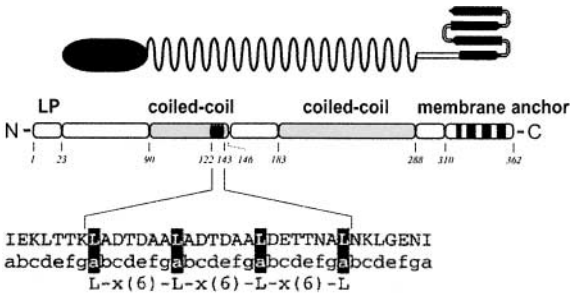

Figure 1. Schematic illustration of the possible NadA topology and corresponding domain structure. The globular amino (N) termini and the amphipatic COOH (C) termini are indicated. The positions of the leader peptide (LP), the coiled-coil structure and the membrane anchor are shown. Leucine zipper is indicated by black ovals and the β strands by dark vertical lines. The amino acid sequence below shows the leucine zipper motif and the leucine repeat highlighted in black.

A closer examination of the amino acid homology of NadA with YadA and UspA2 revealed that similarity is mainly clustered in the COOH terminal region (56–63% identity in the last 70 amino acids, respectively). Outside this region the level of identity drops to 23–25%. Interestingly, the overall similarity is conserved at secondary structure level. In fact, YadA and UspA2 have a COOH terminal membrane anchor made by four amphipatic β-strands (41), and an α-helical internal region with a propensity to form coiled-coils. Two putative leucine zippers are present in UspA2 (36), but they are absent in YadA. In the case of YadA and UspA2 it has been shown that the α-helices form coiled-coils regions, which mediate oligomerization of monomers (41, 42). The absence of cysteine residues in the mature forms of NadA is another feature common to its homologues.

A Foreign Gene Present in a Subset of Hypervirulent Strains. The 1,086 base pairs *nadA* coding region is flanked at the 3′ end by a terminator sequence while at the 5′ end (Fig. 2 A) it shows a putative ribosome-binding site (5′-AAGG-3′) and a putative promoter region located 8 and 47 base pairs, respectively, upstream the ATG start codon. 130 nucleotides upstream the coding region we find nine repeats of the tetranucleotide TAAA, preceded by a second putative promoter with –10 and –35 regions. Because of the presence of the TAAA repeats, the gene had been listed as one of those that may undergo phase variation, even though the repeats are not in the coding region (23). The G+C content of the *nadA* gene and its upstream region is lower than average (45% against an average of the rest of the genome, 51.5%), suggesting acquisition of the gene by horizontal transfer. Remarkably, the gene and the upstream region are not present in the published sequence of the genome of serogroup A, strain Z2491 (24). In this genome, a short sequence of 16 nucleotides with no homologies in the database, replaces the *nadA* gene (Fig. 2 B), whereas the upstream and downstream genes (*nmb1993* and *nmb1995*) are well conserved (91 and 97% identity). Analysis of the sequences immediately adjacent to the *nadA* region and absent in the Z2491 A strain shows that the segment is flanked by the TCAGAC direct repeats. This may indicate a mechanism of recombination. In the A strain, the stretch of 16 nucleotides has a disrupted pair of TCAGAC repeats flanking it.

To assess the presence of the *nadA* gene in the *N. meningitidis* population, 150 isolates, representative of the five disease-associated serogroups (A, B, C, Y, and W-135), were screened by PCR and/or dot blot hybridization. In addition, 25 strains isolated from healthy carriers were tested (Online Supplementary Data). The analysis also included one strain each of *N. gonorrhoeae*, *N. cinerea*, and *N. lactamica*. 47% of the isolates examined were positive for the presence of *nadA*. The gene was present in 52 out of 53 strains of the hypervirulent lineages ET5, ET37, and cluster A4, whereas it was absent in all the tested lineage III strains. Seven of the 25 carrier strains were positive (unpublished data. *nadA* was absent in *N. gonorrhoeae* and in the commensal species *N. lactamica* and *N. cinerea*.

Three Well-Conserved Alleles of nadA. PCR amplification was performed by using primers located in the conserved regions upstream and downstream *nadA*. PCR products of roughly 1,800 nucleotides, slightly different in size depending on strains, were obtained when the *nadA* gene is present (Fig. 3, lanes 1–3), whereas PCR products of 400 nucleotides were obtained in the absence of the gene (Fig. 3, lane 5). Four strains generated an atypical PCR product higher than that expected (Fig. 3, lane 4).

We determined the nucleotide sequence of 36 strains representative of each size: 26 positive strains; four strains generating an atypical PCR product; and six negative strains. In the negative strains, we found the nucleotide sequence of 16 base pairs, identical to the one present in the published sequence of the genome of serogroup A, strain Z2491. The positive strains have a *nadA* gene size ranging from 1,086 to 1,215 base pairs with consequent variation of the amino acid sequences of the encoded proteins from 362 to 405 amino acids. It is possible to cluster

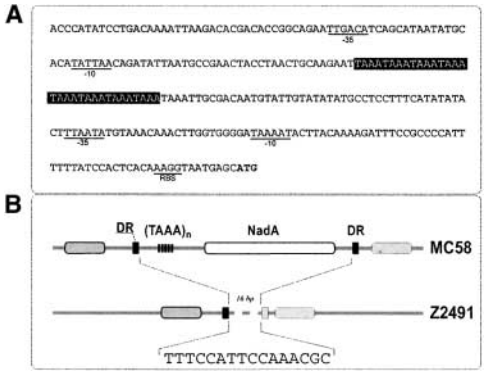

Figure 2. (A) Nucleotide sequence of the *nadA* upstream region. Putative –35 and –10 domains and a possible ribosomal binding site (RBS) are indicated. The translation start codon is reported in bold. The nine repeats of the tetranucleotide TAAA are highlighted in black. (B) Schematic comparison of the genome region organization in B strain MC58 and A strain Z2491. DR, direct repeat.

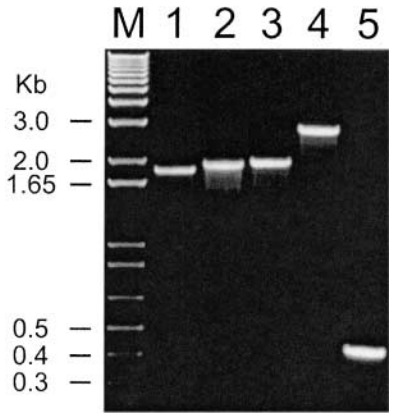

Figure 3. PCR products from positive strains (MC58, 90/18311, 2996; lanes 1–3), negative strains (NG3/88; lane 5), and from strains giving an atypical result (L93/4286; lane 4).

22 of the 26 *nadA* genes into three alleles (Table I and Fig. 4). The sequence of the gene within each allele is identical and the overall identity between the alleles ranges from 96 to 99%. Allele 1, coding for a protein of 362 amino acids, is contained by all the ET5 strains sequenced and recent isolates for which ET-type is not yet available. However, their serotype and serosubtype are typical of the ET5 complex. Allele 2, coding a protein of 398 aa, is contained by all the ET37 strains, one strain of cluster A4, and three additional not ET-typed serogroup C strains. Allele 3, coding a protein of 405 aa, is contained by serogroup A, B, and C strains. As shown in Fig. 4, the three alleles differ by a few amino acid changes and by a 7 or 43 amino acid deletion/insertion.

Interestingly, all deletion/insertion happen in the coiled-coil region and involves 7 or 43 amino acids which, representing two or six turns of the α-helix, allow for variations in length of the coiled-coil region without disturbing the overall structure. Furthermore, the 7 aa deletion in the allele 2 results in the loss of the first heptad repeat of the leucine zipper domain but does not destroy the domain because leucine residues at a fixed spacing of seven residues can be replaced mostly by Met, Val, or Ile. In this case allele 2 could use the Ile upstream or downstream to form the leucine zipper motif (Fig. 1).

The remaining four positive strains are minor variants of the alleles (indicated by asterisks in Table I). Two of them have a single amino acid mutation in the leader peptide, one has an additional 7 amino acids deletion and one is a recombinant (chimera) of alleles 1 and 2.

Finally, the four strains, which generated an atypical PCR product, contain one copy of the insertion sequence IS*1301*. The insertion site was identical, and the increase in length of the PCR sequence was in keeping with the size of the insertion element. The orientation of IS*1301* differed in the four isolates indicating an independent event. The target consensus for IS*1301*, 5′-AYTAG-3′ (43), was found within the *nadA* gene at nucleotide 472

Table I. *Characteristics of 26 N. meningitidis and their NadA Gene Allele*

Strain	Serogroup type: subtype	Clonal group	NadA allele	(TAAA) repeats	NadA expression
64/69	NG:15:P1.7,16	ET5	1	4	+
BZ83	B:15	ET5	1	5	+++
CU385	B:4:P1.15	ET5	1	6	++
MC58	B:15:P1.7,16b	ET5	1	9	+
BZ169	B:15:P1.16	ET5	1	12	++
95330[a]	B:4:P1.15	ET5	1	9	ND
ISS1104	B:15:P1.7,16	ND	1	4	+
ISS1071	B:15:P1.7,16	ND	1	5	+++
ISS832	B:15:P1.7	ND	1	5	++
NM119	B:4.P1.15	ND	1	6	ND
NM066	B:15:P1.7,16	ND	1	12	ND
90/18311	C:NT:P1.5	ET37	2	9	++
NGP165	B:NT:P1.2	ET37	2	9	++
FAM18	C:2a:P1.5,2	ET37	2	9	ND
M986	B:2a:P1.5,2	ET37	2	12	++
ISS1024[a]	C:2b:P1.5	ND	2	9	++
ISS838	C:2a:P1.5,2	ND	2	6	++
PMC8	C	ND	2	10	++++
961-5945	B:2b:P1.21,16	A4	2	12	+++
ISS759[a]	C:2b:P1.2	ND	3	8	++++
F6124	A	subgroup III	3	9	+
NMB	B:2b:P1.5,2	ND	3	12	++
8047	B:2b:P1.2	ND	3	12	+++
2996	B:2b:P1.5-1,2	ND	3	12	+++
C11	C:NT:P1.1	ND	3	12	+++
973-1720[a]	C:2b:P1.2	A4	3	12	+++

[a]Minor variants of the alleles.
ND: not done.

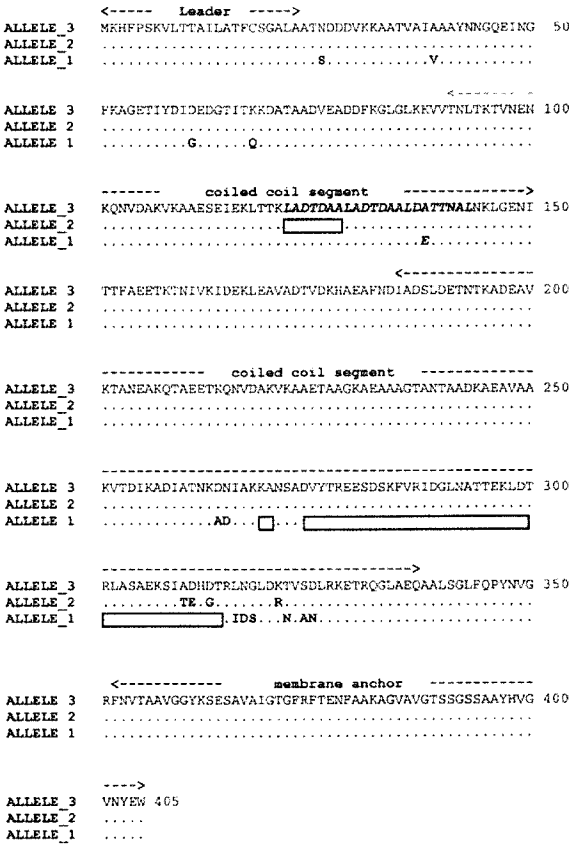

Figure 4. Sequence comparison of NadA polypeptides representative of each of the genetic variants. Differences in amino acids are indicated. Empty boxes represent deletions. The leader peptide, the coiled-coil structure, and the membrane anchor are shown.

generated by an A to G mutation, and was accompanied by a TA duplication.

Sequence analysis of the putative promoter region shows that the number of the tetranucleotide repeat (TAAA) varies from 4 to 12 in different strains (Table I). The sequence polymorphism of the *nadA* flanking regions (50 bases upstream the putative promoter and 350 bases downstream the terminator regions) segregates with each allele, respectively.

The Protein Forms High Molecular Weight Oligomers. Western blots of whole-cell lysates of all *N. meningitidis*, probed with anti-NadA antibodies, showed a high molecular weight reactive band in all strains containing the gene (Fig. 5, lanes 1–3), absent in the negative strains (Fig. 5, lane 5). Boiling of the sample buffer up to 40 min did not change the pattern (data not shown). The different size of the proteins was consistent with the size of the alleles. Given the expected size ranging from 35 to 40 kD of the monomeric proteins, the high molecular weight of the observed band could be explained by the presence of an oligomeric form of NadA. This possibility is supported by the fact that in a strain containing the IS*1301*, coding for a shorter protein of 162 amino acids and lacking most of the coiled-coil region, the high molecular weight reactive band was absent and replaced by a band of 14.5 kD (Fig. 5, lane

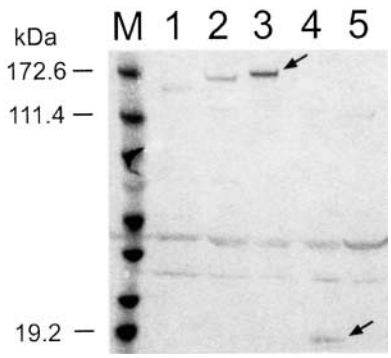

Figure 5. Western immunoblot of whole-cell lysates of strains representative of each NadA allele. M, molecular size marker; lane 1, strain MC58 (allele 1); lane 2, strain 90/18311 (allele 2); lane 3, strain 2996 (allele 3); lane 4, strain L93/4286 (IS*1301*); lane 5, strain NG3/88 (negative). Arrows indicate the diverse forms of the NadA protein.

4), consistent with the predicted molecular weight of the processed monomeric protein. Whereas the oligomeric protein was found in all strains containing a functional gene, the expression level varied from strain to strain (Table I). Moreover, the amount of NadA protein varied within the same strain during growth.

Four different strains (MC58, 2996, C11, and F6124), chosen as representative of diverse overall NadA expression level, were followed during growth up to stationary phase. The growth of two of the tested strains (MC58 and 2996, low and high NadA expression level, respectively) is shown in Fig. 6. Western immunoblot of samples corresponding to each time point of the bacterial growth curve showed that the NadA band was barely visible at the beginning of the growth and became more intense during growth, up to its maximum, at stationary phase. All strains analyzed showed the same growth phase–dependent behavior.

The Protein Is Surface-exposed and Represents a Target for Bactericidal Antibodies. Western blot of outer membrane vesicles fraction (data not shown) revealed the presence of a high molecular weight band corresponding to the oligomeric form of NadA supporting the hypothesis that the oligomer is anchored to the outer membrane. Furthermore, FACS® analysis showed that NadA was available for antibody binding on the surface of bacteria. The analysis was performed on live bacteria during the logarithmic phase of growth, using antibodies against the recombinant protein. The encapsulated NMB and the nonencapsulated isogenic mutant M7 were used. As shown in Fig. 7, polyclonal anti-NadA antibodies were able to bind to both strains, although in the nonencapsulated M7 strain the peak of fluorescence was one log higher than in the capsulated strain. These data indicate that the protein is exposed on the surface of the bacterium and available for binding both in the presence and in the absence of the polysaccharide capsule. However, the presence of the capsule hinders the binding of the antibody.

We also tested whether the anti-NadA serum had bactericidal activity against a panel of strains. As shown in Ta-

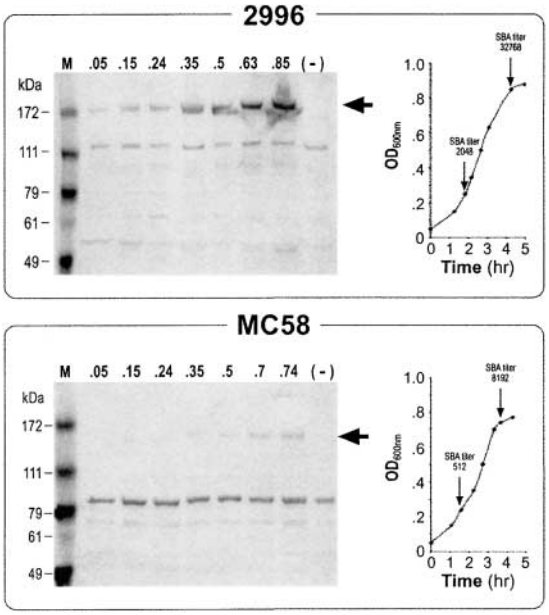

Figure 6. Growth phase–dependent NadA expression of MC58 and 2996 strains. (Left) Western immunoblot of samples corresponding to each time point of the bacterial growth curve (right). M, molecular size marker; absorbance at 600 nm at different time during growth; NG3/88, negative control. Arrows indicate the multimeric forms of the NadA protein. (Right) Growth curves and bactericidal titers determined in the early and late phase of growth.

ble II, the serum induced complement-mediated killing of all strains that have the *nadA* gene, and was inactive against the strains that do not have the gene. However, the bactericidal titers varied between strains. Titers were higher against strains expressing higher amounts of protein. This result was confirmed when bactericidal titers were determined in the early and late phase of growth (Fig. 6). To check whether the differences in the bactericidal activity were due to different allele sequences, immune sera, raised against the three NadA types, were produced and used in a cross-bactericidal assay. The results obtained with the antisera were similar to those shown in Table II, suggesting that the bactericidal activity is not influenced by the allele diversity but rather to the antigen expression level. We also tested the ability of the immune sera to protect animals during infection using the infant rat model and found that the serum is highly protective in this assay (Table III).

The NadA Protein Binds to Epithelial Cells. To investigate the biological function of NadA and its ability to interact with host cells in vitro, we used FACS® analysis to test whether NadA could bind to Chang epithelial cells. Purified recombinant protein was added at increasing concentration to Chang epithelial cells and binding was measured. As shown in Fig. 8 A, cells incubated with NadA exhibited a peak of fluorescence, which was absent when cells were incubated with medium alone. Binding of NadA was concentration dependent, reaching the plateau at 200 μg/ml (Fig. 8 B). Unrelated recombinant protein

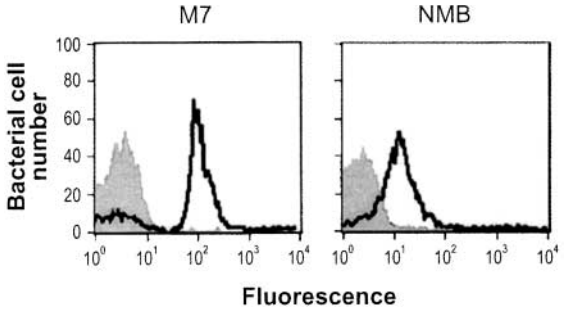

Figure 7. FACS® analysis showing binding of mouse polyclonal anti-rNadA antiserum to live heterologous nonencapsulated (M7) or encapsulated (NMB) *N. meningitidis* strains. Gray profiles show binding of preimmune serum; black profiles show binding of immune serum.

GNA2132 (20) failed to bind cells even at a concentration of 400 μg/ml.

Discussion

We have identified a novel meningococcal antigen, NadA, which has a predicted molecular structure strikingly similar to the known virulence-associated factors YadA and UspA2 (41). NadA has the capacity of forming oligomers anchored to the outer membrane, the ability to interact with host cells in vitro, and evokes a strong bactericidal activity. Furthermore, the gene is present in three of the four hypervirulent lineages of serogroup B and C strains. All these features suggest an important role of NadA in the multifactorial virulence expressed by meningococci.

Gene Presence and Evolution. The gene is present in ~50% of *N. meningitidis* strains surveyed, representative of all the major disease-associated serogroups. Sequence analysis of the *nadA* gene reveals the presence of three well-defined alleles, which are well conserved among each other. Given the extreme variability of surface-exposed proteins in *N. meningitidis*, the conservation among the alleles is surprising and suggests a weak selective pressure. The low GC content of the region is consistent with horizontal gene transfer. It would be interesting to know when the gene was acquired by meningococcus and follow the evolution of this gene. Our data show the presence of the gene since

Table II. *Bactericidal Activity of Mouse Polyclonal Anti-rNadA (Allele 3) Antisera Against Different N. meningitidis Strains*

Strain	NadA expression	Allele	Bactericidal titer
2996	+++	3	32768
C11	+++	3	16384
F6124	+	3	4096
MC58	+	1	8192
BZ232	−	-	<4
NGH38	−	-	<4

Table III. *Anti-NadA Antibody Passive Protection in Infant Rats Challenged with N. meningitidis Serogroup B Strains 8047 or 2996*

Exp.	Strain (challenge CFU per rat)	Treatment	Serum dilution or dose of mAb (μg) per rat	Blood culture at 18 h	
				No. positive/total	CFU/ml (geo. mean,10^3)
1	8047	anticapsular mAb	2	0/4	<0.001
	(7×10^3)	anti-NadA antiserum	1:5	0/4	<0.001
		PBS plus 1% BSA		5/5	40.17
2	2996	anticapsular mAb	20	1/6	0.003
	(5.6 × 103)	anti-NadA antiserum	1:5	1/6	0.002
		anti-NadA antiserum	1:25	3/6	0.035
		preimmune NadA serum	1:5	6/6	1.683

1963, and basically, there is not any sequence variation within the same alleles in strains isolated in the year 2000 (Online Supplemental Material).

Interestingly, the percentage of *nadA* presence rises to ~100% in a subset of the hypervirulent lineages. The gene is always present in the ET5 complex and cluster A4, and nearly always present in the ET37 complex, whereas lineage III strains are always negative. Lineage III has only recently been introduced in Europe and the U.S. and the geographic segregation in New Zealand for many years

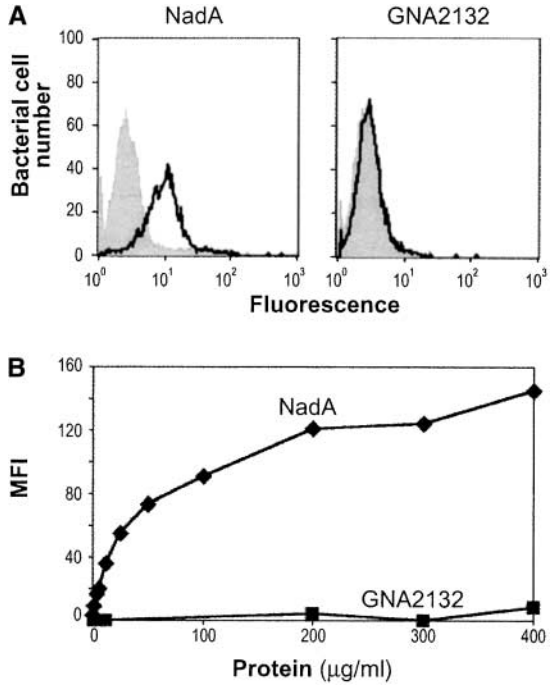

Figure 8. (A) FACS® analysis showing binding of rNadA or an unrelated protein GNA2132, to human Chang cells. Gray profiles show binding of cells incubated with medium; black profiles show binding of recombinant proteins. (B) Concentration-dependent binding of NadA expressed as net mean fluorescence intensity.

could have impaired its ability to acquire novel genes. The sequence polymorphism of the chromosomal flanking regions segregates differentially with each allele (data not shown). Assuming that recombination at *nadA* locus uses the chromosomal flanking regions as recognition sites, we can hypothesize that mutations have occurred in these regions preventing lineage III from further recombination events. Another possible explanation is that ET5, ET37, and cluster A4 strains need *nadA* to achieve their fitness peak, whereas lineage III isolates could not derive any significant benefit from *nadA* insertion, thus undergoing a negative selection.

Gene and Protein Features. The different number of the DNA tetranucleotide repeat elements (TAAA) located in the putative promoter region, could associate with phase variation and influence transcription of the gene, hence its expression. Indeed, the protein is always expressed even if at different levels in different strains, but this does not correlate in an obvious manner to the different number of repeats. Interestingly, the homologous gene *UspA2* has a tetranucleotide repeat (AGAT), located in the same position as *nadA*, which varies in different strains (42). The overall expression of NadA varies during growth reaching its maximum at cellular stationary phase.

NadA forms surface-exposed oligomers, which are stable to heat, SDS, and reduction with β-mercaptoethanol. Since the mature form of these proteins lack cysteine residues, disulphide bond formation cannot be involved in this phenomenon; rather this is consistent with the predicted coiled-coil structure and the presence of leucine zipper motifs that might mediate intermolecular interactions between monomers (44, 45). The size of the oligomers is ~170 kD, suggesting a tetrameric structure. However, a rigid coiled-coil structure is likely to have an anomalous migration is SDS-PAGE and therefore, trimeric forms are also possible.

Antigen Features. The predicted molecular structure, the capacity of forming oligomers anchored to the outer membrane, and the ability of the purified protein to inter-

act with host cells in vitro, suggest that NadA could belong to a novel class of adhesins that are established pathogenicity factors as YadA and UspAs. Furthermore, NadA evokes a strong antibactericidal antibody response.

In conclusion, NadA has several attributes of being a good vaccine candidate: (i) it is a surface-exposed molecule, possibly involved in bacterial adhesion; (ii) it is present in at least 50% of the disease-associated strains and in ~100% of three hypervirulent lineages; (iii) it elicits protective and bactericidal antibodies in laboratory animals; and (iv) each allele induces cross-bactericidal antibodies. A vaccine based on NadA will have some potential for controlling outbreaks of meningococcal disease caused by the three hypervirulent lineages.

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