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Molecular and Biological Analysis of Eight Genetic Islands That Distinguish *Neisseria meningitidis* from the Closely Related Pathogen *Neisseria gonorrhoeae*

SILKE R. KLEE,[1] XAVIER NASSIF,[2] BARICA KUSECEK,[1] PETRA MERKER,[1] JEAN-LUC BERETTI,[2] MARK ACHTMAN[1]* AND COLIN R. TINSLEY[2]

Max-Planck Institut für Molekulare Genetik, 14195 Berlin, Germany,[1] and Laboratoire de Microbiologie, INSERM Unité 411, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cedex 15, France[2]

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The pathogenic species *Neisseria meningitidis* and *Neisseria gonorrhoeae* cause dramatically different diseases despite strong relatedness at the genetic and biochemical levels. *N. meningitidis* can cross the blood-brain barrier to cause meningitis and has a propensity for toxic septicemia unlike *N. gonorrhoeae*. We previously used subtractive hybridization to identify DNA sequences which might encode functions specific to bacteremia and invasion of the meninges because they are specific to *N. meningitidis* and absent from *N. gonorrhoeae*. In this report we show that these sequences mark eight genetic islands that range in size from 1.8 to 40 kb and whose chromosomal location is constant. Five of these genetic islands were conserved within a representative set of strains and/or carried genes with homologies to known virulence factors in other species. These were deleted, and the mutants were tested for correlates of virulence in vitro and in vivo. This strategy identified one island, region 8, which is needed to induce bacteremia in an infant rat model of meningococcal infection. Region 8 encodes a putative siderophore receptor and a disulfide oxidoreductase. None of the deleted mutants was modified in its resistance to the bactericidal effect of serum. Neither were the mutant strains altered in their ability to interact with endothelial cells, suggesting that such interactions are not encoded by large genetic islands in *N. meningitidis*.

Neisseria meningitidis colonizes the nasopharynx, from which it can seed the bloodstream before crossing the blood-brain barrier (BBB) to cause meningitis. In contrast, *Neisseria gonorrhoeae* colonizes and invades the epithelium of the genitourinary tract, where it can cause a localized inflammation; bacteremia, though frequent, is asymptomatic and dissemination is rare. Thus, both species are capable of crossing a cellular barrier at their port-of-entry but they differ in their abilities to subsequently disseminate in the blood. The ability to induce intense and prolonged bacteremia is one of the prerequisites for a bacterial pathogen to cross the BBB. In contrast, the details of specific interactions with the cellular components of the BBB remain unclear. Therefore, in order to understand the mechanisms that allow *N. meningitidis* to cross the BBB, it will be necessary to identify the genes that are involved in bloodstream dissemination and/or specific interaction with the cellular components of the BBB. Such genes might be present in both *N. meningitidis* and *N. gonorrhoeae* but differ subtly in sequence or regulation, or they might be present in only one of the two species.

Results from in vitro models have shown that most of the mechanisms mediating cellular interactions are common to both *N. meningitidis* and *N. gonorrhoeae*. On the other hand, several determinants have been identified that are specific to *N. meningitidis*: the polysaccharide capsule (8), the enzyme rotamase (26), the RTX toxin-like Frp proteins (29, 30), and a glutathione peroxidase (20). Of these, the capsule locus is required for systemic dissemination and bloodstream survival (34), whereas a role in virulence has not been demonstrated for the other genes.

* Corresponding author. Mailing address: Max-Planck Institut für molekulare Genetik, Ihnestrasse 73, 14195 Berlin, Germany. Phone: 49 30 8413 1262. Fax: 49 30 8413 1385. E-mail: achtman@molgen.mpg.de.

We have recently created a bank of *N. meningitidis*-specific sequences after subtractive hybridization between *N. meningitidis* and *N. gonorrhoeae* in order to identify genes which are present only in *N. meningitidis* and might therefore account for its differential pathogenesis (32). Some of the clones mapped closely together, suggesting that they may have been derived from larger regions of *N. meningitidis*-specific DNA. One region containing such clones (region 1) corresponds to the locus of capsule synthesis which had previously been well characterized (8, 12, 13). However, the significance of the other regions was unknown. We have now investigated the other regions of *N. meningitidis*-specific DNA in order to obtain details on the differences between *N. meningitidis* and *N. gonorrhoeae* and to possibly identify mechanisms responsible for the specificity of *N. meningitidis* pathogenesis. Our data identify eight novel DNA islands that are specifically present in *N. meningitidis* and absent from *N. gonorrhoeae*. Those islands that were conserved among a representative set of meningococcal strains and/or showed homologies with known virulence factors were deleted, and the resulting strains were tested for phenotypes that are associated with crossing the BBB. The results show that one of the eight islands is required for high levels of bacteremia.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *N. meningitidis* were tested that represent the genetic diversity of this species according to multilocus sequence typing (MLST) (18). Their MLST assignments were: ST1 (subgroup I, strain B40), ST2 (subgroup VI, Z6835), ST4 (subgroup IV-1, Z5463, Z2491 [27]), ST5 (subgroup III, Z3524), ST8 (A4 cluster, BZ 10), ST11 (ET-37 complex, serogroup C: FAM18; serogroup W135: ROU [24]), ST25 (NG G40), ST30 (NG 4/88), ST32 (ET-5 complex, 44/76), ST41 (lineage 3, BZ 198); ST48 (BZ147), ST49 (297-0), ST60 (subgroup IX, 890592), and ST74 (ET-5 complex, MC58 [33]). Additional strains were *N. meningitidis* 8013, *N. gonorrhoeae* FA1090 and two strains of *Neisseria lactamica* (Z6793 and Z6784). *N. meningitidis* strains were grown on GC agar (GCB; Difco), with the addition of Kellogg's defined supplement plus ferric nitrate (14) for 12 to 20 h at 37°C in a moist atmosphere containing 5% CO_2. Liquid media were GC-PO_4 (1.5% Proteose Peptone number 3 [Difco], 0.5% NaCl, 30 mM potassium phosphate; pH 7.5) and GC-HEPES

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)[a]
Primers to amplify each region from flanking sequences	
Reg2-for	TTGGCGAAGAGCAACGAACCTGTTTATCTCGTG
Reg2-rev	GGCGCAATGTTCGATTTTTCCTCTATCCGGTTC
Reg3-for	GACAAACTGATGACTTGGGGCTTCTGGCTGCTC
Reg3-rev	ATATGCCCGACATCGACGGCGGCATTTTGTTCC
Reg4-for	GCAAATGGTTGGAAGTCGGCGGTTGCGGTATGG
Reg4-rev	GCAATGCCCTTAACACTCAAGCAATCAGCGCGG
Reg5-for	CGAAGCCATGTACCTTGCCAATACTGCGGCCGG
Reg5-rev	ACCGCCGCACTGGAGGCATAAAGGAAGGGGATG
Reg6-for	GATGGCGAAAGTGATGAGTTTCGGCGTGTGGTG
Reg6-rev	AACTGCCGTGCCTCATACGATGCGGCGCAAATC
Reg7-for	CCAAAATGGACGCGACCGTATAGGACGTCTTCC
Reg7-rev	AAAAGTACTGGACGCAAGTTGGGCGGCGTTGCA
Reg8-for	AGCAGTTCCCTGCTTATGCCGCCAACTTTCCCG
Reg8-rev	GAAACAAATCCCCGAAGGTACGGCTGAAAAAGC
Reg9-for	ATTTGAAAGGAAGACGGCAGGCCTGCGTCGCGG
Reg9-rev	CAGTATCCGCAACTCTTGGACAACTACGGCAAC
Primers from Fig. 1A	
T1	GATCCGAAAAGCAGCCGTCTGAAAC**AGATCT**GCA
T2	GATCTGTTTCAGACGGCTGCTTTTCG (5′ PO_4)
T3	TGTTTCAGACGGCTGCTTTTG (5′ PO_4)
T4	GATCCAAAAGCAGCCGTCTGAAACATGCA
BE1	GATCGCAGGG (5′ PO_4)
BE2	AATTCCCTGC
Primers to amplify flanking sequences as in Fig. 1B	
R3001	GTCGGC**AGATCT**TACGCGGGCAACTTCTT
R3002	CGTTTG**GAATTC**CAAACGCCGTTCAATTCAA
R3003	TTTACC**GAATTC**CTCGTCAACCGCGACGGCGA
R3004	GTTACG**GGTACC**TCGATTTGGGACGTTTCT
R6001	CCGTCT**GAATTC**TTTCAGACGGCATTTTTGCCGA
R6002	TGCAAA**GGATCC**AAAGGCCCTCACAACTGTTTT
R6003	TTTTGC**GGATCC**CGTCTGAAACAGGGTATGTTT
R6004	TTCGGC**TCTAGA**TGCCCCACGCCGATACCGA
R7001	TCCCAC**GGATCC**TCGCCAGCCTCGGCGACGTCA
R7002	TAAAGC**AGATCT**GAAACGGTTATGAAATTCCCAA
R7003	CGTGTA**AGATCT**TTTGGAAGACGAAGATTTTAT
R7004	TTTTTT**GGATCC**TTGTGTGATTAAACGTCTTT
R8001	CGTCGA**AGATCT**TCGCCTTCCCCATTGAAATG
R8002	ACAACC**GAATTC**TATTGCCTCACGGAGGAAATGA
R8003	AAATAG**GAATTC**AGACGGCCTTTTGTATTTAGGCT
R8004	CGACG**GGTACC**GCCCTGCCGCGATTGAA
R9001	CTGCGC**GAATTC**CAGCACGCCCAAGTCTTCCGT
R9002	ATTCTT**AGATCT**GTTCCAACCACTAATACACTA
R9003	GATCTC**AGATCT**CATTTGTTGTTCATTTTGGTT
R9004	GGGCGCGTCGATGTG**GAATTC**T

[a] Restriction sites are is indicated in boldface.

(like GC-PO_4 but the potassium phosphate was replaced by 30 mM HEPES [pH 7.5]), both supplemented as for the solid medium. *Escherichia coli* were grown on Luria-Bertani (LB) agar or in LB liquid medium. Antibiotics used were: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml (*N. meningitidis*) or 100 μg/ml (*E. coli*); nalidixic acid, 20 μg/ml; and spectinomycin, 40 μg/ml.

Oligonucleotide primers and PCR conditions. The sequences of the primers used to amplify the individual regions and to construct deletions are listed in Table 1; the other primer sequences are available on request. Template chromosomal DNA was isolated as described elsewhere (27).

The PCR conditions used depended on the length of the desired product. For products up to 3 kb, the reaction mixture contained template DNA (1 μg ml^{-1}); reaction buffer (10 mM Tris-Cl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin); dATP, dCTP, dGTP, and dTTP (200 μM concentrations of each); dimethyl sulfoxide (5%); forward and reverse primers (100 nM concentrations of each); and *Taq* polymerase. The PCR reactions were incubated 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 5°C below the T_m of the oligonucleotide primers, and 2 min at 72°C, followed by incubation for 5 min at 72°C. For PCR products between 3 and 8 kb, semi-long-range PCR was performed by using the Expand Long Template PCR System (Boehringer Mannheim) under the same conditions except that the mixture contained Buffer 1 and the polymerase mix (0.75 μl) supplied with the kit. The thermocycling conditions were 1 min at 94°C, 30 cycles of 45 s at 94°C, 1 min at 65°C, and 3 min at 68°C, and a final incubation for 5 min at 68°C. Template DNA longer than 8 kb was amplified by using the same kit and conditions except that higher concentrations of dATP, dCTP, dGTP, and dTTP (350 μM concentrations of each) and oligonucleotide primers (300 nM) and 2 μl of the polymerase mix were used. Incubation was for 1 min at 94°C, 30 cycles of 10 s at 94°C, 30 s at 65°C, and 20 min at 68°C, followed by 7 min at 68°C.

Sequencing of the eight regions. Chromosomal DNA of *N. meningitidis* Z2491 was restricted by partial *Sau*3AI digestion and fragments of 12- to 23-kb size fractionated by gel electrophoresis were cloned into the *Bam*HI site of the Lambda DASH II (Stratagene) phage vector by using *E. coli* XL1-Blue MRA (Stratagene). Details of the following steps were according to the DIG System Users Guide (Boehringer Mannheim). Plaques were transferred to nylon membranes (Hybond N; Amersham). *N. meningitidis*-specific clones (32) were digoxigenin labeled during PCR amplification and used as probes for plaque hybridization under stringent conditions. Phages containing hybridizing sequences were detected colorimetrically, and single plaques were purified before lysates were prepared. Two microliters of each lysate was used as a template for long-range PCR with primers in the phage vector immediately flanking the inserts. Then, 15 μg of PCR product was randomly sheared by nebulization (no. 4100 Nebuliser; Inhalation Plastics) for 20 min at 0.7 atm as described elsewhere (http://bric.postech.ac.kr/resources/rprotocol/rprotocol_partii.html). The sheared fragments were precipitated, end repaired with T4 DNA polymerase and Klenow DNA polymerase (New England Biolabs), and size fractionated on a 0.8% agarose gel. Fragments of between 0.4 and 0.6 kb and between 0.8 and 1 kb were separately eluted from the gel by using the Qiaquick Gel Extraction Kit (Qiagen). dATP overhangs were added to the fragments and ligated with the TA cloning vector pCR2.1 (Invitrogen). These preparations were transformed into *E. coli* XL1-Blue by electroporation. A total of 96 recombinant colonies were picked per transformation and grown in LB medium with ampicillin, and their inserts were amplified by PCR by using primers complementary to the flanking vector sequences. The PCR products were purified and sequenced by using the M13 reverse primer, a dRhodamine terminator cycle sequencing kit, and ABI Prism 377 DNA sequencers (Perkin-Elmer Applied Biosystems). Raw data from the ABI sequencer were prepared for assembly by using the ASP program (http://www.sanger.ac.uk/Software/Sequencing/ASD/asp/MODULES.shtml), and sequences were assembled with GAP4 from the Staden sequence analysis package (28).

Sequences that were 100% identical to those available in the public domain (Sanger Center; http://www.sanger.ac.uk/Projects/N_meningitidis/) at that time were accepted as correct, whereas all discrepancies were resequenced as follows using PCR products from the chromosomal DNA of strain Z2491. Fragments of approximately 5 kb were amplified by semi-long-range PCR by using primers designed from the sequences of the phage inserts. The PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) and sequenced from both strands with appropriate primers as described above. Additional smaller PCR products from bacterial chromosomes were sequenced from other strains of *N. meningitidis* using the same strategy.

Region 2 was sequenced from both strands by using a different strategy than for the other regions. Primers were designed according to the sequences of the clones isolated by Tinsley and Nassif (32), and products were obtained from chromosomal DNA by semi-long-range PCR. Sequence walking was used to complete the sequences of each of these products.

Analysis of nucleotide sequences. Open reading frames (ORFs) were recognized by using the Codon Use program written by Conrad Halling, which supplies a graphical output for a sliding window of the codon adaptation index in all six frames. The permitted start codons were ATG and GTG, and the permitted stop codons were TAA, TAG, and TGA. Homology searches at the nucleotide level were performed by using BLASTN (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?) and at the amino acid level by using PSI-BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast). Repetitive nucleotide sequences were detected by using Miropeats (22).

DNA dot blot and Southern hybridization. DNA dot blot hybridization was performed according to the DIG System Users Guide (Boehringer) by spotting 1 μl containing 100 ng of denatured chromosomal DNA from each strain onto nylon membranes (Hybond N; Amersham). For Southern hybridization analysis, chromosomal DNA was digested with restriction endonucleases and separated by conventional electrophoresis or by pulsed-field gel electrophoresis (PFGE) and then transferred to nylon membranes. The DNA dot blots were hybridized with digoxigenin-labeled probes obtained by PCR amplification of each ORF, and Southern blots were hybridized with labeled probes corresponding to ORFs or entire regions. For the analysis of the distribution of the regions among meningococcal strains, hybridizations were performed at 37°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 50 mM sodium phosphate, 7% sodium dodecyl sulfate (SDS), 2% Blocking Reagent (Boehringer-Mannheim), 0.1% *N*-lauroylsarcosine, and 50% formamide, and the last washing step was with 0.5× SSC–0.1% SDS at 50°C in order to allow approximately 30% mismatch. Positive hybridization signals were detected by chemiluminescence. For verification of the mutants by Southern blotting, probes were labeled with [α-^{32}P]dCTP. Hybridization in 500 mM sodium phosphate (pH 7.2) containing 7% SDS and 1 mM EDTA and washing in 40 mM sodium phosphate (pH 7.2), containing 1% SDS and 1 mM EDTA were performed at 65°C.

PCR analysis of DNA islands in different *N. meningitidis* strains. The sizes of the eight islands were determined by PCR amplification with primers Reg2-for to Reg9-rev (Table 1) that are complementary to the 5′ and 3′ flanking sequences, respectively, in both *N. meningitidis* and *N. gonorrhoeae*. Semi-long-range PCR was used for all regions. Region 3 containing Pnm1 was amplified by eight sets

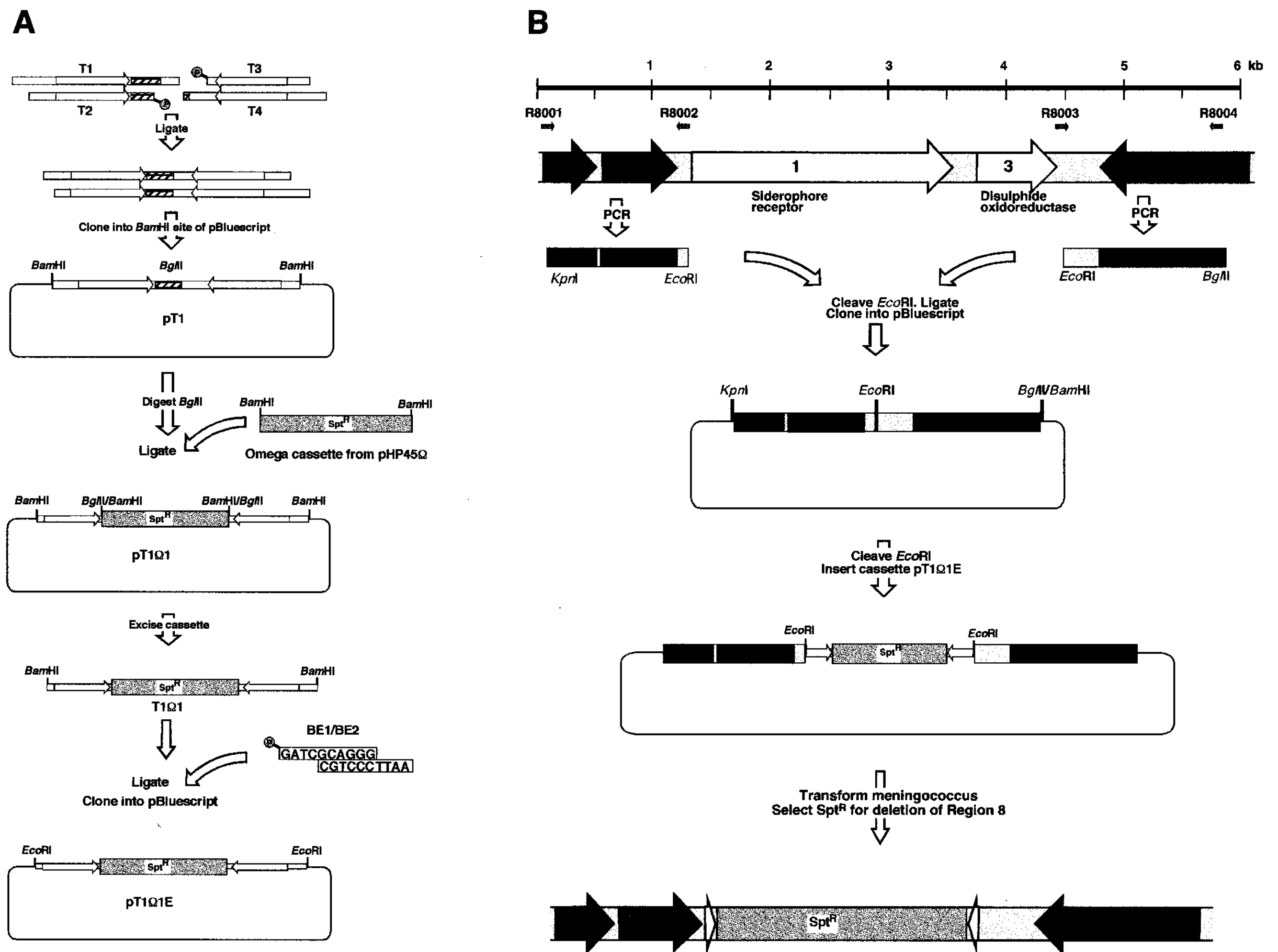

FIG. 1. Production of a cassette, flanked by neisserial uptake sequences, and deletion of the *N. meningitidis*-specific islands. (A) For production of the cassette, an inverted repeat containing two neisserial uptake sequences (arrows) and an internal *Bgl*II site (shaded) was constructed by ligating synthetic oligonucleotides T1 to T4 (Table 1). This molecule was cloned into pBluescript, and an Ω spectinomycin resistance cassette was cloned into the *Bgl*II site to yield a cassette that may be excised with *Bam*HI. (B) Deletion of region 8. For replacement of the *N. meningitidis*-specific islands with the cassette, the flanking regions of the islands were PCR amplified by using the oligonucleotides in Table 1. These were ligated together at an internal restriction enzyme site (*Eco*RI), reamplified, and cloned into pBluescript. The construct was reopened at the *Eco*RI site, and the cassette was inserted between the flanking sequences. This plasmid was then used to transform *N. meningitidis* and replace the chromosomal island with the resistance cassette by homologous recombination.

of long-range PCRs. The locations of each island were confirmed by additional PCR reactions by using each forward primer (Reg2-for, Reg3-for, etc.) with a reverse primer in the leftmost ORF and each reverse primer (Reg2-rev, Reg3-rev, etc.) with a forward primer in the rightmost ORF of the corresponding island. Additionally, each ORF was separately amplified from strains that reacted in dot blot hybridization by primers specific to its 5′ and 3′ ends, and the sizes were confirmed by gel electrophoresis.

Production of deletion mutants in the *N. meningitidis*-specific islands. Briefly, deletions were produced by transforming *N. meningitidis* ROU, MC58, and Z5463 with plasmid DNA into which sequences flanking an island had been cloned, such that they were separated by an antibiotic resistance cassette in place of the *N. meningitidis*-specific island. After transformation of *N. meningitidis*, DNA from a single colony expressing the selected antibiotic resistance was used to retransform the same strain, and a mixture of several hundred transformants was pooled and used for biological assays.

Figure 1B outlines the procedure for the production of the plasmids used for the deletions, taking region 8 as its example. The PCR products of the flanking regions (approximately 1 kb) were amplified using the primers shown in Table 1. The primers were designed so that both PCR products contained either a *Bam*HI or a *Eco*RI restriction site at the internal end and one of a variety of different restriction sites at the external end. The PCR products were digested with *Bam*HI or *Eco*RI, purified, and ligated. The ligation products were reamplified by using the "external" primers, cleaved at the ends with the appropriate enzyme(s) and cloned into pBluescript KS(+) (Stratagene). A resistance cassette (see below) flanked by two neisserial uptake sequences was then cloned into the *Bam*HI or the *Eco*RI site.

Construction of the resistance cassette. The resistance cassette contains the omega fragment (23), which encodes resistance to spectinomycin due to *aadA* (aminoglycoside adenyltransferase) and interrupts both translation and transcription, flanked on each side in inverted orientation by the neisserial uptake sequence GCCGTCTGAA. Due to the degree of secondary structure in the omega fragment, the construct was made without using PCR technology, as outlined in Fig. 1A. An artificial inverted repeat of uptake sequences was produced as follows. The oligonucleotides T1 and T2 (Table 1) were mixed (25 μM, 30 μl in T4 DNA ligase buffer), heated at 75°C for 10 min, and allowed to cool to room temperature for 1 h. This results in a double-stranded DNA containing one copy of the neisserial uptake sequence, with a 5′ overhang of GATC (compatible with *Bam*HI) and a 3′ overhang of TGCA. A similar hybridization of oligonucleotides T3 and T4 also produced a DNA with the same overhangs. After mixing and ligation, only the desired internal join (TGCA) can be ligated due to the 5′ phosphate groups. The resulting mixture was cloned into the *Bam*HI site in pBluescript KS(+), and the correct construct (plasmid pT1) was detected by its possession of a *Bgl*II site (present in oligonucleotide T1).

The omega fragment was excised from plasmid pHP45Ω by using *Bam*HI and inserted into the unique *Bgl*II site of pT1, resulting in plasmid pT1Ω1, where the omega cassette is flanked by neisserial uptake sequences and can be excised by digestion with *Bam*HI. We also constructed plasmid pT1Ω1E, where the fragment can be excised by digestion with *Eco*RI. To this end, the *Bam*HI fragment of pT1Ω1 was ligated to the adaptors BE1 and BE2 and cloned into the *Eco*RI site of pBluescript KS(+).

Transformation of *N. meningitidis*. After overnight growth on GC agar plates, bacteria were resuspended in GC-HEPES containing 1 mM K_2HPO_4 and

Kellogg supplement 1 (14) to an optical density at 600 nm (OD_{600}) of 0.1. $MgCl_2$ was added to a final concentration of 10 mM, and transforming DNA (0.5 to 5 μg of linearized plasmid or 0.5 μg of chromosome) was added to 500 μl of the suspension. After incubation without shaking at 37°C for 30 min, 4.5 ml of GC-PO_4 was added, and the mixture was incubated with shaking for a further 2 h. The bacteria were plated onto GC agar containing 40 μg of spectinomycin per ml for selection of transformants.

Complement-dependent serum bactericidal assay. Antiserum against strains ROU and MC58 was obtained by immunizing rabbits with a mixture of paraformaldehyde-treated and sonicated bacteria with Freund's adjuvant and boosting three times using Freund's incomplete adjuvant. After overnight growth on GC agar plates, bacteria were resuspended to a final concentration of 10^6 CFU/ml in phosphate-buffered saline (PBS) containing 5 mM $MgCl_2$ and 0.25 mM $CaCl_2$ (PBSB). Then, 10 μl of bacterial suspension was mixed with 360 μl of antiserum (decomplemented by heating at 56°C for 30 min) diluted in PBSB and 40 μl of freshly thawed guinea pig serum (Gibco-BRL) as a complement source. Killing was measured by determining colony counts after 45 min incubation at 37°C, and the survival was compared to bacteria incubated in the absence of antiserum. In each experiment, a positive killing control used a serum-sensitive polyphosphate kinase mutant (*ppk*) (31), while as a negative control the *ppk* bacteria were incubated with decomplemented guinea pig serum in the presence of opsonizing antiserum.

Adhesion and invasion assays. Bacteria from GC agar plates were grown in RPMI (Gibco-BRL) containing 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL) with gentle shaking for 2 h to an OD_{600} of 0.1. Then, 1 ml of a 100-fold dilution was added to a confluent monolayer of human umbilical vein endothelial cells (HUVEC) in 2-cm^2 tissue culture wells (Costar). One well was used per *N. meningitidis* strain to test adherence, and two wells were used to assay invasion.

Adhesion. After incubation for 1 h at 37°C in 5% CO_2, the supernatant was removed (nonadherent bacteria) and the monolayer was washed three times with RPMI. The adherent bacteria were released by adding 1 ml of PBS–1% saponin and scraping the bottom of the wells with a micropipette tip. The numbers of adherent and nonadherent bacteria were determined after plating them on supplemented GC agar. Adherence was calculated as the number of adherent bacteria divided by the total number of adherent plus nonadherent bacteria.

Invasion. The wells were washed every hour as described above for 6 h and then filled with RPMI-FCS containing 150 μg of gentamicin per ml. After 1 h of incubation to kill external bacteria, internalized bacteria were harvested and enumerated as described above. Invasion was calculated as the number of internalized bacteria divided by the total bacteria at 1 h after infection.

Infant rat model of meningococcal infection. Bacteria grown on GC agar plates for 14 h were resuspended in pyrogen-free 0.9% NaCl to an OD_{600} of 0.06. Four- to five-day-old Lewis rats (IFFA Credo, L'Arbresle, France) anesthetised with diethyl ether were injected intraperitoneally with 100 μl of bacterial suspension. Half of each litter (usually 10 animals) was injected with the parental strain and half was injected with the mutant strain. Samples of blood (5 μl) were taken from an incision in the tail after 1, 3, 6, 9, and 24 h. The blood samples were diluted in GC-PO_4 and plated on GC agar for enumeration.

Nucleotide sequence accession numbers. The DNA sequences described in this work have been deposited in the EMBL database under the following accession numbers: for region 2 of *N. meningitidis* Z2491 (*fhaB* and *fhaC* homologues and genes of unknown function) and flanking genes, AJ391255; for region 3 (prophage Pnm1 and *gpxIA*) and flanking genes, AJ391256; for region 4 (genes of unknown function) and flanking genes, AJ391257; for region 5 (restriction/modification system) and flanking gene, AJ391258; for region 6 (pseudogene with homology to siderophore receptor genes) and flanking genes, AJ391259; for region 7 (homology to type I secretion system) and flanking genes, AJ391260; for region 8 (*fhuA* and *dsbA* homologues) and flanking genes, AJ391261; for region 9 (cluster of putative ORFs and insertion element IS4351N2) and flanking genes, AJ391262; for strain FAM18 *hlyD* gene (putative component of type I secretion system), AJ391263; for strain FAM18 *tolC* gene (putative component of type I secretion system), AJ391264; for strain NG 4/88 *tolC* gene (putative component of type I secretion system), AJ391265; for strain 297-0 *tolC* pseudogene (putative component of type I secretion system), AJ391266; for strain FAM18 *fhuA* gene for putative siderophore receptor, AJ391267; for strain NG 4/88 *fhuA* gene for putative siderophore receptor, AJ391268; for strain MC58 *fhuA* gene for putative siderophore receptor, AJ391269; for strain ROU *fhuA* gene for putative siderophore receptor, AJ391270; for strain BZ 10 *fhuA* pseudogene for putative siderophore receptor, AJ391271; for strain BZ 147 *fhuA* pseudogene for putative siderophore receptor, AJ391272; for strain BZ 198 *fhuA* pseudogene for putative siderophore receptor, AJ391273; for strain B40 *fhuA* pseudogene for putative siderophore receptor, AJ391274; for strain Z3524 *fhuA* pseudogene for putative siderophore receptor, AJ391275; for strain 297-0 *fhuA* pseudogene for putative siderophore receptor, AJ391276; for strain 44/76 *fhuA* pseudogene, AJ391277; for strain FAM18 *dsbA* gene for putative disulfide oxidoreductase, AJ391278; for strain BZ10 *dsbA* gene for putative disulfide oxidoreductase, AJ391279; for strain NG 4/88 *dsbA* gene for putative disulfide oxidoreductase, AJ391280; for strain 44/76 DNA for region 6 (*rsi1* pseudogene) and flanking *fnr* and *dinP* genes (partial), AJ391281; for strain BZ 198 DNA for region 6, insertion sequence, partial *rsi1* pseudogene and flanking *fnr* and *dinP* genes (partial), AJ391282; for strain 297-0 partial *fnr* gene for putative ferredoxin-$NADP^+$ reductase and partial *dinP* gene for putative DNA-damage inducible protein P, AJ391283; and for strain FAM18 DNA for region 2 (*fhaB* and *fhaC* homologues and genes of unknown function) and flanking genes, AJ391284.

RESULTS

Identification of *N. meningitidis*-specific regions in strain Z2491. Twenty-eight subtractive clones from *N. meningitidis* serogroup A, subgroup IV-1 strain Z2491 have been described that have no homologies in *N. gonorrhoeae* (32). DNA probes corresponding to these clones were used to screen a Lambda Dash II library containing 12- to 23-kb DNA fragments of strain Z2491. Other clones comprising "region 1" were not investigated further because they correspond to the well-characterized locus of capsule production.

A minimal set of Lambda Dash II recombinant phages were identified whose inserts hybridized with 25 of the probes (all except B305, B333, and E103), and these inserts were sequenced. Comparison of these *N. meningitidis* sequences with that of *N. gonorrhoeae* strain FA1090 (University of Oklahoma; http://dna1.chem.ou.edu/gono.html) identified the flanking ends that were homologous in both species and, hence, the extent of eight regions of *N. meningitidis*-specific sequences. The eight regions were designated regions 2 to 9 and are shown in Fig. 2, 3, and 4. They contain 85 ORFs, most with codon usage typical of *N. meningitidis*, of which 43 are homologous to previously described ORFs in other species (Table 2). ORFs with no significant homology to genes of known function were named according to the region (*rtw* [region two], *rth* [region three], *rfo* [region four], *rfi* [region five], *rsi* [region six], *rse* [region seven], *rei* [region eight], and *rni* [region nine]) plus a sequential number corresponding to the position of the ORF (*rtw1*, *rth52*, etc.).

The *N. meningitidis*-specific regions are imported DNA islands in *N. gonorrhoeae* and *N. meningitidis*. The GC contents of most of the ORFs in regions 2 to 3 and 6 to 9 were close to the 51% value typical of *N. meningitidis*. However, *rei1* of region 8 has a GC content of 40%, and regions 4 and 5 have average GC contents of only 33%. The ends of the eight regions are defined by longer stretches of flanking DNA that are 80 to 96% homologous between the two species. Except for region 2 (Fig. 3), the regions begin and end in intergenic stretches. No repeat structures similar to those flanking pathogenicity islands in *Enterobacteriaceae* were found, except possibly for region 9. In region 9, 39 bp at the 5′ end are repeated 180 bp to the left of the 3′ end of the *N. meningitidis*-specific sequence. Furthermore, the right end of region 9 includes a putative IS element, IS*4351*N2, containing a transposase that might have been involved in the insertion of this region.

The comparison between *N. meningitidis* and *N. gonorrhoeae* shows that the regions do not correspond to simple insertions but rather replace alternative sequences, 44 bp to over 12 kb in size, in *N. gonorrhoeae* FA1090 (Table 3). Thus, the *N. meningitidis* specific regions correspond to DNA islands that are species specific. The following data suggest that some or all of these islands may have arisen by import from other species via recombination in the homologous flanking DNA, similar to the mechanism deduced for three small islands described elsewhere (39). First, DNA uptake sequences (DUS) (GCCGTC TGAA) (9, 10) were found in all the *N. meningitidis* islands except regions 4 and 5, where they were found in their immediate borders. The presence of a DUS has been associated with import of DNA into *N. meningitidis* from *Haemophilus influenzae* (15) and would facilitate the import of such islands from unrelated bacteria. Second, regions of 70 to 88% homology over 75 to 1,100 bp were found at one of the two borders in regions 4 and 6 to 8, whereas the homology between *N. meningitidis* and *N. gonorrhoeae* is normally at least 90%. These

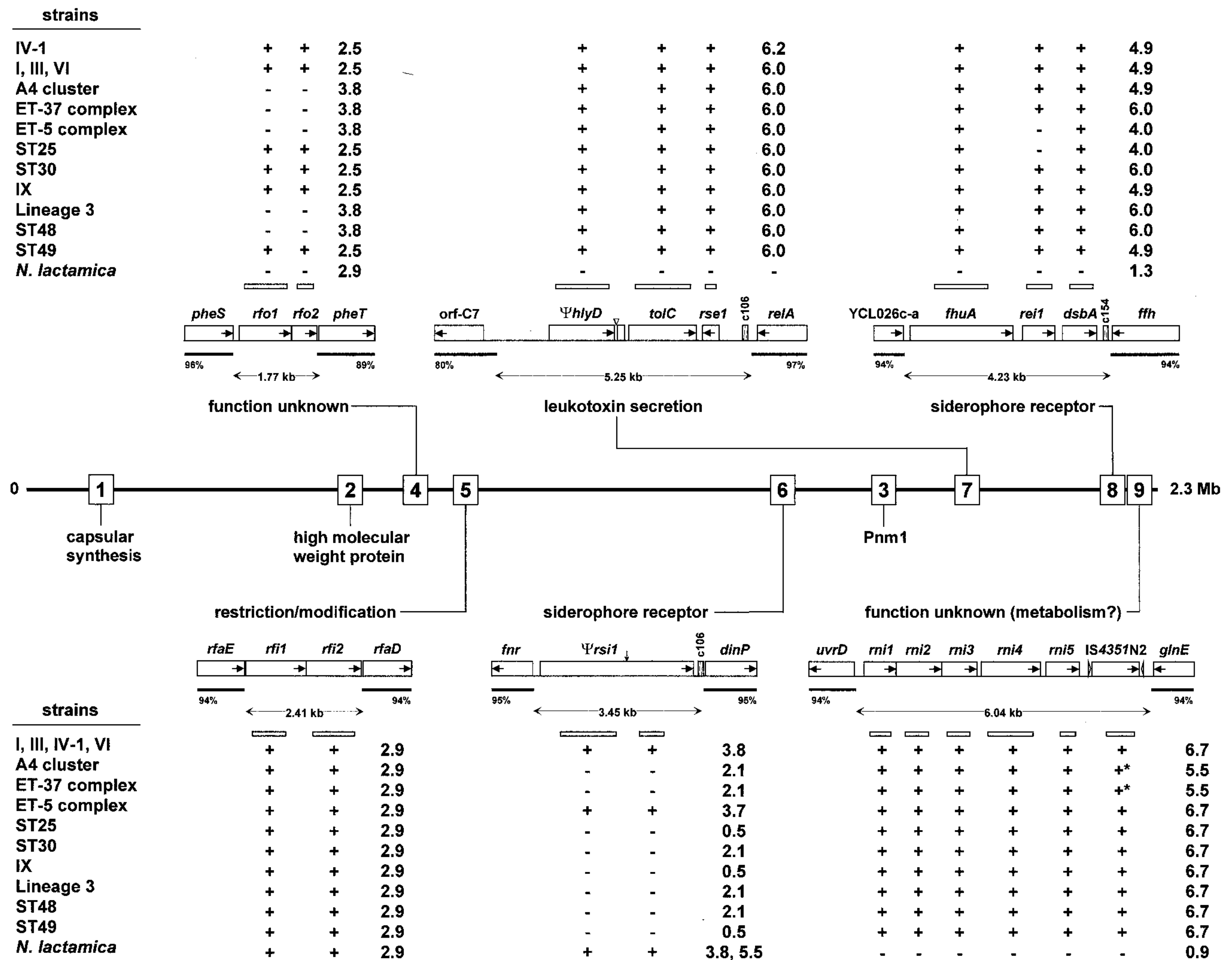

FIG. 2. Relative genetic locations of *N. meningitidis*-specific regions 1 to 9 in the serogroup A subgroup IV-1 strain Z2491. For the six smaller regions, insets indicate the size of ORFs (open rectangles) and the orientation of transcription (internal arrows). Flanking sequences that are homologous in *N. meningitidis* and *N. gonorrhoeae* are indicated by black bars, and the numbers show the percent homology between *N. meningitidis* and *N. gonorrhoeae*. c106 and c154 in regions 6, 7, and 8 refer to 106- and 154-bp Correia elements, respectively, whereas IS*4351*N2 in region 9 is an IS element with flanking inverted repeats (open arrowheads). The PCR probes used for hybridization are indicated by gray bars above and below the map. The results from dot blot hybridization and PCR analyses with diverse strains of *N. meningitidis* and *N. lactamica* are summarized at the top and bottom of the figure by "+" and "−". *, positive hybridization due to other copies of IS*4351*N2 present at other locations in the chromosome. The numbers indicate the sizes (in kilobases) of the PCR products obtained after amplification of the regions with primers complementary to the 5′ and 3′ flanking sequences. An explanation of the genetic designations is in Table 2. ▽, duplication in *hlyD* of region 7; ↓, frameshift in Ψ*rsi1* of region 6.

borders of low homology might represent the remnants of the recombination with foreign DNA (39). Taken together, all of these results are compatible with the import of these specific sequences from different foreign species into *N. meningitidis* and/or *N. gonorrhoeae* and justify using the term DNA island for the eight regions.

Conservation of the eight islands among diverse *N. meningitidis*. Twelve variant capsular polysaccharides are expressed by different *N. meningitidis*, suggesting the existence of at least as many variants of region 1. The data presented above for regions 2 to 9 were based on the sequence analysis of one serogroup A strain of *N. meningitidis* and do not indicate whether the eight islands are generally present at the same location in diverse strains of *N. meningitidis* or whether their genetic content is constant. To address this issue, three kinds of experiments were performed with 13 representative *N. meningitidis* strains, *N. gonorrhoeae* FA1090, and two commensal *N. lactamica* strains. The 13 *N. meningitidis* strains were chosen to represent the genetic diversity of this species according to MLST (18) and include members of the eight hypervirulent clonal groupings called subgroups I, III, IV-1, and VI, ET-5 and ET-37 complex, the A4 cluster, and lineage 3, as well as unrelated endemic strains (serogroup A subgroup IX, STs 25, 30, 48, and 49). (i) To confirm the localization of each island, PCR amplifications were performed by using one primer in each flanking sequence plus a matching reverse primer in the neighboring gene within the island. (ii) The length of each region was determined after PCR amplification by using primers located in the flanking sequences. (iii) Dot blot and Southern hybridizations against chromosomal DNA were performed using PCR products amplified from each of the 85 ORFs as probes. Representative examples of the Southern and dot blot analyses are shown in Fig. 5. Finally, the regions were sequenced at least in part from some of the 13 *N. meningitidis* strains.

As expected, none of the probes hybridized with the *N. gonorrhoeae* strain in dot blots. The other results are summarized in Fig. 2, 3, and 4. They show that, when present, the islands are flanked by the same sequences in all *N. meningitidis*

FIG. 3. Contents of region 2. Region 2 is located within a homologue of the *E. coli cvaB* gene that is a pseudogene in *N. gonorrhoeae* inactivated by the insertion of a Correia element (4). In *N. meningitidis*, region 2 has replaced the Correia element plus 693 bp of the *cvaB* gene. Symbols are as described for Fig. 2. Repetitive stretches within the region from *fhaB* to *rtw4* are indicated by R1 to R4. A stop codon in the Ψ*cvaA* gene is indicated by an asterisk. c106, a 106-bp Correia element.

strains tested and are therefore located at the same chromosomal locations. Furthermore, regions 2, 5, 7, 8, and 9 were present in all representative *N. meningitidis* strains (Fig. 2 and 3), with little apparent variation in content, except that the central and 3′ end of *fhaB* in region 2 and *rei1* in region 8 did not hybridize with some strains. On the other hand, region 4 was absent in about half of the strains tested and region 6 showed considerable interstrain variation (Fig. 2) due to replacement by an IS element and/or deletions (data not shown). Probes from regions 5 and 6 and two probes from region 3 hybridized with the *N. lactamica* strains, showing that these genes are not totally specific to *N. meningitidis*.

Data presented below show that region 3 consists of the *gpxA* gene encoding glutathione peroxidase (19, 20) plus an integrated 39.3-kb prophage that we have called Pnm1 (prophage in *N. meningitidis* 1). Pnm1 was present only in serogroup A strains of the epidemic subgroups I, III, IV-1, and VI (Fig. 4) and was lacking in serogroup A subgroup IX, which is unrelated to the epidemic subgroups of serogroup A (1). Furthermore, Pnm1 was also lacking in all other *N. meningitidis* strains tested. From *N. meningitidis* strains outside the epidemic serogroup A subgroups, primers specific for the flanking *norZ* and *mccF* genes amplified a 1.4-kb product whose sequence contained only *gpxA* plus small, flanking intergenic regions. Within Pnm1, only probes from *rth17* to *rth21* hybridized with *N. meningitidis* strains outside the epidemic serogroup A subgroups. Homology searches in the Sanger Centre genome project for serogroup A strain Z2491 (http://www.sanger.ac.uk/Projects/N_meningitidis/) revealed that these five genes had homologues within a second prophage containing at least 22 ORFs. Furthermore, Southern blots revealed that *rth17* to *rth21* hybridized with two pulsed-field DNA fragments in strains possessing Pnm1 (Fig. 5). Thus, the hybridization of *rth17* to *rth21* with all strains of *N. meningitidis* probably reflects the universal presence of a second prophage.

As with *rth17* to *rth21*, some of the region 2 genes (*fhaC*, *fhaB*, *rtw2*, and *rtw4* to *rtw8*) also hybridized with two pulsed-field DNA fragments in Southern blots from strains of the ET-5 complex, lineage 3, ST25, and ST48 and are therefore present twice in these bacteria (Fig. 5).

FIG. 4. Region 3 and the Pnm1 prophage. Details are as given in Fig. 2. The numbers 1 to 52 above the ORFs indicate genes *rth1* to *rth52*. The transcription orientation is from left to right for *rth2* to *rth52*. Dark gray ORFs are homologous to both phage genes and to clustered genes in *H. influenzae*. ORFs shaded in light gray are only homologous to clustered genes in *H. influenzae*. ORFs in hatched boxes are only homologous to phage proteins (*rth17*, *rth40*, and *rth43*). Blocks of ORFs that yielded identical results in dot blot analysis are delineated by paired vertical lines above the map. +/−, positive result with one of two strains of *N. lactamica*.

TABLE 2. ORFs in the eight DNA islands and their homologies to other bacterial proteins[a]

ORF (length; probes)	Length (aa)	Homologous protein					
		Function	Species	Length (aa)	Statistical significance (P)	% Identity/ % similarity	Accession no.
Region 2 (19.45 kb; B322, B220, B132, E139, B233, E145, B328, B108, and B101)							
Region 2, 5′ flanking							
Ψ*cvaA* pseudogene	411	Colicin V secretion protein	*E. coli*	413	7e-31	25/46	P25519
orf1	100	Unknown					
cvaB left end	79	Colicin V secretion ATP-binding protein	*E. coli*	698	4e-05	48/68	P22520
Region 2[b]							
fhaC	580	Hemolysin activator-like protein precursor	*B. pertussis*	584	5e-27	25/41	P35077
fhaB	2,015	FHA B precursor	*B. pertussis*	3,591	1e-50	25/42	P12255
Region 2, 3′ flanking							
cvaB right end	93	Colicin V secretion ATP-binding protein	*E. coli*	698	8e-23	54/73	P22520
orf2 (fragment)	303	Unknown protein	*Vitreoscilla* sp.	376	2e-47	39/53	AF067083
Region 3 (40.0 kb; E142, E137, E107, E120, E146, E115, E114, B306, and E124)							
Region 3, 5′ flanking							
norZ (fragment)	677	Nitric oxide reductase	*R. eutropha*	762	0.0	58/69	AF002217
Region 3[c]							
rth1	249	Repressor protein	Phage D3112	240	2e-15	36/56	S13498
		Transcriptional regulatory protein HI1476	*H. influenzae*	239	5e-06	25/45	P44207
rth2	87	DNA-binding protein Ner	Phage Mu	75	9e-14	50/66	P06020
		Ner protein homolog HI1477	*H. influenzae*	89	2e-17	53/70	P46496
rth3	681	Transposase	Phage D3112	690	2e-64	30/46	S62728
		Transposase A (MuA)-homolog HI1478	*H. influenzae*	687	4e-07	21/36	B64126
rth4	304	MuB (DNA transposition protein)	Phage Mu	312	9e-16	26/44	P03763
		MuB protein homolog HI1481	*H. influenzae*	287	2e-25	29/52	C64126
rth13	91	DNA-binding protein HU-beta	*S. typhimurium*	90	5e-24	58/75	P05515
rth14	144	muE16	Phage Mu	195	4e-06	40/61	M64097
		E16 homolog HI1488	*H. influenzae*	185	0.002	35/53	E64126
rth17	181	Gene 25	Phage SPP1	271	4e-04	28/39	X97918
rth27	519	HI1501	*H. influenzae*	520	1e-118	47/63	P44225
rth28	448	HI1502	*H. influenzae*	414	7e-22	31/46	P44226
rth29	165	MuG (virion morphogenesis)	Phage Mu	156	0.004	29/45	Q01261
		MuG homolog HI1568	*H. influenzae*	138	2e-19	37/50	P45255
rth30	354	MuI homolog HI1504	*H. influenzae*	355	4e-19	26/38	I64126
rth31	300	HI1505	*H. influenzae*	308	3e-83	52/65	P44227
rth33	141	HI1508	*H. influenzae*	141	3e-13	32/51	P44230
rth34	222	HI1509	*H. influenzae*	194	0.81	22/41	P44231
rth35	475	Sheath protein gpL	Phage Mu	495	2e-23	26/37	AB000833
		gpL (MuL) homolog HI1511	*H. influenzae*	487	6e-27	25/38	P44233
rth40	559	ORF15	Phage phi PVL	694	5e-07	22/38	AB009866
rth42	455	64-kDa virion protein muN	Phage Mu	491	0.011	21/38	P08557
		MuN homolog HI1515	*H. influenzae*	457	7e-05	22/41	A64127
rth43	379	43-kDa tail protein	Phage Mu	379	5e-16	23/40	P08558
rth44	209	Baseplate assembly protein V (gpV)	Phage P2	211	3e-06	25/40	P31340
		HI1518	*H. influenzae*	182	6e-09	26/44	P44238
rth45	117	HI1519	*H. influenzae*	135	2e-04	37/46	P44239
rth46	161	HI1520 (N terminus)	*H. influenzae*	355	4e-06	29/44	P44240
rth47	191	Protein xkdT (C terminus)	PBSX prophage	348	1e-07	33/48	P54339
		HI1520 (C terminus)	*H. influenzae*	355	8e-10	28/44	P44240
rth48	188	Hypothetical protein YmfQ (27% identical to HI1521)	*E. coli*	194	6e-08	25/43	P75982
rth49	760	HI1522	*H. influenzae*	623	3e-14	36/50	P44242
rth52	279	HI1523	*H. influenzae*	296	3e-71	47/65	P44243
gpxA	177	Glutathione peroxidase	*N. meningitidis*	177	0.0	100	P52036
Region 3, 3′ flanking							
mccF	394	Microcin immunity	*E. coli*	344	1e-16	27/42	X57583
Region 4 (1.77 kb; B342)							
Region 4, 5′ flanking *pheS*	330	Phenylalanine-tRNA synthetase alpha chain	*E. coli*	327	1e-111	59/73	P08312
Region 4							
rfo2	172	No homologies					
rfo1	354	No homologies					

Continued on following page

TABLE 2—*Continued*

ORF (length; probes)	Length (aa)	Homologous protein					
		Function	Species	Length (aa)	Statistical significance (P)	% Identity/ % similarity	Accession no.
Region 4, 3′ flanking							
pheT (fragment)	693	Phenylalanine-tRNA synthetase beta chain	*E. coli*	795	1e-148	41/59	P07395
Region 5 (2.41 kb; E136)							
Region 5, 5′ flanking							
rfaE	323	ADP-heptose synthase	*H. influenzae*	342	6e-77	49/64	U17642
Region 5							
rfi1	411	*scrFI-a*, modification methylase	*L. lactis*	389	1e-46	31/48	P34877
rfi2	376	*dcrH*, type II restriction enzyme	*N. gonorrhoeae*	374	6e-08	35/55	AF001598
Region 5, 3′ flanking							
rfaD	334	ADP-L-glycero-D-mannoheptose epimerase	*N. gonorrhoeae*	334	0.0	97/99	L07845
Region 6 (3.45 kb; B208)							
Region 6, 5′ flanking							
fnr	259	ferredoxin-$NADP^+$ reductase	*A. vinelandii*	258	9e-32	32/53	A57432
Region 6							
Ψ*rsi1* pseudogene	1045	*fpvA*, ferripyoveridine receptor precursor	*P. aeruginosa*	813	5e-38	33/51	P48632
Region 6, 3′ flanking							
dinP	352	DNA-damage-inducible protein P	*E. coli*	351	2e-65	41/58	Q47155
Region 7 (5.25 kb; E116)							
Region 7, 5′ flanking							
orf-C7	333	ORF on plasmid pJTPS1	*R. solanacearum*	444	8e-20	32/47	AB015669
Region 7							
Ψ*hlyD* pseudogene	454 (+54)	Hemolysin secretion protein D	*E. coli*	478	3e-77	35/56	P09986
tolC	467	Outer membrane protein	*E. coli*	495	5e-20	23/40	P02930
rse1	111	sll0201, putative transposase	*Synechocystis* sp.	164	4e-12	42/64	D64000
Region 7, 3′ flanking							
relA (fragment)	345	GTP-pyrophosphokinase	*E. coli*	744	5e-58	36/57	P11585
Region 8 (4.23 kb; B313 and B341)							
Region 8, 5′ flanking							
YCL026c-a	201	Hypothetical protein on chromosome III	*S. cerevisiae*	192	9e-20	31/53	P37261
Region 8							
fhuA	703	Ferrichrome iron receptor	*E. coli*	747	5e-26	23/40	P06971
rei1	219	HP1334, hypothetical protein	*H. pylori*	224	2e-46	47/61	AE000635
dsbA	231	Disulfide oxidoreductase	*P. syringae*	214	3e-18	28/47	AF036929
Region 8, 3′ flanking							
ffh	456	Signal recognition particle protein	*E. coli*	453	1e-174	64/76	P07019
Region 9 (6.04 kb; E102)							
Region 9, 5′ flanking							
uvrD	735	DNA helicase II	*E. coli*	720	0.0	47/65	P03018
Region 9							
rni1	215	HI1731, urea amidolyase homolog	*H. influenzae*	213	6e-69	58/72	P44299
rni2	309	HI1730, urea amidolyase homolog	*H. influenzae*	309	1e-101	59/71	P44298
rni3	245	HI1729, lactam utilization protein homolog	*H. influenzae*	257	3e-80	59/77	P45347
rni4	397	HI1728, *braB* homolog	*H. influenzae*	397	1e-162	72/84	G64138
rni5	230	MTH939, unknown function	*Methanobacterium*	188	1e-7	27/47	AE000868
rni6	321	*tra4*, transposase for IS element IS*4351*	*B. fragilis*	326	3e-62	41/60	P37247
Region 9, 5′ flanking							
glnE (fragment)	719	Glutamine-synthetase adenyltransferase	*E. coli*	946	1e-130	37/54	P30870

[a] aa, amino acids. Flanking genes designated as fragments were only partially sequenced. Genes indicated as pseudogenes contain stop codons compared with their homologues in other bacteria.
[b] Region 2 ORFs with no homologies (length in aa): *rtw1* (127), *rtw2* (893), *rtw3* (143), *rtw4* (833), *rtw5* (162), *rtw6* (90), *rtw7* (313), *rtw8* (311), *rtw9* (116), and *rtw10* (98).
[c] Region 3 ORFs with no homologies (length in aa): *rth5* (80), *rth6* (99), *rth7* (71), *rth8* (122), *rth9* (97), *rth10* (151), *rth11* (203), *rth12* (261), *rth15* (148), *rth16* (225), *rth18* (78), *rth19* (155), *rth20* (101), *rth21* (115), *rth22* (113), *rth23* (93), *rth24* (71), *rth25* (168), *rth26* (539), *rth32* (157), *rth36* (124), *rth37* (128), *rth38* (155), *rth39* (129), *rth41* (238), *rth50* (207), and *rth51* (162).

TABLE 3. Sizes of the eight islands in *N. meningitidis* and *N. gonorrhoeae*[a]

Region	Flanking genes		Size of region (kb)		Contents of *N. gonorrhoeae* region (homologies)
	Left	Right	*N. meningitidis*	*N. gonorrhoeae*	
2	*cvaBΔ1*	*cvaBΔ2*	19.5	0.746	*cvaBΔ3*, Correia element
3	*norZ*	*mccF*	40.0	0.047	
4	*pheS*	*pheT*	1.8	1.841	*hpaIIV*, *hpaIIM*, *hphIR*
5	*rfaE*	*rfaD*	2.4	0.044	
6	*fnr*	*dinP*	3.5	0.755	ORF98
7	orf-C7	*relA*	5.3	>12	Unknown
8	YCL026c-a	*ffh*	4.2	0.640	ORF151
9	*uvrD*	*glnE*	6.0	0.328	ORF58

[a] *hpaIIV*, gene for very-short-patch-repair enzyme from *H. parainfluenzae*; *hpaIIM*, gene for modification enzyme from *Haemophilus parainfluenzae*; *hphIR*, gene for restriction enzyme from *Haemophilus parahaemolyticus*. ORF98, ORF151, and ORF58 are ORFs of unknown function whose numbers indicate the length of the putatively encoded protein. The genes flanking region 7 in *N. meningitidis* are on separate contigs in *N. gonorrhoeae*. Those *N. gonorrhoeae* contigs contain a total of 12 kb of DNA at the position where region 7 is located in *N. meningitidis*, as confirmed by PCR analysis.

Together, these data show that the *N. meningitidis*-specific islands 2, 5, 7, 8, and 9 are fairly well conserved among representative strains of *N. meningitidis* and that Pnm1 is conserved among epidemic serogroup A strains.

Sequence analysis of the *N. meningitidis*-specific islands. The possible functions of the ORFs in the eight islands were investigated by protein homology searches and computer analysis of the sequences (Table 2). The following description concentrates on those homologies that might be relevant to the virulence potential of *N. meningitidis*.

The two ORFs at the left of region 2 are homologous to and approximately the same size as genes in *Bordetella pertussis* encoding the filamentous hemagglutinin (FHA) precursor, FhaB, and the accessory protein, FhaC, involved in the secretion of FHA (17). In *B. pertussis*, FhaB (367 kDa) is processed during secretion, yielding the 220 kDa FHA protein (5, 6). FHA is thought to be one of the major adhesins of *B. pertussis* and is also a component of several vaccines against whooping cough. The homology between FhaB in *N. meningitidis* and *B. pertussis* is largely restricted to the N-terminal 900 amino acids, including the NPNGI(S/T) sequence involved in secretion and the signal peptide cleavage site (HA ↓ Q), at the end of an unusually long signal peptide. The central and 3′ end of the *fhaB* gene are variable between strains according to the dot blot hybridization results (Fig. 3). In addition, the sequencing of region 2 was complicated by the presence of direct repeats (>95% similarity) within *fhaB*, *rtw2*, and *rtw4* that were designated R1 (431 bp), R2 (271 bp), R3 (160 bp), and R4 (185 bp) (Fig. 3). An interesting hypothesis is that recombination between these repeats could provide for variation in the C-terminal portion of the FhaB protein, a feature that is common among virulence factors in pathogenic neisseriae.

Integrated prophages are known to encode virulence factors in other bacteria (3, 35). Prophage Pnm1 in region 3 contains 52 ORFs, of which 16 are homologous to Mu and related phages (including genes for the transposase and repressor protein). A total of 13 of these 16 ORFs, as well as 7 others, also show homologies to a group of genes from *H. influenzae* in section 140 to 143 of the genome (7) (Table 2), suggesting that homologous prophages have integrated into both serogroup A *N. meningitidis* and *H. influenzae*. In *N. meningitidis* Z2491, Pnm1 is flanked by direct repeats of AACT (21 bp downstream of *norZ* and 153 bp upstream of *gpxA*) which are present only once in nonepidemic serogroup A strains. Pnm1 is the first example of a neisserial prophage, although *N. meningitidis* bacteriophages have previously been reported (2).

Region 7 contains the pseudogene Ψ*hlyD* and the *tolC* gene (Fig. 2) in *N. meningitidis* Z2491. There is a 61-bp insertion containing a stop codon 1,311 bp after the ATG start codon of the Ψ*hlyD* pseudogene in *N. meningitidis* strain Z2491, followed by a direct duplication of the 73 bp preceding the insertion. On the other hand, sequencing region 7 from the ET-37 complex strain revealed that this strain contains a complete *hlyD* ORF. In the other *N. meningitidis* strains, region 7 was the same size as from the ET-37 complex (6.0 kb) (Fig. 2). Furthermore, sequences of the 3′ end of *hlyD* revealed that none of these other strains contained the insertion from strain Z2491. Thus, with the exception of Z2491, *hlyD* is normally not a pseudogene. Considering the homologies of *hlyD* and *tolC* with genes of the type 1 secretion apparatus in other bacteria, it seems possible that region 7 might be involved in the virulence of most *N. meningitidis* strains.

Region 8 contains the *fhuA*, *rei1*, and *dsbA* genes in Z2491 (Fig. 2). PCR analysis of region 8 revealed size variations in half of the representative *N. meningitidis* strains. This region was subsequently sequenced from several strains to determine the basis for this variability. The data revealed that *rei1* is deleted in the ET-5 complex, whereas in four other strains (ET-37 complex, lineage 3, ST30, and ST48) an IS element, IS*4351*N1 (1,076 bp), has been inserted between *dsbA* and the Correia element, c154 (4). However, *fhuA* and *dsbA* were present in all strains. The *fhuA* gene is a homologue of an *E. coli* ferrichrome-iron receptor that is involved in the uptake of siderophore-bound iron. *fhuA* was a pseudogene in some of the strains (subgroup I, subgroup III, A4 cluster, lineage 3, ST48, and ST49) due to stop codons introduced by base changes, insertions, or deletions, but the reading frame was intact in strains belonging to the ET-37 and ET-5 complex, subgroup IV-1, and in ST30. *dsbA* is homologous to genes in *E. coli* and *Pseudomonas syringae* that encode a disulfide oxidoreductase. The predicted *N. meningitidis* DsbA protein contains the motif C-X-X-C (residues 76 to 79), characteristically present at the active sites of DsbA enzymes. The *N. meningitidis* DsbA protein also contains a typical lipoprotein signal peptide and cleavage site between A_{18} and C_{19} (25) within the motif L-X-A-C. The amino acid following the LXAC motif is S, indicating that the *N. meningitidis* DsbA is sorted to the outer membrane.

Region 9 contains five ORFs of unknown function plus an IS element, IS*4351*N2, homologous to that inserted in region 8 in some strains. Sequence analysis revealed that the size differences in region 9 (Fig. 2) were due to the lack of IS*4351*N2 in strains of the A4 cluster and ET37 complex. The other five genes in region 9 were present in all bacteria, and therefore region 9 was retained for further study of its possible role in virulence.

Regions 4 and 5 are probably not important in *N. meningi-*

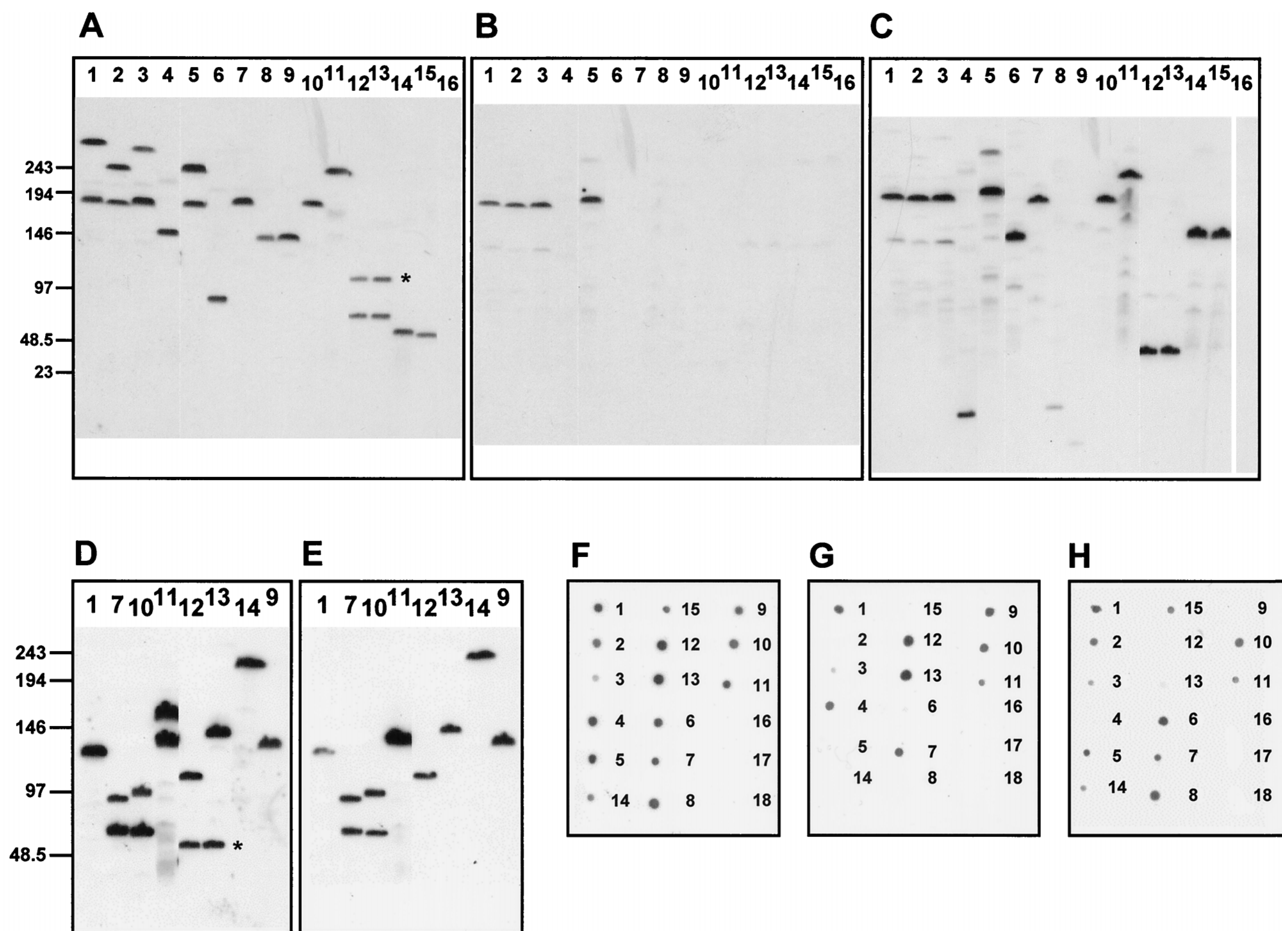

FIG. 5. Southern analysis of pulsed-field gels of *Spe*I-digested chromosomal DNA (A to E) and DNA dot blot analysis (F to H) of regions 2 and 3. The numeration of bacterial strains is the same in all panels: 1, subgroup IV-1; 2, subgroup I; 3, subgroup III; 4, subgroup IX; 5, subgroup VI; 6, A4 cluster; 7, lineage 3; 8, ST30; 9, ST49; 10, ST48; 11, ST25; 12, ET-5 complex (44/76); 13, ET-5 complex (MC58); 14, ET-37 complex (FAM18); 15, ET-37 complex (ROU); 16, *N. gonorrhoeae* FA1090; 17, *N. lactamica* Z6793; 18, *N. lactamica* Z6784. (A to C) Southern analysis of region 3 with probes corresponding to *rth18* (A), *rth33* (B), and *gpxA* (C). Results identical to those shown in panel A were obtained with probes for *rth17* and *rth19*. Hybridization with probes for *rth20* and *rth21* resulted in a similar pattern except for lanes 12 and 13, where the upper signal (marked by an asterisk) disappeared. The same results as in panel B were obtained with probes for *rth1*, *rth4*, *rth28*, and *rth30*. The other ORFs of Pnm1 were not tested by Southern analysis, but similar results can be expected according to dot blot analysis except for *rth26*, which is also present in strains of the ET-5 complex, ST25, and ST48. (D and E) Southern analysis of region 2 with probes for *fhaC* (D) and *rtw7* (E). The same results as in panel D were obtained with probes for *fhaB* (5′-part), *rtw2*, and *rtw4*. With a probe for *rtw5*, the lower signals in lanes 12 and 13 (marked by an asterisk) disappeared. The result of hybridization with a probe for *rtw8* was the same as with *rtw7* (E). Positions of the molecular size markers are shown in kilobases. (F to H) DNA dot blot analysis with probes for the 5′ end (F), the central part (G), and the 3′ end (H) of *fhaB*.

tidis virulence. The former region contains two genes of unknown function and is absent from a large proportion of *N. meningitidis* strains, and the latter region encodes a restriction-modification system and is also present in the commensal *N. lactamica*. Region 6 encodes a pseudogene having homologies with a siderophore receptor in Z2491 but is deleted in most of the strains. Regions 4, 5, and 6 were not investigated further.

Virulence analysis of the *N. meningitidis*-specific islands. Based on the results described above, regions 2, 3, 7, 8, and 9 were chosen for subsequent investigation of their possible roles in virulence. The entire regions were inactivated by deletion (Fig. 1) and confirmed by Southern blot (Fig. 6). These regions had been sequenced from *N. meningitidis* strain Z2491, but this strain is not competent for DNA transformation and could not be used to construct the deletion mutants. Therefore, regions 7, 8, and 9 were deleted within *N. meningitidis* strains MC58 (ET-5 complex) and ROU (ET-37 complex). These strains were chosen to give consistent results in the tests for the different biological phenomena and have been tested previously by several models related to bacterial infection. Neither of these strains contained pseudogenes in any of these regions. Region 2 was deleted only within strain ROU because the chromosome of MC58 contains at least two copies at distinct locations of several of the ORFs from region 2 (see above). The deletions were constructed by using cloned PCR products from the chromosome of strain MC58, except that region 2 was deleted using cloned PCR products from strain ROU. The existence of the desired deletion mutations was confirmed by PCR and Southern blotting. All of these mutants grew well on GC agar and were as transformable as are their parents, indicating that they are piliated.

First, the deletion mutants of strain ROU were assessed for their ability to adhere to and to invade HUVEC, a model for interactions with the endothelial cells of the BBB. No differences were detected between the parental strain and the deletion mutants (Table 4), indicating that regions 2, 7, 8, and 9 do not affect interactions with endothelial cells.

Second, the deletion mutants were tested for sensitivity to

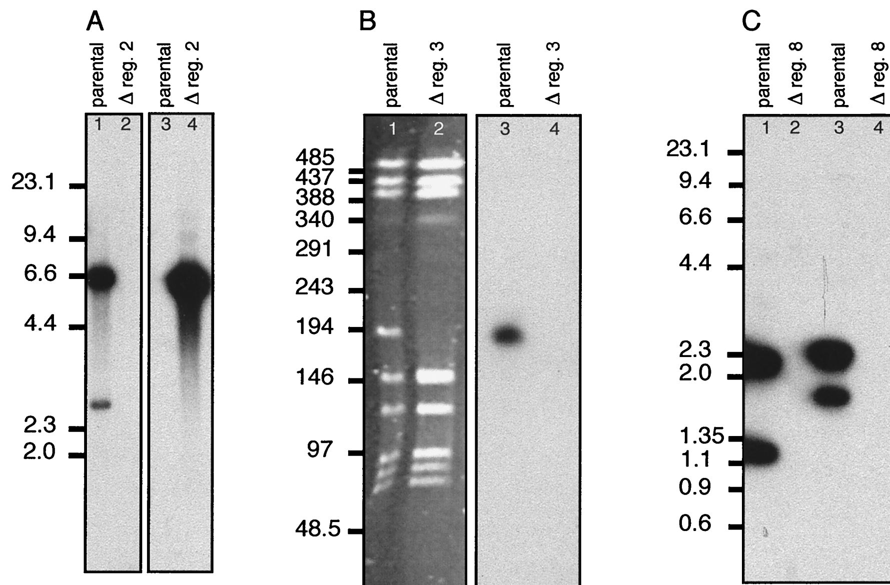

FIG. 6. Verification of the deletion mutants. Examples are shown of the methods used to verify the deletion of the regions. Positions of the molecular size markers are shown in kilobases for each gel. (A) Deletion of region 2 from strain ROU. Chromosomal DNA from the parental strain (lanes 1 and 3) and the region 2 deletion mutant (lanes 2 and 4) was digested with *Cla*I. Lanes 1 and 2 were probed with a PCR product corresponding to the gene *fhaC*; lanes 3 and 4 were probed with the cassette "omega" used to replace the region. Due to its size (about 25 kb) region 2 encompassed several *Cla*I fragments; similar results were obtained after probing with PCR products corresponding to several of the other ORFs. (B) Deletion of region 3 from strain Z5463. Chromosomal DNA from the parental strain (lanes 1 and 3) and the region 2 deletion mutant (lanes 2 and 4) was digested with *Sgf*I. Lanes 1 and 2 are the pulsed-field gel electrophoresis analysis of the deletion. Note the disappearance of a band at about 194 kb in the mutant and the new band appearing with a size of about 146 kb; this corresponds to the deletion of about 50 kb (region 3). Lanes 3 and 4 were probed with a PCR product corresponding to a portion of the phage transposase gene. (C) Deletion of region 8 from strains MC58 and ROU. Lane 1, MC58 parental strain; lane 2, MC58 region 8 deletion; lane 3, ROU; lane 4, ROU region 8 deletion. Chromosomal DNA was digested with *Cla*I, and Southern blots were probed with a PCR product corresponding to the entire region 8 from strain Z2491.

the bactericidal activity of complement (Table 5). For these bactericidal assays, meningococci were incubated with 10% guinea pig serum as a complement source supplemented with different dilutions of antiserum raised against the homologous parental strain as an antibody source. The deletion mutants of region 2, 7, 8, or 9 were as resistant to the bactericidal action of the immune serum as their parents in contrast to the serum-sensitive *ppk* mutant control. Thus, these regions do not seem to be involved in serum resistance.

Finally, the mutants were tested for their ability to multiply in the bloodstream in an infant rat model. These tests were performed with the mutants deleted for regions 7, 8, and 9 of strain MC58, which causes high levels of bacteremia in this model. Region 2 could not be tested in this strain because ORFs in region 2 are present at two distinct locations in MC58, the flanking sequences are not known in this strain, and the sequence of region 2 is highly variable between strains. For each experiment, half of the animals in each litter were injected intraperitoneally with the parental strain, and the other half were injected with the deletion mutant; the numbers of bacteria in the blood were then quantitated for 24 h. No differences were detected with deletion mutants of regions 7 or 9 (data not shown), showing that these regions are not important for causing bacteremia. On the other hand, lower numbers of bacteria in blood samples than with the parental control were consistently found with the MC58 deletion mutant lacking region 8 (Fig. 7). Furthermore, only 2 of 15 infant rats died within 48 h after injection of the region 8 deletion mutant, whereas 11 of 14 rats died after injection of the parental con-

TABLE 4. Percent adherence and invasion by deletion mutants of strain ROU[a]

Strain	% Adherence	% Invasion
Parental	2	0.24
ΔRegion 2	2	0.21
ΔRegion 7	2.6	0.29
ΔRegion 8	2.7	0.29
ΔRegion 9	2.9	0.27

[a] Percent adherence is the number of bacteria adherent to HUVEC × 100, divided by the total number of bacteria present after 1 h of infection. Percent invasion is the number of internalized bacteria after 6 h × 100, divided by the number of bacteria present 1 h after infection.

TABLE 5. Complement-dependent percent killing of deletion mutants[a]

Strain	% Killing of strain at (serum dilution):			
	MC58		ROU	
	1:3,000	1:10,000	1:3,000	1:10,000
Parent	85	9	93	28
ΔRegion 2	77	7	88	34
ΔRegion 7	80	−7	81	18
ΔRegion 8	86	12	86	44
ΔRegion 9	88	8	85	31
Ppk$^-$ control	100	79	100	98
Ppk$^-$ without complement	6	3	33	13

[a] The data show the average percent killing from two experiments with two dilutions of homologous sera against strains MC58 and ROU. As a positive killing control, a serum-sensitive polyphosphate kinase mutant (Ppk$^-$) was tested while, as a negative control, decomplemented guinea pig serum was tested with the Ppk$^-$ mutant rather than normal guinea pig serum.

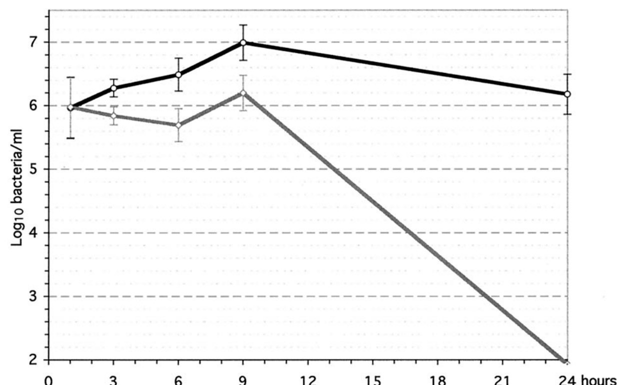

FIG. 7. Bacterial concentrations in sequential blood samples after the intraperitoneal infection of infant rats. One typical experiment is shown with average counts and standard deviations. ○, MC58 parental strain; ◇, MC58 Δregion 8.

trol. These data demonstrate that region 8 is important for bacterial survival in the bloodstream.

Virulence determinants may be carried on bacterial prophages (3, 35). Though region 3, including Pnm1, is only present in epidemic serogroup A strains, we deleted this region in order to investigate its possible role in the pathogenesis of these strains. We identified a subgroup IV-1 strain, Z5463, that is transformable and then constructed a region 3 deletion of this strain. Z5463 does not yield high levels of bacteremia in infant rats, and we could not carry out this assay. Because of this strain's sensitivity to complement, bactericidal assays were performed with guinea pig complement in the absence of opsonizing antiserum. The deletion of region 3 did not affect the resistance to complement killing; both the wild-type and mutant strains were killed at concentrations of serum of >1%. Neither did deletion of region 3 affect the interaction of Z5463 with monolayers of HUVEC in the adhesion and invasion assay (data not shown).

DISCUSSION

We investigated genes within eight DNA islands that comprise approximately 5% of the chromosome (100 kb) of *N. meningitidis* and are lacking in *N. gonorrhoeae* (Table 6). Many of the genes in these islands seem to be irrelevant to the differences in pathogenicity between these species. However, the islands designated as regions 2, 7, 8, and 9 contained genes homologous to genes from other pathogenic bacteria that are involved in virulence and were present in diverse, representative *N. meningitidis* strains. Deletion mutants lacking region 8 were impaired in their ability to cause bacteremia in an infant rat model. A ninth island containing genes encoding the synthesis of capsular polysaccharide is also known to be necessary for dissemination in the bloodstream. Thus, two of the nine known islands in *N. meningitidis* are important for bloodstream dissemination, a prerequisite for causing meningitis. It remains possible that the other seven islands may play a role in virulence that was not detected by the assays used here.

Region 8 contains homologues of *fhuA* and *dsbA*, as well as a new gene called *rei1*. However, *rei1* is deleted within MC58, the *N. meningitidis* strain that was used in the infant rat model, leaving only *fhuA* and *dsbA* as candidates for genes involved in bacteremia. FhuA is a siderophore-iron receptor in *E. coli*. The *fhuA* gene was defective in several of the *N. meningitidis* strains, suggesting that it is not important for virulence. The DsbA homologue is present in diverse *N. meningitidis* strains and is probably sorted to the outer membrane. DsbA proteins in other pathogenic bacteria have been shown to play an indirect role in virulence. They are involved in the correct folding of proteins responsible for both the secretion of invasion proteins by *S. flexneri* (36) and for its intracellular survival (37), as well as for the formation of type IV fimbriae in enteropathogenic *E. coli* (38). The DsbA homologue in region 8 does not affect piliation because mutants deleted for region 8 were normally transformable and adhered to endothelial cells in a pilus-dependent assay as efficiently as their parent.

In several bacterial species (for example, *E. coli*, *Helicobacter pylori*, *Salmonella enterica*, and *Yersinia enterocolitica*), genes encoding increased pathogenicity are clustered in so-called pathogenicity islands (PAIs) (11). PAIs are usually large (50 to 200 kb) and often have a different GC-content from that of the host chromosome. PAIs can be genetically unstable due to flanking repetitive sequences or IS elements and, within the *Enterobacteriaceae*, tRNA loci often serve as targets for their integration and excision. None of the *N. meningitidis*-specific islands in regions 2 to 9 fulfills all of these criteria, and most fulfill none of them. Thus, the generic term "DNA island" seems more appropriate for these sequences in the neisseriae than the term PAI. We note that some of the differences

TABLE 6. Features of the eight islands in *N. meningitidis*

Region	Size (kb)	Important homologies	Distribution	Comments
2	19.5	*fhaB* (FHA of *B. pertussis*); *fhaC* (FHA activator protein)	Present in all *N. meningitidis* strains; high variability of *fhaB*	Some region 2 genes are present twice in some serogroup B strains
3 (Pnm1)	40	Prophage in *H. influenzae gpxA* (glutathione peroxidase)	Pnm1 in clonally related serogroup A strains only; *rth17–rth21* and *gpxA* in all *N. meningitidis* strains	Southern blot data suggests a second prophage present in all *N. meningitidis* strains
4	1.8	None	Present in only 60% of the *N. meningitidis* strains	Replaced by restriction/modification system in *N. gonorrhoeae*
5	2.4	Restriction-modification system	Present in all *N. meningitidis* strains	Also present in *N. lactamica*
6	3.5	Siderophore receptor (Ψ*rsi1*)	Pseudogene, replaced by IS element or deleted in all *N. meningitidis* strains	
7	5.3	Leukotoxin secretion (*hlyD*, *tolC*)	Present in all *N. meningitidis* strains	*hlyD* is a pseudogene in serogroup A, subgroup IV-1
8	4.2	Siderophore receptor (*fhuA*), disulfide oxidoreductase (*dsbA*)	Present in all *N. meningitidis* strains, but *fhuA* is a pseudogene in half of the strains	Deletion of region 8 results in reduced bacteremia in the infant rat
9	6.0	Four proteins of *H. influenzae* with putative metabolic functions	Present in all *N. meningitidis* strains	

between these islands and PAIs may reflect the fact that the neisseriae are naturally transformable and readily undergo genetic exchange via homologous recombination after DNA transformation, whereas mobile genetic elements are often more important for the *Enterobacteriaceae*.

N. meningitidis probably contains more DNA islands than the eight described here. For example, upstream of region 2 in *N. meningitidis* is another small island containing *orf1* plus a 106-bp long Correia element (Fig. 3), whereas two small ORFs are present at that location in *N. gonorrhoeae*. Similarly, three other islands in the *opcA* and Ψ*opcB* regions were described elsewhere (39), and the regions corresponding to three *N. meningitidis*-specific clones (32) were also not identified here. Finally, region 1 encodes genes involved in capsular biosynthesis and was not investigated further in this report. Thus, a considerable proportion of the differences between *N. meningitidis* and *N. gonorrhoeae* may be encoded by species-specific DNA islands, only some of which have been analyzed here.

The eight islands were present in most of the strains studied, and their overall organization was conserved, despite the presence of a number of pseudogenes. The conservation of region 5 (restriction-modification system) can be explained, since functional restriction-modification systems act as selfish genetic units (21). Similarly, region 4 of *N. meningitidis* is replaced by a restriction-modification system in *N. gonorrhoeae* FA1090 (see Table 3, *hpaIIM* and *hphIR*), which has no homologue in *N. meningitidis* and whose corresponding genes exhibit a low GC content. The presence of some of the other islands might be accounted for by the selfish operon theory (16), whereby the integrity of a group of genes linked by a common function is favored by stochastic mechanisms. On the other hand, conserved, functional genes are classically the result of natural selection by the environment, and those in the *N. meningitidis*-specific islands should therefore be beneficial to *N. meningitidis* in those environments which it specifically inhabits. *N. meningitidis* and *N. gonorrhoeae* differ not only in their characteristic pathogenicities but also in the anatomical sites they colonize. Thus, these islands might be relevant to multiplication under the particular biochemical conditions and in the presence of the microbial competition experienced by the meningococcus in its natural habitat. Region 2 encodes *fhaB* and *fhaC* homologues, as well as a number of ORFs with no obvious homologies, and is present in all *N. meningitidis* strains analyzed. The FHA protein is an adhesin of *B. pertussis*. Although the *N. meningitidis fhaB* homologue is considerably shorter than that in *B. pertussis*, it could produce a 224-kDa protein after cleavage of the signal peptide that is of comparable size to the processed FHA. The conservation of an amino-terminal asparagine-rich domain plus the conserved NPNGI motif might reflect a similar secretion mechanism. However, the C termini differ extensively between *B. pertussis* and *N. meningitidis*; such sequence variability might reflect functional differences or diversifying selection due to the human immune system. Serum sensitivity and adhesion were not affected by deletion of region 2, indicating that it is not essential for these phenotypes in *N. meningitidis*. It was not possible to test whether region 2 is important for blood dissemination because MC58, the strain used for these tests, possesses two copies of ORFs from region 2 at distinct locations, and ROU, the other strain in which a deletion of region 2 was introduced, does not replicate in the infant rat for unknown reasons, although it is a clinical isolate from a case of meningitis with septicemia. Notwithstanding the lack of sequence information, we were able to produce a double mutation in the gene *fhaB* in the two region 2 loci of another strain, 8013, by the deletion of one region 2 followed by the insertional inactivation of the *fhaB* gene in the other. The double mutant had no alteration with respect to the in vitro or in vivo assays (data not shown); however, it is not possible to extrapolate this result to consider the whole of region 2.

In summary, we describe the characteristics and distribution of eight DNA islands that are specific to *N. meningitidis* and show that genes on one of these islands are important for virulence. These results will form the basis of additional experiments to develop new protein vaccine candidates and to define factors that are important for bacterial invasion of the bloodstream.

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ADDENDUM IN PROOF

During the course of this work, sequences corresponding to two of the regions have been submitted to GenBank by other groups. Region 5 with its flanking *rfaE* and *rfaD* genes has been sequenced in the *N. meningitidis* serogroup B strain CDC 8201085 (C. M. Kahler and D. S. Stephens, GenBank accession number AF125564), where it is 98.6% identical to region 5 of Z2491. The *rfi1* and *rfi2* genes are designated *nmg*II and *nmg*I, respectively. Region 7 was sequenced in the *N. meningitidis* serogroup C strain IR1075 (I. Stojiljkovic, GenBank accession number AF121772), where it contains *natC*, which is 99.8% identical to *tolC* of Z2491, and *natD*, with 99% identity to *hlyD* of Z2491 over the first 1,310 bases.

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