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Mu-Like Prophage in Serogroup B *Neisseria meningitidis* Coding for Surface-Exposed Antigens

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Sequence analysis of the genome of *Neisseria meningititdis* serogroup B revealed the presence of an ~35-kb region inserted within a putative gene coding for an ABC-type transporter. The region contains 46 open reading frames, 29 of which are colinear and homologous to the genes of *Escherichia coli* Mu phage. Two prophages with similar organizations were also found in serogroup A meningococcus, and one was found in *Haemophilus influenzae*. Early and late phage functions are well preserved in this family of Mu-like prophages. Several regions of atypical nucleotide content were identified. These likely represent genes acquired by horizontal transfer. Three of the acquired genes are shown to code for surface-associated antigens, and the encoded proteins are able to induce bactericidal antibodies.

Many mobile DNA elements transpose from one chromosomal location to another by a fundamentally similar mechanism. They include IS elements (25), transposons (20), phages (4), and more recently the so-called pathogenicity islands (8). These elements contribute substantially to genetic diversity and genome plasticity. Particularly, in pathogenic bacteria some of these elements may contribute to the exchange of genetic material coding for virulence traits. This mechanism may increase the fitness of bacterial strains through acquisition of virulence factors. Among the mechanisms for transfer of DNA, lysogenic conversion by bacteriophages appears to be advantageous; in fact, bacteriophages can carry large blocks of DNA and can survive harsh conditions. Bacteriophages may also code for virulence factors that allow the host bacterium to enlarge its host range and provide mechanisms to evade immune response. Examples of bacterial virulence factors carried on bacteriophages include the well-studied diphtheria toxin of *Corynebacterium diphtheriae* (1), cholera toxin (CTX) of *Vibrio cholerae* (11), the pore-forming toxin CTX of *Pseudomonas aeruginosa* (9), the erythrogenic toxins of *Streptococcus pyogenes* (24), the *Clostridium botulinum* neurotoxin (1), and the Shiga-like toxins and enterohemolysin produced by *Escherichia coli* (2, 15).

Neisseria meningitidis, a gram-negative capsulated bacterium, is a major cause of septicemia and meningitis that can kill children and young adults within hours. There are five pathogenic *N. meningitidis* serogroups (A, B, C, Y, and W135) as determined by capsular polysaccharide typing (26). Very recently, the genomic sequences of *N. meningitidis* serogroup B strain MC58 (22) and serogroup A strain Z2491 (17) have been determined, showing, among other features, a number of open reading frames (ORFs) with homology to phage functions. We analyzed the chromosomal region of serogroup B strain MC58 coding for these genes and compared it to the genomes of *N. meningitidis* serogroup A strain Z2491 (17) and the closely related bacterium *Haemophilus influenzae* strain Rd (5). Our analysis indicates that these genomes contain chromosomal regions with similarities to Mu-like phages. These phage DNA regions are clearly mosaic with obvious sequence similarity to phage Mu interspersed with segments that are apparently unrelated. We show that some genes mapping within the phage regions code for surface-exposed proteins capable of eliciting serum bactericidal response. A possible role of these proteins in bacterial virulence and vaccine development is discussed.

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MATERIALS AND METHODS

Computer analysis. The region spanning positions 1,099,626 to 1,134,164 of the serogroup B *N. meningitidis* genome strain MC58 (22) was analyzed for coding capacity by using databases and computer programs included in the Wisconsin Package (version 10.0; Genetics Computer Group [GCG], Madison, Wis.). We revisited each single ORF in order to assign the correct start codon on the basis of ribosomal binding sequence and promoter regions. Subsequently, the programs Psi-BLAST, FASTA, MOTIFS, FINDPATTERNS, and PSORT (http://psort.nibb.ac.jp), as well as the databases ProDom, Pfam, and Blocks were used to predict protein features and to assign putative functions. The selected region containing a hypothetical Mu-like prophage was screened for conservation against the complete genomes of *N. meningitidis* serogroup A available at the Sanger Center (17, http://www.genome.ou.edu/gono.html) and *H. influenzae* Rd available at The Institute for Genomic Research (TIGR) (5) (http://www.tigr.org/tdb/CMR/ghi/htmls/SplashPage.html) and against the partial *Neisseria gonorrhoeae* genomic sequences available at the Advanced Center for Genome Technology, University of Oklahoma (http://www.genome.ou.edu/gono.html). Identified prophage regions map within positions 1,768,530 to 1,807,766 (PNM1) and 1,207,176 to 1,236,496 (PNM2) of the serogroup A strain Z2491 genome and within positions 1,559,960 to 1,594,298 of the *H. influenzae* complete genome. The same analysis on coding capacity, ORF reassignments and functional predictions described for MuMenB has been carried out for DNA segments defining PNM1, PNM2, and MuHi.

Nucleotide composition study has been performed using the programs WINDOW and STATPLOT available in the GCG Package. For this analysis we have used a window size of 500 nucleotides with a shift increment of 3 nucleotides.

Cloning, expression, and protein purification. ORFs were amplified by PCR on chromosomal DNA from strain 2996 (23), with synthetic oligonucleotides used as primers. The amplified DNA fragments were cloned into pGEX-KG vector (7) to express the proteins as NH_2-terminal glutathione-*S*-transferase fusions. Expression of recombinant proteins was evaluated according to the appearance of protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant fusion proteins were purified by affinity chromatogra-

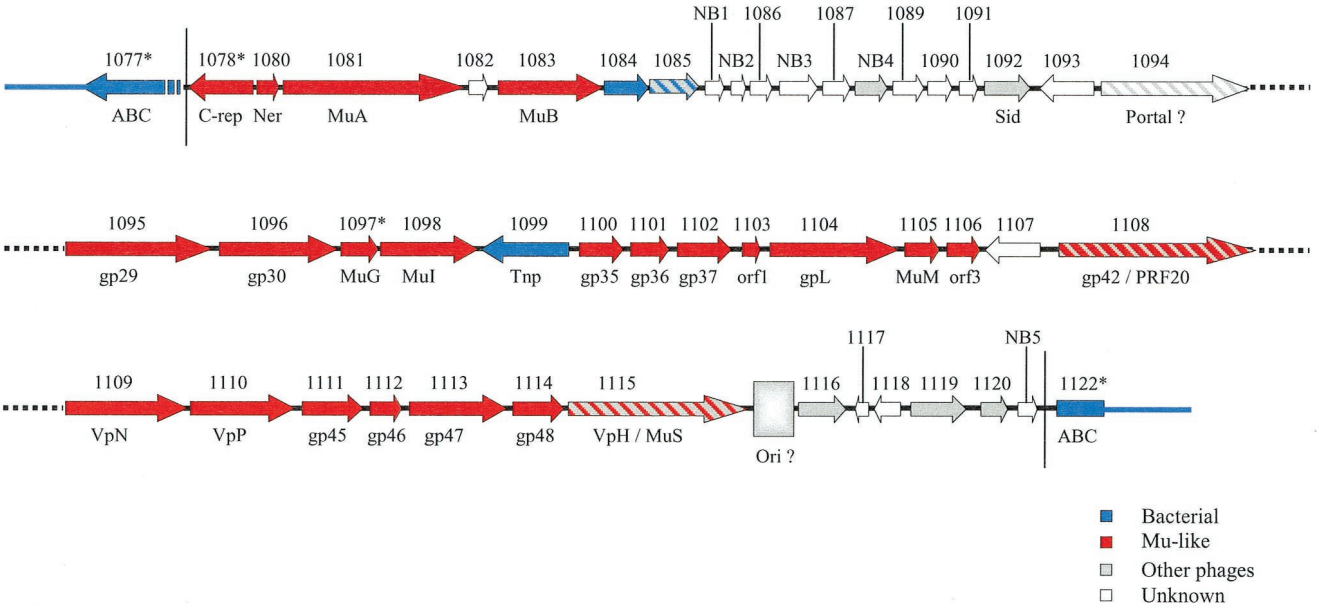

FIG. 1. Schematic representation of gene organization in prophage MuMenB of *N. meningitidis* strain MC58. For each gene, arrows indicate the direction of transcription and are scaled according to gene length. Numbers above arrows correspond to TIGR annotation (the suffix NMB has been omitted for simplicity in all cases). Newly annotated ORFs are marked NB1 to NB5 (for new in serogroup B). Putative functional assignments and correspondences to Mu homologues are reported below the arrows. Hypothetical sources of genes are color coded as indicated below the map. Hatched colored arrows represent genes for which source assignment may fall into two categories.

phy on glutathione-Sepharose 4B resin (Pharmacia). Twenty micrograms of each purified protein was mixed with Freund's adjuvant and used to immunize mice at days 1, 21, and 35. Blood samples were taken at days 34 and 49.

Serum analysis. (i) FACScan bacterium binding assay. *N. meningitidis* strain M7 (acapsulated) was grown on chocolate agar plates overnight at 37°C with 5% CO_2. Bacterial colonies were collected with a sterile Dacron swab and used to inoculate four tubes (8 ml each) of Mueller-Hinton broth (Difco) containing 0.25% glucose. Cells were harvested at an optical density at 620 nm (OD_{620}) of 0.35 to 0.5, washed, and resuspended in blocking buffer (1% bovine serum albumin in phosphate-buffered saline, 0.4% NaN_3) at an OD_{620} of 0.05. One hundred microliters of diluted sera (1:100, 1:200, 1:400) was added to 100 μl of bacterial cells in a 96-well plate (Costar), and incubated for 2 h at 4°C, washed with blocking buffer (200 μl/well), and 100 μl of 1:100 dilution of *R*-phycoerythrin-conjugated $F(ab')_2$ goat anti-mouse was added to each well and incubated for 1 h at 4°C. Cells were collected, washed, resuspended in PBS (200 μl/well)–phosphate-buffered saline 0.25% formaldehyde and transferred to FACScan tubes.

(ii) Bactericidal assay. *N. meningitidis* strain 2996 was cultivated overnight at 37°C on chocolate agar plates with 5% CO_2. Colonies were collected and used to inoculate 7 ml of Mueller-Hinton broth, containing 0.25% glucose, grown at 37°C with shaking to an OD_{620} of 0.23 to 0.24, and diluted to 10^5 CFU/ml in assay buffer (50 mM phosphate buffer [pH 7.2] containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.5% [wt/vol] bovine serum albumin). Serum bactericidal activity determination (18) was carried out in a final volume of 50 μl with 25 μl of serial twofold dilutions of test serum, 12.5 μl of bacteria at the working dilution, and 12.5 μl of baby rabbit complement (final concentration, 25%). Controls included bacteria incubated with complement serum and immune sera incubated with bacteria and with complement inactivated by heating at 56°C for 30 min. Immediately after the addition of the baby rabbit complement, 10 μl of the controls was plated on Mueller-Hinton agar plates using the tilt method (time zero). The 96-well plate was incubated for 1 h at 37°C with rotation. Seven microliters of each sample was plated on Mueller-Hinton agar plates as spots, whereas 10 μl of the controls was plated on Mueller-Hinton agar plates using the tilt method (time one). Agar plates were incubated for 18 h at 37°C, and the colonies corresponding to time zero and time one were counted.

RESULTS AND DISCUSSION

Identification of a Mu-like prophage in the genome of *N. meningitidis* serogroup B strain MC58. The annotation of the complete genome sequence of *N. meningitidis* serogroup B strain MC58 revealed the existence of 10 ORFs with striking amino acid similarities (identities ranging from 28.1 to 70.3%) to phage functions (22). These ORFs, interspersed within a genomic region of 20,995 bp spanning coordinates 1,101,155 to 1,131,150, include genes coding for regulatory functions of phage Mu (Ner, MuA, MuB) as well as genes coding for baseplate and tail functions of this phage (MuG, MuI, GpL, VpN, VpP, gp45, VpH). With the exception of a transposase of the IS*30* family (NMB1099 in reference 22), these phage functions are surrounded by ORFs of unknown functions (Fig. 1). Moreover, two partial ORFs, NMB1077 and NMB1122, with homologies to ABC transporters map upstream and downstream of the region under study, respectively. Reunion of these truncated ORFs gives rise to a complete ORF which shows 55% amino acid identity to a hypothetical ABC transporter ATP-binding protein of *H. influenzae* (5). This indicates that the ABC transporter-encoding gene was split upon integration of a DNA segment. Nucleotide sequence analysis of the region flanking the split transporter gene revealed two imperfect septamer direct repeats, 5′-CTCA(A/G)CA-3′. This repeated sequence might arise from a duplication event following integration of a large DNA segment of 34,539 bp spanning positions 1,099,626 to 1,134,164 of the *N. meningitidis* genome strain MC58 (22). A schematic representation of this DNA region is shown in Fig. 1. This ~35-kb DNA region includes 46 ORFs, most of which have been recently annotated (22), whereas 5 additional ORFs, named NB1 to NB5 (for new in serogroup B) (Fig. 1), have been identified, including previously unseen duplicated genes (see below).

A further analysis of the ORFs contained in this region highlighted an additional 19 ORFs displaying significant amino acid identities to phage proteins (Table 1). A total of 29 ORFs

TABLE 1. ORFs encoded within the prophage region of MuMenB and their genome positions[a]

MuMenB ORFs	Genome position	Length (aa[b])	ORF in:			
			Mu	MuHi	PNM1	PNM2
NMB1077*	1099075–1099616 (−)[c]					Not annotated
NMB1078*	1100312–1099875 (−)	235	C repressor	HI1476	NMA1884	
NMB1080*	1100822–1101061	80	Ner	HI1477	NMA1883	
NMB1081	1101126–1103108	661	MuA	HI1478	NMA1882	NMA1284
NMB1082	1103120–1103317	67				NMA1285*
NMB1083	1103481–1104650	390	MuB	HI1481	NMA1881*	NMA1286
NMB1084°	1104700–1105173	158				
NMB1085	1105319–1105861	181			NMA1864	NMA1303
NB1	1105861–1106062	68				
NB2	1106068–1106230	55			NMA1863	NMA1304
NMB1086	1106234–1106467	79			NMA1862	NMA1305
NB3	1106442–1106859	139			NMA1861	NMA1306
NMB1087	1106758–1107060	101			NMA1860*	NMA1307
NB4	1107160–1107506	115			NMA1858	
NMB1089	1107506–1107841	112			NMA1857	NMA1308/NMA1309*
NMB1090	1107856–1108119	88			NMA1856	NMA1310
NMB1091	1108119–1108313	65			NMA1855	NMA1311
NMB1092	1108319–1108822	168			NMA1854	NMA1312
NMB1093	1109412–1108825 (−)	196				
NMB1094*	1109425–1111045	540			NMA1852*	NMA1313
NMB1095	1111048–1112612	522	gp29	HI1501	NMA1851	NMA1314
NMB1096	1112602–1113894	431	gp30	HI1502	NMA1850	NMA1315
NMB1097*	1114006–114417	137	MuG	HI1503	NMA1849	NMA1316
NMB1098	1114653–1115711	353	Mul	HI1504	NMA1848	Not annotated*
NMB1099	1116767–1115805 (−)	321				
NMB1100°	1116795–1117274	160	gp35	HI1506	NMA1845	
NMB1101	1117277–1117696	140	gp36	HI1508	NMA1844	
NMB1102	1117746–1118336	197	gp37	HI1509	NMA1843	
NMB1103	1118336–1118530	65	gp38	HI1510	NMA1842	
NMB1104	1118536–1119942	469	gpL	HI1511	NMA1841	
NMB1105	1120010–1120384	125	MuM	HI1512	NMA1840	
NMB1106	1120391–1120753	121	ORF3	HI1513		
NMB1107	1121610–1121011 (−)	200				
NMB1108	1121780–1123933	723	gp42	HI1514	NMA1833 (N terminal)	
NMB1109	1123936–1125264	443	VpN	HI1515	NMA1831	NMA1319 (C terminal)
NMB1110	1125257–1126399	381	VpP	HI1516/fs	NMA1830	NMA1320
NMB1111	1126399–1127064	222	gp45	HI1518	NMA1829	NMA1321
NMB1112	1127168–1127512	115	gp46	HI1519	NMA1828	NMA1322
NMB1113*	1127528–1128580	350	gp47	HI1520	NMA1827/NMA1826*	NMA1323
NMB1114	1128580–1129137	186	gp48	HI1521	NMA1825	NMA1324
NMB1115	1129151–1131121	657	MuS (N terminal)	HI1522	NMA1824 (N terminal)	NMA1325
NMB1116	1131560–1132084	175				
NMB1117	1132350–1132204 (−)	49				NMA1326
NMB1118	1132762–1132478 (−)	95				NMA1327
NMB1119	1132842–1133444	201			NMA1823	NMA1328
NMB1120	1133426–1133719	98				NMA1329
NB5	1133719–1133926	69		HI1523 (C terminal)	NMA1821 (C terminal)	NMA1330
NMB1122*	1134173–1135151 (−)	326				Not annotated

[a] Corresponding annotated (5, 17, 22) and not annotated ORFs on serogroup A meningococcus (prophages PNM1 and PNM2) and *H. influenzae* (prophage MuHi) are also reported. Symbols: *, frame-shifted ORF; °, different start codon as compared to TIGR annotation; NB, new ORF in serogroup B meningococcus.

[b] aa, amino acid.

[c] (−), negative sense.

[d] ?, undetermined or uncertain.

TABLE 1—*Continued*

Homology(ies)	% Amino acid identity	Function or remarks
YE67 (*H. influenzae*) (C terminus)	58 (on 179 aa)	ABC transporter, putative
Repressor protein, Mu-like phage D3112	34	Putative repressor
Ner protein phage Mu	76	Negative regulator of transcription
Transposase A, phage D3112	30	Transposase
Transposase A, phage Mu	22	
None		?[d]
DNA transposition protein B, phage Mu	29	Bacteriophage integration and replication
LCND_LACLA (*Lactococcus lactis*)	29 (on 84 aa)	Coded on a plasmid, lipoprotein, secretion of lactococcin A
AMIB_ECOLI	28 (on 88 aa)	Cell wall hydrolase, lytic enzyme
None		Outer membrane, periplasmic
None		?
None		?
None		Outer membrane, periplasmic
None		Lipoprotein
10-kDa protein plasmid prfl (*Plectonema* sp.)	31 (on 77 aa)	?
None		?
None		?
None		?
Sid protein phage phi-R73	34 (on 70 aa)	Head size determination?
Outer surface protein OspC (*Borrelia* spp.)	30 (on 95 aa)	Antigenic protein
None		Portal protein? (deduced by location, size, and aa composition)
gp29 phage Mu	38	Head assembly
gp30 phage Mu	35 (on 250 aa)	Head assembly
ORF240 (*Dichelobacter nodosus*)	30 (on 177 aa)	
G protein phage Mu	30	Virion morphogenesis
I protein phage Mu	35	Virion morphogenesis
Transposase for IS1655	99	Transposase
gp35 phage Mu	35 (on 60 aa)	?
gp36 phage Mu	35 (on 140 aa)	Head-tail junction?
Hypothetical protein (*Pasteurella multocida*)	35 (on 112 aa)	
gp37 phage Mu	24 (on 95 aa)	Helicase?
Ban protein phage HP1	28 (on 94 aa)	
gp38 phage Mu (orf1)	30	?
gpL protein phage Mu	29	Sheath protein, major tail subunit
M protein phage Mu	24 (on 78 aa)	Tube gene
ORF3 phage Mu	27	?
None		Lipoprotein
PRF20 (*P. aeruginosa*)	25 (on 139 aa)	Tail length determination putative
ORF25 (phi-CTX)	21 (on 580 aa)	
gp42 phage Mu	21 (on 722 aa)	
VpN phage Mu	26 (on 157 aa)	Tail, DNA circulation, virion protein
VpP phage Mu	26	Tail protein
gp45 phage Mu	27	Baseplate assembly
gp46 phage Mu	41	Tail
gp47 phage Mu	30	?
PBSX prophage ORF xkdT	24	
gp48 phage Mu	25	?
Vph bacteriophage HP1	50 (on 528 aa)	Tail fiber
Protein S phage Mu (N terminus)	23 (on 293 aa)	
ORF20 (phi-CTX)	20 (on 412 aa)	
ORF35 (phi-CTX)	25 (on 103 aa)	?
None		?
None		?
ORF21 (phi-CTX) (N terminus)	46 (on 54 aa)	Tail assembly
ORF17 (phi-CTX)	32 (on 67 aa)	Baseplate?
None		?
YE67 (*H. influenzae*) (N terminus)	30 (on 64 aa)	ABC transporter, putative

out of 46 (63%) show homologies to phage functions, and 23 of these ORFs (50%) show homologies to functions of phage Mu. We conclude that this region was likely acquired by *N. meningitidis* strain MC58 upon infection with a Mu-like phage, subsequently referred to as MuMenB phage.

Similarities of the deduced MuMenB gene products to known sequences and functional assignments. By comparing the genetic map of phage Mu and the genetic map of the newly identified phage MuMenB (Fig. 2), we observed a certain degree of resemblance in the number of ORFs, their amino acid

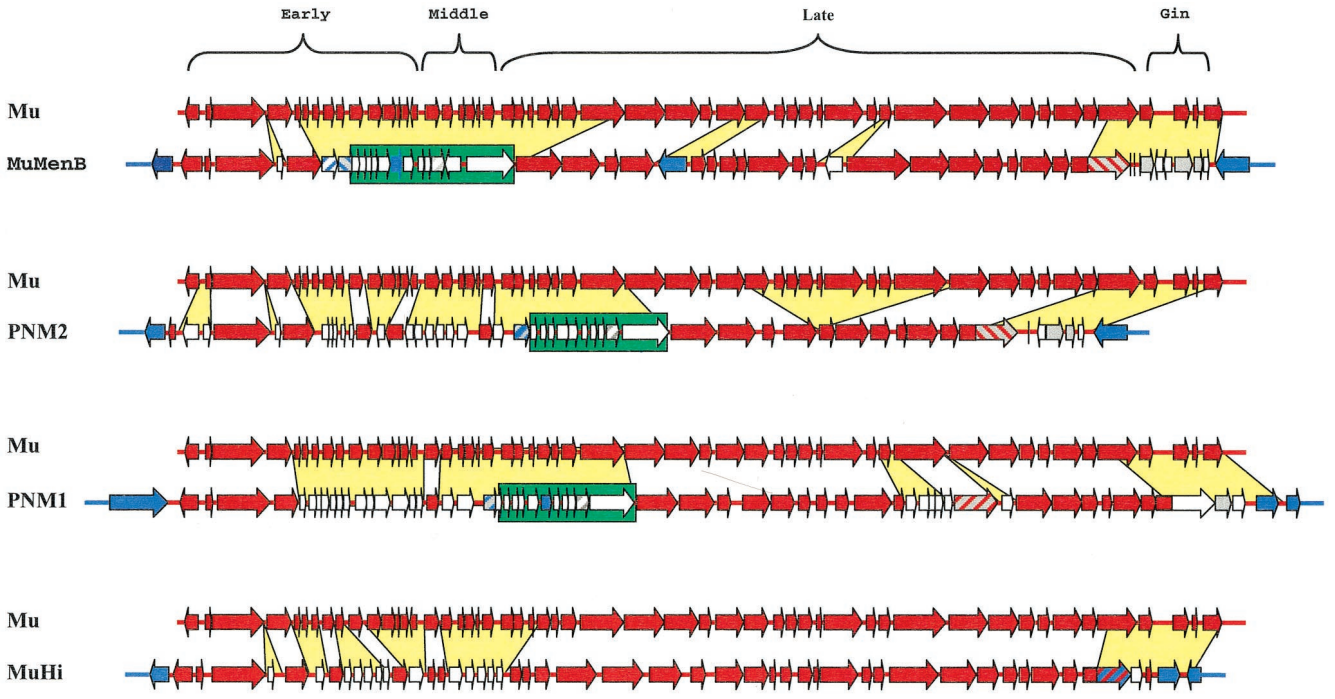

FIG. 2. Pairwise comparison between the structure of Mu (36,717 bp) and the indicated related Mu-like bacteriophages MuMenB (34,538 bp), PNM1 (39,237 bp), PNM2 (29,321 bp), and MuHi (34,339 bp). Green boxes highlight a group of ORFs specifically acquired by neisserial prophages, whereas yellow spaces indicate genes, which either have been inserted or differ from the corresponding region of Mu. Color codes are as described in the legend to Fig. 1 and on Fig. 1 itself.

length, and map positions between the two phages. Therefore, the amino acid sequences of the products deduced from the MuMenB ORFs were screened for similarities with sequences from the available databases and between each pair of the corresponding proteins from the two phages. The basic characteristics of the predicted gene products and the significant homologies found that allowed hypothetical functional assignments are described below and summarized in Table 1.

(i) Early region. Only 4 (NMB1078, NMB1080, NMB1081, and NMB1083) out of 19 early functions are conserved between MuMenB and Mu bacteriophage genomes (Fig. 1; Table 1). Probably, the missing early functions have been lost during or after phage integration. By contrast, the map positions and orientation of transcription of the conserved genes and of the downstream genes are identical between the two phages.

(ii) Middle region. A region of about 6 kb that includes 14 ORFs (NMB1084 to NMB1094 and NB1 to NB4) separates hypothetical early and late Mu-related functions. Of these 14 ORFs, NMB1092 shows homology (34% identity on 70 amino acids) to the Sid protein from phage phi-R73 (Fig. 1; Table 1). This protein has been suggested to function as determining head size with a DNA binding activity (21). While ORF NB4 and NMB1084 show similarity to proteins encoded by plasmid-borne genes, the other ORFs detected in this DNA region show no amino acid similarity to known protein. Moreover, it is worth noting that, whereas ORFs NB1 and NB2 are identical to ORFs NMB0988 and NMB0989, respectively, NB3 has a point mutation compared to NMB0990. Therefore, these three ORFs represent duplicated genes with one copy on the bacterial genome and one copy on the phage genome.

(iii) Late region. The major number of conserved functions between the MuMenB and Mu phages are mainly related to late functions such as to head assembly and to virion morphogenesis and tail proteins (Fig. 1; Table 1). This region spans ORFs NMB1095 to NMB1115. With the exception of ORFs NMB1099, which codes for the IS*1655* Tnp transposase, and NMB1107 (of unknown function), the map gene order and direction of transcription parallel those of phage Mu.

The rightmost region of the MuMenB genome contains six ORFs (NMB1116 to -1120 and NB5) either with unknown function or with functions homologous to those of phages different from Mu (Fig. 1; Table 1).

An apparent missing phage function is the lytic enzyme Lys, essential for host cell lysis to release mature phage particles from the cell wall, by breaking down the peptidoglycan. Nevertheless, NMB1085 displays homologies to a number of bacterial hydrolases; thus, it might be involved in bacterial lysis. Completely missing from the MuMenB genome are those functions related to immunity, proteins Mor and C, which function as positive regulators of middle and late transcription, respectively, and the Gin region (Fig. 2).

We conclude that most of the deduced functions mapping within this 35-kb DNA region of the *N. meningitidis* strain MC58 genome are similar to functions encoded by the bacteriophage Mu genome and, therefore, this region may represent the remnant DNA region evolved with the bacterial genome upon the Mu-like phage infection MuMenB. Evolution of the MuMenB prophage may account for the loss of some Mu functions and for the acquisition of functions related to other infecting phages. Likely, one of these acquired regions lies in

TABLE 2. Hypothetical location for ORFs mapping within the conserved region acquired by the *N. meningitidis* phages. Symbols are as in Table 1.

Features	MenB		MenA		
	MuMenB	Genome-encoded	PNM1	PNM2	Genome-encoded
Outer/periplasmic	NB1	NMB0988	NA1	NA1'	
	NB2	NMB0989	NMA1863	NMA1304	
	NMB1086		NMA1862	NMA1305	NMA1190
Outer/periplasmic	NB3	NMB0990*	NMA1861	NMA1306	
Lipoprotein	NMB1087		NMA1860*	NMA1307*	NMA1192*
Outer/periplasmic	NB4		NMA1858		
	NMB1089		NMA1857	NMA1308	
	NMB1090		NMA1856	NMA1310	
	NMB1091		NMA1855	NMA1311	NMA1197
DNA binding	NMB1092		NMA1854	NMA1312	NMA1198
	NMB1093*				
Portal protein	NMB1094		NMA1852	NMA1313	

the rightmost part of the MuMenB prophage and includes ORFs NMB1116 to NB5. Similarly, a wide region with ORFs of unknown functions (NMB1084 to NMB1094), including chromosomal duplicated genes, has replaced the missing Mu-related early-middle functions. Therefore, the structure of this phage genome is clearly mosaic, with regions of obvious sequence similarity interspersed with segments that are apparently unrelated. This argues not only for the existence of extensive horizontal genetic exchange among members of the Mu phages but also for extensive genetic exchange among phages from different families and with the bacterial genome (10).

Comparison of MuMenB with prophages in group A meningococcus and *H. influenzae*. We compared the genetic structure of the Mu functions with those of the two major regions PNM1 and PNM2 of the *N. meningitidis* serogroup A strain Z2491, which encode putative phage functions (17) as well as with the region coding for phage functions in *H. influenzae* strain Rd (5), which we call MuHi. Surprisingly, search for a similar region on the partial genomic sequence of *N. gonorrhoeae* strain FA1090 (http://www.genome.ou.edu/gono.html) revealed no corresponding clusters of Mu-related functions. Accordingly, it has been recently reported (12), that a region corresponding to phage PNM1 of *N. meningitidis* serogroup A represents a specific genetic island missing in *N. gonorrhoeae*.

As schematized in Fig. 2, the Mu-like prophages MuMenB, PNM1, PNM2, and MuHi share a similar overall gene organization with most of the phage Mu functions. Interestingly, colinearity of gene order is preferentially and extensively interrupted within the early-middle region of these phages. This region of phage Mu, starting downstream from *muB*, includes 21 ORFs, which have been described as coding for nonessential or growth-enhancing functions (16). The functions that are not Mu related that were detected in this region include 14 ORFs for MuMenB, 22 ORFs for PNM1, 28 ORFs for PNM2, and 12 ORFs for MuHi.

The genome segment corresponding to the *gin* invertase region of phage Mu appears to be replaced by functions likely acquired from other phages. Twenty-nine tandem repeats of 13 bp that could represent an origin of DNA replication are detected in this region of the MuMenB genome (Fig. 1) but not in the other phages. By contrast, late functions could represent the target for gene variability by means of gene insertions and/or deletions and/or substitutions (Fig. 2). While most of the acquired ORFs display no homology to known proteins in databases and are not conserved among the phages, a subset of 12 ORFs mapping within the early-middle region are found in the three phages of *N. meningitidis* (Fig. 2). Their features, as deduced by computer algorithms (Motifs and PSORT) are reported in Table 2. Intriguingly, some of these ORFs are predicted to encode membrane-associated proteins, and some of them are duplicated within the *N. meningitidis* genomes. From the evolutionary point of view, some of these ORFs may have been acquired simultaneously as a cluster of genes, with others being acquired as a single gene acquisition. Therefore, evolution of these phages could have been achieved by a stepwise mechanism of gene acquisition, thus generating a mosaic genome structure whose products may contribute to *N. meningitidis* pathogenicity.

Horizontally acquired regions. Horizontal transfer of DNA between species is well documented and is often associated with evolution of pathogenicity and drug resistance (6, 13). Bacteriophages may play an important role in acquisition of new genetic information, acting either as carriers of DNA fragments or as specialized systems for virulence-related genes (3, 4). Regions of DNA that have been acquired by horizontal transfer are often characterized by atypical DNA composition relative to the rest of the genome. One example is the cytotoxin-converting phage phi-CTX of *P. aeruginosa* (9). This phage shows an extensive homology to and a gene arrangement similar to that of coliphage P2 and P2-related Mu-like phages, and it carries the cytotoxin gene coding for the pore-forming toxin inserted within a region of atypical nucleotide content (14). Therefore, G+C composition study was used to identify recently acquired regions within neisserial prophages with results reported in Fig. 3.

In all bacteriophages here reported, as well as in phage Mu, the region located at the leftmost terminus displays a lower G+C content relative to the overall corresponding phage genomes and includes the genes coding for the C-repressor and Ner proteins (Fig. 1 and 2). The intrinsic property of a low G+C content in this region may indicate an evolutionary constraint unrelated to DNA transfer.

The early-middle regions of MuMenB and PNM2 display a low G+C content, with peaks corresponding to a few specific DNA fragments. In MuMenB, these regions include ORFs NMB1084 and NMB1093, with a G+C value of 38 and 40%,

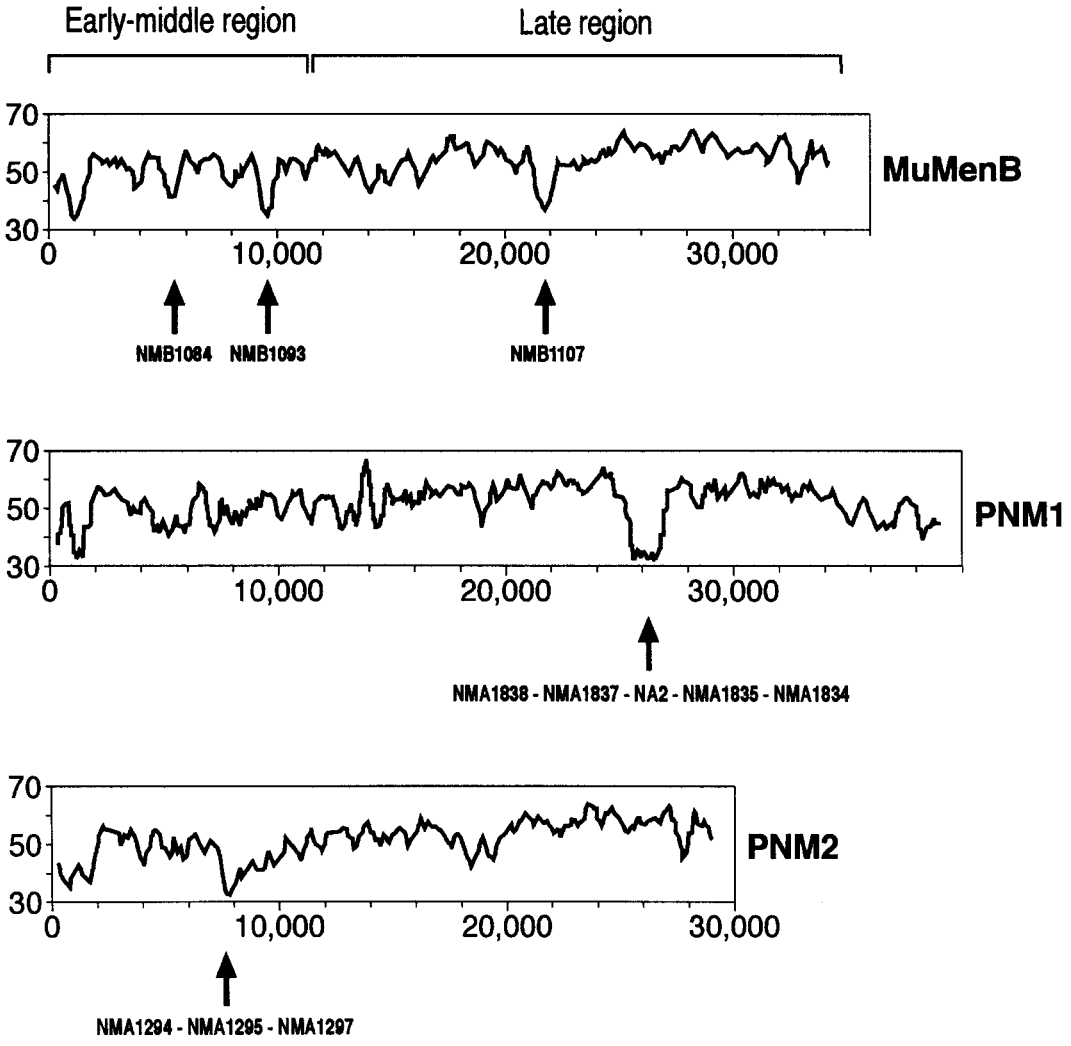

FIG. 3. G+C content plots derived for neisserial prophages. The *x* and *y* axes report base positions and percentage of G+C, respectively. Arrows indicate peaks of atypical G+C composition along with the ORFs encoded therein. The graphs were obtained by using the computer programs WINDOWS and STATPLOT of the GCG Package.

respectively. Noteworthy, NMB1084 shows a putative leader peptide characteristic of lipoproteins and shares no amino acid homologies to known proteins, whereas NMB1093, although lacking a predicted signal peptide, shows significant similarity (Table 1) to the variable outer surface protein C (OspC) of *Borrelia* species. Both ORFs are absent from the corresponding early-middle region of PNM2, thus explaining why no peaks of low GC content are present within this region of PNM2. Nevertheless, another segment of atypical composition (G+C = 36.8%) is evident in the early-middle portion of PNM2, and this includes ORFs NMA1294, NMA1295, and NMA1297, specific for prophage PNM2. Interestingly, the three ORFs share a significant (44 to 54% identity) degree of amino acid similarity to each other, thus suggesting that these genes have evolved from a common ancestor gene. By contrast, the early-middle region of PNM1 shows a nucleotide composition that, on the whole, approximates the average value calculated for the whole genome (G+C = 52%).

Another region displaying an atypical nucleotide content maps within the middle portion of the late transcriptional unit and is present in MuMenB and PNM1 prophages (Fig. 3). In MuMenB the inserted DNA fragment (G+C = 39%) contains a single gene (*nmb1107*) in the reverse orientation, which codes for a predicted lipoprotein with no significant homologies to know proteins. The corresponding region of PNM1 shows a G+C content of 38.6% and codes for five ORFs (NMA1838, NMA1837, NA2 [for new in serogroup A], NMA1835, and NMA1834), including one which was not reported in the previous annotation (17). Of these, NMA1838 shows a homology to a regulatory protein of *Streptomyces coelicolor*, NMA1837 is characterized by a zinc-metalloendopeptidase motif, and NA2 is predicted to be a membrane protein. These segments of the two MuMenB and PNM1 phages very likely correspond to recent insertion events occurring upstream of the genes coding for the tail length determination proteins.

Some genes acquired by MuMenB encode surface-exposed antigens. The mosaic genetic architecture of the neisserial

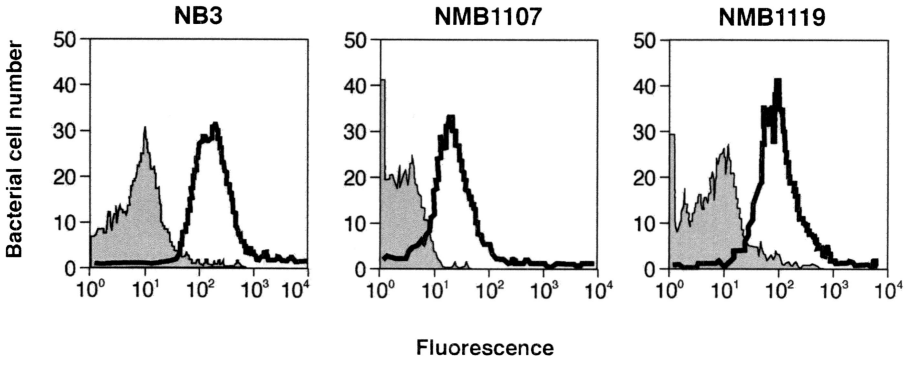

FIG. 4. FACS analyses showing binding of polyclonal NB3, NMB1107, and NMB1119 antisera to the ethanol-treated homologous strain 2996. Gray profiles show binding of preimmune sera; white profiles show binding of immune sera.

phages indicates the existence of acquired conserved genes as well as of genes unique to each phage with hypothetical assigned or unknown functions (Fig. 1, Fig. 2, and Table 1). We selected three ORFs mapping to different positions in the phage genome for further characterization. ORF NB3 is in common with the three phages and maps within the region of conserved ORFs into the hypervariable early-middle region (Fig. 2); it was originated by gene duplication from the genome, and it may code for an outer membrane protein (Table 2). NMB1107 is likely to represent a recently acquired gene (Fig. 3), it is MuMenB specific (Fig. 2), and it may code for a lipoprotein (Table 1). NMB1119 is in common to MuMenB and PNM1 and has a homolog in PNM2 (27% amino acid identity); it maps within the 3′ end of the genomes, with features similar to a phage function different from that of Mu, and it may code for a tail assembly protein. These proteins are likely to be membrane associated on the bacterial envelope.

To assess whether these proteins are exposed on the bacterial surface, we raised antibodies against recombinant proteins in mice and used the immune sera in fluorescence-activated cell sorter (FACS) analysis (Materials and Methods). As shown in Fig. 4, the three immune sera recognized the heterologous *N. meningitidis* strain M7, suggesting that these proteins are exposed on the surface of the cell, therefore confirming the predicted computer search and analyses.

Very recently, we have reported that surface-exposed proteins can be used as vaccine components against group B *N. meningitidis* strains (19). Therefore, FACS results prompted us to test whether immune sera obtained against proteins NB3, NMB1107, and NMB1119 can exert bactericidal activity, which in turn correlates with protection in humans. Immune sera have been tested for complement-mediated bacteriolysis against strain 2996 as described by Pizza et al. (19) and in Materials and Methods of the present work. Interestingly, antisera against proteins NB3, NMB1107, and NMB1119 showed a bacterial killing activity, reducing to 50% the number of viable bacterial cells, at 1:32, 1:32, and 1:64 dilutions, respectively. We conclude that these recombinant proteins may elicit bactericidal immune response, and therefore, these should be considered for vaccine development studies.

This conclusion is further substantiated by preliminary data obtained on the high degree of amino acid conservation for protein NMB1119 among meningococcus strains. Deduced amino acid sequences of gene NMB1119 from five different serogroup B strains (MC58, 1000, 2996, BZ133, and NGH38) and two serogroup A strains (Z2491 and F6124) (17, 22) revealed an amino acid conservation ranging from 93.7 to 97.0% amino acid identity (data not shown). This, suggests that at least this protein is conserved among serogroup B strains.

Conclusions. We have reported the identification of chromosomal DNA regions of *N. meningitidis* strains that represent remnants of a Mu-like phage infection. Likely, this phage originally infected the bacterium and subsequently acquired specific genes to spread itself among a population of different strains. This is supported by the observation that a few of these genes are duplicated genes with one copy still residing within the bacterial chromosome. By contrast, some of the acquired genes seem to be unique to a given strain, thus suggesting a peculiar function in the host strain. Interestingly, computer search and comparison analyses suggest that both specific and common genes might code for membrane-associated proteins. This suggests that these proteins contribute to the variability in envelope structure and composition and may influence virulence and pathogenicity. Also, three of these proteins can be added to the list of vaccine candidates recently discovered among the meningococcal genome by Pizza and coworkers (19).

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REFERENCES

1. **Barksdale, L., and S. B. Arden.** 1974. Persisting bacteriophage infections, lysogeny, and phage conversions. Annu. Rev. Microbiol. **28:**265–299.
2. **Beutin, L., U. H. Stroeher, and P. A. Manning.** 1993. Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. Gene **132:**95–99.
3. **Bishai, W. R., and J. R. Murphy.** 1988. Bacteriophage gene products that cause human disease, p. 683–724. *In* R. Calendar (ed.), The bacteriophages, vol. 2. Plenum Press, New York, N.Y.
4. **Cheetham, B. F., and M. E. Katz.** 1995. A role for bacteriophages in the evolution and transfer of bacterial virulence determinants. Mol. Microbiol. **18:**201–208.
5. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
6. **Fuchs, T. M.** 1998. Molecular mechanisms of bacterial pathogenicity. Naturwissenschaften **85:**99–108.

7. **Guan, K. L., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:**262–267.
8. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
9. **Hayashi, T., H. Matsumoto, M. Ohnishi, and Y. Terawaki.** 1993. Molecular analysis of a cytotoxin-converting phage, phiCTX, of *Pseudomonas aeruginosa*: structure of the *attP-cos-ctx* region and integration into the serine tRNA gene. Mol. Microbiol. **7:**657–667.
10. **Hendrix, R. W., M. C. M. Smith, R. N. Burns, M. E. Ford, and G. F. Hatfull.** 1999. Evolutionary relationships among bacteriophages and prophages: all the world's a phage. Proc. Natl. Acad. Sci. USA **96:**2192–2197.
11. **Karaolis, D. K., S. Somara, D. R. Maneval, Jr., J. A. Johnson, and J. B. Kaper.** 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature **399:**375–379.
12. **Klee, S. R., X. Nassif, B. Kusecek, P. Merker, J.-L. Beretti, M. Achtman, and C. R. Tinsley.** 2000. Molecular and biological analysis of eight genetic islands that distinguish *Neisseria meningitidis* from the closely related pathogen *Neisseria gonorrhoeae*. Infect. Immun. **68:**2082–2095.
13. **Morschhauser, J., G. Kohler, W. Ziebuhr, G. Blum-Oehler, U. Dobrindt, and J. Hacker.** 2000. Evolution of microbial pathogens. Phil. Trans. R. Soc. Lond. B Biol. Sci. **355:**695–704.
14. **Nakayama, K., S. Kanaya, M. Ohnishi, Y. Terawaki, and T. Hayashi.** 1999. The complete nucleotide sequence of phi CTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa*: implications for phage evolution and horizontal gene transfer via bacteriophages. Mol. Microbiol. **31:**399–419.
15. **Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. Science **230:**179–181.
16. **Paolozzi, L., and N. Symonds.** 1987. The SE region, p. 53–62. *In* N. Symonds, A. Toussaint, P. van de Putte, and M. M. Howe (ed.), Phage Mu. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R. M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, M. A. Rajandream, K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt, and B. G. Barrel.** 2000. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. Nature **404:**502–506.
18. **Peeters, C. C., I. J. Claassen, M. Schuller, G. F. Kersten, E. M. van der Voort, and J. T. Poolman.** 1999. Immunogenicity of various presentation forms of PorA outer membrane protein of *Neisseria meningitidis* in mice. Vaccine **17:**2702–2712.
19. **Pizza, M., V. Scarlato, V. Masignani, M. M. Giuliani, B. Aricò, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C. L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, E. Blair, T. Mason, H. Tettelin, D. W. Hood, A. C. Jeffries, N. J. Saunders, D. M. Granoff, C. J. Venter, E. R. Moxon, G. Grandi, and R. Rappuoli.** 2000. Whole genome sequencing to identify novel vaccine candidates against serogroup B meningococcus. Science **287:**1816–1820.
20. **Salyers, A. A., N. B. Shoemaker, A. M. Stevens, and L. Y. Li.** 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. **59:**579–590.
21. **Sun, J., M. Inouye, and S. Inouye.** 1991. Association of a retroelement with a P4-like cryptic prophage (retronphage phi R73) integrated into the selenocystyl tRNA gene of *Escherichia coli*. J. Bacteriol. **173:**4171–4181.
22. **Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D. Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, H. Cittone, E. B. Clark, M. D. Cotton, T. R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H. O. Smith, C. M. Fraser, E. R. Moxon, R. Rappuoli, and J. C. Venter.** 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science **287:**1809–1815.
23. **van der Ley, P., and J. T. Poolman.** 1992. Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. Infect. Immun. **60:**3156–3161.
24. **Weeks, C. R., and N. N. Ferretti.** 1986. Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T12. Infect. Immun. **52:**144–150.
25. **Weinert, T. A., N. A. Schaus, and N. D. Grindley.** 1983. Insertion sequence duplication in transpositional recombination. Science **222:**755–765.
26. **Yakubu, D. E., F. J. Abadi, and T. H. Pennington.** 1999. Molecular typing methods for *Neisseria meningitidis*. J. Med. Microbiol. **48:**1055–1064.

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