A chromosomally integrated bacteriophage in invasive meningococci

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Cerebrospinal meningitis is a feared disease that can cause the death of a previously healthy individual within hours. Paradoxically, the causative agent, *Neisseria meningitidis*, is a common inhabitant of the human nasopharynx, and as such, may be considered a normal, commensal organism. Only in a small proportion of colonized people do the bacteria invade the bloodstream, from where they can cross the blood–brain barrier to cause meningitis. Furthermore, most meningococcal disease is caused by bacteria belonging to only a few of the phylogenetic groups among the large number that constitute the population structure of this genetically variable organism. However, the genetic basis for the differences in pathogenic potential remains elusive. By performing whole genome comparisons of a large collection of meningococcal isolates of defined pathogenic potential we brought to light a meningococcal prophage present in disease-causing bacteria. The phage, of the filamentous family, excises from the chromosome and is secreted from the bacteria via the type IV pilin secretin. Therefore, this element, by spreading among the population, may promote the development of new epidemic clones of *N. meningitidis* that are capable of breaking the normal commensal relationship with humans and causing invasive disease.

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Abbreviations used: MDA, meningococcal disease associated; ORF, open reading frame; ST, sequence type.

The normal relationship of the meningococcus with humans is one of asymptomatic carriage. The reasons why some strains of meningococcus disseminate from their niche to cause septicemia and meningitis are not well-understood, but certainly depend on host and bacterial factors. The most important host factors are the presence of specific antibodies (1) and a functional complement system (2). The importance of bacterial factors—suggested by early epidemiological studies—was confirmed by the introduction of multilocus enzyme electrophoresis (3, 4), and later, multilocus sequence typing (5), which demonstrated that the large majority of meningococcal disease worldwide is caused by a small minority of "hyperinvasive" phylogenetic groups (clonal complexes, defined on the basis of the sequence type (ST), which are associated with a high case/carrier ratio). Factors demonstrated to be necessary for disease, such as the protective capsular polysaccharide and the type IV pilus adhesin, are distributed widely among meningococci, irrespective of their association with disease. These suggest the existence of undiscovered attributes that contribute to the capacity of some meningococci to cause disease.

In the case of this specifically human disease, animal and cell culture models generally address only a single aspect of pathogenesis, and are not well-adapted to search for new factors that might change the habit of the meningococcus from one of a commensal to a pathogenic organism. To gain insight into the factors that influence this aspect of the human–meningococcal interaction, we compared the genomes of a large collection of strains of differing pathogenic potentials; this allowed the identification of genes on the basis of their association with disease. This technique detected an element corresponding to a novel prophage of the filamentous bacteriophage family that is present

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specifically in strains that have the potential to cause disease.

RESULTS

An 8-kb genetic island is specific to hyperinvasive meningococcal complexes

To investigate the genetic basis of the increased pathogenic potential of disease-causing meningococci, we performed whole genome comparisons between representatives of the major hyperinvasive clonal complexes that are responsible for disease worldwide (29 isolates belonging to nine separate STs), and those belonging to clonal groups that have no association with disease (20 isolates belonging to nine STs; Table S1, available at http://www.jem.org/cgi/content/full/jem.20050112/DC1). The DNA arrays corresponded to the genome of isolate Z2491, a commonly used laboratory strain isolated in 1983 from an epidemic occurring in the Gambia (6) (http://www.sanger.ac.uk/Projects/N_meningitidis/seroA/strain.shtml). Data are deposited in the Array Express database (http://www.ebi.ac.uk/arrayexpress/query/entry) with accession no. (E-MEXP-343). Of the 1950 amplicons (representing 92% of the 2121 predicted open reading frames (ORFs) for isolate Z2491) represented on the arrays, 1532 (79%) were present in all meningococcal isolates examined. The percentage of isolate Z2491 sequences present in the strains tested was 94% (±0.9) for the hypervirulent lineages, and 93% (±1.3) for the nonvirulent lineages; these figures were consistent with the known levels of diversity of this organism. However, isolates with the same sequence type had similar genomic content profiles, and demonstrated congruence between the DNA array technique and the clonal complexes defined by multilocus sequence typing.

No gene satisfied the condition that it be present in all 29 invasive isolates and none of the 20 noninvasive isolates. However, a single group of genes (*NMA1792–NMA1799*) of 8 kb (Fig. 1 A) was associated significantly with the hypervirulent isolates (Table S2, available at http://www.jem.org/

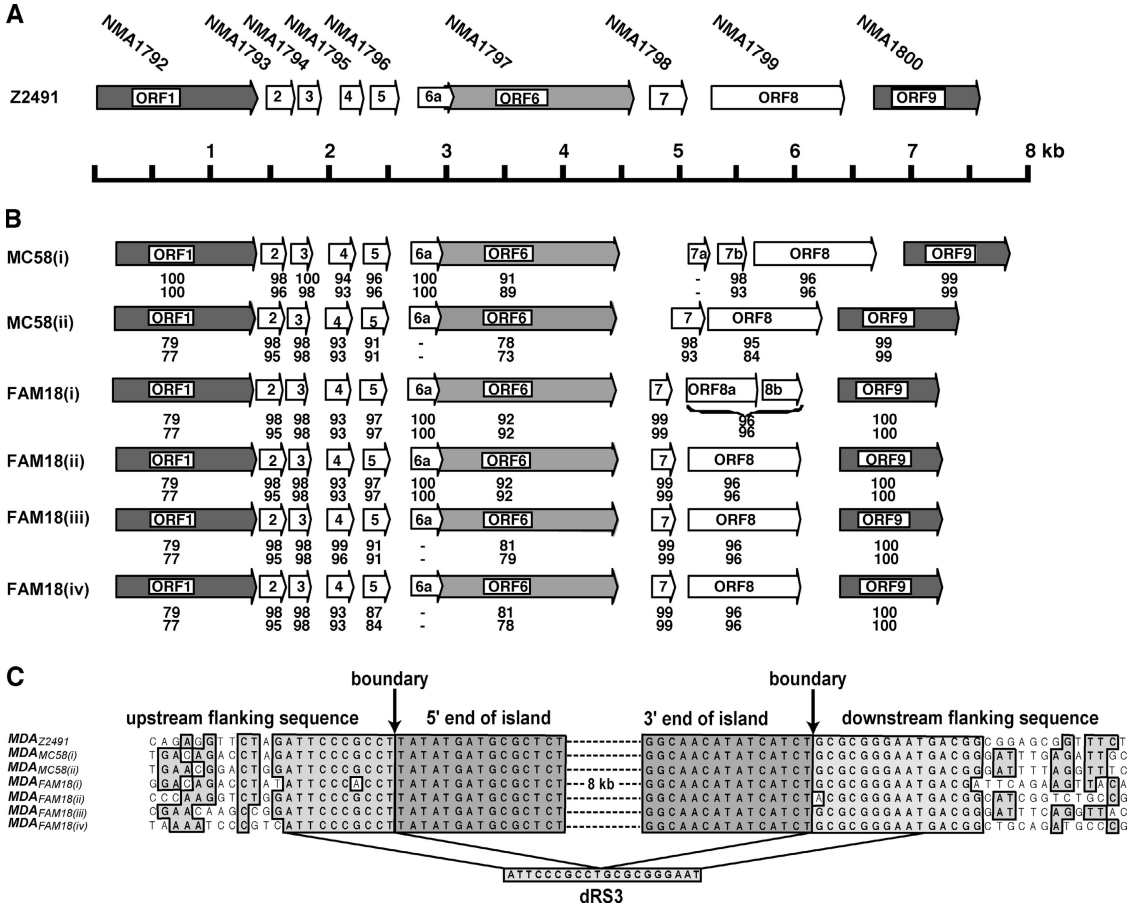

Figure 1. Organization of the genetic island in *N. meningitidis*. (A) Genes are numbered according to the Z2491 genome sequencing project (9). (B) Genetic organization of the islands of other sequenced meningococcal genomes (two in strain MC58 and four in strain FAM18). Percentage protein (upper) and nucleotide (lower) identities to Z2491 are indicated; a dash (-) shows an absence of homology with proteins of the Z2491 island. (C) The sequences (ATTCCCNC and GNGGGGAAT), present several hundred times in the meningococcal chromosome as part of a 20-bp inverted repeat (dRS3: ATTCCCNNNNNNNNGGGAAT), constitute the chromosomal point of insertion of the island, which was confirmed by sequencing of the circular form of the element.

Table I. Properties of the island's ORFs

Gene	Protein length	Presence in meningococcal strains: In 29 "invasive"	Presence in meningococcal strains: In 20 "noninvasive	Protein homologies (BlastP)[a]	Conserved domains (BlastP)[b]
ORF1, NMA1792	429	27	0	ORF C7 [*Ralstonia solanacearum* plasmid] (NP_052309.1), 9×10^{-24}; RstA1 protein [*Vibrio cholerae* prophage] (NP_231106.1), 1×10^{-11}	replication initiation factor (pfam02486), 6×10^{-42}; phage replication protein RstA (COG2946), 4×10^{-143}
ORF2, NMA1793	104	29	1	NS[c]	NS
ORF3, NMA1794	67	29	1	NS	NS
ORF4, NMA1795	77	29	1	NS	NS
ORF5, NMA1796	102	29	1	NS	NS
ORF6, NMA1797	547	29	2	NS	Neisserial TspB proteins
ORF7, NMA1798	95	29	2	NS	NS
ORF8, NMA1799	401	29	2	ORF C6 [*Ralstonia solanacearum* plasmid] (NP_052316.1), 2×10^{-31}; phage-related protein [*Xylella fastidiosa*] (NP_779131.1), 3×10^{-18}	zonular occludens toxin (Zot) (pfam05707), 7×10^{-47}; NS
ORF9, NMA1800	323	26	0	transposase [*Escherichia coli* IS621] (BAC76887.1), 3×10^{-37}	transposase (COG3547), 1×10^{-17}

[a]This column's data are organized as "protein [species] (identifier), significance."
[b]This column's data are organized as "family (identifier), significance."
[c]NS, no significant homologies were found.

cgi/content/full/jem.20050112/DC1). This gene cluster was present in 100% (29/29) of these isolates and absent from 90% (18/20) of the noninvasive isolates. Another amplicon corresponding to a frame (*NMA0776*) that was highly similar (42% DNA base identity, 53% amino acid identity) to one of the genes (*NMA1797*) in the above cluster showed the same distribution. It was striking that these two groups were the only genes to have this extreme distribution. The genes showing the next highest degree of association with the virulent strains (*NMA1283–NMA1285*, part of a possible prophage pnm2; reference 6) were present in 9 out of 20 of the noninvasive isolates. These were the only genes to have this extreme distribution; the probability of finding a gene having as significant an association with the invasive isolates as the above group by chance alone was calculated as 0.007. Thus, the 8-kb genetic island is specifically present in isolates belonging to the invasive complexes of *N. meningitidis*. The island was equally present as a defined genetic unit in the published genomes of two other isolates, MC58 and FAM18, of invasive clonal complexes (ST-32 and ST-11, respectively; Fig. 1 B). Although located in different places on the chromosomes of the sequenced isolates, the elements were highly homologous at their 5′ and 3′ ends; this allowed the definition of a site of insertion (Fig. 1 C) that corresponded to the "dRS3 repeats" (6) which are present several hundred times in the meningococcal genome.

The genetic island corresponds to a filamentous bacteriophage genome

DNA sequence analysis revealed little homology to genes of known function. However, the genetic island did demonstrate (a) a low G+C content (42%, compared with 52% for the chromosome in total); (b) homology of the first ORF to bacteriophage replication proteins (RstA of CTX-phi of *Vibrio cholerae*; Table I); and (c) a resemblance in size and arrangement of ORFs to f1- or M13-type bacteriophages (Fig. 2 A), a group that includes the phage CTX-phi, was shown to carry the cholera toxin (7). It seemed probable that the island was a mobile genetic element that had been acquired horizontally, and moreover, in view of the different locations of these islands in the chromosome, by independent events in different invasive lineages. This idea was reinforced by the discovery of a circular, double-stranded form in the cytoplasm, and a nuclease-resistant circular form of the element in the supernatant of bacterial cultures that were prepared by standard bacteriophage purification procedures (Fig. 2, B and C; references 8 and 9). Dot blots of the extracellular material reacted with a ^{32}P-labeled negative—but not positive—strand probe (unpublished data). The result was supported by PCR analysis which demonstrated specific amplification by a primer corresponding to the negative—but not the positive—strand DNA (Fig. 3). We were unable to detect the DNA on Southern blots using single-stranded probes which suggested that the element was secreted in low quantities. Together, these data provide evidence that the element corresponds to an integrated phage genome that is secreted as nuclease-resistant, positive, single-stranded DNA from the bacteria that carry it.

Mutational analysis of selected ORFs was performed to test the hypothesis of homology to the filamentous bacteriophage family. The mutations, interpreted in light of the functions of the homologous proteins in bacteriophage M13,

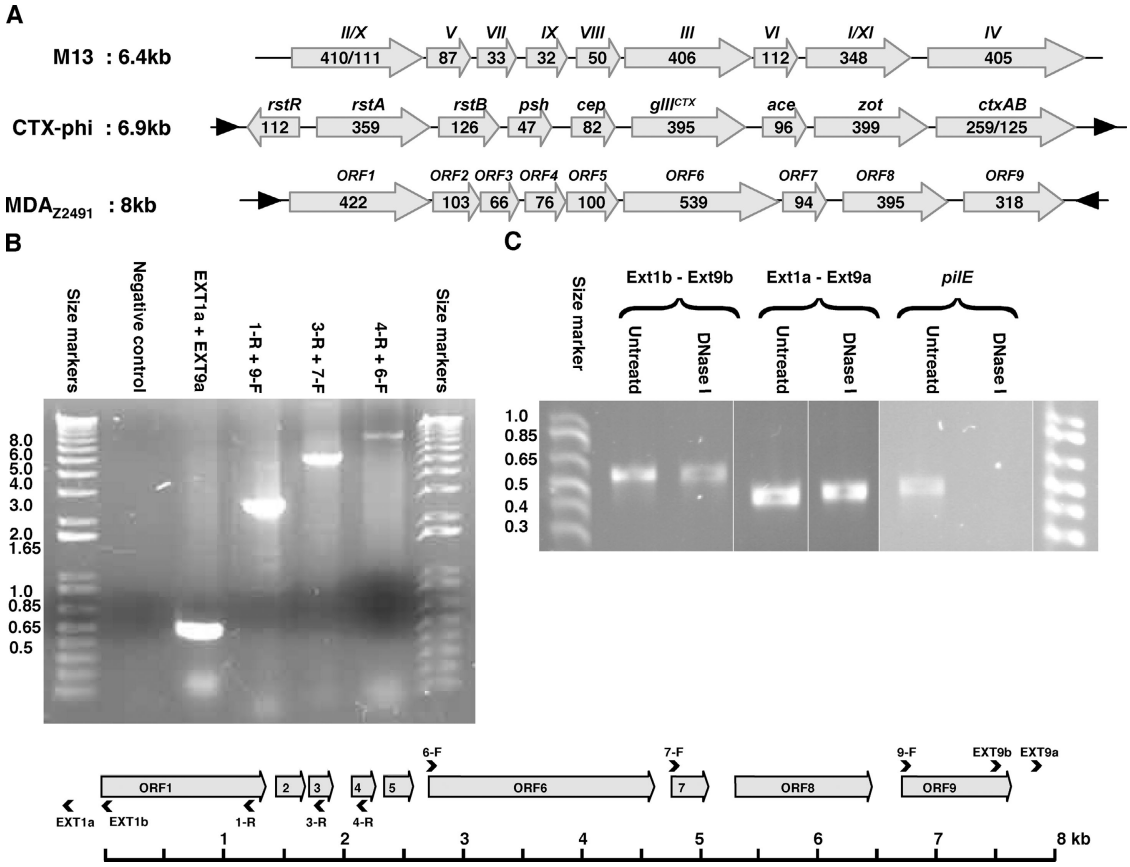

Figure 2. Preliminary characterization of the genetic island. (A) Comparison of the organization of the genetic island with that of coliphage M13 and *V. cholerae* bacteriophage CTX-phi (after reference 20). (B) The island exists in an extrachromosomal, closed-circular form. PCR amplifications of total DNA of strain Z2491 with oligonucleotide pairs directed toward opposite ends of the element yielded fragments of sizes expected from a circular form of the island (EXT1a + EXT9a, 0.43 kb; EXT1b + EXT9b, 0,66 kb; 1-R + 9-F, 2.4 kb; 3-R + 7-F, 5.0 kb; 4-R + 6-F, 7.4 kb). Positions of the oligonucleotides are shown on the map below the gel figures. (C) The element is secreted in a nuclease-resistant form. Treatment with DNase I had no significant effect on the material amplifiable by the circular form–specific primer pairs EXT1a-EXT9a or EXT1b-EXT9b (note that the difference in intensities of the products EXT1b-EXT9b is due to the typical experimental variation). In contrast, the signal due to contaminating chromosomal DNA (the pilin gene pilE, amplified with pilin-specific primers) was abolished by nuclease treatment.

had the predicted effects on production of the replicative (cytoplasmic) or the extracellular form (Fig. 4, A–C). Inactivation of *ORF1*, encoding the replication protein, abolished the intracellular and extracellular circular forms. Inactivation of *orf6*, phage M13 gene III homologue, reduced secretion of the element (10), whereas that of *ORF8*, homologue of gene I/XI, a protein necessary for phage secretion (11), abolished the extracellular form. Filamentous phages are secreted through a channel formed by a protein known as a secretin. In the case of CTX-phi, the phage uses a chromosomally encoded secretin involved in protein secretion (12). The only identified secretin in *N. meningitidis* is PilQ, responsible for extrusion of the type IV pilus through the outer membrane; as expected, inactivation of the chromosomal gene *pilQ* abolished secretion of the element (Fig. 4 D), thus linking the process of phage production with that of type IV piliation. (Most meningococci express PilQ because pili are necessary for effective colonization). However, the secretion of the element seems to be independent of the pilus extrusion mechanism. A nonpiliated mutant secreted the genetic island DNA in similar quantities to the wild-type strain, and, in contrast to the *ORF8* mutant (Fig. 4), a *pilF* mutant did not produce diminished quantities of the secreted form (not depicted).

The genetic island is associated with disease

Because of the necessary simplification of the population studied in the initial microarray analysis (we considered only the extremes of pathogenic potential), we now needed to investigate whether the reasons for the association were clonal or causal (i.e., whether the element was present by chance in each of a set of clonal complexes which are invasive, or whether the presence of the element rendered these complexes invasive). Therefore, we performed a more detailed analysis of the presence of the island in a large strain collection from the Czech Republic (13, 14) where carried and disease-causing bacteria were collected as part of an epidemi-

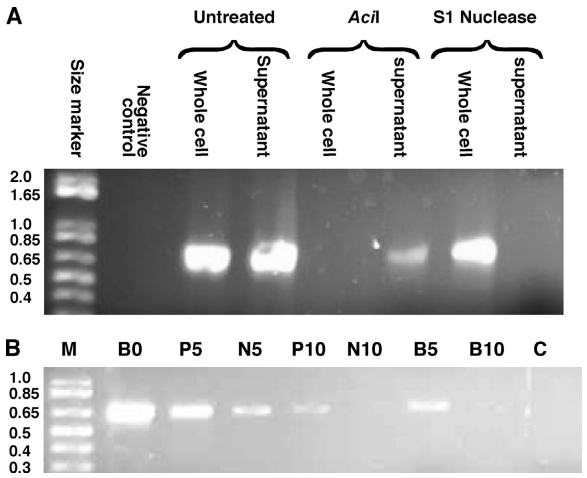

Figure 3. The extracellular form is positive, single-stranded DNA. (A) PCR amplifications were performed on DNA prepared from whole cells and from "crude phage preparations." DNA from whole cells was sensitive to digestion by the double strand–specific restriction enzyme, *AciI*, and thus, corresponds to the double-stranded "replicative form" of the element. DNA from the phage preparations was resistant to *AciI* but sensitive to the single strand–specific endonuclease S1. (B) The extracellular form is positive-strand DNA. DNA isolated from culture supernatants was amplified with oligonucleotides EXT1b (negative strand; primer Neg.) and EXT9b (positive strand; positions of oligonucleotides are as in Fig. 2 B). Equal quantities of supernatant were amplified for 30 cycles. Lanes: M, molecular size standards; B0, primers Neg. and Pos. added at the start; P5, primer Pos. added after cycle 5, primer Neg. present from the start; N5, primer Neg. added after cycle 5, primer Pos. present from the start; P10, primer Pos. added after cycle 10, primer Neg. present from the start; N10, primer Neg. added after cycle 10, primer Pos. present from the start; B5, both primers added after cycle 5; B10, both primers added after cycle 10; C, no template DNA added, both primers present from the start. Note that intensities P5 > N5, P10 > N10, P5 > B5, and P10 > B10 (primer Neg. acting by itself amplified the signal); B5~N5 (the presence of primer Pos during the first five cycles had little effect on the intensity).

ological study, and which represented the entire population of meningococci present at that time in the Czech Republic. Thus, 293 isolates (Table S3, available at http://www.jem.org/cgi/content/full/jem.20050112/DC1) were tested by PCR for the presence of the island; negative results were confirmed by Southern blotting. A multivariate statistical analysis (15, 16) of the results revealed that the element was associated very significantly with disease ($P = 0.0013$; Table II), even after taking into account the confounding clonal association with invasive complexes. Bacteria carrying the island were more likely to cause disease. Therefore, we propose to designate it "meningococcal disease associated (MDA) island."

In addition, a further analysis of the epidemiological data shows a marked difference in the age distribution of patients depending on the presence or absence of the island (Fig. 5). The classic age profile (1) shows a peak of susceptibility between 1 and 2 yr of age and a smaller peak of disease at approximately 20 yr that are characteristic of meningitis caused

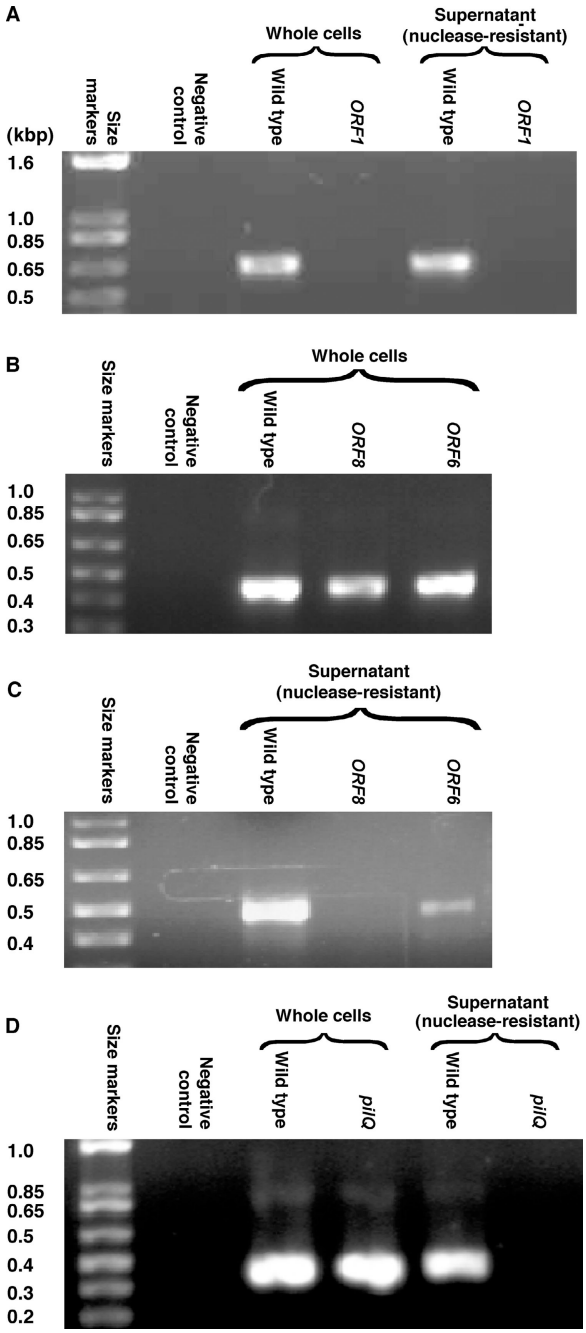

Figure 4. Mutational analysis of the genetic island. Production of the extrachromosomal form of the element was determined by PCR amplification (primers EXT1b and EXT9b) of DNA purified from whole cells or from phage preparations. (A) Mutants in the integrase homologue gene, *ORF1*, produced neither the cytoplasmic nor the secreted circular form of the element. Production of the cytoplasmic (B) and extracellular (C) circular forms was modified in *ORF6* and *ORF8* mutants. Inactivation of *ORF8*, which corresponds to gene I/IX of M13 (important in phage assembly), had no effect on production of the cytoplasmic form but prevented secretion. Inactivation of *ORF6*, which corresponds to gene III of M13 (a minor coat protein involved in adhesion to host bacteria), had a moderate effect on secretion of the element. (D) Inactivation of the chromosomal gene, *pilQ*, encoding the type IV pilin secretin, abolished secretion of the circular form to the extracellular medium.

Table II. Epidemiological analysis of the association of the genetic island with disease

Clonal complex	Presence of the island	Patients (81)[a]	Carriers (212)
Invasive[b]	present	36	61
	absent	2	2
Others	present	28	51
	absent	15	98
Association	island: disease[c]	0.0013	
	disease: "invasive" complex	0.2231	
	"invasive" complex: island	0.0001	

[a]The presence or absence of the element in a given isolate was determined by PCR amplification using primers, MDA-F and MDA-R, corresponding to conserved sequences near the 5′ and 3′ ends of the island.
[b]"Invasive" are bacteria that belong to recognized "invasive lineages" defined on the basis of their MLST designation (ST-4, ST-11, ST-32, and ST-41/44 complexes). "Others" are bacteria that belong to phylogenetic groups other than those recognized as invasive. Because of the genetic variability of the meningococcus, some of the members of groups defined as Others are capable of causing disease.
[c]Statistical associations (expressed as p-value) are the significance of the association of two variables. Hence, in the first Association row, "island: disease" is the statistical significance of the relation between possession of the genetic island and the causing of disease, taking into account the confounding effects of the (clonal) association between possession of the island and belonging to an "invasive clonal complex" and the association (by definition) between belonging to an invasive clonal complex and the causing of disease.

by the meningococcus. The peak in young children corresponds to a period of susceptibility that is due to the depletion of circulating maternal antibodies before the development of natural immunity. The reasons for the latter peak are less clear, but the incidence in this age range increases in epidemic situations, and generally has increased in recent years (17, 18). Meningococci that lack the island follow the classic profile of age dependence. This is in contrast with meningococci that carry the island, where 60% of cases of disease were seen in young adults. Indeed, >90% of cases in this age group were caused by MDA-containing meningococci.

DISCUSSION

The identification of factors that are involved in bacterial pathogenesis relies mostly on animal or tissue culture models. However, these models rarely mimic the natural disease, especially for human-specific pathogens, such as *N. meningitidis*. Furthermore, they generally are designed to investigate the mechanisms of systemic infection, and do not address the questions of multiplication of the agent at the port of entry or the balance between a commensal and pathogenic relationship. Here, a comparative genomic strategy using a large collection of clinical strains circumvents the need for animal or tissue culture models and detects virulence determinants on the basis of data from human disease and carriage. The great potential of the meningococcus for genetic exchange, coupled with the low case/carrier ratio—even in the case of epidemic strains—led us to begin the study with a simplified population of strains at the extremes of the spectrum of pathogenic potential. Thus, we were able to identify a genetic element associated with the invasive clonal groups. To confirm that this element was responsible for the invasive character, we compared possession of the island in disease-causing and asymptomatically carried meningococci in a larger, nonbiased population taken from an epidemiological study. The result demonstrated that the prophage is associated with the ability of the bacteria to cause meningococcal disease.

The carriage of virulence determinants by phages is not an uncommon situation in bacterial pathogens. The cholera toxin is carried by a prophage of the f1/M13 family (7), whereas the shiga and diphtheria toxins are carried on lysogenic lambdoid phages (for review see reference 19). The rapid evolution of bacteriophages results in great variation in primary sequence within a conserved structural framework imposed by the necessity to produce functional virions. Although the similarity between the predicted proteins encoded on the MDA and those of M13 or of CTX-phi was limited, the mutational analysis revealed functional homologies. The intriguing similarity of ORF8 to the Zot protein of *V. cholerae* deserves further attention, although the homology was to the N-terminal moiety of the protein in-

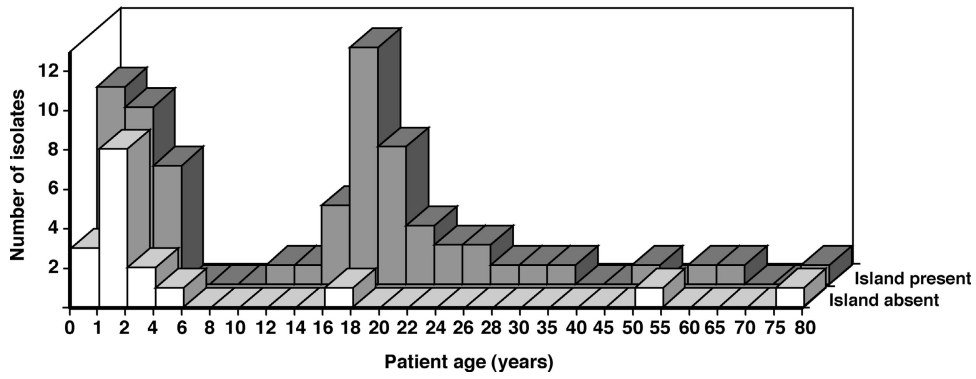

Figure 5. Age distribution of disease cases caused by meningococci possessing or lacking the MDA island. Patients were classified according to their age and whether their disease was caused by meningococci that did or did not contain the MDA island (Table S2).

volved in phage morphogenesis, rather than to the COOH terminus which is implicated in disorganization of the intestinal epithelium. Database searches did not show any other homologies to described virulence factors. However, a predicted membrane protein gene, *ORF6*, is interesting as a potential vaccine candidate. Its expression is increased in conditions of iron limitation, a frequent characteristic of bacterial virulence factors, and it demonstrates an interstrain sequence variability that is typical of surface-exposed antigens (unpublished data).

The excreted form apparently is produced in small quantities, at least by the strain Z2491. A similar situation was described in *V. cholerae*, where efficient production of phage particles requires tandem integration of prophages in the chromosome (20). Increased phage secretion might be achieved by engineering tandem MDA islands or by investigation of a panel of meningococcal isolates. In addition, varying the growth conditions or the use of DNA-damaging agents might increase phage production. The presence of multiple MDA islands within a genome (strains MC58 and FAM18) is intriguing; several explanations are possible. The presence of one island might not induce immunity to superinfection with another element having significant regions of sequence difference (compare Fig. 1). Another possible mechanism that could lead to the presence of multiple insertions is genomic rearrangements associated with repeated sequences which result in duplication of the element. *N. meningitidis* is a naturally transformable bacterium, a property which allows a separate mechanism of horizontal transfer of the prophage. In contrast to integrase- or transposase-mediated insertion of the element into the chromosome, transformation might be expected to favor insertion at a single chromosomal sequence by homologous recombination within genes flanking the island. However, repeats of dRS3-containing sequences, frequent in the meningococcal genome, might constitute a target for homologous recombination and result in relatively dispersed and multiple insertions. The MDA island may be transferred—as any other meningococcal DNA fragment—by transformation, but also may have the capacity to spread to other meningococci by infection.

The simplest explanation for the role of the MDA island is that a phage protein increases the pathogenic potential of the bacteria. An alternative is that the increased pathogenicity of strains carrying the MDA island may be a consequence of an enhanced genetic plasticity induced by the mobile element. Preliminary investigations of the phenotype of meningococci that harbor deletions of the MDA have not demonstrated deficiencies in commonly used laboratory assays for correlates of pathogenicity (growth in serum [rabbit], adhesion to T84 human cells, virulence in an immunocompromised [influenza A-treated] mouse model; reference 21). These support the idea that a classic approach would not have allowed the identification of this prophage as important for the invasiveness of *N. meningitidis*. The usual commensal relationship of *N. meningitidis* with humans suggests that in the presence of the classic virulence factors (e.g., capsule, pili, iron-acquisition proteins) that are distributed widely among pathogenic and avirulent strains, the bacterial factors that are responsible for disease—rather than asymptomatic carriage—may be linked to unknown or little investigated phenotypes. Thus, the virulence factors that are encoded by this phage may be associated with the ability of the bacteria to colonize the nasopharyngeal membranes, or with their ability to leave the commensal state and to become virulent (e.g., by modulating the interaction with the mucosal innate immunity or affecting the reaction of the bacteria to changes in the host environment). Because disease is disadvantageous for the host and the meningococcus (it kills the bacteria's only host and does not favor transmission, the outcome being to eliminate the bacteria), factors that are important in disease should have a primary function other than the promotion of invasiveness. A molecular epidemiological analysis allowed us to identify a novel mobile element which modulates the relationship of the bacteria with its human host, and may tip the balance from one of commensalism to invasive disease.

MATERIALS AND METHODS

Recombinant DNA techniques, PCR methods, and oligonucleotide primers. For testing the presence of the MDA island, long-range PCR reactions were performed using the Ex-Taq PCR System of TAKARA Bio Inc., according to the manufacturer's instructions. The reaction mixture (25 μl) contained template chromosomal DNA (1 μg); reaction buffer; dATP, dCTP, dGTP, and dTTP (0.2 mM each); forward and reverse primers (0.2 μM each); and 0.5 units of polymerase. Sequences of the primers used to amplify the genetic island from each strain were (5′ to 3′) TTATATGATGCGCTCTATCAAAG and CAGATGATATGTTGCCCGTCAAC, conserved sequences based on alignments of the seven islands present in meningococcal chromosomal sequences. PCR conditions were as follows: 1 min at 94°C, followed by 14 amplification cycles of 20 s at 98°C, 15 s at 55°C, and 20 min at 68°C, then by 13 amplification cycles of 20 s at 98°C, 15 s at 55°C and *n* min at 68°C (*n* increased linearly from 20 min at cycle 1 to 24 min at cycle 13). Finally, the reactions were held for 10 min at 72°C.

For deletion of the MDA, chromosomal sequences flanking the island were amplified and cloned sequentially in the plasmid pBluescript in such a way that the island was replaced by an BamHI restriction site. A spectinomycin resistance cassette was inserted into this site and the resulting plasmid was used to transform *N. meningitidis* Z2491, selecting for replacement of the island with the resistance cassette (22). To mutagenize the island genes PCR products, produced under standard conditions using primer pairs listed in Table S4 (available at http://www.jem.org/cgi/content/full/jem.20050112/DC1), were mutagenized by in vitro transposition using a modified mariner transposon carrying a gene encoding resistance to kanamycin (23). For inactivation of pilin genes, mutations created in *N. meningitidis* strain 8013-2C43 by in vitro transposition (24) were amplified by PCR. In both cases, mutations were introduced into *N. meningitidis* by transformation with the PCR products, and verified by Southern blotting and sequencing (22).

The secreted form of the MDA island was prepared by standard phage preparation techniques (8, 9). Bacteria were sedimented by centrifugation from 200 ml of exponentially growing culture in GC-phosphate (22), and total DNA that was extracted from the resulting cells was redissolved in 200 μl of 10 mM Tris-Hcl, 1 mM EDTA-Na, pH 8, buffer. After filtration at 0.45 μm, the supernatant was treated for 3 h at 20°C with DNase I and RNase A, 25 μg/ml each. Particles were precipitated by addition of NaCl to 1 M and polyethylene glycol 8000 to 10%, incubation at 4°C overnight, and

centrifugation at 11,000 *g* for 30 min. DNA (i.e., the secreted form of the island) phenol that was extracted from the precipitated material was redissolved in 200 μl of 10 mM Tris-Hcl, 1 mM EDTA-Na, pH 8, buffer.

For dot blot analysis, aliquots were spotted onto positively charged nylon membranes, that were fixed or not with 0.5M NaOH, and reacted with ^{32}P-labeled 36-mer probes (Table S4) under standard Southern blotting conditions, and washed finally for 30 min in 2× SSC; all manipulations were performed at room temperature.

Whole genome comparisons by DNA array. DNA arrays were nylon membranes onto which PCR products corresponding to portions of the chromosome of *N. meningitidis* strain Z2491 were bound (http://www.sanger.ac.uk/Projects/N_meningitidis/; reference 6). 1950 of the 2121 Z2491 genes were represented on the membranes. The presence or absence of a gene in the test was determined by hybridization of its labeled chromosomal DNA with the membrane and comparison with the intensity of hybridization obtained with the reference strain Z2491. Techniques and data analysis were described previously (25).

Computer-assisted genomic analysis. Chromosome sequence data were from the following web sites: *N. meningitidis* Z2491: http://www.sanger.ac.uk/Projects/N_meningitidis; *N. meningitidis* MC58: http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gnm; and *N. meningitidis* FAM18: http://www.sanger.ac.uk/Projects/N_meningitidis/seroC.shtml. Other comparisons were performed using the BLAST tools at the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST). Data shown are BLASTP results, using default settings with filtering for low complexity regions disabled.

Meningococcal isolates. For the initial screen, isolates were chosen to represent well-defined invasive and noninvasive meningococcal clonal complexes (Table S1). Several of the meningococcal strains were the gift of M. Achtman (Max-Planck Institut für Infektionsbiologie, Berlin, Germany), P. Nicolas (Meningococcal Reference Centre, Marseilles, France), or D. Caugant (National Institute of Public Health, Oslo, Norway). The 29 representatives of the invasive clonal complexes (recognized hyperinvasive, lineages) included 1 member of the ST-4 complex (subgroup IV; Z2491); 9 of the ST-11 (ET-37) complex, including representatives of serogroups C and W-135 and those of ET-37 and ET-15; 6 of the ST-32 (ET-5) complex; and 13 of the ST-41/ST-44 complex (lineage 3). Noninvasive meningococci were taken from an epidemiological study in the Czech Republic during 1993 (13, 14). Isolates were considered as belonging to a noninvasive group if no isolate of the same clonal complex had been isolated from a patient during the study, and consisted of 20 isolates belonging to nine distinct STs for which at least 3 isolates had been obtained from different individuals. This same study also furnished some, but not all, of the bacteria of the hyperinvasive sequence types. The 293 strains that were used in the larger scale investigation are described in Table S3.

Epidemiology and statistical methods. In the initial analysis of the DNA array results, the significance of the discovery of the MDA island as being associated with invasive isolates was evaluated by estimating the probability of finding a false positive (i.e., a gene showing as strong an association as does the MDA island by chance alone; see supplemental Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20050112/DC1). The data were subjected to multivariate analysis to determine the presence or absence of the element in a large number of strains (15, 16). This allows the determination of the association of two variables, A (presence of the element) and B (causing disease), which also are associated with a third variable, C (belonging to an invasive lineage). The data are discrete and each class of variables is treated as interchangeable. The log-linear model (CATMOD procedure from the SAS package, SAS Inc.), was applied to determine the statistical significance of the interactions between the variables.

Online supplemental material. The characteristics of the strains used in this study, the DNA array data and transformations thereof, and the results of PCR analysis of the presence of the MDA island are presented as online supplemental material. Table S1 describes the isolates that were used in the whole genome DNA array experiments and the results concerning the presence or absence of the genetic island described in this publication. Table S2 summarizes the distribution of each gene between the invasive and the noninvasive groups of strains, as determined from the DNA array results. Table S3 gives similar information to that in Table S1 for the larger number of strains tested for the presence of the genetic island by PCR amplification. Table S4 lists the oligonucleotide PCR primers that were used in the study. The statistical analysis of the DNA array results also is described in an online supplemental document. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050112/DC1.

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