Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11109–11114, October 1996
Microbiology

Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*: Two closely related bacteria expressing two different pathogenicities

C. R. TINSLEY* AND X. NASSIF

Laboratoire de Microbiologie, Institut National de la Santé et de la Recherche Médicale Unité 411, Faculté de Médecine Necker-Enfants Malades, 156 rue de Vaugirard, 75730 Paris Cedex 15, France

Communicated by Emil C. Gotschlich, The Rockefeller University, New York, NY, June 17, 1996 (received for review April 3, 1996)

ABSTRACT **We have investigated genetic differences between the closely related pathogenic *Neisseria* species, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, as a novel approach to the elucidation of the genetic basis for their different pathogenicities. *N. meningitidis* is a major cause of cerebrospinal meningitis, whereas *N. gonorrhoeae* is the agent of gonorrhoea. The technique of representational difference analysis was adapted to the search for genes present in the meningococcus but absent from the gonococcus. The libraries achieved are comprehensive and specific in that they contain sequences corresponding to the presently identified meningococcus-specific genes (capsule, *frp*, rotamase, and *opc*) but lack genes more or less homologous between the two species, e.g., *ppk* and *pilC1*. Of 35 randomly chosen clones specific to *N. meningitidis*, DNA sequence analysis has confirmed that the large majority have no homology with published neisserial sequences. Mapping of the cloned DNA fragments onto the chromosome of *N. meningitidis* strain Z2491 has revealed a nonrandom distribution of meningococcus-specific sequences. Most of the genetic differences between the meningococcus and gonococcus appear to be clustered in three distinct regions, one of which (region 1) contains the capsule-related genes. Region 3 was found only in strains of serogroup A, whereas region 2 is present in a variety of meningococci belonging to different serogroups. At a time when bacterial genomes are being sequenced, we believe that this technique is a powerful tool for a rapid and directed analysis of the genetic basis of inter- or intraspecific phenotypic variations.**

The study of bacterial pathogenicity has greatly benefited from tools such as transposon mutagenesis, which have made possible the identification of virulence genes. However, there exist many bacteria for which mutagenesis is inefficient. One way to study virulence in such organisms is to take advantage of naturally occurring differences in pathogenicity between variants of the same species or between closely related species. In such a case, the differences in DNA sequence, including genes responsible for the differential pathogenicities, may be isolated from the generally similar genetic background by a subtractive technique. We set out to define genes or loci that may be responsible for the pathogenesis of meningococcal meningitis by comparing the chromosome of the meningococcus [*Neisseria meningitidis* (Nm)] with that of the gonococcus [*Neisseria gonorrhoeae* (Ng)]. These two human pathogens are very closely related, but cause notably different diseases. While Ng is generally responsible for localized inflammation of the urogenital tract, the meningococcus is the cause of life-threatening disease, meningitis, that follows penetration of the blood-brain barrier and colonization of the meningeal membranes by the bacteria.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

In contrast to the great differences in pathogenic potential of the gonococcus and the meningococcus, the organisms are closely related at the level of genetic organization and DNA sequence. Estimates based on DNA–DNA hybridization (1, 2) have suggested degrees of homology of DNA primary sequence between 80 and 90%. Most of the genes studied in either one of these bacteria have been found to have their homologues in the other. Similarly most of the virulence factors so far identified in Nm have a counterpart in Ng, i.e., pilin, PilC proteins, porins, opacity proteins, and the lactoferrin and transferrin receptors. Previously characterized attributes that are specific to the meningococcus are the capsule, RTX toxin-like Frp proteins (3, 4), the Opc outer membrane proteins (5), glutathione peroxidase (6), the porin PorA (7), and the rotamase gene (8). Of these, only the capsule is invariably present in virulent strains of Nm. However, a capsule is present in many extracellular pathogens that do not cross the blood-brain barrier. Therefore, some yet unidentified attributes must be responsible for the specificity of meningococcal pathogenesis. These attributes are likely to be encoded by genes located in DNA sequences found in the meningococcus but not in the gonococcus.

The aim of this work was the identification of regions of the Nm DNA which do not have a counterpart in Ng and which may therefore determine factors responsible for the pathogenesis of meningococcal meningitis. We used for this purpose a method based on the procedure representational difference analysis that was recently described by Lisitsyn *et al.* (9) for the isolation of probes specific for restriction fragment length polymorphisms in human DNA. In this work, we have adapted the technique to the production of a comprehensive and specific library of sequences present only in the meningococcus. Mapping of the locations of 35 randomly isolated Nm-specific clones onto the chromosome of strain Z2491 has indicated regions that are present in a variety of Nm strains, but absent from Ng and commensal *Neisseria* species. Though not all Nm-specific sequences will necessarily be related to pathogenesis, it is likely that some of the clones will correspond to as yet undiscovered genetic determinants of virulence with distinguish Nm from Ng, and that these will help to elucidate the differences in pathogenicity between the two organisms.

MATERIALS AND METHODS

Bacterial Strains. Strains used for the subtractive library were Nm strain Z2491, a serogroup A isolate from the Gambia (10) obtained from M. Achtman (Max-Planck-Institut für Molekulare Genetik, Berlin), and Ng MS11 (11). Ng strains

Abbreviations: Nm, *Neisseria meningitidis*; Ng, *Neisseria gonorrhoeae*; Nl, *Neisseria lactamica*; Nc, *Neisseria cinerea*.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U56738–U56772).

*To whom reprint requests should be addressed. e-mail: tinsley@citi2.fr.

MS11, 403, and 6934 were from M.-K. Taha (Institut Pasteur, Paris). Other neisserial strains were Nm 8013 (group C), Nm 1121 (nongroupable), Nm 1912 (group A), Nm 7972 (group A), Nm 8216 (group B), Ng WI (isolated from a disseminated gonococcal infection), *Neisseria lactamica* (Nl) 8064, and *Neisseria cinerea* (Nc) 32165, coming from the collection of X.N.

Molecular Genetic Techniques. Unless otherwise specified, techniques and reagents were as recommended by Sambrook *et al.* (12). Oligodeoxynucleotides used in this study were as follows: RBam12, GATCCTCGGTGA; RBam24, AGCACTCTCCAGCCTCTCACCGAG; JBam 12, GATCCGTTCATG; JBam24, ACCGACGTCGACTATCCATGAACG; REco12, AATTCTCGGTGA; REco24, AGCACTCTCCAGCCTCTCACCGAG; JEco12, AATTCGTTCATG; JEco24, ACCGACGTCGACTATCCATGAACG; NEco12, AATTCTCCCTCG; NEco24, AGGCAACTGTGCTATCCGAGGGAG.

Southern Blotting. Southern blotting was performed by capillary transfer onto positively charged nylon membranes (Boehringer Mannheim). Hybridizations were performed at 65°C in 0.5 M NaPi pH 7.2/1 mM EDTA/7% SDS/1% BSA (13). Washing of the membranes was accomplished in 40 mM NaPi pH 7.2/1 mM EDTA/1% SDS; the final wash was at 65°C for 5 min.

The *frp* probe, produced with oligonucleotides based on the sequence of *frpA* (3), corresponded to 2.4 kb of the 5′ end of the gene from Z2491. The *opc* (14) and rotamase (8) probes corresponding to the whole genes were produced from strain Z2491 using oligonucleotides designed on the basis of the published sequences. The *pilC1* and *ppk* (polyphosphate kinase) probes corresponded to the inserts in plasmids pJL1 (15) and pBlueppk6001 (16), respectively.

Representational Difference Analysis. The procedure was modified from that of Lisitsyn *et al.* (9). Chromosomal DNA from Ng strain MS11 was sheared by repeated passage through a hypodermic needle to give fragments of length ranging between 3 and 10 kb. This DNA was purified by phenol extraction. Chromosomal DNA from Nm strain Z2491 was cleaved with restriction endonuclease *Mbo*I. This DNA (20 μg) was ligated with 10 nmol of annealed oligonucleotides RBam12 and RBam24. The meningococcal DNA was separated from excess primers by electrophoresis through a 2% low-melting-point agarose gel. The portion of the gel containing amplified fragments of size above 200 bp was excised and digested with β-agarase. This DNA was then purified by phenol extraction.

For the subtractive hybridization (first round), 0.2 μg of Nm DNA, ligated to RBam oligonucleotides, was mixed with 40 μg of sheared Ng DNA in a total volume of 8 μl of 3× EE buffer [1× EE buffer = 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid)/1 mM EDTA, pH 8.0]. The solution was overlayered with mineral oil and the DNA denatured by heating at 100°C for 2 min; 2 μl of 5 M NaCl was added and the mixture was allowed to hybridize at 55°C for 48 hr. The reaction was diluted 10-fold with preheated 3× EE buffer/1 M NaCl and immediately placed on ice. A portion of the dilution (10 μl) was added to 400 μl of PCR reaction mix (10 mM Tris·HCl, pH 9.0/50 mM KCl/1.5 mM $MgCl_2$/0.1% Triton X-100/0.25 mM of each dNTP/50 units/ml of *Taq* polymerase) and incubated for 3 min at 70°C to fill in the ends of the reannealed meningococcal fragments. After denaturation at 94°C for 5 min and addition of the oligonucleotide RBam24 (0.1 nmol per 100 μl), the hybridizations were amplified by PCR (30 cycles of 1 min at 94°C, 1 min at 70°C, and 3 min at 72°C followed by 1 min at 94°C and 10 min at 72°C; Perkin–Elmer GeneAmp 9600 thermal cycler). The PCR products were gel-purified to separate amplified meningococcal fragments from the primer and high-molecular-weight gonococcal DNA. They were digested with *Mbo*I and new oligonucleotides JBam12 and JBam24 were ligated. The ligated DNA was again gel-purified and phenol-extracted. A second round of subtractive hybridization was performed using 40 μg of sheared Ng DNA and 25 ng of the JBam-ligated DNA obtained from the first round. In this case, amplification of the self-annealed Nm DNA was realized using the JBam24 oligonucleotide.

Another library was produced using *Tsp*509I-digested Nm DNA instead of the *Mbo*I fragments. This enzyme recognizes the sequence AATT, leaving a four-base, 5′ overhang compatible with *Eco*RI. The oligonucleotides used were REco, JEco, and Neco. The experiment was performed as described above except that, in compensation for the larger number of species of low-molecular-weight fragments produced by *Tsp*509I, more of the meningococcal DNA was used in the first subtractive hybridization. In the first round, 400 ng of Nm DNA fragments were subtracted, and in the second round 25 ng of Nm fragments were subtracted against 40 μg of sheared Ng DNA. In this case, a third round of subtraction was performed, using 40 μg of sheared Ng DNA and 0.2 ng of the fragments obtained from the second round, having been digested with *Tsp*509I and religated to NEco adaptors.

Analysis of Clones from the Subtractive Libraries. DNA from the subtractive libraries was cloned into the *Bam*HI (*Mbo*I library) or *Eco*RI (*Tsp*509I library) site of pBluescript (Stratagene), then transformed into *Escherichia coli* DH5α. The inserts were amplified by PCR reactions performed on transformant colonies, using the primers M13-50 and M13-40, the latter being biotinylated at its 5′ end.

(i) DNA sequencing. Sequencing was performed on each PCR product after separation of the biotinylated and nonbiotinylated strands using the Dynabeads M-280 streptavidin system (Dynal, Oslo). Sequences were screened for homologies with previously-published sequences using the computer programs BLASTN and BLASTX at National Center for Biotechnology Information (Bethesda) and Fasta (17).

(ii) Southern blot hybridization. The PCR products from the colonies of transformants, using primers M13-40 and M13-50 as described above, were labeled by random-primed incorporation of [α-^{32}P]dCTP, and were used as probes for Southern blots of *Cla*I-digested chromosomal DNA from strains Z2491 and MS11 to check their specificity.

(iii) Pulsed-field gel electrophoresis and mapping of the clones onto the chromosome of Z2491. The positions of the DNA sequences corresponding to the cloned difference products was determined relative to the map of Nm Z2491 (18) by probing Southern blots of pulsed-field agarose gels. DNA of strain Z2491 was digested using *Pac*I, *Pme*I, *Sgf*I, *Bgl*II, *Spe*I, or *Nhe*I, and subjected to pulsed-field gel electrophoresis. Gels (20 × 20 cm) were 1% agarose in 0.5 × TBE buffer, and were subjected to electrophoresis at 6 V/cm for 36 hr with pulse times varying linearly between 5 and 35 s. Southern blot hybridizations were carried out as described above. The positions on the chromosome of Z2491 of sequences reactive with each of the clones was determined by comparison of the recognized restriction fragments with the published macrorestriction map.

RESULTS

Production of Libraries of Fragments of Nm Z2491 DNA Not Found in the Genome of Ng MS11. DNA from Nm Z2491 was cleaved with restriction endonuclease *Mbo*I and subjected to two rounds of subtractive hybridization against an excess of sheared chromosome from Ng MS11 followed by PCR amplification of those meningococcal sequences that, being absent from or having no significant homology with the Ng DNA, were able to reanneal.

To confirm that the sequences amplified from the second round of subtraction were Nm specific, the difference product was labeled and used as probe against *Cla*I-digested DNA from a panel of six Nm, four Ng, and one each of Nl and Nc (Fig.

1*A*). In contrast to the strong reactivity with multiple bands in all the meningococci, there is little or no signal in the cases of Ng or the commensal *Neisseria* species, demonstrating that the library is generally specific to Nm. The weak reactivity toward a variable band in Ng of about 4.5 kb is probably due to minor contamination by sequences corresponding to the gonococcal plasmid, harbored by each of these Ng strains.

The second consideration is the comprehensiveness of the library, i.e., it should ideally contain sequences representative of all the genes specific to Nm. To address this question, the whole of the amplified difference products from the first and second round of subtraction (Fig. 1*B*) were probed with genes known to exist only in the meningococcus: *frp*, *opc*, and rotamase (Fig. 1 *C–E*). Though the Nm-specific *frp* gene was represented by a fragment of 600 bp, no signal was obtained with the rotamase and the *opc* genes. Hence, the library was not considered to be comprehensive. Nevertheless, considering their high specificity, the products of the second round of amplification were cloned into the *Bam*HI site of pBluescript.

The inclusion in the subtractive library of a sequence corresponding to any Nm-specific gene depends on its being contained on a restriction fragment of suitable size. This condition is a function of two factors. Firstly, larger fragments are less likely to be totally Nm specific. Secondly, even if they exist, larger Nm specific fragments will be underrepresented in the library because of the limitations of the PCR technique, which result in increasingly inefficient amplification of larger fragments. Fragments of size greater than about 600 bp were not found in the library. For the rotamase and *opc* genes, the absence of suitably sized *Mbo*I fragments in the Z2491 chromosome precluded their inclusion in the library. Since any one enzyme may not produce a small fragment corresponding to any Nm-specific gene, one restriction enzyme is unlikey to suffice to produce a comprehensive library, and therefore another library was produced using a restriction enzyme, *Tsp*509I, with a different specificity. The enzyme *Tsp*509I has the advantage that it produces smaller fragments from the Nm chromosome, having an average size between 0.3 and 0.6 kbp, compared with an average size of 2 to 4 kbp produced by *Mbo*I.

As for the previous library, the product of the second round of subtraction/amplification was labeled and used as probe against a panel of *Neisseria* strains. As expected (Fig. 2*A*), in contrast to the strong reactivity with all the Nm strains, there was little or no signal with Ng and the commensal neisseria species. The specificity of the library was investigated further by reacting Southern blots of the products from the three rounds of subtraction with probes corresponding to *pilC1* and *ppk*, both of which are common to Nm and Ng (Fig. 2 *C* and *D*). Sequences from these genes were completely removed from the library after the second round of subtraction. The comprehensiveness of the library was investigated by reaction of the subtraction products with probes corresponding to three genes found only in Nm (*frp*, rotamase, and *opc*). These Nm-specific probes did react with the amplification products of the first and second rounds of subtraction (Fig. 2 *E–G*). A third round of subtraction, however, resulted in the loss of some Nm-specific sequences because the rotamase- and *opc*-reactive fragments were absent from this round of amplification. Taken together, these data indicate that the subtraction products from the second round of subtraction/amplification are both specific to Nm and comprehensive for Nm-specific sequences. The second round products were cloned into the *Eco*RI site of pBluescript.

Position of Meningococcus-Specific Sequences on the Chromosome. The availability of a physical map of the chromosome of Nm Z2491 (18) made possible an investigation of the distribution of randomly isolated Nm-specific sequences. First, to confirm the specificity of each of the clones toward the Nm strain, the corresponding inserts were used to probe Southern blots of *Cla*I-digested DNA from strains MS11 and Z2491. Subsequent sequence analysis allowed identification of dupli-

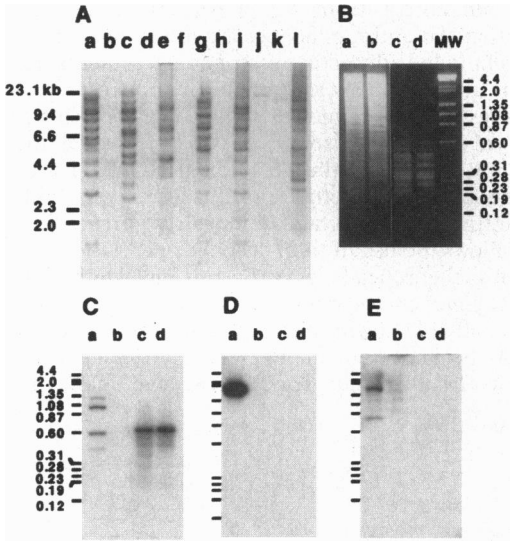

FIG. 1. Analysis of the *Mbo*I subtractive library. (*A*) The products of the second round of subtractions were used as probes against Southern blots of *Cla*I-digested chromosomal DNA from the following strains. Lanes: a, Z2491 (Nm; group A); b, MS11 (Ng); c, 8013 (Nm; group C); d, 403 (Ng); e, 1121 (Nm; nongroupable); f, 6934 (Ng); g, 1912 (Nm; group A); h, WI (Ng; DGI strain); i, 7972 (Nm; group A); j, 8064 (Nl); k, 32165 (Nc); l, 8216 (Nm; group B). (*B*) Agarose gel electrophoresis of the chromosomes of Nm strain Z2491 and Ng MS11, digested with *Mbo*I, and of both rounds of subtractive hybridization and PCR amplification. Lanes: a, Nm chromosome (1 μg); b, Ng chromosome (1 μg); c, first round of subtraction/amplification (0.1 μg); d, second round of subtraction/amplification (0.1 μg); MW, molecular size markers: *Hin*dIII digest of phage lambda plus *Hae*III digest of phage ΦX174. Gels were subjected to Southern blotting and probed with: *frpA* (*C*), rotamase (*D*), and *opc* (*E*). Lanes are as in *B*.

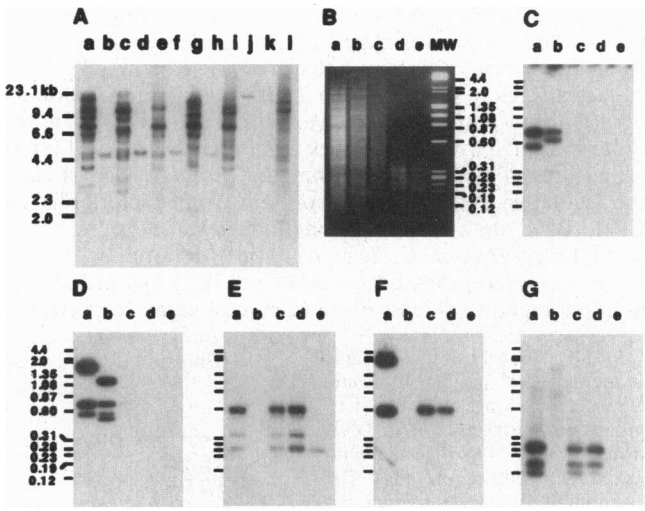

FIG. 2. Analysis of the *Tsp*509I subtractive library. (*A*) The products of the second round of subtractions were used as probes against Southern blots of *Cla*I-digested chromosomal DNA as described for Fig. 1. Lanes: a, Nm Z2491; b Ng MS11; c, Nm 8013; d, Ng 403; e, Nm 1121; f, Ng 6934; g, Nm 1912; h, Ng WI; i, Nm 7972; j, Nl 8064; k, Nc 32165; l, Nm 8216. (*B*) Agarose gel electrophoresis of the chromosomes of Nm Z2491 and Ng Ms11, digested with *Tsp*509I, and of each round of subtractive hybridization and PCR amplification. Lanes: a, Nm chromosome (1 μg); b, Ng chromosome (1 μg); c, first round of subtraction/amplification (0.15 μg); d, second round (0.1 μg); e, third round (0.05 μg); MW, molecular size markers. Gels were subjected to Southern blotting and probed with: *pilC1* (*C*), *ppk* (*D*), *frpA* (*E*), rotamase (*F*), *opc* (*G*). Lanes are as in *B*.

cate clones and hence the unambiguous definition of the different types of clone recovered from the subtractive libraries. 19 clones from the *Mbo*I library (designated with the letter "B") and 16 clones from the *Tsp*509I library (designated with the letter "E") were kept for further study; each one of these clones has a unique sequence with no counterpart in Ng.

PCR products from these clones were labeled and used to probe Southern blots of DNA from Z2491 digested with infrequently cutting enzymes. The positions of the reactive bands enabled us to localize the clones to within ±20 kb relative to the published map (Fig. 3 and Table 1). This mapping revealed a nonrandom distribution of the Nm-specific sequences. The majority of the Nm-specific sequences are clustered in three distinct groups, one of which (region 1) corresponds in position to the previously described capsule-related genes. (We may define region 1 as E109, E138, B230, and B323; region 2 as B322, B220, B108, B132, B233, B328, E139, E145, and B101; and region 3 as B306, E114, E115, E124, E146, E120, E107, E137, and E142.) Of the sequences identified as specific to the meningococcus, 63% map within these small, distinct regions. This clustering is in contrast to the distribution of the previously reported Nm-specific genes *frpA* and *C*, *porA*, *opc*, and the capsule-related region, which had suggested that the Nm-specific genes might be found, with the exception of the functionally related capsule genes, scattered around the chromosome.

When the PCR products corresponding to the insert of the clones from each of these regions respectively were pooled and used as probes on Southern blots of the panel of neisserial strains (Fig. 4), regions 1 and 2 produced a limited number of bands in each of the meningococci, suggesting that these regions are both Nm specific and common to all meningococci. Interestingly, region 3 showed reactivity only with meningococci of serogroup A; this region therefore appears to be specific to a subgroup of Nm.

Comparison with reported sequences in the data banks was performed to give insights as to the possible function of the regions cloned (Table 1). First, it is seen that the clones in region 1 all correspond to genes involved in capsule biosynthesis, previously investigated in Nm serogroup B (19, 20). Apart from some homology with hemolysin proteins of *Serratia marcescens*, clones from region 2 show no significant homologies with any published sequences, either at the DNA or at the protein level. Two of the clones from region 3 showed interesting homologies with DNA-binding proteins, in particular the regulatory and transposase proteins from bacteriophages. Clone B208 showed strong homology with one of the conserved regions in a class of TonB-dependent ferric-siderophore receptors. Clones B134 and B339 hybridized with multiple regions (at least five and at least eight, respectively) of the chromosome. Sequence data showed that B339 corresponded to insertion sequence IS*1106* (21); the translation of B143 showed limited homology to the transposase of an *Aeromonas* insertion sequence IS*AS2* (22) and was found by Southern blotting to be an Nm-specific entity, present in multiple copies in the chromosomes of each meningococcus of the panel. The other clones showed no significant homology with neisserial or indeed any published sequences and therefore constitute, together with the majority of the other isolated clones, a bank of previously undiscovered Nm-specific loci.

DISCUSSION

We have adapted the subtractive technique of representational difference analysis to allow us to isolate a large number of probes specific for the meningococcus but absent from the closely related gonococcus. The utility of this procedure is demonstrated by the generation of new and potentially important information on the genetic organization of the meningococcus.

Several modifications were made to the original procedure, appropriate to the different application. The relatively small size of bacterial chromosomes makes the procedure technically easier, and such genomes may even be said to be ideally suited to this kind of analysis. However, the known antigenic variability of bacterial proteins leads to a problem of genes that are homologous but not identical between the two organisms and could well be isolated together with the truly specific genes. For this reason, we lowered the temperature of the hybridizations to render the subtractions more stringent. That the libraries are not "contaminated" with such variable genes is shown by the exclusion even of the *pilC1* sequence (Fig. 2), which has only 77% homology with the analogous gonococcal gene (unpublished data).

Since the aim of this work was to isolate only genes present in Nm but absent from Ng, it was preferable to use for subtraction the entire gonococcal chromosome. The unmodified technique, subtracting restriction fragments against restriction fragments, has recently been applied to the production of restriction fragment length polymorphisms as epidemiological markers for isolates of Nm (23), though their use is apparently limited by the genetic variability of the species. Another subtractive method (24), using repeated subtraction with biotinylated DNA and removal by streptavidin-coated beads, allows the use of total chromosomal DNA for subtraction, but is theoretically less efficient. This method, first used to isolate genes corresponding to deletion mutants of yeast, has very recently (25) been used to produce probes for deletions that may be important in the loss of virulence of the BCG strain of *Mycobacterium bovis*. These two studies have pro-

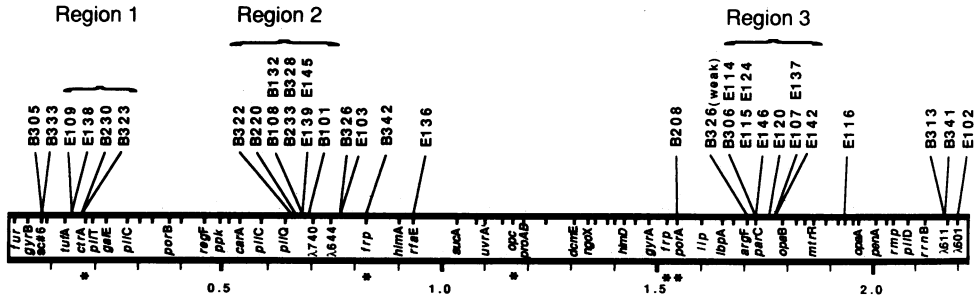

FIG. 3. Distribution of specific sequences on the chromosome. Nm-specific clones were used as probes on Southern blots of DNA from strain Z2491 digested with infrequently cutting enzymes. The enzymes used were *Pac*I, *Pme*I, *Bg*/II, *Spe*I, *Nhe*I, and *Sgf*I. Reactivity was localized by comparison with the positions of fragments of the corresponding size on the published map. Distances are shown below the map in units of 0.1 Mb. The positions of all of the genetic markers mapped by Dempsey *et al.* (18) are shown as points on the linearized chromosomal map. Certain are named to help to align our data with their more detailed map. The previously reported Nm-specific genes are marked with asterisks. The two loci labeled "*frp*" are the *frpA* and *frpC* genes, those labeled "*pilC*" are the two genes *pilC1* and *pilC2*; both of these are pairs of homologous genes that are not distinguished on the map. The positions of our Nm-specific clones are accurate to a degree depending on the overlap of the reactive restriction fragments. The average is about ±20 kb; however, one clone, B342, could be mapped only to a region of about 140 kb, centered on the frp marker. The clone B326 gave two bands, a weak and a strong, with each restriction enzyme. The strong signal gave a position near to the marker λ644, while the weak signal was located close to *argF*. Clones B134 and B339 are not located on the map because they were found to correspond to sequences located in at least five and eight regions of the Z2491 chromosome, respectively.

Table 1. Map positions of the specific clones and homologies with known sequences

Clone name*	Insert size	Reactive fragments†						Position in Z2491	Protein sequence homologies‡	GenBank accession no.
		Pac	Pme	Bgl	Spe	Nhe	Sgf			
B305	259	18–20	15–17	22–23	18	11–13	2	λ736		U56748
B333	235		15–17	22–23	18	11–13	2	λ736		U56753
E109[1,§]	212		6–7	11–15	10	11–13	2	*tufA/ctrA*	LipB protein. *N. meningitidis* (3×10^{-26})	U56759
E138[1,§]	316	1	6–7	11–15	10	11–13	2	*tufA/ctrA*	LipB protein. *N. meningitidis* (4×10^{-75})	U56767
B230[1]	356	1–3	6–7	1	10	11–13	2	*ctrA*	KpsC protein. *E. coli* (3×10^{-53})	U56746
B323[1]	363	1	6–7	1	10	11–13	2	*ctrA*	CtrB protein. *N. meningitidis* (2×10^{-64})	U56750
B322[2]	210		2	16–18	6	1	5	*pilQ*/λ740	Hemolysin B. *S. marcescens* (8×10^{-5})	U56747
B220[2]	341		2	16–18	6	≥18	5	*pilQ*/λ740		U56743
B108[2]	276		2	19–21	6	>18	5	*pilQ*/λ740		U56739
B132[2]	390	2	2	19–21	6	≥18	5	*pilQ*/λ740	Hemolysin A. *S. marcescens* (6×10^{-2})	U56741
B222[2]	164	1–3	2	19–21	6	≥18	5	*pilQ*/λ740		U56744
B328[2]	256	1–3	2	22–23	6	≥18	5	*pilQ*/λ740		U56752
E139[2]	324	2	2	19–21	6	≥18	5	*pilQ*/λ740		U56768
E145[2]	343	2	2	19–21	6	≥18	5	*pilQ*/λ740		U56771
B101[2]	257	≤20	2	19–21	6	≥18	5	*pilQ*/λ740		U56738
E103**	334		2	11–15	3–5	10	3	λ644		U56756
B326[‖]	314		2	11–15	3–4	10	3	λ644		U56751
B326 (Weak reactivity)			5	6	16	2	1	*argF*		U56751
B342	167		2	19	3–4	6–7	3	*iga*		U56761
E136	249		2	7	1	3	3	*lepA*		U56770
B208	177		1	2	3–4	2	1	*porA*	FeIII-pyochelin receptor. *P. aeruginosa* (5×10^{-4})	U56742
B306[3,¶]	219	11	5	11–12	5	2	4	*parC*		U56745
E114[3]	227	11	5	11–12	5	2	4	*parC*		U56760
E115[3,¶]	251		5	11–15	6	2	4	*parC*		U56762
E124[3]	207		5	11–12	5	2	4	*parC*		U56763
E146[3]	184		5	11–15	5		4	*parC*		U56772
E120[3]	263		5	3–4	5	16	4	*opaB*		U56766
E107[3]	249	11	14–17	3–4	5	16	4	*opaB*		U56758
E137[3]	274		14–17	3–4	5	16	4	*opaB*	Transposase. Bacteriophage D3112 (6×10^{-12})	U56765
E142[3]	230		14–17	3–4	5	16	4	*opaB*	Ner-like protein. *H. influenzae* (6×10^{-23}) Ner protein. Phage mu (3×10^{-18})	U56769
E116	379	5–7	11–13	3–4	2	6–7	8	λ375		U56764
B313	436	9	9	3–4	13–14	5	2	λ611		U56749
B341	201	8–10	9	3–4	13–14	5	2	λ611		U56755
E102	238		11–13	3–4	19	5	2	λ601	Hypothetical protein H11730. *H. influenzae* (7×10^{-24})	U56757
B134	428		Multiple						*ISAS2* transposase, *Aeromonas salmonicida* (3×10^{-4})	U56740
B339	259		Multiple						IS*1106* transposase. *N. meningitidis* (6×10^{-45})	U56754

*Clones marked with "1," "2," or "3" belong to regions 1, 2, or 3, as defined in Fig. 3.
†Numbers are those assigned to the bands on pulsed-field gel electrophoresis, in order of decreasing size (18). Where a range is shown, the signal could not be assigned to a specific band, but to a number of comigrating or closely-spaced bands.
‡The values in parenthesis are the significance of the homologies, as given by the program BLASTX.
§E109 and E138 are contiguous clones.
¶B306 and E115 overlap.
‖B236 also shows a weak reactivity in the region of *argF*.
**Clone E103 contains a *Tsp*5091 site and hence may contain two inserts. However, since it reacts with a single *Cla*I fragment (6 kb) of the chromosome and maps to one position only, it is included here.

duced mixtures of difference products capable of recognizing clones corresponding to deletions in an otherwise extremely homologous genetic environment. By contrast, in this work, we have set out to show that a representative library of specific clones may be produced in the face of the known background of genetic variability in the pathogenic *Neisseria* species.

Using our technique, a representative pool of clones can be achieved by analysis of the representational difference analysis subtraction products after only two rounds. Both the library produced with *Mbo*I and that produced with *Tsp*509I were specific for Nm. However, the library produced by *Tsp*509I was more comprehensive, as expected from theoretical considerations based on the enzyme's production of smaller restriction fragments. In this regard, it should also be pointed out that the *Tsp*509I library is less redundant than the *Mbo*I library, i.e., there is less duplication of clones. Most (86%) of the clones from the *Tsp*509I library correspond to distinct sequences, compared with 43% from the *Mbo*I library (data not shown). Further evidence that the *Tsp*509I library is not exhausted as a source of Nm-specific clones is that a probe reacting in the

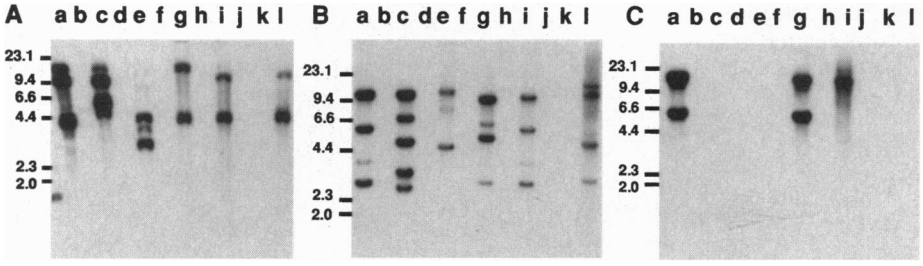

FIG. 4. Reactivity of the clones from the three defined regions toward a panel of neisserial strains. Pooled clones from region 1 (*A*), region 2 (*B*), and region 3 (*C*) were used as probes against a panel of meningococci, gonococci, and commensal *Neisseria* species. Lanes are as described for Fig. 1: a, NM Z2491; b Ng MS11; c, Nm 8013; d, Ng 403; e, Nm 1121; f, Ng 6934; g, Nm 1912; h, Ng WI; i, Nm 7972; j, Nl 8064; k, Nc 32165; l, Nm 8216.

region of *opc* has not yet been cloned, though the subtraction products obviously contain such sequences (Fig. 2*G*).

In our experiments with *Mbo*I and *Tsp*509I, we have found that although the three potentially important regions (Fig. 3) were detected by both of the libraries, several loci were represented only in one of the two. Hence, notwithstanding the potential of the *Tsp*509I library to produce more probes, it seems that at least two different libraries would be preferable to achieve a representative number of specific clones. In this regard, the success of our modification of the *Bam*HI adaptor sequences of Lisitsyn *et al.* (9) to suit the enzyme *Tsp*509I (and *Eco*RI) suggests that libraries may be constructed using any enzyme leaving cohesive ends and producing fragments of a suitable size.

Mapping of the clones to the chromosome revealed a nonrandom distribution of Nm-specific sequences. The majority of the genetic differences between the meningococcal and gonococcal strains used were clustered in three distinct regions. Region 1 must be the capsule gene cluster of group A meningococcus. The function of the genes in the other regions is not known, but homologies with published sequences (Table 1) suggest similarities between some of the genes of region 3 and bacteriophage transposases and regulatory proteins. No meningococcal viruses have been characterized and it is tempting to imagine that these sequences may be of phage origin. Interestingly, the *Haemophilus influenzae* genome also contains a sequence homologous to that of the Ner regulatory protein from phage Mu, but it is not known whether this is a functional gene. Clone B208 showed strong homology with one of the conserved regions (domain III) in the class of TonB-dependent ferric-siderophore binding-proteins. The homology was much better (46% identity, 91% homology in 33 amino acids) with the domains of receptors produced by *Pseudomonas* (26) than with those of the TonB-dependent receptors for host iron-binding proteins previously described from *Neisseria* (27). The translated sequence is too short to allow searches for the other characteristic conserved domains in this class of receptor. However, the proximity of this clone to the Nm-specific genes *porA* and the iron-regulated *frp*, and in particular the possibility that it is an Nm-specific receptor protein exposed on the outer membrane make it an attractive candidate for further investigation. Two clones were present in multiple copies. Clone B339 corresponds to the insertion sequence IS*1106* (21). The weak homology between clone B134 and the *Aeromonas* insertion sequence, together with its presence in a variety of Nm strains in multiple copies, suggest that it might represent a new type of Nm-specific insertion sequence.

Of particular interest is the possibility that the regions containing the Nm-specific clones might correspond to "pathogenicity islands," as have been described in *Escherichia coli* and *Yersinia pestis*. We know already that region 1 is such an island since it carries the genes of the capsule complex. The representational difference analysis procedure has indicated other regions of high concentrations of Nm-specific sequences that might repay a more detailed investigation, in addition to localizing a number of other candidate loci. The clones isolated in this work will aid in the further understanding of the relevance of regions specific to Nm, by allowing cloning and sequencing of larger chromosomal fragments, and directly by their use in mutation of the loci. Finally, the detection of meningococcus-specific genes, perhaps involved in the pathogenesis of the organism, may help to target antigens suitable for use in an anti-meningococcal vaccine.

The efficacy and rapidity of the method described lend it to use in a wide range of situations where it is desired to investigate the genetic differences responsible for any phenotype peculiar to one of a pair of closely related bacteria.

We thank P. Berche for his constant support during this work and J. Cannon for generously making available the map of *N. meningitidis* strain Z2491 before publication. We also thank Eric Abachin, J.-L. Beretti, and A. Perrin for freely given technical help. The gift of neisserial strains and support from M. Achtman and M. K. Taha are gratefully acknowledged. C.R.T. was the recipient of fellowships from the European Economic Community, under the Human Capital and Mobility Programme, and the "Fondation pour la Recherche Médicale." This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Université Paris V René Descartes, and the "Association pour Gille."

1. Hoke, C. & Vedros, N. A. (1982) *Int. J. Syst. Bacteriol.* **32,** 57–66.
2. Guibourdenche, M., Popoff, J. Y. & Riou, J. Y. (1986) *Ann. Inst. Pasteur Microbiol. (Paris)* **137B,** 177–185.
3. Thompson, S. A., Wang, L. L., West, A. & Sparling, P. F. (1993) *J. Bacteriol.* **175,** 811–818.
4. Thompson, S. A., Wang, L. L. & Sparling, P. F. (1993) *Mol. Microbiol.* **9,** 85–96.
5. Achtman, M., Neibert, M., Crowe, B. A., Strittmatter, W., Kusecek, B., Weyse, E., Walsh, M. J., Slawig, B., Morelli, G., Moll, A. & Blake, M. (1988) *J. Exp. Med.* **168,** 507–525.
6. Moore, T. D. E. & Sparling, P. F. (1995) *Infect. Immun.* **63,** 1603–1607.
7. Gotschlich, E. C. (1990) in *Microbiology*, eds. Davis, B. D., Dulbecco, R., Eisen, H. N. & Ginsberg, H. S. (Lippincott, Philadelphia), pp. 551–560.
8. Sampson, B. A. & Gotschlich, E. C. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1164–1168.
9. Lisitsyn, N., Lisitsyn, N. & Wigler, M. (1993) *Science* **259,** 946–951.
10. Achtman, M., Wall, R. A., Bopp, M., Kusecek, B., Morelli, G., Saken, E. & Hassan-King, M. (1991) *J. Infect. Dis.* **164,** 375–382.
11. Swanson, J., Sparks, E., Zeligs, B., Siam, M. A. & Parrott, C. (1974) *Infect. Immun.* **10,** 633–644.
12. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
13. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
14. Olyhoek, A. J. M., Sarkari, J., Bopp, M., Morelli, G. & Achtman, M. (1991) *Microb. Pathog.* **11,** 249–257.
15. Nassif, X., Beretti, J., Lowy, J., Stenberg, P., O'Gaora, P., Pfeifer, J., Normark, S. & So, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3769–3773.
16. Tinsley, C. R. & Gotschlich, E. C. (1995) *Infect. Immun.* **63,** 1624–1630.
17. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2444–2448.
18. Dempsey, J. F., Wallace, A. B. & Cannon, J. G. (1995) *J. Bacteriol.* **177,** 6390–6400.
19. Frosch, M., Weisgerber, C. & Meyer, T. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1669–1673.
20. Frosch, M. & Muller, A. (1993) *Mol. Microbiol.* **8,** 483–493.
21. Knight, A. I., Ni, H., Cartwright, K. A. V. & McFadden, J. J. (1992) *Mol. Microbiol.* **6,** 1565–1573.
22. Gustafson, C. E., Chu, S. & Trust, T. J. (1994) *J. Mol. Biol.* **237,** 452–463.
23. Strathdee, C. A. & Johnson, W. M. (1995) *Gene* **166,** 105–110.
24. Straus, D. & Ausubel, F. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1889–1893.
25. Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C. & Stover, C. K. (1996) *J. Bacteriol.* **178,** 1274–1282.
26. Ankenbauer, R. G. & Quan, H. N. (1994) *J. Bacteriol.* **176,** 307–319.
27. Cornelissen, C. N. & Sparling, P. F. (1994) *Mol. Microbiol.* **14,** 843–850.