Genes Associated with the Meningococcal Capsule Complex Are Also Found in *Neisseria gonorrhoeae*

HOLGER PETERING,¹ SVEN HAMMERSCHMIDT,¹ MATTHIAS FROSCH,¹
JOS P. M. VAN PUTTEN,^{2†} CATHERINE A. ISON,³
AND BRIAN D. ROBERTSON^{2,3*}

*Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30623 Hannover,¹ and
Max-Planck-Institut für Biologie, Infektionsbiologie, 72076 Tübingen,² Germany, and
Department of Medical Microbiology, Imperial College School of Medicine
at St. Mary's, London W2 1PG, United Kingdom³*

Received 4 December 1995/Accepted 18 March 1996

A homolog of the meningococcal *cps* locus region E has been identified in *Neisseria gonorrhoeae* immediately upstream of the gonococcal region D locus. Region E has no detectable function in capsule biosynthesis in *Neisseria meningitidis* or in lipopolysaccharide biosynthesis in either organism. The open reading frame is homologous to proteins of unknown function in *Escherichia coli* and *Haemophilus influenzae*. Further analysis of the *N. meningitidis* *cps* cluster has identified a second copy of region D encoding three additional open reading frames, including homologs of DNA methyltransferases. The organization of the region D and E genes in *N. gonorrhoeae* and *N. meningitidis* in relation to the *cps* genes provides some insight into the evolutionary origin of encapsulation in *N. meningitidis*.

One of the striking structural features that distinguishes *Neisseria gonorrhoeae* from the closely related pathogen *Neisseria meningitidis* is that the gonococcus lacks a polysaccharide capsule. The role of the capsule in meningococcal biology has been extensively studied, but it remains unclear exactly to what extent the capsule determines the kind of infection caused and whether it is implicated in the contrasting clinical presentations and sequelae seen for these two infections. Both organisms are obligate pathogens of humans, causing mainly localized mucosal infections. Meningococcal colonization of the nasopharynx is asymptomatic, while gonococcal infection of the genitourinary tract is usually characterized by a localized inflammatory response. However, under rare, poorly defined conditions, pathogenic *Neisseria* spp. may disseminate to cause severe and, in some cases, life-threatening conditions, including meningitis (*N. meningitidis*), bacteremia (*N. meningitidis* and *N. gonorrhoeae*), pelvic inflammatory disease, and septic arthritis (*N. gonorrhoeae*). Treatment of gonorrhoea, which is rarely fatal, is usually successful but does not preclude reinfection, and there is no apparent acquired immunity. Meningococcal meningitis is associated with a 10% mortality rate, which rises to 40% for patients with severe septicemia and shock (15).

The composition of the meningococcal capsule varies, depending on the serogroup concerned. The group B capsule is a homopolymer of α -(2,8)-linked *N*-acetyl-neuraminic acid; the cluster of genes involved in its biosynthesis, lipid substitution, and export have been identified and characterized (5, 7, 8, 10), and their organization is the same in all the sialic acid-containing serogroups (7). The genes required for capsular polysaccharide synthesis are located in a central region A, which is immediately flanked on the left side by the transporter region C and then region E, which was thought to participate in the

regulation of capsule expression (10). On the right side of region A is region D, containing genes from the meningococcal lipopolysaccharide (LPS) biosynthesis pathway, including *galE* (13), followed by region B, which is involved in phospholipid substitution of the polysaccharide chains (8). Southern blot analysis demonstrated that there is a duplication of region D elsewhere in the genome (10, 13), which has been termed region D'.

As an approach to explain the absence of capsule expression in *N. gonorrhoeae* and to gain insight into the evolutionary origin of encapsulation in *N. meningitidis*, we have investigated the molecular differences which preclude the expression of a capsule by the gonococcus.

Nucleotide sequence analysis of the meningococcal region D' and region E. We have analyzed genes associated with the meningococcal *cps* locus and determined the nucleotide sequence of the meningococcal region E in strain B1940 (10) (the sequence of region E and that of region D' described below are available from the EMBL and GenBank databases under accession number L09189). Southern blots using the isolated meningococcal region D and the region E gene fragments as a probe indicated that both region D' and region E were located on the same *EcoRI-EcoRV* fragment with a size of about 9 kb (10) (data not shown). For nucleotide sequence determination of the *EcoRI-EcoRV* fragment, several subclones were constructed in the pBluescript plasmid vector (see Fig. 1). The open reading frame (ORF) of the region E gene spanning 2,272 nucleotides started 64 bp after the stop codon of *ctrD* of region C (7). The derived amino acid sequence for this gene, consisting of 757 amino acids, would specify a polypeptide of 83 kDa. The homologous sequences found in the GenBank and EMBL databases (releases 90 and 44, respectively) are given in Table 1. The region E gene is separated from the truncated *galE'* (13) by 1,430 nucleotides. In this spacer region there are two ORFs on the same strand as the region E gene and the truncated *galE'* (Fig. 1). The protein encoded by ORF1 showed significant homology to DNA methyltransferases from *Haemophilus parahaemolyticus* and *Haemophilus aegyptius*. The ORF2 gene product is homologous to

* Corresponding author. Mailing address: Department of Medical Microbiology, Imperial College School of Medicine at St. Mary's, Norfolk Pl., London W2 1PG, United Kingdom. Phone: 171-594-3965. Fax: 171-262-6299. Electronic mail address: b.robertson@ic.ac.uk.

† Present address: Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840-2999.

TABLE 1. Proteins with homology to the meningococcal region E sequences^a

Homolog	Identical residues (%)		Reference	Accession no. ^b
	<i>N. gonorrhoeae</i> ORF region E	<i>N. meningitidis</i> ORF region E		
<i>E. coli</i> ORF o622	59	71, 61	2	U18997
<i>H. influenzae</i> ORF HI0568	84	56, 74, 47	6	U32738

^a Homologs were identified by searching the GenBank and EMBL databases with the program TFASTA (3). Optimal alignments were made with BESTFIT (3).

^b Alignments with these sequences indicate the presence of frameshifts, and values are given for the sections of homologous sequence between apparent frameshifts.

H. influenzae and *Campylobacter jejuni* proteins of unknown origin (Table 2). Downstream of the truncated *galE'*_{Nm} (*Nm*, *N. meningitidis*) gene are ORFs homologous to *rfbB*, *rfbA*, and *rfbC*. There is no homolog of the fifth ORF found in region D. A third ORF without any homology to known sequences was located on the opposite strand downstream of *rfbC*. A map of the entire clone is presented in Fig. 1B, and the homologies are listed in Table 2.

Functional analysis of the region E-encoded gene product.

For construction of a meningococcal mutant with a deletion in region E, the *NotI*-*XmaIII* fragment (between positions 682 and 2209) in plasmid pAO1 (Fig. 1) was replaced by a chloramphenicol acetyltransferase cartridge excised as a *NotI* fragment from plasmid TnMax5 (11) (kindly provided by R. Haas, Max-Planck-Institut für Biologie, Tübingen, Germany). This construct (pMH1) was introduced into *Neisseria* spp. by trans-

formation as described previously (17). Transformants were selected on gonococcal (GC)-agar plates containing chloramphenicol (10 µg/ml), and the correct genomic replacement was monitored by PCR and Southern blot analysis (19).

Since region E is closely linked to LPS biosynthesis genes, we analyzed the LPS of the region E mutant by polyacrylamide gel electrophoresis (14) and subsequent silver staining (21) and compared the LPS phenotype with that of the parent strain. No difference between the two strains was observed (data not shown). We also analyzed whether the ability to exogenously sialylate the terminal galactose residue of the LPS was affected in the region E mutant (16) but, again, could not demonstrate any difference between the two strains (data not shown). This suggests that the region E gene product is not involved in either LPS biosynthesis or sialylation. In addition, quantitative analysis of capsular polysaccharide production (9) showed no differences between the wild type and the region E mutant (data not shown).

The gonococcus has a homolog of the meningococcal region E.

The sequence upstream of *galE*_{Ng} (*Ng*, *N. gonorrhoeae*) on clone pBDR8.11, which has been described previously (17, 18), was determined and is available from the EMBL and GenBank databases under accession number Z21508. These sequence data include the sequence for *galE*_{Ng} (17) and *rfbB* *AC*_{Ng} (18) of *N. gonorrhoeae* MS11-E1 (12). An ORF starting 115 nucleotides upstream of *galE*_{Ng} at position 1429, extending on the other strand to the end of the clone, and encoding 351 amino acid residues was found during analysis of the sequence. No stop codon is encountered, making it likely that the ORF extends beyond this clone (see below). Comparison of this sequence with the Genbank and EMBL databases (releases 90 and 44, respectively) and with other meningococcal sequences,

FIG. 1. (A) Map of the *N. gonorrhoeae* homologs of meningococcal region D (17, 18) and region E (this study). The genes identified in this and previous studies (17, 18) are shown in detail. The sequence of the whole region is available from the EMBL and GenBank databases under accession number Z21508. (B) Map of the chromosomal *cps* locus of *N. meningitidis* of 24 kb. The constructs (pAO1, pSHX, pHP1, and pHP2) used for nucleotide sequence determination are shown below the physical map. The regions investigated in this and a previous study (13) are expanded to show the genes identified in detail. The sequences of regions D' and E are available from the EMBL and GenBank databases under accession number L09189.

TABLE 2. Proteins with homology to the ORFs identified in meningococcal region D^a

<i>N. meningitidis</i> homolog	Identical residues (%)	Reference	Accession no.
ORF1			
<i>H. parahaemolyticus</i> cyto-sine-specific DNA methyl-transferase HphIM(C)	64	Unpublished	X85374
<i>H. aegyptius</i> HaeIII methyl-transferase	32	20	M24625
ORF2			
<i>H. influenzae</i> ORF HI0560	59	6	L45201
<i>C. jejuni</i> ORF J01.1	51	Unpublished	X76062

^a Homologs were identified by searching the GenBank and EMBL databases with the program TFASTA (3). Optimal alignments were made with BESTFIT (3).

using the programs FASTA and TFASTA from the Genetics Computer Group software (3), demonstrated that it was 95% homologous to region E of *N. meningitidis*. A detailed map of clone pBDR8.11 is presented in Fig. 1 and shows the positions of the gonococcal region D genes previously described (17, 18) and the region E homolog.

To confirm that the observed arrangement of gonococcal regions D and E was not a cloning artifact or a peculiarity of strain MS11, PCR was used to amplify the region spanning nucleotides 1629 (*galE*_{NG}; primer BDR17; 5'-GTGGCAGGTA AGGTTCCAGCAGTT-3') to 1042 (region E; primer BDR27; 5'-CGGCAAGCAACACGTCCGCC-3') from 17 *N. gonorrhoeae* isolates collected from patients attending the sexually transmitted diseases clinic at St. Mary's Hospital, London, United Kingdom, presenting with symptoms of gonorrhoea. In all the isolates, a PCR product of the size predicted for clone pBDR8.11 (587 bp) was generated (data not shown).

Conclusions. The arrangements of regions D and E in *N. gonorrhoeae* and in *N. meningitidis* were shown in this study to be identical. In the latter species, however, regions E and D are interrupted by regions C and A, which contribute to capsule formation by polysialic acid synthesis and the transport of the polysaccharide chains to the cell surface (5, 7). From the common organization of regions D and E in both gonococci and meningococci, we can propose two possible scenarios, which led either to the loss of encapsulation in gonococci or, alternatively, to the gain of the capability to form a capsule in meningococci.

(i) If we assume that *N. gonorrhoeae* did at one time have all the genes required for capsule production, then during the divergence of these organisms *cps* regions A, B, and C must have been deleted from the gonococcal chromosome by recombination. Whether or not this occurred before or after the duplication of region D in *N. meningitidis* is not known. The fact that the gonococcus does not express a capsule may indicate that it is not required for the colonization of the niches inhabited by *N. gonorrhoeae* or for the dissemination of the organism, whereas it apparently is a requirement for meningococcal disease although not for nasopharyngeal colonization (1). The arrangement of regions D and E described here was found in all the gonococcal isolates examined and could suggest that modern gonococcal strains are derived from one ancestral source. Alternatively, the events resulting in this arrangement could be favored and may have occurred many times. Experimental evidence to support either of these hypotheses is lacking.

(ii) Alternatively, we could assume that the gonococcus has never synthesized a capsule and that regions D, D', and E are not part of the capsule complex per se in *N. meningitidis*. Support for this hypothesis comes from our analysis of strains carrying null mutations in regions D' and E, which failed to show any effect on either LPS or capsule expression. Earlier studies suggested that regions D and E were involved in the regulation of capsule expression, since *Escherichia coli* clones harboring the complete *cps* cluster expressed less capsular polysaccharide than did mutants with deletions in either region. Neither mutation had any effect on capsular polysaccharide synthesis in meningococci. An explanation for this is that there is a competition between two distinct biosynthesis pathways (capsular polysaccharide and LPS) for one carbohydrate source (13) in *E. coli* cells that is not observed in meningococci.

If the second scenario (gain of encapsulation in meningococci) is correct, one would predict that gene cassettes were introduced within the region D-E locus. Support for this hypothesis comes from the analysis of the *cps* nucleotide sequence, since the region A genes have a GC content of only 30% (5), which is significantly below the average GC content of *Neisseria* spp. A similar low GC content was also found for capsule biosynthesis genes in *E. coli* K1 (22). These observations support the idea that encapsulation is the result of horizontal DNA transfer to meningococci in the region D-E locus.

The region D' in meningococci seems to be the result of an incomplete duplication, and the *galE'* is nonfunctional, with a deletion at the amino terminus of 126 amino acids (13). However, the other genes are duplicates of those downstream of the functional *galE*, for which there is no obvious function. The presence of the additional ORFs between region E and the truncated *galE* and downstream of *rfbC* suggests that the recombinational event has occurred between ORF2 and ORF3. The function of these ORFs and ORF1, which encodes a putative methyltransferase, may provide a selective pressure to maintain this part of the chromosome, including the nonfunctional *galE'-rfb* gene cassette.

We are grateful to Maïke Hartmann for expert technical assistance.

B.D.R. is supported by a Research Career Development Fellowship from the Wellcome Trust. M.F. is supported by grants from the Deutsche Forschungsgemeinschaft (Fr689/2-3 and Fr689/8-1) and gratefully acknowledges the receipt of a professorship from the Hermann and Lilly Schilling Foundation.

REFERENCES

1. Cartwright, K. 1995. Meningococcal carriage and disease, p. 115-146. In K. Cartwright (ed.), *Meningococcal disease*. John Wiley and Sons, Chichester, United Kingdom.
2. Daniels, D. L., G. D. Plunkett, V. Burland, and F. R. Blattner. 1992. Analysis of the *Escherichia coli* genome: DNA sequence of the region from 84.5 to 86.5 minutes. *Science* 257:771-778.
3. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
4. DeVoe, I. W. 1982. The meningococcus and mechanisms of pathogenicity. *Microbiol. Rev.* 46:162-190.
5. Edwards, U., A. Mueller, S. Hammerschmidt, R. Gerardy-Schahn, and M. Frosch. 1994. Molecular analysis of the biosynthesis pathway of the α -2,8 polysialic acid capsule by *Neisseria meningitidis* serogroup B. *Mol. Microbiol.* 14:141-149.
6. 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, K. McKenney, G. Sutton, W. FitzHugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269:496-512.
7. Frosch, M., U. Edwards, K. Bousset, B. Krausse, and C. Weisgerber. 1991. Evidence for a common molecular origin of the capsule gene loci in gram-

- negative bacteria expressing group II capsular polysaccharides. *Mol. Microbiol.* **5**:1251–1263.
8. **Frosch, M., and A. Mueller.** 1993. Phospholipid substitution of capsular polysaccharides and mechanisms of capsule formation in *Neisseria meningitidis*. *Mol. Microbiol.* **8**:483–493.
 9. **Frosch, M., I. Roberts, I. Gorgen, S. Metzger, G. J. Boulnois, and D. Bitter Suermann.** 1987. Serotyping and genotyping of encapsulated *Escherichia coli* K1 sepsis isolates with a monoclonal IgG anti K1 antibody and K1 gene probes. *Microb. Pathog.* **2**:319–326.
 10. **Frosch, M., C. Weisgerber, and T. F. Meyer.** 1989. Molecular characterization and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria gonorrhoeae* group B. *Proc. Natl. Acad. Sci. USA* **86**:1669–1673.
 11. **Haas, R., A. F. Kahrs, D. Facius, H. Allmeier, R. Schmitt, and T. F. Meyer.** 1993. TnMax—a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. *Gene* **130**:23–31.
 12. **Haas, R., H. Schwarz, and T. F. Meyer.** 1987. Release of soluble pilin antigen coupled with gene conversion in *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. USA* **84**:9079–9083.
 13. **Hammerschmidt, S., C. Birkholz, U. Zähringer, B. D. Robertson, J. van Putten, O. Ebeling, and M. Frosch.** 1994. Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis*. *Mol. Microbiol.* **11**:885–896.
 14. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269–277.
 15. **Klein, N. J., R. S. Heyderman, and M. Levin.** 1993. Management of meningococcal infections. *Br. J. Hosp. Med.* **50**:42–49.
 16. **Mandrell, R. E., H. Smith, G. A. Jarvis, J. M. Griffiss, and J. A. Cole.** 1993. Detection and some properties of the sialyltransferase implicated in the sialylation of lipopolysaccharide of *Neisseria gonorrhoeae*. *Microb. Pathog.* **14**:307–313.
 17. **Robertson, B. D., M. Frosch, and J. P. van Putten.** 1993. The role of *galE* in the biosynthesis and function of gonococcal lipopolysaccharide. *Mol. Microbiol.* **8**:891–901.
 18. **Robertson, B. D., M. Frosch, and J. P. M. van Putten.** 1994. The identification of cryptic rhamnose biosynthesis genes in *Neisseria gonorrhoeae* and their relationship to lipopolysaccharide biosynthesis. *J. Bacteriol.* **176**:6915–6920.
 19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 20. **Slatko, B. E., R. Croft, L. S. Moran, and G. G. Wilson.** 1988. Cloning and analysis of the *HaeIII* and *HaeII* methyltransferase genes. *Gene* **74**:45–50.
 21. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
 22. **Weisgerber, C., A. Hansen, and M. Frosch.** 1991. Complete nucleotide and deduced protein sequence of CMP-NeuNAc: poly- α -2,8-sialosyl sialyltransferase of *Escherichia coli* K1. *Glycobiology* **1**:357–365.