INFECTION AND IMMUNITY, Oct. 1989, p. 3045–3052
0019-9567/89/103045-08$02.00/0

Vol. 57, No. 10

Identification of a Chromosomal Locus for Expression of Lipopolysaccharide Epitopes in *Haemophilus influenzae*

JEFFREY N. WEISER,[1]* ALF A. LINDBERG,[2] E. JANE MANNING,[1] E. J. HANSEN,[3] AND E. RICHARD MOXON[1]

Oxford University Department of Paediatrics and Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom[1]; Department of Clinical Bacteriology, Karolinska Institute, Huddinge University Hospital, S-141, 86 Huddinge, Sweden[2]; and Department of Microbiology, Southwestern Graduate School of Biomedical Sciences, Southwestern Medical School, University of Texas Health Science Center, Dallas, Texas 75235[3]

Received 14 March 1989/Accepted 3 May 1989

Lipopolysaccharide (LPS) is a major virulence determinant of *Haemophilus influenzae*. The organism is able to display an extensive repertoire of different LPS structures through the loss and acquisition of multiple oligosaccharide epitopes in various combinations. This marked heterogeneity of LPS molecules has complicated the analysis of the structure of LPS and its role in pathogenesis. A genomic library was screened for the ability to transform *H. influenzae* to express novel LPS epitopes defined by reactivity with oligosaccharide specific monoclonal antibodies. A chromosomal locus, *lic-1*, involved in expression of at least three different epitopes (recognized by monoclonal antibodies 4C4, 12D9, and 6A2), was identified on a 5.6-kilobase restriction endonuclease fragment. Transformation of *H. influenzae* with subclones from within *lic-1* was used to generate a series of isogenic and phenotypic variants. All transformants displayed phase variation for their newly acquired epitopes. Altered binding specificities of LPS with monoclonal antibodies correlated with changes in sugar compositional analysis. The expression of two epitopes was eliminated by introduction of site-specific mutations in *lic-1*, confirming the role of *lic-1* in oligosaccharide biosynthesis.

Haemophilus influenzae remains a leading cause of bacterial meningitis in children in developed countries as well as a common etiologic agent in respiratory tract infection worldwide. Much attention is now being focused on the surface antigens other than capsule, such as lipopolysaccharide (LPS), outer membrane proteins and fimbriae. LPS contributes to the pathogenicity of a number of gram-negative bacteria (1, 15, 17); in *H. influenzae*, there is evidence that LPS is a major virulence determinant (5, 11, 12, 24).

The LPS of *H. influenzae* is rough, resembling that of the *Neisseria* species and *Bordetella pertussis*, and is sometimes referred to as lipooligosaccharide. Unlike members of the family *Enterobacteriaceae*, which possess LPS polysaccharide chains built of repeating oligosaccharide units, *H. influenzae* LPS contains antigenic determinants that reside on the equivalent of the core region, which consists of a short chain of covalently linked neutral sugar residues (11). The configuration of the LPS core region has been resolved for *Salmonella typhimurium* through the analysis of a series of LPS mutants that define the *rfa* genetic locus (14). In contrast, there is only a single well-defined *H. influenzae* LPS mutant reported (21, 23); otherwise there is no information about its genetics or structure.

The oligosaccharide of *H. influenzae* LPS differs from the core region of *S. typhimurium* in its composition (9) and ability to vary its structure. When *H. influenzae* LPS is characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining, there is heterogeneity among LPS molecules from different strains (8, 19). The recent availability of monoclonal antibodies (MAbs) to the oligosaccharide has made it possible to define a number of epitopes and to order these strains into antigenic groups. These MAbs were used to characterize 126 clinical isolates, all of which were recognized by one or the other of two MAbs (5). Using a colony blot radioimmunoassay, Kimura et al. subsequently showed that individual strains are capable of demonstrating antigenic variation that occurs at a high frequency and involves both spontaneous loss and acquisition of MAb reactivity, i.e., phase variation; when reactive and nonreactive variants were tested in a rat model, differences in virulence were shown to be associated with LPS phenotype (12). However, associations between virulence or other characteristics with LPS are difficult to establish since the inherent variability results in a heterogeneous population of organisms. Structural analysis has been hampered by difficulties in obtaining homogeneous samples of LPS molecules.

For these reasons we sought a genetic approach to investigate further the structure and function of LPS in *H. influenzae* pathogenicity. We report here the use of MAbs to isolate a region of the chromosome responsible for the expression of several LPS antigenic determinants that display phase variation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. influenzae* RM.7004 is an encapsulated type b clinical isolate generously provided from the collection of Loek Van Alphen (strain 760705) and previously characterized (20). RM.118 (Rd^-) is a capsule-deficient type d strain that can be made highly competent for transformation. *Escherichia coli* Q358 (16) was used to propagate λEMBL3. Constructs in plasmid pUC13 (Pharmacia Fine Chemicals) were maintained in *E. coli* DH5α (Bethesda Research Laboratories, Inc.).

H. influenzae strains were grown in brain heart infusion (BHI) broth (Oxoid Ltd.) supplemented with 2 μg of NAD (Boehringer Mannheim Diagnostics) and 10 μg of hemin per ml. BHI plates were prepared with 1% agar and supplemented with 10% Levinthal base. Bacterial cells used for extraction of LPS were grown to the late log phase in

* Corresponding author.

supplemented BHI broth with 3% yeast extract (Difco Laboratories) in a 12-liter fermentor. The cells were washed in phosphate-buffered saline (pH 7.2; PBS) and then lyophilized.

Preparation and analysis of genomic DNA. Total cellular DNA was prepared from 3-ml broth cultures as previously described (7). DNA for use in transformation experiments was digested to completion with different restriction endonucleases purchased from Amersham International. DNA for analysis by Southern hybridization was digested and separated electrophoretically on 0.7% agarose–Tris borate gels, transferred to nitrocellulose filters, and hybridized at 42°C in 40% formamide with nick-translated [α-^{32}P]dCTP-labeled DNA probes (16). Nitrocellulose filters were then washed at a stringency calculated to allow for a 12% mismatch.

DNA transformation. *H. influenzae* was grown to the early log phase in supplemented heart infusion broth (Difco) before washed cells were transferred to Herriot MIV medium (6) and incubation at 37°C for 100 min with shaking at 90 rpm. Competent cells were incubated in the presence of either total genomic or cloned DNA for 30 min at 37°C. After DNA uptake, cells were plated to yield 500 to 1,000 colonies per BHI plate after overnight incubation.

Construction of gene banks λEMBL3. A library of total cellular DNA from strain RM.7004 was constructed in λEMBL3 (Amersham). A partial *Sau*3A1 digest was fractionated by centrifugation through a sucrose density gradient, and the fractions containing 15- to 20-kilobase (kb) fragments were selected. These were ligated into the *Bam*HI sites on λEMBL3 and packaged to form infectious particles by using a packaging kit (Amersham). Approximately 90% of the resulting phage represented recombinants.

Screening of the library in λEMBL3. Total genomic DNA from RM.7004 digested to completion of *Pvu*II (6 μg) was size fractionated by centrifugation through a 36-ml 10 to 40% sucrose density gradient. The fraction containing DNA in the size range of 15 to 22 kb was ethanol precipitated, [α-^{32}P]dCTP labeled by nick translation, and used as a hybridization probe on nitrocellulose lifts of plaques from the λEMBL3 library. Positively hybridized plaques were selected, and samples were pooled in groups. The phage were propagated in L broth, and then DNA was prepared by centrifugation in polyethelene glycol as described previously (16). When DNA from a pooled group of clones was shown to possess transforming activity, it was further subdivided until a single clone was identified.

Plasmid constructions. DNA fragments for subcloning were separated on 1% agarose–Tris borate gels, removed, and purified with Gene Clean (Stratagene) for ligation into the cloning site of pUC13. Recombinants were distinguished from nonrecombinants by their failure to generate blue colonies in the presence of isopropyl-β-D-thiogalactopyranoside (US Biochemical Corp.) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Bethesda Research Laboratories). Site-specific mutations were created by digestion of plasmid DNA with either restriction endonuclease *Xba*I or *Ava*I followed by ligation of blunted ends filled in with the Klenow fragment of *E. coli* polymerase I (Amersham). λEMBL3 or plasmid DNA for incubation with competent *H. influenzae* was digested with restriction endonucleases to separate the cloned insert intact from the vector.

Colony immunoblotting. The LPS phenotype was determined by reactivity with MAbs specific for the oligosaccharide portion of *H. influenzae* LPS. The characteristics of MAbs 4C4, 5G8, 12D9, and 6A2 have been previously reported (4, 5). Murine MAb A1-12E-5G-8E was raised by using purified LPS from *H. influenzae* Morgan A.

H. influenzae colonies were transferred to nitrocellulose filters and incubated with PBS containing 3% chicken egg ovalbumin (Sigma Chemical Co.) for 30 min, and MAbs were added at final dilutions of 1:80 (4C4), 1:50 (5G8), 1:150 (12D9), 1:80 (6A2), and 1:10,000 (A1-12E-5G-8E) for an additional 60 min at 37°C with gentle shaking. After filters were washed thoroughly with PBS, they were placed in 1:800 dilution of peroxidase-conjugated rabbit anti-mouse globulins (Jansen) for 120 min at room temperature. The filters were again washed with PBS, and reactivity was visualized by incubation for 10 min in diamino-benzidine and hydrogen peroxide. Colonies were grown after immunoblotting by incubating for 8 h at 37°C.

Immunoperoxidase staining. A single *H. influenzae* colony was suspended in 10 μl of PBS and air dried on a glass slide. Cells were gently fixed in 0.25% glutaraldehyde in PBS for 20 min and then quenched with 0.25 M glycine in PBS for 20 min. Undiluted MAb was applied for 90 min at room temperature. After cells were washed with PBS, a 1:30 dilution of the conjugated antibody in PBS was applied for 60 min. Slides were stained with diaminobenzidine and hydrogen peroxide and then counterstained for 20 min with 10% Giemsa stain in Sorensen buffer.

Sugar analysis of LPS. LPS was extracted with phenol-chloroform-light petroleum as described by Galanos et al. (2). After flash evaporation of the organic solvents, LPS was precipitated with 9 volumes of ice-cold ethanol (96%, vol/vol) and left at −20°C for 18 h. The precipitate was recovered and lyophilized. The LPS was subsequently suspended in distilled water (10 mg/ml) and centrifuged in an angle rotor at 105,000 × *g* at 4°C for 4 h. The pellet was recovered, and the ultracentrifugation was repeated twice. The final pellet was lyophilized and treated for 72 h with hydrofluoric acid (48%, vol/vol) at 0°C (10 mg/ml) to remove phosphate groups. The LPS was then dialyzed against distilled water and finally hydrolyzed in 1.0% acetic acid (10 mg/ml) at 100°C for 1 h. The solution was neutralized with 0.1 M NaOH and desalted on a Bio-Gel P4 column (Bio-Rad Laboratories). The effluent was monitored with a differential refractometer (model 403; Waters Associates, Inc.), and the fractions were assayed for carbohydrates by the phenol-sulfuric acid method. The peak fraction was collected, lyophilized, and used for further analyses.

RESULTS

LPS phenotype is genetically determined. We utilized the natural transformability of *H. influenzae* to show that the LPS phenotype of a recipient strain can be altered by the uptake of DNA from a heterologous donor strain. LPS phenotype was assessed by reactivity with LPS-specific MAbs in a colony immunoblot assay. Initial experiments used MAb 4C4 because the expression of this epitope was associated with increased virulence (11). RM.118 (Rd$^-$) was selected as the recipient for transformation because it was constitutively nonreactive with 4C4 and could be made highly competent. The donor strain, RM.7004, a type b clinical isolate, was chosen because it bound 4C4 avidly and showed a high frequency of phase variation for this epitope. When total cellular DNA from RM.7004 was added to competent RM.118, 0.1 to 0.2% of colonies acquired reactivity with MAb 4C4. When a reactive colony was inoculated into BHI broth, growth to the midlog phase, and replated, the progeny displayed both 4C4$^-$ and 4C4$^+$ phenotypes.

TABLE 1. Reactivity with LPS-specific MAbs by colony immunoblotting[a]

H. influenzae strain	Reactivity with MAb:				
	12D9	6A2	4C4	5G8	A1-12E-5G-8E
RM.7004	+	+	+	+	+
RM.118	−	−	−	−	+
RM.118-1	−	−	+	−	+
RM.118-26	+*	+	−	−	+
RM.118-28	+	+*	−	−	+
RM.118-102	+*	−	−	−	+
RM.118-26H4	−	−	−	−	+
RM.7004-H7	−	−	+	+	+
RM.7004-A1	−	−	+	+	+

[a] −, Constitutively nonreactive; +, phase variation for this epitope, with greater than 99% of variants reactive; +*, phase variation for this epitope with less than 5% of variants reactive.

Growth of either $4C4^+$ or $4C4^-$ progeny again gave rise to a mixed population of phenotypes. Thus, transformation of RM.118 by donor DNA from RM.7004 resulted in both the acquisition of a novel epitope and the ability to display phase variation for this novel epitope. These observations indicated the feasibility of identifying relevant transforming activity in cloned DNA fragments.

Identification of *lic-1* from a library in λEMBL3. A chromosomal locus for the expression of LPS epitopes, designated *lic-1* (lipopolysaccharide core), was identified in a λEMBL3 library of total genomic DNA from RM.7004. Initial attempts to detect $4C4^+$ transformants of RM.118 by using pooled DNA from 500 λEMBL3 recombinants were unsuccessful, and we concluded that the frequency of transformation of RM.118 to $4C4^+$ was too low to be detected by the colony immunoblot technique.

An alternative method for screening the λEMBL3 library was employed. Further investigation showed that endonuclease digestion of total genomic DNA markedly reduced the transformation frequency, perhaps due to either cleavage of genes of interest or preferential selection of sized fragments not possessing transforming activity. However, digestion of donor DNA with a single endonuclease, *Pvu*II, showed no diminution in transforming activity. *Pvu*II-digested RM.7004 DNA was size fractionated to obtain a population of fragments enriched in transforming activity. When the fraction with 15- to 22-kb fragments was added to competent cells, 1.3% became $4C4^+$. This enriched fraction was then used as a hybridization probe for the EMBL3 library. Of 118 hybridizing clones, 1, labeled λ2239A, was found to possess 4C4-transforming activity.

Surprisingly, the frequency of transformation with λ2239A averaged only 1.0%, which was no higher than the frequency of transformation obtained with the 15- to 22-kb *Pvu*II fragments of total genome DNA. A transformant, RM.118-1, (Table 1), bound MAb 4C4 avidly and displayed variability in expression for this epitope.

Transformation experiments mapping *lic-1*. The transforming activity for 4C4 reactivity was localized to a 5.6-kb *Bgl*II subclone of λ2239A. To determine whether genes for the expression of LPS epitopes, other than 4C4, might be physically linked, as is the case in the *rfa* locus (10), three other MAbs, 12D9, 6A2 and 5G8, previously shown to recognize different LPS epitopes (5), were examined. RM.7004 was reactive with each of these MAbs, whereas RM.118 was nonreactive (Table 1). An additional LPS-specific MAb, A1-12E-5G-8E, which bound both strains, showed that both were capable of displaying phase variation. When DNA from the subcloned *Bgl*II fragment (pGA6) was used to transform RM.118, up to 50% of colonies acquired reactivity with MAbs 12D9 and 6A2; no 5G8-reactive transformants were observed. A DNA fragment capable of transforming RM.118 to bind MAbs 12D9 and 6A2 was further localized to a 1.2-kb *Hin*dIII-*Nru*I fragment of pGA6, designated pHN1. Two transformants generated with pHN1, RM.118-26 and RM.118-28, were reactive with both 12D9 and 6A2. A cloned 0.5-kb *Eco*RV fragment, pRV2, from within pHN1 was able to transform RM.118 to bind MAb 12D9 but not MAb 6A2 (RM.118-102)). This suggests that genes responsible for expression of the epitope specified by MAb 12D9 map to this 0.5-kb fragment, whereas those specific to MAb 6A2 reside in the neighboring 0.7 kb. To date, attempts to further localize a region for expression of the 4C4 determinant have been unsuccessful.

Subcloned DNA from λ2239A was used as a hybridization probe to map the corresponding region in RM.118 and RM.7004 (Fig. 1). The map of RM.118, based on the position of endonuclease cleavage sites, was similar to that of RM.7004 within the two *Bgl*II sites, despite differences between strains in expression of at least four epitopes. The degree of homology at the sequence level was sufficient to allow for the homologous recombination of small fragments in the transformations described. The region in RM.7004 and RM.118 was mapped with a total of 17 restriction endonucleases. Over a span of 5.6 kb the two strains differed only in the presence of unique *Xba*I and *Ava*I cleavage sites in RM7004.

Southern hybridization (Fig. 2) with pGA6 as a probe showed that DNA containing one or both of these restriction sites had been incorporated into chromosomal DNA of

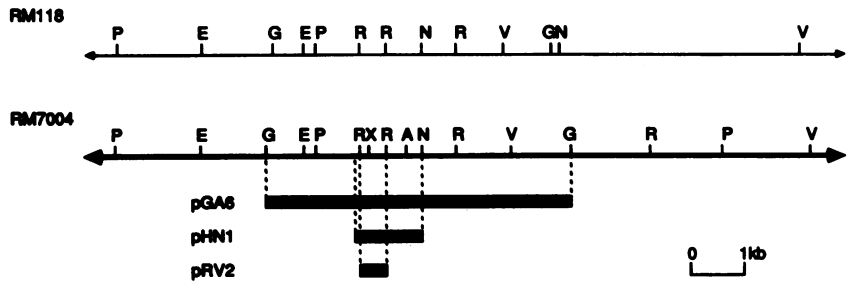

FIG. 1. Physical map of strain RM.7004 spanning 13.0 kb of the chromosome. Also shown is the corresponding physical map of strain RM.118, utilized as a recipient in transformation experiments. A locus for the expression of LPS epitopes, *lic-1*, has been localized to a 5.6-kb subclone, pGA6. Other subclones are labeled pHN1 and pRV2. Restriction sites: A, *Ava*I; E, *Eco*RI; R, *Eco*RV; G, *Bgl*II; N, *Nru*I; P, *Pst*I; V, *Pvu*II; X, and *Xba*I.

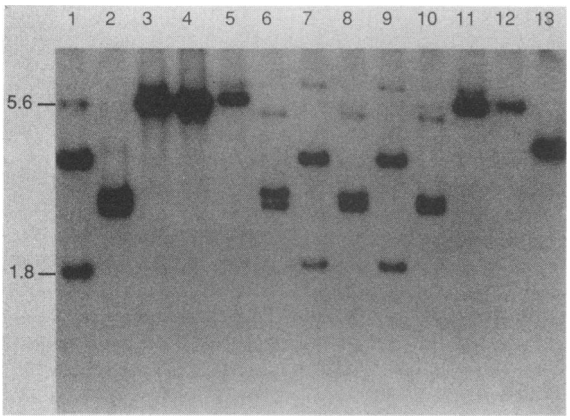

FIG. 2. Southern blot of chromosomal DNA from strains with specific alterations in LPS epitopes, generated by transformation with various constructs from *lic-1* as described in the text. DNA was digested with *Bgl*II in addition to either *Ava*I (lanes 2, 4, 6, 8, 10, and 13) or *Xba*I (lanes 1, 3, 5, 7, 9, 11, and 12) and probed with pGA6. Lanes: 1 and 2, RM.7004; 3 and 4, RM.118; 5 and 6, RM.118-1; 7 and 8, RM.18-26; 9 and 10, RM.118-28; 11, RM.118-26H4; 12, RM.7004-H7; 13, RM.7004-A1. Sizes are marked in kilobases.

transformants RM.118-1 (*Ava*I) and RM.118-26 and RM.118-28 (*Xba*I and *Ava*I). A *Xba*I site was not incorporated from pRV2 in the chromosome of RM.118-102, and no other restriction site polymorphisms were identifiable in this transformant.

The possibility that the *Xba*I (present on pHN1 and pRV2) and *Ava*I (present on pHN1 but not pRV2) sites marked DNA regions specific, respectively, for 12D9 and 6A2 epitopes was tested. Frameshift mutations were introduced into these sites in pGA6. When DNA containing the *Xba*I site mutation was transformed into RM.118-26 and colonies nonreactive with MAb 6A2 were selected, strain RM.118-26H4 was identified. This strain was no longer reactive with either MAb 6A2 or 12D9. Similarly, introduction of either the *Xba*I site mutation (RM.7004-H7) or the *Ava*I site mutation (RM.7004-A1) into RM.7004 eliminated reactivity with both MAbs 12D9 and 6A2. Southern hybridization with pGA6 as a probe confirmed the chromosomal loss of an *Xba*I site (RM.118-26H4, RM.7004-H7) or *Ava*I site (RM.7004-A1) in the *lic-1*. The altered phenotype of the RM.7004 mutants provided further evidence that *lic-1* is responsible for biosynthesis of LPS oligosaccharide components and that expression of 12D9- and 6A2-specific epitopes is genetically linked.

LPS sugar analysis. LPS was extracted from strains RM.118, RM.118-01, RM.118-26, and RM.118-28 by the phenol-chloroform-light petroleum method. Extracted LPS was precipitated from the phenol phase with cold ethanol, which gave a more complete precipitation than the mere addition of water. To facilitate a quantitative determination of the sugars, the LPS was dephosphorylated by hydrofluoric acid treatment. Sugars subsequently released by acid hydrolysis were analyzed as their alditol acetates by gas-liquid chromatography–mass spectroscopy (Table 2). Although these data did not allow for detailed structural analysis, clear differences in the oligosaccharide between strains could be shown by the relative differences of glucose, galactose, and heptose. Basically all had D-glucose and L-glycero-D-heptose (low amount in RM.118-26). The most pronounced variation was seen with the D-galactose content. Methylation analyses of the oligosaccharides have shown

TABLE 2. LPS sugar analysis[a]

H. influenzae strain	Sugar ratios		
	Glucose	Galactose	Heptose
RM.118	2.0	1.0	2.7
RM.118-1	2.0	1.1	1.0
RM.118-26	2.0	0.7	0.1
RM.118-28	2.0	0.1	1.6

[a] Sugar composition of LPS oligosaccharide obtained after hydrogen fluoride and acetic acid treatment. Sugars released by hydrolysis were analysed as their alditol acetate by gas-liquid chromatography–mass spectometry, yielding the molecular ratios shown.

the presence of different glucose and galactose methyl ethers (unpublished data) correlating with the phenotypic changes shown by reactivity with the LPS-specific MAbs.

Phase variation of LPS epitopes. When a single well-isolated colony of RM.7004 was inoculated into BHI broth, grown to mid-log phases, and plated, the resultant colonies displayed both reactive and nonreactive phenotypes for each of five MAbs recognizing distinct epitopes. Phase variation, as shown by the sectored appearance of the progeny colonies in the immunoblot assay, occurred at a high frequency (Fig. 3). All the transformants underwent phase variation for their newly acquired epitopes, although the frequency of reversion varied from strain to strain. Phase variants of RM.118-26 were less than 5% $12D9^+$ and greater than 99% $6A2^+$, whereas variants of RM.118-28 were less than 5% $6A2^+$ and greater than 99% $12D9^+$. Interestingly, both of these transformants were derived by using the same 1.2-kb donor DNA fragment (pHN1).

We used an immunoperoxidase staining procedure to look at the expression of LPS epitopes on individual organisms. Cells, fixed on a glass slide, were incubated with MAb and then a second antibody conjugated to peroxidase to yield a brown color in the presence of diaminobenzidine-hydrogen peroxide. Nonreactive organisms were visualized by counterstaining purple with Giemsa. Figure 4A shows phase variation for the 4C4-specific epitope on individual organisms of strain RM.7004. Two adjacent organisms, one reactive and the other nonreactive, were seen. The expression of the relevant epitope appeared to be either detectable or nondetectable; there were no intermediates.

Immunoperoxidase staining of RM.118 (Fig. 4C) confirmed its nonreactivity with MAb 4C4. RM.118-1 (Fig. 4B), a transformant of RM.118, was reactive with MAb 4C4 and displayed variable expression of this epitope, confirming results obtained with the immunoblot assay.

The technique of sodium dodecyl sulfate-polyacrylamide gel electrophoresis is capable of showing only major structural variations in the LPS oligosaccharides. Using MAbs to follow defined epitopes suggests that variation in LPS structure is a complicated phenomenon that confers on the organisms a large array of possible LPS configurations. We looked at the relationship between epitopes in 500 colonies of RM.7004 of a clonal origin that were replica plated and immunoblotted. An illustrative example of this data is provided in Fig. 5. RM.7004 displayed phase variation for at least five LPS epitopes. The majority of organisms were able to react with all five MAbs. The epitope recognized by MAb 6A2, which was only expressed in conjunction with the epitope recognized by MAb 12D9. Loss of a single epitope (5G8) is shown in position 18. The repertoire of unique LPS configurations is greatly multiplied when one also considers the coordinate on-off switching of more than a single epitope

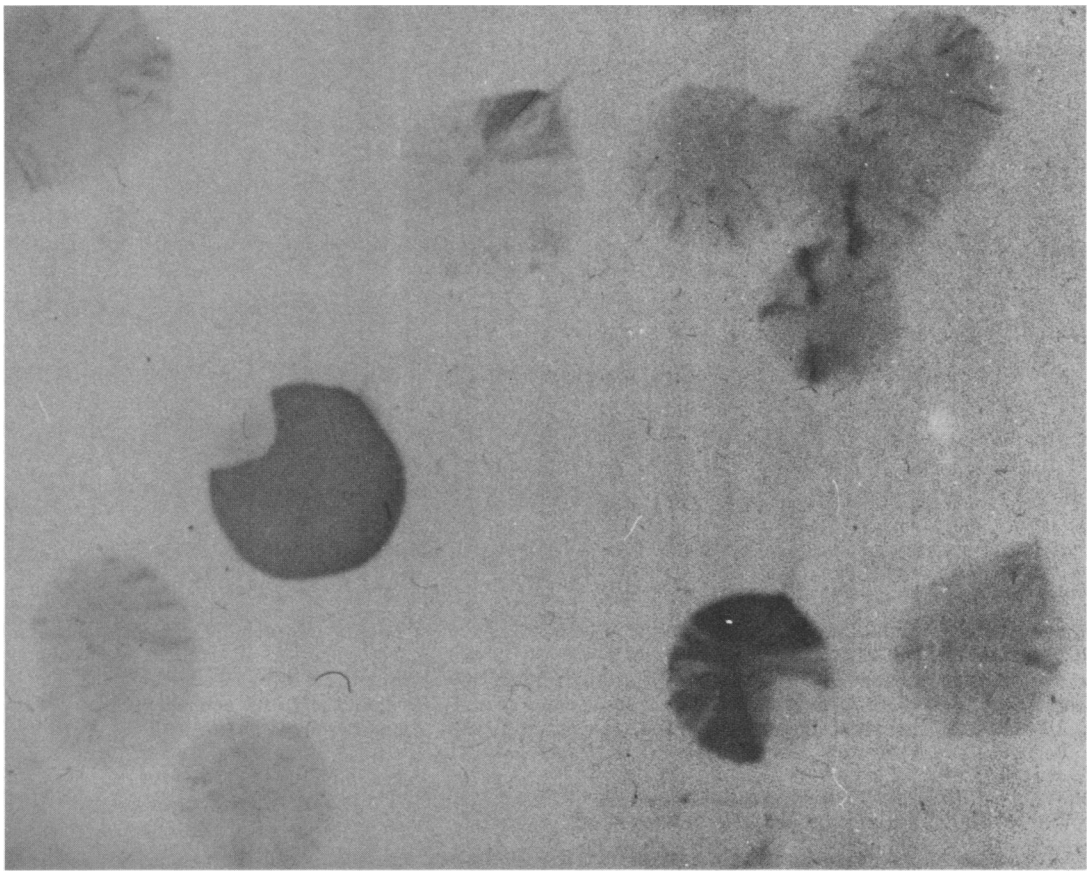

FIG. 3. Sectored colonies of RM.7004 due to phase variation. A single colony, nonreactive with MAb 12D9, was inoculated into supplemented BHI broth, grown to the midlog phase, and plated on BHI agar. These colonies were immunoblotted with MAb 12D9 and photographed.

in various combinations as demonstrated in position 21 by the loss of reactivity with 6A2, 4C4, 5G8, and A1-12E-5G-8E.

DISCUSSION

The LPS of *H. influenzae* is an important determinant of virulence. The core oligosaccharides have been shown to contain the neutral sugars heptose, glucose, and galactose in various molar ratios and linkages, so that there is considerable interstrain heterogeneity among clinical isolates (22). Previous studies have reported that the LPS molecules of single strains show high-frequency, spontaneous variation as a result of sequential loss and gain of oligosaccharide epitopes (i.e., phase variation). This phenomenon, first described by Kimura and Hansen (11), has been clearly and conveniently demonstrated by using several MAbs specific for oligosaccharide epitopes. The availability of these MAbs was pivotal to obtaining the results of the present study of the cloning and endonuclease mapping of a chromosomal locus (*lic-1*). The cloned and subcloned fragments of *lic-1* have facilitated the isolation of isogenic transformants that express different LPSs as shown by immunoblotting, immunoperoxidase staining of individual bacterial cells, and compositional analysis of extracted oligosaccharides. Endonuclease mapping and site-directed mutagenesis have shown that sequences within *lic-1* are involved in the expression of oligosaccharide epitopes defined by MAbs 4C4, 12D9, and 6A2 and have also indicated the likely location of genes essential for the expression of the epitopes defined by MAbs 12D9 and 6A2.

Since the molecular basis of the biogenesis of *H. influenzae* LPS is unknown, the strategy for cloning genes involved in the expression of these complex microbial surface-exposed molecules (tertiary gene products) was not straightforward. The successful search of a gene library (constructed from a strain expressing several defined oligosaccharide epitopes) for donor DNA clones that could transform a recipient strain (constitutively lacking the expression of these epitopes) proved possible because of the efficiency with which competent *H. influenzae* cells take up and recombine (18) DNA into their own genome and the use of the simple, sensitive, and specific colony blot assay. The use of cloned RM.7004 DNA to transform RM.118, which is constitutively lacking expression of epitopes defined by four of five MAbs used in this study (Table 1), provides clear evidence that *lic-1* is necessary for the expression of these altered oligosaccharide structures but leaves unspecified the nature of the permissive genetic event involved in the transformation of RM.118. The physical (endonuclease) map of the chromosomal region of RM.118 corresponding to *lic-1* (RM.7004) shows extensive sequence identity, as evidenced by the results of Southern hybridization and the corresponding presence and absence of many identical endonuclease restriction sites. The failure of RM.118 to express epitopes that could be conferred by transformation might suggest that the locus is cryptic, owing to one or more mutations or the

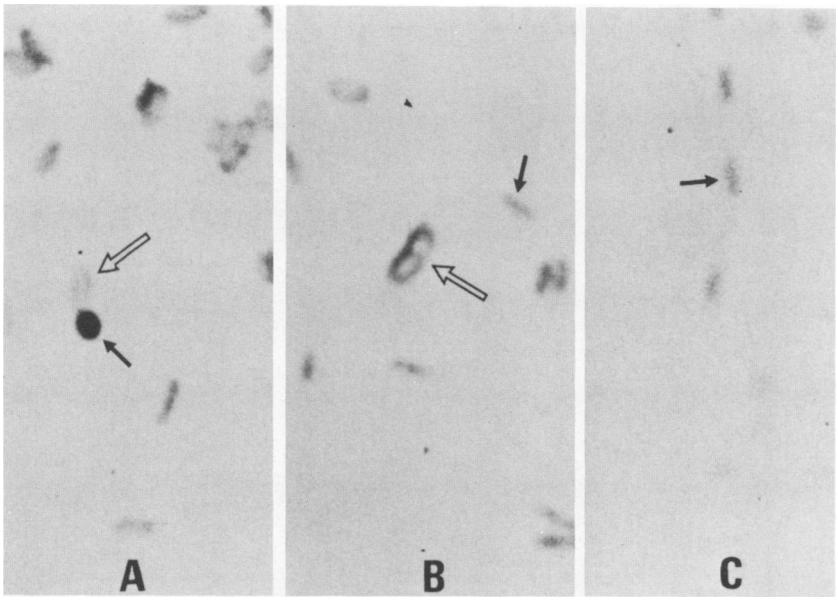

FIG. 4. Reactivity of individual organisms by LPS-specific MAb 4C4 by an immunoperoxidase staining technique. Bacteria from a single colony were incubated with MAb 4C4 before initial staining by use of peroxidase-conjugated second antibody then counterstaining with Giemsa as described in Materials and Methods. Reactive cells (open arrows) appear larger, with an enhanced outer ring. The counterstain is absorbed by nonreactive cells (solid arrows) and stains encapsulated organisms more intensely than nonencapsulated cells. (A) RM.7004 (encapsulated), (B) RM.118-1 (nonencapsulated), (C) RM.118 (nonencapsulated). Magnification, ca. ×4,650.

lack of appropriate transcriptional or regulatory signals. Each of the different transformants of RM.118 that acquired the ability to express novel epitopes also showed phase variation of these structural refinements. However, it is not yet apparent whether the genetic basis of this antigenic variation (as opposed to the expression of novel epitopes) was acquired during transformation or was an intrinsic property of the RM.118 before its transformation. The latter possibility seems more likely, since transformation of RM.118 with a fragment of less than 0.5 kb in size (pRV2) is capable of inducing a novel epitope; it would seem unlikely that such a small sequence could carry the information for both expression and antigenic variation of the epitope defined by 12D9.

The homologous recombination of either genomic or cloned donor DNA from RM.7004, resulting in the transformation of RM.118 to express the epitope defined by MAb 4C4, appears to be complicated and cannot be adequately explained by the current data. Compared with the relatively high frequency of transformation to 12D9 and 6A2 epitopes, $4C4^+$ transformants occurred at significantly lower frequency. Indeed, there was no difference in transformation frequency with cloned (λ2239A) DNA as compared to unselected, sized genomic fragments. Furthermore, Southern hybridization analysis of the $4C4^+$ transformant indicates that more than one recombinational event may have occurred (data not shown), so that changes in more than one locus may be required for the aquisition of the $4C4^+$ epitope, perhaps involving the provision of more than one functional gene product or regulatory sequence. In contrast, since a 1.2-kb fragment (pHN1) of DNA subcloned from *lic-1* could transform RM.118 ($12D9^-$ $6A2^-$) to $12D9^+$ $6A2^+$ and a smaller fragment (pRV2) was alone able to confer 12D9 reactivity, the present data provide a reasonable basis for locating two genes, possibly encoding glycosyl transferases, involved in the expression of the epitopes defined by MAbs 12D9 and 6A2. No homology was observed when the *S.*

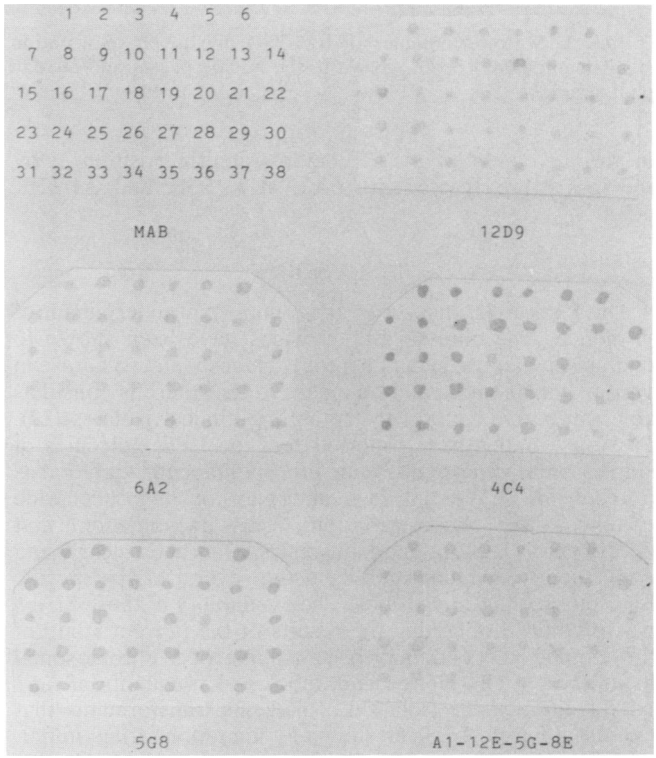

FIG. 5. Relationship between five LPS epitopes that display phase variation as assessed by MAb reactivity. Single colonies of strain RM.7004 were replica plated to five identical plates, and the resulting colonies were immunoblotted to MAbs 12D9, 6A2, 4C4, 5G8, and A1-12E-5G-8E. Coordinate loss of four or five epitopes is shown in position 21. Loss of a single epitope is shown in position 18.

typhimurium glycosyl transferase genes (*rfaG*, *B*, *I*, and *J*) (10) were used to probe *lic-1* under conditions allowing for 33% base pair mismatch in Southern hybridizations. The *E. coli* hosts for the *lic-1* constructs did not acquire reactivity with any of the *H. influenzae* MAbs tested.

There are two aspects of the data that merit further comment. First, the phenotypes of RM.118-26 and RM.118-28 were unusual. Although both transformants showed antigenic (phase) variation, the majority (95%) of RM.118-26 colonies were $6A2^+$, whereas only 5% were positive for MAb 12D9. RM.118-28 showed a similar phenomenon, only the pattern of reactivity showed the opposite preference; 95% $12D9^+$ and 5% $6A2^+$. We speculate that these transformations may have resulted from recombination events in which there was incomplete restoration of one gene (or its transcriptional signals), resulting in reduced activity of the relevant gene products. Second, frameshift mutations introduced at either the *Xba*I or *Ava*I endonuclease sites, corresponding to the loci for 12D9 and 6A2 epitopes, respectively, abolished binding to both MAbs 12D9 and 6A2. A possible explanation is as follows. Reactivity with MAb 6A2 does not occur in RM.7004 in the absence of the 12D9 epitope, suggesting that in this strain the structure of the oligosaccharide is such that the latter is a prerequisite for the expression of the 6A2 epitope. The effect of the *Ava*I frameshift mutation may be polar, i.e., neither gene product is translated. In the case of the *Xba*I frameshift mutation, the permissive structural feature for the expression of the 6A2 epitope is not available, and therefore neither of the epitopes is expressed. Evidently, the dependence of the 6A2 upon 12D9 epitope expression does not apply to all strains (e.g., RM.118-26).

Finally, *lic-1* does not appear to be in close proximity to two other regions of the *H. influenzae* chromosome that have been implicated in LPS expression. One of these loci (I69) has been recognized in association with strains having a deep rough LPS phenotype (23). The other locus is apparently linked to *cap* b (24). Genomic mapping of the *H. influenzae* chromosome with hybridization probes and DNA separated by pulsed-field gel electrophoresis shows that *lic-1* is not linked to either *cap* b or the I69 locus in either RM.118 (13) or strain Eagan (P. Butler, personal communication).

The antigenic variation of oligosaccharide epitopes may explain much of the phenotypic heterogeneity that has been reported in *H. influenzae* LPS. Present findings extend the observations of Kimura and Hansen (11) by demonstrating that the individual organisms of a clonal population are heterogeneous owing to high-frequency on-off switching, which occurs in vitro. The extent of this variation would not be detected by analysis of large numbers of organisms in structural studies or with techniques such as colony blotting or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The use of the simple immunoperoxidase staining procedure described here in, which permits characterization of the oligosaccharide epitopes of single cells, should prove valuable in further studies of both in vitro- and in vivo-derived organisms.

The potential repertoire of variant oligosaccharide structures is extensive; individual epitopes undergo phase variation independently, coordinately, and in various combinations. The variation of oligosaccharide structures within a clonal population may provide a mechanism by which *H. influenzae* can adapt to the different environments and host cell phenotypes that it encounters in humans. For example, such variation may facilitate attachment to epithelial cells or allow the organism to mimic host glycolipids or glycoproteins so as to constrain host responses and thereby abolish or lessen specific immune clearance mechanisms as has been proposed for the *Neisseria* species (3). Although the genetic mechanism responsible for phase variation is not yet understood, preliminary results suggest that the control of the on-off switching is regulated by sequences within or adjacent to *lic-1*.

ACKNOWLEDGMENTS

We acknowledge K. Sanderson for plasmids containing the *rfa* genes and G. Milon for guidance with immunostaining.

This work was funded by a grants from The Meningitis Trust, United Kingdom, the Medical Research Council (PG.8325352), and the Swedish Board for Technical Development (85-4311P). J.N.W. is a Fellow of the Institute for Pediatric Service, Johnson and Johnson Baby Products Co.

LITERATURE CITED

1. **Cryz, S. J., Jr., T. L. Pitt, E. Furer, and R. Germanier.** 1984. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. Infect. Immun. **44**:508–513.
2. **Galanos, C., O. Loderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. **9**:245–249.
3. **Griffiss, J. M., H. Schneider, R. E. Mandrell, R. Yamasaki, G. A. Jarvis, J. J. Kim, B. W. Gibson, R. Hamadeh, and M. A. Apicella.** 1988. Lipooligosaccharides: the principal glycolipids of the Neisserial outer membrane. Rev. Infect. Dis. **10**(Suppl.): S287–S295.
4. **Gulig, P. A., and E. J. Hansen.** 1983. Co-precipitation of lipopolysaccharide and the 39K major outer membrane protein of *Haemophilus influenzae* type b by lipopolysaccharide-directed monoclonal antibody. Infect. Immun. **42**:516–524.
5. **Gulig, P. A., C. C. Patrick, L. Hermanstorfer, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Conservation of epitopes in the oligosaccharide portion of the lipooligosaccharide of *Haemophilus influenzae* type b. Infect. Immun. **55**:513–520.
6. **Herriot, R. M., E. M. Meyer, and M. J. Vogt.** 1970. Defined non-growth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101**:517–524.
7. **Hoiseth, S. K., C. Connelly, and E. R. Moxon.** 1985. Genetics of spontaneous high-frequency loss of b capsule in *Haemophilus influenzae*. Infect. Immun. **49**:389–395.
8. **Inzana, T. J.** 1983. Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. J. Infect. Dis. **148**:492–499.
9. **Inzana, T. J., W. E. Seifert, Jr., and R. P. Williams.** 1985. Composition and antigenic activity of the oligosaccharide moiety of *Haemophilus influenzae* type b lipooligosaccharide. Infect. Immun. **48**:324–330.
10. **Kadam, S. K., A. Rehemtulla, and K. E. Sanderson.** 1985. Cloning of *rfaG*, *B*, *I*, and *J* genes for glycosyltransferase enzymes for synthesis of the lipopolysaccharide core of *Salmonella typhimurium*. J. Bacteriol. **161**:277–284.
11. **Kimura, A., and E. J. Hansen.** 1986. Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. Infect. Immun. **51**:69–79.
12. **Kimura, A., C. C. Patrick, E. E. Miller, L. D. Cope, G. H. McCracken, Jr., and E. J. Hansen.** 1987. *Haemophilus influenzae* type b lipo-oligosaccharide: stability of expression and association with virulence. Infect. Immun. **55**:1979–1986.
13. **Lee, J. L., H. O. Smith, and R. J. Redfield.** 1989. Organization of the *Haemophilus influenzae* Rd genome. J. Bacteriol. **171**: 3016–3024.
14. **Makela, P. H., and B. A. D. Stocker.** 1984. Genetics of lipopolysaccharide, p. 59–82. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1. Elsevier Science Publishing, Inc., New York.
15. **Makela, P. H., V. V. Valtonen, and M. Valtonen.** 1973. Role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella*. J. Infect. Dis. **128**(Suppl.):S81–S85.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Pluschke, G., A. Mercer, B. Kusecck, A. Pohl, and M. Actman.** 1983. Induction of bacteremia in newborn rats by *Escherichia coli* K1 is correlated with only O (lipopolysaccharide) antigen types. Infect. Immun. **39**:599–608.
18. **Smith, H. O., D. B. Danner, and R. A. Deich.** 1981. Genetic transformation. Annu. Rev. Biochem. **50**:41–68.
19. **Tolan, R. W., Jr., R. S. Munson, and D. M. Granoff.** 1986. Lipopolysaccharide gel profiles of *Haemophilus influenzae* type b are not stable epidemiologic markers. J. Clin. Microbiol. **24**:223–227.
20. **Van Alphen, L., T. Riemans, J. Poolman, C. Hopman, and H. C. Zanen.** 1983. Homogeneity of cell envelope protein subtypes, lipopolysaccharide serotypes and biotypes among *Haemophilus influenzae* type b from patients with meningitis in The Netherlands. J. Infect. Dis. **148**:75–81.
21. **Zamze, S. E., M. A. J. Ferguson, E. R. Moxon, R. A. Dwek, and T. W. Rademacher.** 1987. Identification of phosphorylated 3-deoxy-manno-octulosonic acid as a component of *Haemophilus influenzae* lipopolysaccharide. Biochem. J. **245**:583–587.
22. **Zamze, S. E., and E. R. Moxon.** 1987. Composition of the lipopolysaccharide from different capsular serotype strains of *Haemophilus influenzae*. J. Gen. Microbiol. **133**:1443–1451.
23. **Zwahlen, A., L. G. Rubin, C. J. Connelly, T. J. Inzana, and E. R. Moxon.** 1985. Alteration of the cell wall of *Haemophilus influenzae* type b by transformation with cloned DNA: association with attenuated virulence. J. Infect. Dis. **152**:485–492.
24. **Zwahlen, A., L. G. Rubin, and E. R. Moxon.** 1986. Contribution of lipopolysaccharide to pathogenicity of *Haemophilus influenzae*: comparative virulence of genetically-related strains in rats. Microb. Pathogen. **1**:465–473.