

## Cloning and Expression in *Escherichia coli* of a *Haemophilus influenzae* Type b Lipooligosaccharide Synthesis Gene(s) That Encodes a 2-Keto-3-Deoxyoctulosonic Acid Epitope

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The composition of lipooligosaccharide (LOS) can modify the virulence of *Haemophilus influenzae* type b (Hib). A genomic library of Hib strain A2 was constructed in the lambda bacteriophage EMBL3. Twenty-six phage clones expressed a Hib LOS oligosaccharide epitope in *Escherichia coli* that was detected by the monoclonal antibody (MAb) 6E4. None of the clones bound a polyclonal sera specific for Hib A2 LOS or an anti-*H. influenzae* lipid A MAb. One clone, designated EMBLOS-1, assembled an oligosaccharide with an apparent molecular weight of 1,400 (the 1.4K oligosaccharide) on a 4.1K lipopolysaccharide (LPS) species in *E. coli* LE392 and produced a novel 5.5K LPS that bound 6E4. Binding of 6E4 to the 5.5K EMBLOS-1 LPS band was abolished by treatment with sodium metaperiodate but was not affected by digestion with proteinase K, confirming the carbohydrate nature of the epitope. The EMBLOS-1 *Haemophilus* insert hybridized to similar restriction fragments in type b and nontypeable strains regardless of whether they expressed the 6E4 epitope. The 6E4 epitope did not undergo phase variation in Hib strain A2 at a frequency of  $>10^{-3}$ . The oligosaccharide of the *Salmonella minnesota* Re mutant and 2-keto-3-deoxyoctulosonic acid (KDO) inhibited binding of 6E4 to Hib A2 LOS. We conclude that a gene(s) encoding an enzyme(s) that assembles a stable Hib LOS epitope containing KDO is conserved in *H. influenzae* and that the cloned Hib LOS synthesis gene products assemble a Hib LOS epitope on an *E. coli* K-12 LPS core.

*Haemophilus influenzae* type b (Hib) is the leading cause of bacterial meningitis in children (31). Although the capsule of Hib is the major virulence factor of this organism (22, 33), alterations in the lipooligosaccharide (LOS) can modify its pathogenicity (6, 17, 18, 41, 42).

*H. influenzae* LOS is antigenically heterogeneous (2, 3). Hib strains that cause systemic disease express a restricted set of LOS antigenic determinants, and antibodies directed against one of these epitopes are protective in experimental Hib infections (8, 9, 34). Since Hib LOS does not contain repeating O antigen units (13), its antigenicity is probably based on the structure of the oligosaccharides or complex interactions between the oligosaccharides and lipid A moieties of this molecule.

There are few data available on the structure of Hib oligosaccharides other than data on their chemical compositions (10, 11, 14, 18, 26, 39, 40). Since antigenic variation of the oligosaccharide component of the LOS of invasive Hib isolates occurs at high frequencies (17, 18) and since some Hib strains can synthesize two antigenically distinct LOSs simultaneously (27), the structure of Hib LOS may be difficult to elucidate. In order to begin to develop a model for Hib LOS structure and antigenicity and to study their relationship to pathogenesis, we now report the cloning in a lambda bacteriophage of Hib DNA that encodes at least one enzyme involved in Hib LOS synthesis that is expressed in *Escherichia coli*. The cloned Hib LOS synthesis gene(s) directs assembly of an oligosaccharide that has an epitope containing 2-keto-3-deoxyoctulosonic acid (KDO) on the *E. coli* LE392 lipopolysaccharide (LPS) core.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Hib strains A2 and A8 were cerebrospinal fluid isolates obtained from Alaskan children and were provided by Joel Ward of the University of California, Los Angeles. In the infant rat model (32), A2 was as virulent as Hib strain Eagan (data not shown). In a colony blot assay, A2 bound the monoclonal antibodies (MAbs) 4C4 and 5G8 but did not bind 6A2 or 12D9 (MAbs provided by Eric Hansen, University of Texas Health Science Center at Dallas; data not shown) and was therefore classified in LOS group 2a (17, 18).

Hib strain Eagan and the nontypeable *H. influenzae* strains 5657 and 7502 were obtained from our own collection (2). *H. influenzae* Rd/b<sup>+</sup>:01 was provided by E. Richard Moxon, John Radcliff Hospital, Oxford, United Kingdom (42). The identities of the strains were confirmed by colonial morphology and a requirement for X and V factors. Counterimmunoelectrophoresis was used to determine serotype as described previously (2). The strains were grown in supplemented brain heart infusion broth containing 2% Fildes reagent (Difco Laboratories, Detroit, Mich.) or supplemented brain heart infusion plates containing 1.5% agar at 35°C in a 5% CO<sub>2</sub> atmosphere.

*E. coli* LE392 was used for recombinant constructions and was grown in LB medium (20). *Salmonella minnesota* R mutants were provided by H. Schneider, Walter Reed Army Institute. Other *Haemophilus* sp. strains were obtained from our collection.

**Preparation of LOS, LPS, and hydrolyzed oligosaccharides.** *H. influenzae* LOS and *S. minnesota* LPS were prepared by the phenol-water method of Westphal and Jann (37) or by the microphenol method of Inzana (13). LPS was prepared from recombinant phage lysates by centrifugation at 100,000 × g

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for 3 h and by treatment of the pellet by the microphenol method. In some experiments, the LOS or LPS isolated by the microphenol method was treated with proteinase K (50 µg/ml) or oxidized with sodium metaperiodate (5 mM) by published methods (15, 20).

Oligosaccharide from the Re mutant of *S. minnesota* SF1167 was prepared from phenol-water LPS by boiling the LPS in 0.1% acetic acid for 1 h. The supernatant was cleared of lipid A by the semicarbazide method of Droge et al. (4). Estimates of the molarity of hydrolyzed oligosaccharide were based on the assumption that KDO was present in equal concentrations as a monomer, dimer, and trimer after hydrolysis. The symbol  $(KDO)_n$  will be used henceforth to distinguish the hydrolyzed Re oligosaccharide from monomeric KDO (Sigma Chemical Co., St. Louis, Mo.).

**SDS-PAGE, Western blot, and immunodot assays.** LOS or LPS was solubilized, electrophoresed on gels containing 14% acrylamide and 2.5 M urea by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (19) and on gels containing 16% acrylamide with Tricine as a trailing ion (30), and stained with silver (13) or transferred to nitrocellulose (2). Apparent molecular weights were estimated by comparison with LPS isolated from the R mutants of *S. minnesota*. In immunodot assays, a suspension of solubilized whole cells or LOS was applied to nitrocellulose (2). Western blots (immunoblots) and immunodots were probed with antibody, goat anti-mouse immunoglobulin M (IgM) IgG (if necessary), protein A peroxidase, and horseradish peroxidase color developer (Bio-Rad Laboratories, Richmond, Calif.) exactly as described previously (2), except that Tris-saline containing 5% bovine serum albumin (BSA) was used as a blocking agent. Conjugates and second antibodies were purchased from Zymed Laboratories, Burlingame, Calif., and Kirkegaard & Perry Laboratories, Gaithersburg, Md., and used according to the instructions of the manufacturers.

**Development of LOS-directed MAbs and polyclonal sera.** BALB/c mice were immunized intraperitoneally with whole cells of nontypeable *H. influenzae* or Rd/b<sup>+</sup>:01 on days 0 and 28. On day 32, splenic lymphocytes were fused to Sp2/0-Ag14 plasmacytoma cells by a modification of the method of Kennett as described elsewhere (2, 16). Hybridomas were screened in an immunodot assay with whole cells and purified LOS of the immunizing strain used as target antigens. Hybridoma supernatants that reacted with LOS in immunodots were tested in a Western blot assay to confirm their LOS specificity. Clones producing LOS antibodies were implanted into pristane-primed mice for the production of ascites fluid (2). MAbs that bound to oligosaccharide determinants in Western blots were obtained from splenocytes primed with strains 7502 (MAb 3D9) and Rd/b<sup>+</sup>:01 (MAb 6E4). MAb 3D2, which binds to a lipid A determinant of 54% of Hib strains tested, is described elsewhere (2). These MAbs cross-reacted with A2 whole cells and purified A2 LOS in immunodots and Western blots.

New Zealand White rabbits were immunized with heated, acetone-dried A2 organisms by the schedule of Rappuoli (3, 29). Rabbit anti-LOS antiserum was affinity purified by incubation with nitrocellulose that had been coated with A2 LOS. Antibodies directed specifically against LOS were eluted from the nitrocellulose with 200 mM glycine, pH 2.8.

**Colony blot assay.** To examine LOS epitopes for phase variation, we probed colony blots of strain A2 sequentially with MAbs. A single colony of A2 was grown in broth to mid-log phase, and dilutions were spread onto supplemented brain heart infusion agar plates at 200 to 300 CFU per plate.

The colonies were blotted onto a nitrocellulose disk, which was dried at 37°C for 30 min. The nitrocellulose was blocked in phosphate-buffered saline (pH 7.0) containing 0.5% casein and 0.5% bovine serum albumin, incubated with a 1/5,000 dilution of Sp2/0-Ag14 or 3D9 ascites fluid overnight, and incubated with alkaline phosphatase-conjugated protein A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and AP color development reagents (Bio-Rad) according to the instructions of the supplier. The number of colonies that bound 3D9 (stained red) was compared with the number of colonies present on the plates. To elute bound antibody, the disks were washed five times with 150 mM glycine, pH 2.3, and washed 3 times with phosphate-buffered saline by the method of Gotschlich et al. (7). The disks were then blocked in phosphate-buffered saline containing 3% bovine serum albumin and probed with a 1/5,000 dilution of 6E4 or Sp2/0-Ag14 ascites fluid or 5G8 tissue culture supernatant, goat anti-mouse IgM IgG (if necessary), protein A peroxidase, and horseradish peroxidase color developer. The number of colonies that bound 6E4 or 5G8 (overstained blue) was compared with the number of colonies that bound 3D9 (stained red) and the number of colonies present on the plate.

**Competition ELISA.** The specificity of MAbs 3D9 and 6E4 was determined by testing the ability of monosaccharides, disaccharides, and  $(KDO)_n$  to inhibit binding of the MAbs to microdilution wells coated with A2 LOS. Additional studies were performed with enzyme-linked immunosorbent assay (ELISA) plates coated with LPS from *S. minnesota* SF1167. The methods used for these inhibitions were identical to those previously described for the evaluation of monoclonal antibodies to gonococcal LOS (1).

In this assay, binding curves were generated for 3D9 and 6E4 to A2 LOS and SF1167 LPS. Several dilutions of each antibody were incubated at 4°C overnight in glass tubes with concentrations of A2 LOS ranging from 1.5 to 100 µg/ml and concentrations of saccharides varying in 10-fold dilutions from 1 nM to 10 µM. Samples of these mixtures were added to ELISA plates coated with 10 µg of A2 LOS or SF1167 LPS. These plates were incubated at room temperature overnight, washed, and developed as a standard ELISA. The optical density readings obtained with the LOS- or saccharide-incubated MAbs were divided by the optical density readings obtained with MAbs alone, and the results were expressed as the percentage of inhibition. By using Statview 512+ (Brainpower Inc., Ventura, Calif.) on an Apple Macintosh II computer, linear regression curves with 95% confidence limits were generated that compared the concentration of each inhibitor with the percent inhibition of binding of each MAb to Hib A2 LOS or SF1167 LPS. The concentration of LOS or saccharide necessary to achieve 50% inhibition of the antibody binding was recorded from these curves.

**DNA manipulation.** *H. influenzae* chromosomal DNA was prepared by the method of Marmur (21). Bacteriophage DNA was isolated as described by Maniatis et al. (20). T4 DNA ligase and restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and Promega Biotec (Madison, Wis.) and used according to the specifications of the suppliers. Restriction fragments used as Southern hybridization probes were purified by agarose gel electrophoresis and electroelution (20) followed by chromatography on NACS Prepac columns (Bethesda Research Laboratories).

**Cloning and screening.** A genomic library of Hib strain A2 was constructed by ligating 12- to 20-kilobase (kb) *Sau3A*-

digested genomic fragments with EMBL3 *Bam*HI arms (EMBL arms cloning system; Promega Biotec). The recombinant phage was packaged with commercially available reagents (Packagene System; Promega Biotec) and used to transfect *E. coli* LE392. Plaques were screened with a mixture of MAbs and the LOS polyclonal serum in an assay similar to the colony blot assay described above. EMBL3 containing a 16-kb test insert (encoding a human immunoglobulin gene; EMBL arms cloning system; Promega Biotec) was used as a control for screening.

**Southern blotting.** DNA was digested to completion with appropriate restriction enzymes, electrophoresed overnight on 0.8% agarose gels, and transferred to nitrocellulose (20). Southern blots were probed with nick-translated phage DNA restriction fragments as described by Maniatis et al. (20). The blots were washed with a buffer containing  $2\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at room temperature for 1 h and with a buffer containing  $0.2\times$  SSC and 0.1% SDS at 68°C for 2 h and autoradiographed with Kodak XAR-5 film (Eastman Kodak Co., Rochester, N. Y.) and a Cronex intensifying screen (Du Pont Co., Wilmington, Del.).

### RESULTS

A chromosomal library was constructed in the phage EMBL3 from Hib strain A2, which belonged to the LOS group 2a of Hansen et al. (17, 18) and was virulent in the infant rat model. The library was screened for the expression of Hib LPS epitopes by using a mixture of MAbs and a polyclonal serum that bound to oligosaccharide and lipid A epitopes present in A2 LOS. Approximately 5,000 clones were screened, and 26 bound the mixture of antibodies. In plaque blot assays probed with MAbs and polyclonal serum separately, all clones reacted with MAbs 3D9 and 6E4. None of the clones bound the polyclonal serum or the MAb 3D2, which recognizes a lipid A epitope of *H. influenzae* (2).

The MAbs 3D9 and 6E4 were the products of independent fusions that followed immunization with *H. influenzae* 7502 and Rd/b<sup>+</sup>:01, respectively. In colony blots, these MAbs bound to identical *Haemophilus* strains (data not shown). The antibodies also bound to identical Hib A2 LOS bands in Western blots. Competitive-inhibition ELISA studies showed that the MAbs were inhibited from binding to A2 LOS by KDO and (KDO)<sub>n</sub>. We concluded that 3D9 and 6E4 recognized identical or very similar LOS epitopes and will describe the recombinant phage and antibody specificity only with respect to 6E4.

**Characterization of recombinant phage clones.** Of the 26 clones that bound 6E4, one clone, designated EMBLOS-1, was chosen for analysis. LPS was purified from Hib A2, *E. coli* LE392, and lysates of LE392 transfected with EMBLOS-1 and LE392 transfected with EMBL3 control phage. In SDS-PAGE, the LOS of Hib A2 migrated as multiple bands whose molecular weights ranged from 1,900 (the 1.9K band) to 4,900 (Fig. 1). LPS isolated from LE392 and LE392 transfected with control phage migrated as five bands with molecular weights ranging from 2,000 to 4,600. When LPS isolated from LE392 transfected with EMBLOS-1 was compared with that isolated from the LE392 controls, there was a reduction in the intensity of a 4.1K band and the appearance of a novel 5.5K band. SDS-PAGE of whole-cell lysates prepared from LE392 transfected with EMBLOS-1 and control phage did not show any gross differences in Coomassie-stained bands. Therefore, EMBLOS-1 encoded an enzyme(s) that added a 1.4K oligosaccharide to the 4.1K species in LE392 LPS.

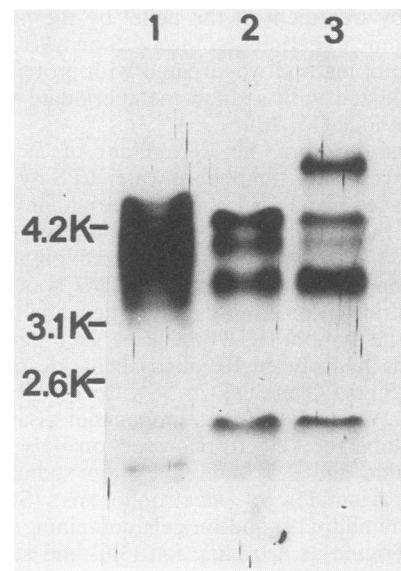


FIG. 1. SDS-PAGE of LOS and LPS resolved on a 16% acrylamide gel. Lane 1, Hib A2; lane 2, LE392 transfected with an EMBL3 control; lane 3, LE392 transfected with EMBLOS-1. The relative migration of the LPS of the Ra, Rc, and Re mutants of *S. minnesota* is shown at the left.

To test the hypothesis that the products of the recombinant phage were enzymes that functioned in oligosaccharide synthesis, LOS and LPS were treated with sodium metaperiodate and proteinase K and probed with 6E4 in a Western blot (Fig. 2). The 6E4 MAb did not bind to LPS isolated from LE392 transfected with a control phage. The MAb bound to 4.9K and 4.2K bands in Hib A2 LOS and to the 5.5K band and several minor bands in LPS isolated from LE392 transfected with EMBLOS-1. Proteinase K treatment did not alter MAb reactivity. Sodium metaperiodate treatment abolished MAb binding to Hib A2 LOS and to the 5.5K band in EMBLOS-1 LPS. Thus, the epitope assembled by the EMBLOS-1 gene products was carbohydrate in nature.

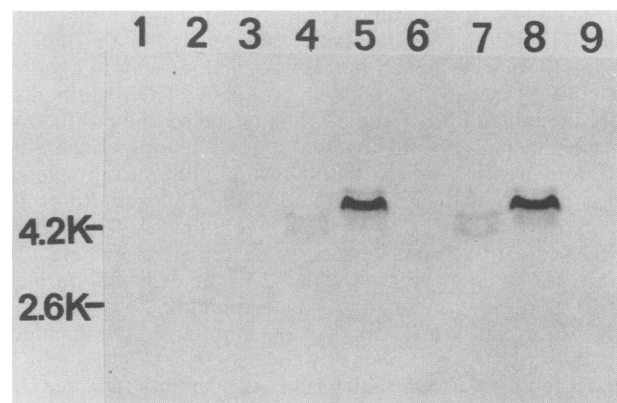


FIG. 2. Western blot of LOS and LPS probed with 6E4. Samples from Hib A2 are in lanes 1, 4, and 7; samples from LE392 transfected with EMBLOS-1 are in lanes 2, 5, and 8; and samples from LE392 transfected with a control phage are in lanes 3, 6, and 9. Lanes 1 to 3, Samples treated with sodium metaperiodate; lanes 4 to 6, samples treated with proteinase K; lanes 7 to 9, untreated samples. The relative migration of the R mutants of *S. minnesota* is shown at the left.

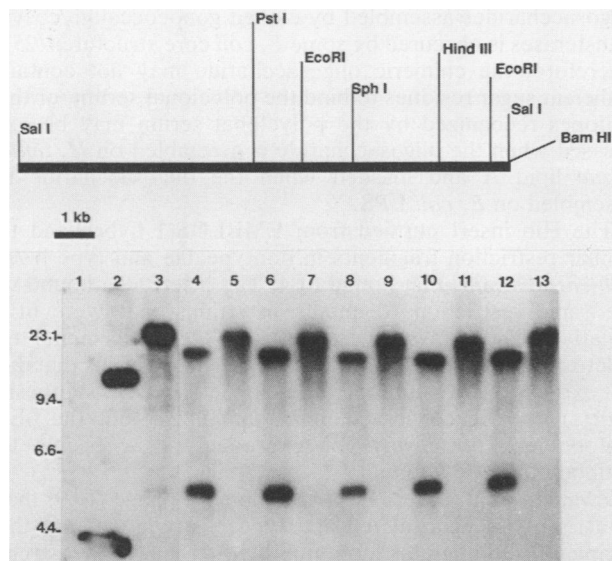


FIG. 3. Restriction map of the EMBLOS-1 insert and hybridization of the insert to phage and chromosomal DNA. The *Bam*HI and *Sal*I sites are contained in the vector. Lane 1, *Eco*RI-digested LE392 DNA; lanes 2 and 3, *Sal*I- and *Eco*RI-digested EMBLOS-1 DNA; lanes 4 and 5, *Eco*RI- and *Xba*I-digested A2 DNA; lanes 6 and 7, *Eco*RI- and *Xba*I-digested A8 DNA; lanes 8 and 9, *Eco*RI- and *Xba*I-digested Eagan DNA; lanes 10 and 11, *Eco*RI- and *Xba*I-digested 5657 DNA; lanes 12 and 13, *Eco*RI- and *Xba*I-digested 7502 DNA. The relative migration of *Hind*III-digested lambda standards is shown at the left.

Sodium metaperiodate treatment also revealed binding of 6E4 to a 2.6K band in LE392 transfected with EMBLOS-1 and with control phage. These bands may represent lipid A-KDO structures that were partially oxidized by the reagent.

#### Restriction map and Southern analysis of EMBLOS-1 DNA.

Restriction mapping of EMBLOS-1 DNA and Southern blot analysis confirmed that the inserted DNA in the recombinant phage originated from Hib A2. The inserted DNA contained an internal 5.6-kb *Eco*RI fragment and no *Xba*I sites (Fig. 3). The insert was released from the vector by digestion with *Sal*I and used to probe *Eco*RI- and *Xba*I-digested A2 and LE392 chromosomal DNA and *Sal*I- and *Eco*RI-digested EMBLOS-1 DNA. The insert hybridized to a single >23-kb *Xba*I fragment of A2 chromosomal DNA and exhibited homology to 5.6- and 21-kb fragments in *Eco*RI-digested A2 chromosomal DNA. The insert did not hybridize to LE392 or EMBL3 DNA. EMBLOS-1 DNA also exhibited homology to >23-kb *Xba*I fragments and to similar *Eco*RI fragments in type b and nontypeable strains that bound 6E4 (strains Eagan and 7502) and those that did not (A8 and 5657).

**Specificity of the 6E4 epitope.** In colony and plaque blots, 6E4 did not bind to *E. coli* LE392 or LE392 transfected with control phage but did bind to the Re mutant of *S. minnesota*. In Western blots, 6E4 bound to a 2.6K band of *S. minnesota* Re LPS. Since the LPS of the Re mutant consists only of lipid A and KDO, these data suggested that 6E4 bound to a KDO epitope and that the KDO present on the Ra LPS of LE392 was not accessible to antibody.

We tested the ability of A2 LOS and a variety of monosaccharides, disaccharides, and (KDO)<sub>n</sub> to inhibit the binding of 6E4 to A2 LOS or *S. minnesota* Re LPS in a competition ELISA. Microdilution wells were coated with LOS or LPS

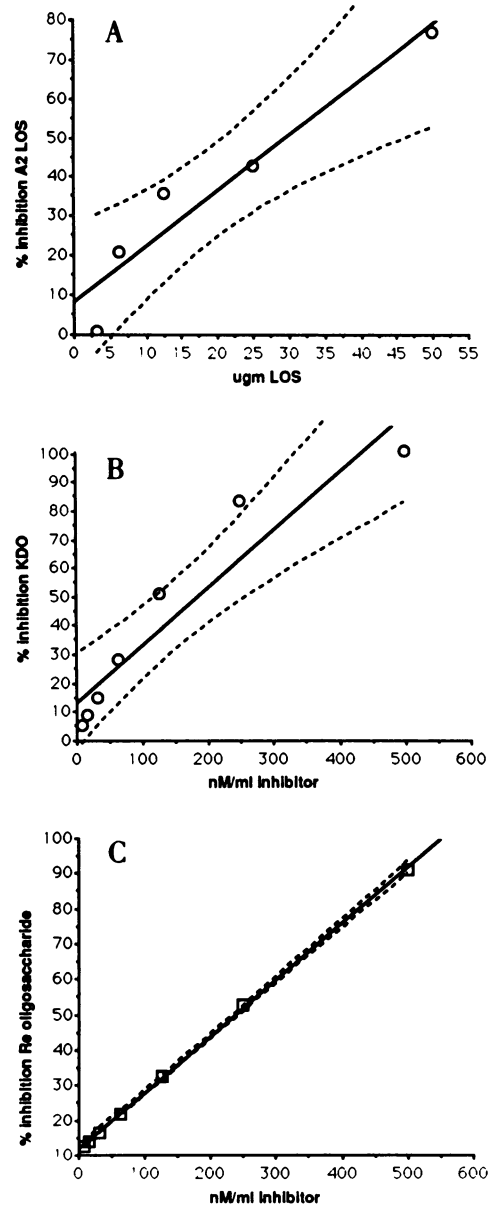


FIG. 4. Competition ELISA demonstrating percent inhibition of 6E4 binding to Hib A2 LOS by Hib A2 LOS (A), by KDO (B), and by the oligosaccharide of the Re mutant of *S. minnesota* (KDO)<sub>n</sub> (C). Each value represents a mean of 6 [LOS and (KDO)<sub>n</sub>] or 24 (KDO) determinations. Ninety-five-percent confidence limits are indicated by dashed lines.

and incubated with 6E4 alone or solutions containing 6E4 and concentrations of saccharides ranging from 1 nmol/ml to 10  $\mu$ mol/ml. A2 LOS was used as an inhibitor in concentrations ranging from 1.5 to 100  $\mu$ g/ml. A2 LOS, KDO, and (KDO)<sub>n</sub> inhibited 6E4 binding to A2 LOS (Fig. 4) and Re LPS (data not shown) in a dose-dependent fashion. Inhibition of 50% of 6E4 binding to A2 LOS was achieved at an A2 LOS concentration of 30  $\mu$ g/ml, a KDO concentration of 180 nmol/ml, and a (KDO)<sub>n</sub> concentration of 250 nmol/ml. Glucose, galactose, fucose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, *N*-acetylmannosamine, galactosamine 1-phosphate, glucosamine 6-phosphate, lactose, and sucrose did not inhibit 6E4 binding

to A2 LOS or Re LPS in the range of concentrations tested. Thus, 6E4 bound to an epitope that contained KDO.

**Phase variation of the 3D9 and 6E4 epitope.** We examined whether the epitope recognized by 3D9 and 6E4 underwent phase variation at a frequency similar to that of an epitope (5G8) that is used in the LOS grouping system of Hansen et al. (17, 18). A single colony of A2 was grown in broth and subcultured to 200 to 300 CFU on agar plates. Colony blots were probed with 3D9, protein A conjugated to alkaline phosphatase, and alkaline phosphatase substrate. We screened 3,867 colonies, and all colonies bound 3D9, as indicated by red color development. Bound antibody was eluted from the colony blots by treatment with 150 mM glycine (pH 2.3), which did not remove the red precipitate. Several of the blots were probed with Sp2/0-Ag14 ascites fluid, 6E4 ascites fluid, or 5G8 tissue culture supernatant, goat anti-mouse IgM IgG, protein A peroxidase, and horseradish peroxidase color developer, which stained antigen-antibody complexes blue. The colonies probed with Sp2/0-Ag14 ascites fluid remained red, indicating that 3D9 had been eluted and that protein A peroxidase did not bind nonspecifically to the colonies. Of 2,552 3D9-positive colonies tested, all bound 6E4 (overstained blue). Of 927 colonies that were 3D9 positive, 902 bound 5G8. Therefore, the 5G8 epitope present in the Hib A2 LOS phase varied at a frequency of 2.6%, while the epitope recognized by 3D9 and 6E4 did not phase vary at a frequency  $>10^{-3}$ .

## DISCUSSION

The LOS of Hib can modify the virulence of this organism. To begin to develop a model that correlates LOS genetics with structure and antigenicity, we isolated a gene(s) encoding an enzyme(s) involved in Hib LOS synthesis that is expressed in *E. coli*. The cloned gene(s) directed the assembly of an oligosaccharide containing a Hib LOS epitope recognized by the MAb 6E4 but did not express a Hib lipid A epitope. The recombinant LOS epitope was sensitive to periodate oxidation but resistant to proteinase K digestion, confirming that the antigen was composed of carbohydrate. Similar results have been obtained by Nano and Caldwell (23) and by Palermo et al. (25), who have cloned glycosyltransferases that assemble LOS epitopes of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* on *E. coli* LPS core structures. Our studies confirm their observations that similar mechanisms for LOS and LPS biosynthesis occur in different genera.

The recombinant phage EMBLOS-1 produced a chimeric LPS by adding a 1.4K oligosaccharide (6 to 8 saccharides) to a 4.1K LPS band in *E. coli* LE392, shifting its molecular weight to 5,500. The apparent molecular weight of the Hib A2 oligosaccharide was calculated to be approximately 3,200 by subtracting the calculated  $M_w$  (1,700) of *H. influenzae* I-69 Rd<sup>-</sup>/b<sup>+</sup> lipid A (10) from the highest-molecular-weight band in Hib A2 LOS (4,900). Since the cloned LOS synthesis gene(s) directed assembly of a 1.4K oligosaccharide on the *E. coli* LPS, the recombinant phage probably does not encode all the enzymes necessary for Hib A2 oligosaccharide synthesis.

Although EMBLOS-1 expressed a Hib LOS epitope recognized by the MAb 6E4, the chimeric oligosaccharide did not contain epitopes recognized by a polyclonal serum raised against Hib A2 organisms. Yamasaki and co-workers have shown that the expression of a gonococcal LOS epitope is significantly influenced by the structure of gonococcal lipid A (38). Palermo et al. have shown that the antigenicity of

oligosaccharides assembled by cloned gonococcal glycosyltransferases is obscured by some *E. coli* core structures (25). Therefore, the chimeric oligosaccharide may not contain sufficient sugar residues to bind the polyclonal serum, or the epitopes recognized by the polyclonal serum may be expressed when the oligosaccharide is assembled on *H. influenzae* lipid A and masked when the oligosaccharide is assembled on *E. coli* LPS.

The Hib insert purified from EMBLOS-1 hybridized to similar restriction fragments in nontypeable and type b *H. influenzae* strains that bound 6E4. The insert also bound to the same restriction fragments in strains that were 6E4 negative. Thus, there were no gross rearrangements or deletions in the chromosome of *H. influenzae* strains that did not express the 6E4 epitope. The cloned Hib LOS synthesis gene(s) may be conserved in *H. influenzae*, but the 6E4 epitope may not always be expressed or accessible to antibody in these strains.

Zwahlen et al. described a recombinant phage (I-69) that transforms unencapsulated and type b strains so that the recipient LOS migrates more rapidly than that of the parent in SDS-PAGE (41). Unfortunately, a detailed restriction map of I-69 has not been published. Since the cloned glycosyltransferase(s) in EMBLOS-1 assembled an oligosaccharide that consists of 6 to 8 saccharides and did not reduce the size of the *E. coli* LPS, EMBLOS-1 may not be identical to the I-69 construction.

Some LOS synthesis genes in strain Eagan may be linked to the *cap b* locus (42). The EMBLOS-1 insert hybridized to similar restriction fragments in Hib strains A2 and Eagan but did not hybridize to cosmid clones (12; provided by S. Hoiseth, Georgetown University, Washington, D.C.) containing flanking sequences 6 kb to the left and 10 kb to the right of the tandem repeats that compose the Eagan *cap b* locus (data not shown).

The 6E4 epitope did not phase vary in strain A2 at a frequency  $>10^{-3}$ . Therefore, expression of the 6E4 epitope is stable relative to the LOS epitopes used in the grouping system of Hansen et al., which phase vary at a frequency of  $10^{-2}$  (9, 17, 18). Weiser et al. recently identified a genetic locus, *lic-1*, that is involved with the expression of three different *H. influenzae* LOS epitopes which exhibit phase variation (35). The restriction map of EMBLOS-1 is clearly different from that of *lic-1* (35). The first gene in the *lic-1* gene cluster contains a variable number of tandem repeats of the tetramer CAAT. This repetitive sequence probably mediates phase variation of LOS epitopes through a translational switch mechanism (36). We probed EMBLOS-1 and Hib A2 chromosomal DNA with an oligonucleotide consisting of five CAAT repeats. The oligonucleotide hybridized to 20- and 8.6-kb *EcoRI* fragments of the A2 chromosome but did not hybridize to the EMBLOS-1 construction (data not shown). Thus, the genes responsible for the assembly of the 6E4 epitope do not contain the same sequences that regulate LOS phase variation at the *lic-1* locus.

The binding of 6E4 to the Re mutant of *S. minnesota* suggested that 6E4 bound to KDO. In a competitive-inhibition ELISA, A2 LOS, KDO, and (KDO)<sub>n</sub> inhibited the binding of 6E4 to A2 LOS, confirming that this MAb bound to KDO. These observations were somewhat surprising in view of a number of studies showing that *H. influenzae* LOS contains small amounts of KDO, usually less than one KDO residue per molecule (14, 40). However, phosphate substitutions at positions C-4 and C-5 may render estimates of KDO residues by standard techniques inaccurate (10, 39, 40). By varying acid hydrolysis conditions, Parr and Bryan

have shown that *H. influenzae* LOS may contain as much KDO as smooth *Salmonella typhimurium* (26).

In Western blots, 6E4 bound to 4.9K and 4.2K Hib A2 LOS bands and did not bind to any lower-molecular-weight bands. In SDS-PAGE, A2 LOS contained greater amounts of high-molecular-weight species than of low-molecular-weight material (Fig. 1), and this may preclude detection of 6E4 binding to low-molecular-weight structures such as lipid A-KDO. The 6E4 epitope in *H. influenzae* may be composed of exposed KDO sugar(s) located in an oligosaccharide side chain, or the oligosaccharides may assume a conformation that exposes a deep core structure such as lipid A-KDO. Similarly, the 6E4 epitope expressed by EMBLOS-1 is probably contained in the oligosaccharide assembled by the cloned LOS synthesis gene(s), but we cannot exclude the possibility that the added oligosaccharide exposed a KDO epitope contained in the *E. coli* K-12 core.

Chemical analysis of the deep rough LOS of the I-69 Hib transformant shows that it consists of lipid A and a single KDO, which is attached to *N*-acetylglucosamine of lipid A via a 2''-6' linkage (10, 39). The structure of LE392 LPS has not been published, but the genotype of LE392 indicates that this strain can synthesize the entire *E. coli* K-12 LPS core (20, 24, 28). The LPS of several *E. coli* K-12 derivatives contains a terminal *N*-acetylglucosamine, and these structures have calculated molecular weights of 4,100 to 4,300 in their unsubstituted forms (28). These structures may correspond to the 4.1K LE392 band that was modified by EMBLOS-1 and may act as acceptor molecules for the Hib enzyme that adds KDO to the *N*-acetylglucosamine of Hib lipid A. To test this hypothesis, we are analyzing Hib A2 LOS, LE392 LPS, and the recombinant LPS by techniques used to determine the structure of *N. gonorrhoeae* oligosaccharides (5).

These studies demonstrate that a gene(s) encoding an enzyme(s) involved in the assembly of a stable Hib LOS epitope can be expressed in *E. coli*. The EMBLOS-1 recombinant should facilitate the construction of genetically defined mutants and the development of a model that correlates mutations with LOS structure, antigenicity, and pathogenesis.

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