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The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants

(pathogenesis/intestinal colonization/inverse PCR)

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ABSTRACT **_Vibrio cholerae_ serogroup O139 emerged on the Indian subcontinent in October 1992 to become the first non-O1 _V. cholerae_ serogroup documented to cause epidemic cholera. Although related to _V. cholerae_ El Tor O1 strains, O139 strains have unique surface structures that include a capsular surface layer and lipopolysaccharide (LPS). Immunoblot analysis of either whole-cell lysates or LPS preparations revealed three electrophoretic forms of the O139 antigen: two slowly migrating forms and one rapidly migrating form that appeared identical to O139 LPS. All three forms of the antigen shared an epitope defined by an O139-specific monoclonal antibody. A serum-sensitive nonencapsulated mutant was isolated that lacks only the slow migrating forms. The slow migrating forms did not stain with silver whereas the rapidly migrating form did, suggesting that the former might constitute highly polymerized O-antigen side-chain molecules that were not covalently bound to core polysaccharide and lipid A (an "O-antigen capsule"). A single transposon insertion resulted in the loss of immunoreactivity of both the LPS and the O-antigen capsule, implying that there are genes common to the biosynthesis of both these macromolecules. The O139 LPS and O-antigen capsule were both important for colonization of the small intestine of the newborn mouse and for serum resistance, demonstrating that both of these forms of the O139 serogroup antigen are virulence factors.**

Cholera is a severe and sometimes lethal secretory diarrheal disease caused by the Gram negative bacterium *Vibrio cholerae*. Historically, only the O1 serogroup of *V. cholerae* has been associated with cholera epidemics. However, in early 1993 a dramatic shift in the cause of cholera was observed with the emergence of a major cholera epidemic in India and Bangladesh caused by a non-O1 serogroup (1, 2). This non-O1 serogroup was named *V. cholerae* O139 synonym Bengal (3). Epidemiologic data from the large O139 epidemics in Bangladesh and India strongly suggest that immunity to *V. cholerae* O1 does not protect against O139 infection and thus support the notion that the O antigen of lipopolysaccharide (LPS) is the major protective antigen of *V. cholerae* (4, 5).

The O1 serogroup of *V. cholerae* is divided into two biotypes—classical and El Tor. Many laboratories have shown that O139 strains are very similar to El Tor O1 strains (4, 6–11). However, O139 strains belong to a distinct serogroup defined by monoclonal antibodies and polyclonal antisera that only recognize O139 strains (3, 12). In *V. cholerae* O1, the chemical basis for the serogroup-defining antigen lies in the O side chain of LPS (13). Biochemical characterization of the LPS from O139 strains has demonstrated that O139 LPS differs from O1 LPS in that it has a short O-side-chain length and different sugar composition (13–15).

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Electron microscopic studies have demonstrated that O139 strains are encapsulated (10, 15). The chemical composition of a capsular polysaccharide has been reported (15), but it is not clear whether this material corresponds to an O139-specific antigen and/or the capsular surface layer seen in the electron microscopic studies. Also, it is not known whether the capsule or the O antigen is important for intestinal colonization and virulence of O139 strains.

In the current study, we have isolated and characterized mutant strains with different forms of the O139 antigen. Immunoblot analyses revealed three electrophoretic forms of the O139 antigen. Our analysis suggests that one of these electrophoretic forms corresponds to LPS and the other two correspond to an O-antigen-related capsular polysaccharide. Both the O-antigen capsule and the LPS O side chains were shown to play an important role in serum resistance and in colonization of the infant mouse small intestine.

MATERIALS AND METHODS

Bacterial Strains and Antisera. Bacterial strains were maintained at −70°C in LB broth (16) containing 20% (vol/vol) glycerol. The phenotypes of the *V. cholerae* strains used in this study are listed in Table 1. Formalin-treated cells of the O139 type strain MO45 were used to raise a polyclonal antiserum in New Zealand White rabbits. Monoclonal antibody 2D12 was raised against the O139 strain SG24 and is a mouse IgG2a (12).

Transposon Mutagenesis and Selection of O139-Negative Mutants. Tn*5lac* (17) was used to mutagenize the O139 strain Bengal-2, a prototype vaccine derivative of MO10 (11). Tn*5lac* mutagenesis of Bengal-2 was performed with plasmid pMW1, in a manner similar to that described for Tn*phoA* mutagenesis of *V. cholerae* O1 strains with plasmid pRT291 (18). Tn*5lac* insertion mutants of Bengal-2 that were O139-negative were enriched from pools of transposon mutants by agglutination of the O139-positive cells with polyclonal anti-O139 antisera. Nonagglutinating cells were purified and retested by slide agglutination with the anti-O139 antisera. Southern blot analysis was used to confirm that each O139-negative strain contained a single transposon insertion.

Biochemical and Immunoblot Analyses. *V. cholerae* strains were grown in LB broth overnight at 37°C on a roller incubator. To prepare whole-cell lysates, cells were collected by centrifugation, resuspended in sample buffer, and boiled for 5 min. LPS was prepared from the same cultures by using the hot phenol/water method as modified by Slauch *et al.* (19). Samples were electrophoresed in 15% polyacrylamide gels in the presence of SDS (SDS/PAGE) and either stained by the Bio-Rad silver staining protocol or electrotransferred to nitrocellulose for Western blot analysis (7). *V. cholerae*

Abbreviations: LPS, lipopolysaccharide; RM, rapid migrating; MM, medium migrating; SM, slow migrating.

Table 1. Serum resistance and intestinal colonization of opaque and translucent strains

Strain	Relevant phenotype	Serum resistance, % survival	Colonization ratio
MO10	O139 opaque wild type	100	2.0
MO10-T4	Spontaneous translucent variant of MO10	2	0.07
MO10-OpR3	Spontaneous opaque revertant of MO10-T4	100	2.0
AI-1837	Opaque wild type	100	33.6
1837-T3	Spontaneous translucent variant of AI-1837	0.8	0.06
Bengal-2	Opaque vaccine derivative of MO10	100	0.9
Bengal-2R1	O139-negative Tn*5lac* derivative of Bengal-2	0.1	0.01
Bengal-2R2	O139-negative Tn*lac* derivative of Bengal-2	0.08	0.002
O395	O1 wild type	90	1.0
O395R-1	O1-negative Tn*5lac* derivative of O395	0.1	0.003

Serum resistance was calculated as 100 × the ratio of the number of colony-forming units found after cells were incubated in guinea pig serum divided by the number of colony-forming units found after cells were incubated in heat-inactivated guinea pig serum. The colonization ratio is the mean ratio of the number of blue (the *lacZ*-positive test strain) to white (the Δ*lacZ* LAC-1 strain) colony-forming units found in small intestinal homogenates after 20 hr of *in vivo* growth. There were seven mice per group.

chromosomal DNA was purified and Southern blot analysis was carried out as described (7).

Serum Resistance and Mouse Colonization Assays. For serum bacteriocidal assays, cells were grown at 37°C to midlogarithmic phase in LB broth, washed, and mixed 1:1 with 16% (vol/vol) guinea pig serum or with 16% (vol/vol) heat-inactivated guinea pig serum in phosphate-buffered saline. Approximately 1×10^6 cells were used in each assay. After incubation at 37°C for 1 hr, LB broth at 4°C was added to each mixture and then viable cells were enumerated by plating on L agar. Identical serum bacteriocidal assays were performed using pooled human sera at a final concentration of 30%.

Colonization of the infant mouse small intestine was assessed by competition assays using 1:1 mixtures of test strains with the O395 ΔlacZ strain LAC-1, as described (7).

Insertional Inactivation of *rfcX*. Inverse polymerase chain reaction (PCR) (20) was used to amplify DNA sequences adjacent to the right end of Tn*5lac* in the O139-negative insertion mutant Bengal-2R1. Chromosomal DNA from Bengal-2R1 was digested with *Taq* I and ligated under dilute conditions to favor intramolecular ligation. The ligated DNA was then used as a template for PCR. The two primers that were used for the PCR are complementary to bp 84–65 (5′-ACCATGTTAGGAGGTCCT-3′, primer 2) and bp 231–250 (5′-CCATCTCATCAGAGGGTAGT-3′, primer 1) of the right end of Tn*5lac*. The product of the PCR was subcloned to yield plasmid pCRII-I3. The cloned insert in pCRII-I3 was subcloned into the *Eco*RI site of pGP704 (21), a plasmid that is unable to replicate in *V. cholerae*. The resultant plasmid pMW704-I3 was grown in *Escherichia coli* SM10λpir (21) and then transferred into O139 strain MO10 by conjugation. The integration of pMW704-I3 into the homologous locus *rfcX* in MO10 was confirmed by Southern blot analysis.

RESULTS

Isolation of Spontaneous and Tn*5lac* Mutant Strains. Initial streaks of some O139 clinical isolates show a mixture of opaque and translucent colonies (10). This phase variation in colony morphology has been observed and studied in non-O1 *V. cholerae* strains (22) and in *Vibrio vulnificus* (23). In non-O1 strains and in *V. vulnificus*, opaque colonies are encapsulated and translucent colonies are nonencapsulated (22, 23). Johnson *et al.* (10) have shown that opaque O139 strains are encapsulated, but, to our knowledge, no analysis of translucent colonies has been reported.

We began our analysis of O139 antigen by characterizing spontaneous translucent variants of opaque O139 strains. A single colony from a translucent sector of the opaque O139 clinical isolate MO10 was isolated and designated MO10-T4. A spontaneous opaque revertant of MO10-T4 was isolated and designated MO10-OpR3. A similar translucent variant of the opaque clinical isolate AI-1837 was designated 1837-T3. Both of these translucent variants were O139-positive in slide agglutination tests with O139 typing sera.

To identify the genes encoding the biosynthesis of the O139 antigen, we constructed a pool of transposon insertion mutants in the opaque strain Bengal-2, a prototype live attenuated vaccine derivative of MO10 (11). The transposon Tn*5lac* (17) was used to mutagenize Bengal-2, and Tn*5lac* insertion mutations that rendered Bengal-2 O139-negative were enriched for by agglutination of O139-positive cells with an anti-O139 antisera. Two such O139-negative Tn*5lac* insertion mutants were designated Bengal-2R1 and Bengal-2R2 and used for subsequent analyses. Southern blot analysis was used to confirm that these two strains each contained a single Tn*5lac* insertion (data not shown).

Multiple Electrophoretic Forms of the O139 Antigen. Immunoblot analysis was used to detect changes in the O139 serogroup antigen in the spontaneous translucent colony morphology mutants and the Tn*5lac* O139-negative insertion mutant strains. Both a polyclonal anti-O139 antisera and an anti-O139 monoclonal antibody, 2D12 (12), were used to analyze whole cell lysates of these strains and the *V. cholerae* O1 strain O395 and its O1-negative derivative of O395-R1.

The anti-O139 polyclonal antisera defined O139-specific bands that were not seen in the O1 strain O395, or in the O139-negative Tn*5lac* insertion mutant strains Bengal-2R1 and Bengal-2R2 (Fig. 1*A*). In all four of the opaque O139 strains, MO10, MO10-OpR3, Bengal-2, and AI-1837, three O139-specific electrophoretic forms of the O139 antigen were observed: a rapidly migrating (RM) form, a medium migrating (MM) form that was observed as a series of fine bands, and a slowly migrating (SM) form that migrated into the stacking gel but not into the running gel (Fig. 1*A*). In MO10-T4, the spontaneous translucent variant of MO10, the SM and MM forms of the antigen were not observed and only the RM form of the O139 antigen was detected (Fig. 1*A*). The SM and MM forms of the antigen were also missing in the other spontaneous translucent mutant strain 1837-T3. The RM form of O139 antigen was seen in 1837-T3, but only in overloaded and over-developed immunoblots (data not shown). Thus immunoblots with anti-O139 polyclonal antisera of whole cell lysates revealed that there are three electrophoretic forms of the O139 antigen of which two, the apparently higher molecular mass forms, are absent in translucent strains.

The absence of the SM and MM forms of the O139 antigen in translucent strains was more apparent when the anti-O139 monoclonal antibody was used in immunoblots of whole cell

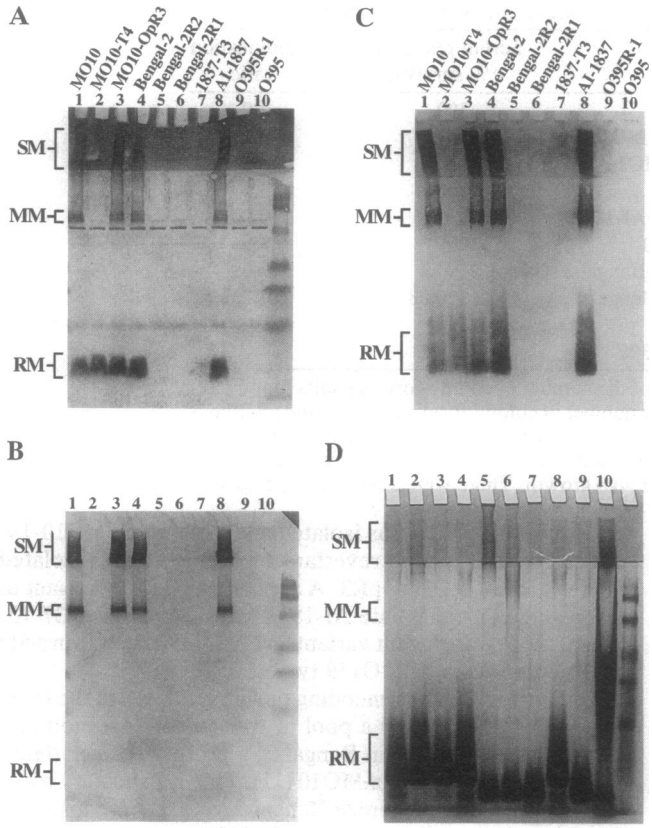

FIG. 1. Electrophoretic forms of the O139 serogroup antigen detected in immunoblots of whole cell lysates (*A* and *B*) or LPS preparations (*C*) of the indicated strains with either anti-O139 polyclonal antisera (*A*) or anti-O139 monoclonal antibodies (*B* and *C*). *C* was developed with a chemiluminescent substrate (7). *D* is a silver-stained SDS/PAGE gel of LPS from the indicated strains. The three electrophoretic forms of the O139 antigen, SM, MM, and RM, are indicated. The Bio-Rad prestained molecular mass markers with apparent molecular masses of 107 kDa, 80 kDa, 49.5 kDa, 32.5 kDa, 27.5 kDa, and 18.5 kDa are shown in unlabeled lanes.

lysates (Fig. 1*B*). However, the monoclonal antibody did not define an epitope unique to the SM and MM forms of O139. The RM form of the antigen was reactive with the monoclonal antibody in immunoblots of whole cell lysates and LPS when the blots were developed with a more sensitive chemiluminescent detection method (see below). Also immunoelectron microscopic studies with this monoclonal antibody have shown that this antibody recognizes the surface of MO10 cells, suggesting that the SM and MM forms of the O139 antigen are surface-localized (data not shown).

LPS preparations were made from the same 10 strains. Immunoblot analysis of SDS/PAGE gels of these LPS preparations with the anti-O139 monoclonal antibody revealed the same three electrophoretic forms of the O139 antigen in the opaque strains that were observed in immunoblots of whole cell lysates (Fig. 1*A* and *C*). Also, as in immunoblots of whole cell lysates, the SM and MM forms were not detected in MO10-T4; only the RM form was apparent in this translucent strain (Fig. 1*C*). Since the monoclonal antibody reacted with all three electrophoretic forms of the O139 antigen, the RM, MM, and SM forms of the antigen must share an epitope.

When duplicate SDS/PAGE gels of LPS preparations were stained with silver, the translucent variant MO10-T4 appeared identical to the opaque strains MO10, MO10-OpR3, Bengal-2, and AI-1837 and different from the O1 strain O395, which is known to express LPS molecules with relatively long O side chains (13) (Fig. 1*D*). The two O139-negative Tn*5lac* insertion mutant strains Bengal-2R1 and Bengal-2R2 lacked the major silver staining band seen in MO10-T4 and the opaque O139 strains. In contrast, the major silver staining band in these two O139-negative strains comigrates with the band seen in the O1-negative derivative of O395, O395R-1, which we have shown carries a Tn*5lac* insertion in *rfbT* (13) (data not shown). This comigrating band in LPS preparations of O1- and O139-negative strains is also seen in the wild-type O1 and O139 strains and apparently corresponds to the core polysaccharide attached to lipid A. Thus, the O139-specific silver staining band seen in MO10, MO10-T4, MO10-OpR3, Bengal-2, and AI-1837 corresponds to the O139 LPS molecules containing an O side chain and comigrates with the RM form of the antigen observed in immunoblots (Fig. 1 *C* and *D*). This is consistent with the observation by Hisatsune *et al.* (14) that O139 strains have relatively short O side chains in their LPS compared with the O1 strains (e.g., Fig. 1*D*, lane 10).

A striking feature of silver-stained gels of the O139 LPS preparations is the relative absence of silver staining material where the SM and MM antigen forms migrate. Moreover, suspensions of MO10, MO10-T4, and Bengal-2R1 in india ink, followed by air-drying and counterstaining with crystal violet, produced smears that differentiated these strains under light microscopy; strain MO10 cells frequently displayed translucent halos whereas MO10-T4 and Bengal-2R1 did not, suggesting that a capsular layer was absent in the latter two strains. Therefore, we propose that the SM and MM species are capsular forms of O139 O antigen that are present in LPS preparations. This O-antigen capsule is apparently not covalently linked to the LPS core and lipid A since SM and MM species do not significantly stain with silver.

Insertional Inactivation of *rfcX*. Tn*5lac* insertion mutants Bengal-2R1 and Bengal-2R2 are deficient in the production of all three electrophoretic forms of the O139 antigen when analyzed with anti-O139 polyclonal antisera or monoclonal antibodies (Fig. 1 *A* and *C*). To establish that this phenotype was linked to the transposon insertion in one of these two strains, we devised a site-directed mutational strategy outlined in Fig. 2. Inverse PCR (20) was used to isolate a DNA

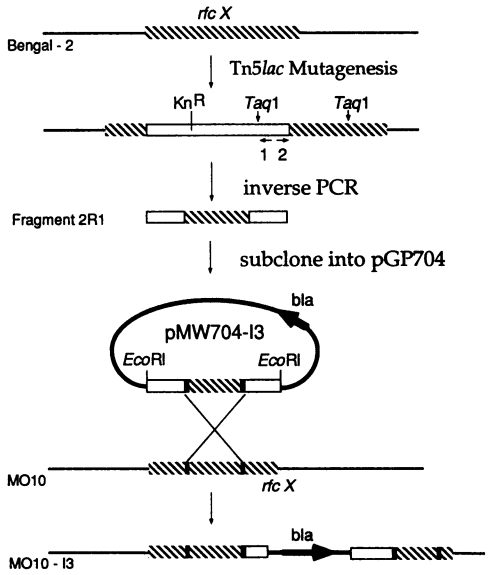

FIG. 2. Schema for cloning the chromosomal DNA adjacent to the right end of the Tn*5lac* insertion in Bengal-2R1 and the insertional inactivation of the homologous locus in MO10, *rfcX*, with this cloned fragment. The open box represents transposon DNA and the hatched box represents chromosomal DNA. Arrows 1 and 2 represent the PCR primers that are complementary to bp 231–250 and bp 84–65, respectively, of the right end of Tn*5lac*.

fragment immediately adjacent to the right end of the Tn*5lac* insertion present in strain Bengal-2R1. This 450-bp PCR product was then subcloned into the suicide plasmid pGP704 (21). The resultant plasmid pMW704-I3 was transferred into MO10 by conjugation and integrated into the chromosome by homologous recombination occurring via the cloned PCR fragment. A representative transconjugate, designated MO10-I3, was O139-negative when tested by slide agglutination, and an immunoblot analysis of a whole cell lysate of MO10-I3 with the anti-O139 polyclonal antisera demonstrated that this strain, like Bengal-2R1, lacked all three electrophoretic forms of the O139 antigen (Fig. 3*A*). The insertion of fragment 2R1 into the homologous locus in MO10, which was designated *rfcX*, was confirmed by Southern blot analysis (Fig. 3*B*). Thus, MO10-I3 reproduces the O139-negative phenotype of Bengal-2R1 and establishes linkage between the Tn*5lac* insertion in Bengal-2R1 and the strain's O139-negative phenotype.

Serum Resistance and Intestinal Colonization of Wild-Type and Mutant strains. The pathogenic significance of the O139 O-antigen capsule and LPS O side chains was investigated by comparing the serum resistance and intestinal colonization properties of mutant and wild-type strains. The spontaneous translucent strains MO10-T4 and 1837-T3 exhibited markedly increased serum sensitivity compared with encapsulated O139 strains (Table 1). The O-antigen-capsule-negative O-side-chain-negative strains Bengal-2R1 and Bengal-2R2 were even more sensitive to serum than MO10-T4 (Table 1). When human sera was used to assess serum resistance, MO10-T4 and Bengal-2R1 were three orders of magnitude and greater than five orders of magnitude more serum-sensitive than wild-type O139 strains, respectively. These results indicate that the LPS O side chains and the O-antigen capsule are important for conferring serum resistance.

Intestinal colonization was assessed in a competition assay using the perorally infected CD-1 suckling mouse. In this assay, suckling mice were coinfected with approximately equal numbers of two strains of *V. cholerae*. One strain (LAC-1) was a Δ*lacZ* derivative of the O1 strain O395 (7); the other strain was one of the wild-type or mutant *lacZ*-positive O139 strains being studied. After *in vivo* growth, homogenates of the small intestine were plated and the ratio of LacZ-positive colonies to LacZ-negative colonies was determined. MO10 colonized the suckling mouse small intestine ≈2-fold better than the O1 strain LAC-1 (Table 1). MO10-T4 was significantly reduced in its ability to colonize the mouse small intestine, whereas its opaque revertant, MO10-OpR3, had wild-type colonization properties (Table 1). Thus, the capsular form of the O139 O antigen that is present in MO10 and MO10-OpR3, but not in MO10-T4, is important for the colonization of the small intestine.

The two O139-negative strains Bengal-2R1 and Bengal-2R2 like the O1-negative strain O395R-1 were severely attenuated *in vivo* and had virtually no capacity to colonize the infant mouse small intestine (Table 1). Thus, the expression of the RM form of the O139 antigen, which is O139 LPS with its short O side chain and which distinguishes Bengal-2R1 and Bengal-2R2 from MO10-T4, also contributes significantly to intestinal colonization and serum resistance.

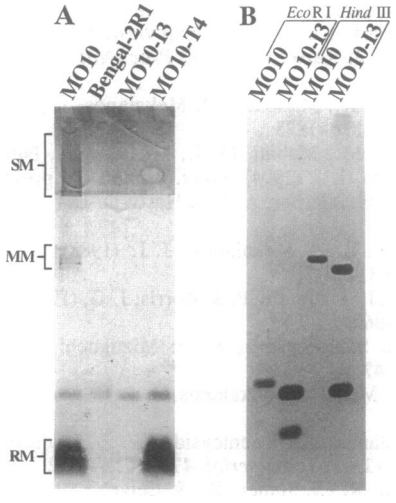

FIG. 3. (*A*) Immunoblot of whole cell lysates of the indicated strains with an anti-O139 polyclonal antisera. MO10-I3, which contains the insertional inactivation of *rfcX* with pMW704-I3, lacks all three forms of the O139 antigen. (*B*) Southern blot of chromosomal DNA from MO10 or MO10-I3. The single *Eco*RI and *Hind*III fragments that hybridize with labeled fragment 2R1 in MO10 are lost in MO10-I3 and two new junction fragments are seen for each enzyme in this strain.

DISCUSSION

By analogy to our understanding of immunity to *V. cholerae* O1 infection, the mucosal immune response directed against the O139 serogroup antigen is likely to be the determinant of protective immunity to *V. cholerae* O139 infection. A genetic and immunologic understanding of the O139 serogroup antigen will aid in constructing vaccines against O139 infection and will help to explain the evolution of this serogroup.

Based on our analysis of wild-type and mutant O139 strains, we propose a model for the O139 serogroup antigen (Fig. 4). Encapsulated opaque strains have two forms of the serogroup antigen—an O-antigen capsule and LPS. The O-antigen capsule corresponds to the two slowly migrating forms of the antigen observed in immunoblots (the SM and MM forms of the O139 antigen). The LPS corresponds to the RM form of the antigen. Immunoblots with the anti-O139 monoclonal antibody demonstrated that there is a shared epitope in the O-antigen capsule and the LPS (represented by the circle in Fig. 4). Since there was virtually no silver staining of the SM and MM forms of the antigen, the O-antigen capsule is not bound to the LPS core and lipid A.

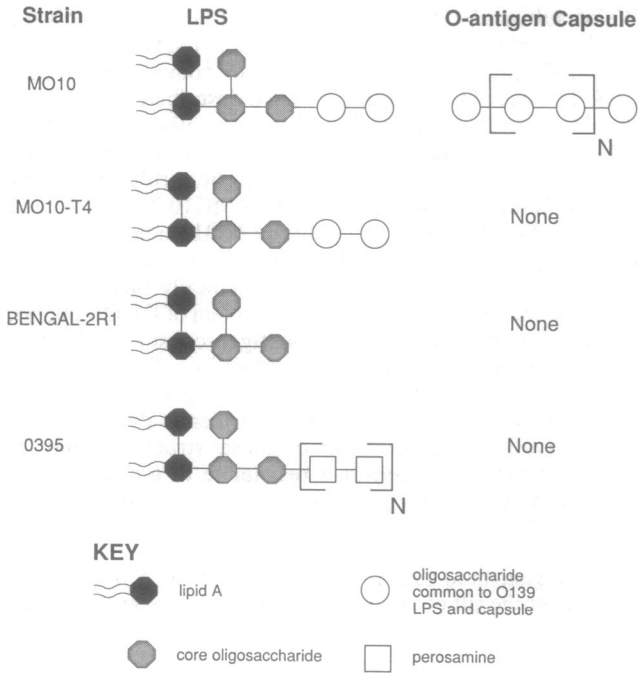

FIG. 4. Schematic representation of a model for the O139 serogroup antigen.

Translucent strains like MO10-T4 lack the O-antigen capsule, lose the SM and MM forms of the antigen on immunoblots, and become more serum-sensitive. All translucent strains are not identical; for example, 1837-T3 appears similar to rough O139-negative strains on silver-stained LPS gels and only expresses very small amounts of the RM form of the antigen in immunoblots.

O139-negative mutant strains, such as Bengal-2R1, lack both the LPS-associated O side chains and the O-antigen capsule. Insertional inactivation of the *rfcX* locus in MO10 with the cloned junctional fragment from Bengal-2R1 (fragment 2R1) established that the O139-negative phenotype of this insertion mutant is indeed associated with the Tn*5lac* insertion in Bengal-2R1. That the *rfcX* locus presumably defines one of the genes required for O139-antigen synthesis is further supported by our observation that fragment 2R1 defines an O139-specific DNA sequence (24). Since the single transposon insertion led to the loss of both the O-antigen capsule and the O side chain of LPS in Bengal-2R1, there must be genes common to the biosynthesis of these structures or, alternatively, the assembly or expression of these two macromolecules is biosynthetically coordinated.

We have defined the SM and MM forms of the O139 antigen as an O-antigen capsule. This is based on the finding that these two slowly migrating forms of the antigen exhibit little if any staining with silver in LPS gels and that, by analogy to other encapsulated vibrios (22, 23), opaque O139 strains produce these forms of the antigen whereas translucent strains do not. Also, similar to encapsulated vibrios (22, 23), opaque O139 strains show enhanced serum resistance and seem to exhibit a capsular surface layer when stained with polycationic ferritin (10) or ruthenium hexamine trichloride (15) and examined with electron microscopy. Our data suggest that the SM and MM forms of the O139 antigen represent highly polymerized O side chains that are not linked to core oligosaccharide and lipid A. These forms of the O139 antigen are, therefore, analogous in structure to certain O-side-chain-related capsules found, for example, in *E. coli* K30 (group I) (25) or in *E. coli* O111 (26). The SM and MM forms of the O139 antigen might correspond to the capsular polysaccharide described by Weintraub *et al.* (15) or could be distinct from this polysaccharide material. It is interesting that while Weintraub *et al.* (15) concluded that O139 capsular polysaccharide contained a 3,6-dideoxyhexose, Hisatsune *et al.* (14) concluded that this sugar was a major component of O139 LPS. The use of the mutants we have described in this report should aid in the clarification of these conflicting results.

Both the O-antigen capsule and LPS-associated O side chains of *V. cholerae* O139 proved to be virulence factors. Thus, colonization of the intestine by MO10-T4 was considerably reduced relative to wild-type MO10, and the O-antigen-capsule-negative and LPS-O-side-chain-negative strains Bengal-2R1 and Bengal-2R2 were essentially unable to colonize the intestine. What is the mechanism of the attenuation of these strains? Is the O139 O-antigen capsule and/or the LPS-associated O side chain important for adherence to the small intestine or does the loss of these antigens interfere with the assembly of other cell surface virulence determinants? Alternatively, either or both of these structures may be important for the bacterium's resistance to a complement-like activity previously proposed to be present in the gastrointestinal tract (27).

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