

The Capsule and O Antigen in *Vibrio cholerae* O139 Bengal Are Associated with a Genetic Region Not Present in *Vibrio cholerae* O1

LAURIE E. COMSTOCK,¹ DAVID MANEVAL, JR.,¹ PINAKI PANIGRAHI,^{2,3} AARON JOSEPH,¹
MYRON M. LEVINE,¹ JAMES B. KAPER,¹ J. GLENN MORRIS, JR.,^{1,3} AND JUDITH A. JOHNSON^{3,4*}

Center for Vaccine Development,¹ Department of Pediatrics,² and Department of Pathology,⁴ University of Maryland School of Medicine, and Veterans Affairs Medical Center of Baltimore,³ Baltimore, Maryland 21201

Received 1 August 1994/Returned for modification 23 September 1994/Accepted 26 October 1994

***Vibrio cholerae* O139 Bengal, although closely related to *V. cholerae* O1 El Tor, produces a polysaccharide capsule and has a distinct O antigen. We have identified a chromosomal region of at least 11 kb, as defined by three *TnphoA* mutations, that is required for the expression of both polysaccharides. Electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis show that these *TnphoA* mutants have lost the abilities both to express capsule and to produce lipopolysaccharide beyond the core oligosaccharide. Reactivity with O139 typing serum and resistance to serum are also lost in the mutants. DNA probes for this region do not hybridize with O1 *V. cholerae* but do react with other vibrios, implying that the region was recently acquired.**

Until 1992, only *Vibrio cholerae* of serogroup O1 was recognized as a cause of epidemic cholera. *V. cholerae* of the other 137 serogroups, also known as non-O1 *V. cholerae*, had never been shown to cause epidemic cholera, although some strains cause sporadic gastrointestinal disease (19). Non-O1 *V. cholerae*, however, can cause extraintestinal disease not seen with O1 strains, including wound infections and septicemia in susceptible hosts (19, 24). The ability to cause extraintestinal disease has been associated with the ability of many non-O1 isolates to produce a polysaccharide capsule (10), which may allow the organism to survive in the blood (11).

Late in 1992, large outbreaks of cholera-like disease occurred in southern and eastern India and southern Bangladesh (9, 21, 22). The etiologic agent of these outbreaks was the newly described O139 *V. cholerae* synonym Bengal. This strain is the first documented non-O1 *V. cholerae* to cause epidemic cholera which is clinically indistinguishable from cholera caused by O1 strains (3, 9). Since the beginning of the outbreak, *V. cholerae* O139 has rapidly spread to many countries in Asia, demonstrating the pandemic potential of this strain (4, 20).

V. cholerae O139 was classified as a new serogroup because it failed to react with antisera to the previously known 138 serogroups (9). Several studies have shown that this strain is phylogenetically and phenotypically very similar to O1 El Tor strains. Like El Tor strains, Bengal strains have tandemly repeated chromosomal cholera toxin genes (5, 12). Bengal strains also have zonula occludens toxin (*zot*) and accessory cholera toxin (*ace*) (12) and have genes for and express the TcpA pilus (6, 20). Chromosomal DNAs from Bengal and El Tor strains give similar banding patterns by pulsed-field electrophoresis and by Southern blot analysis with labeled rRNA (ribotyping) (20). They also are identical by multilocus enzyme electrophoresis analysis (12). However, at least two important differences exist between O1 El Tor and Bengal strains. Manning et al. have demonstrated that O139 *V. cholerae* possesses a truncated lipopolysaccharide (LPS) O side chain which is unreactive with O1-specific antiserum and that a large portion of

DNA corresponding to the *rfb* region of O1 strains is missing from the O139 strain (17). In addition, O139 *V. cholerae* expresses a polysaccharide capsule (12, 27). These differences induce distinct immune responses, with prior exposure to O1 strains affording no protection to disease due to the Bengal strain (1, 2, 9).

The present study was undertaken to begin to understand the genetic basis of capsule and LPS O side chain of *V. cholerae* O139 and to determine if this strain contains genetic material which is not present in the closely related O1 El Tor strains.

MATERIALS AND METHODS

Bacteria and media. *V. cholerae* O139 Bengal strain AI1837, isolated from a Bangladeshi patient with severe cholera in early 1993, was a gift of M. J. Albert, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). O139 strains for the probe experiments were kindly provided by Peter Echeverria, Bangkok, Thailand. Additional strains of *V. cholerae* and other *Vibrio* spp. were from collections at the Center for Vaccine Development and Veterans Affairs Medical Center and represent both clinical and environmental isolates. Frozen stocks were maintained at -70°C in LB broth containing 50% glycerol. Unless otherwise noted, antibiotic concentrations were as follows: ampicillin, 100 $\mu\text{g/ml}$; gentamicin, 30 $\mu\text{g/ml}$; kanamycin, 50 $\mu\text{g/ml}$; polymyxin B, 50 IU/ml; and tetracycline, 9 $\mu\text{g/ml}$.

Transposon mutagenesis. *TnphoA* is a Tn5 derivative containing a gene for alkaline phosphatase lacking the promoter and signal sequences; in-frame fusion of this gene to genes encoding a secreted protein results in an active alkaline phosphatase enzyme (18). To determine the region of the O139 genome which encodes the polysaccharide capsule, *TnphoA* was introduced into AI1837 by conjugation with *Escherichia coli* SM10 bearing the tetracycline-resistant, *TnphoA*-containing plasmid pRT291. *V. cholerae* cells carrying the plasmid were selected on LB agar plates containing polymyxin B and tetracycline. Loss of the vector plasmid was induced by introduction of a second IncP plasmid pHII (gentamicin resistant) by mating with *E. coli* MM294 bearing this plasmid. The mating was plated on LB agar containing polymyxin B, kanamycin, and gentamicin. These antibiotics select for *V. cholerae* containing the *TnphoA* inserted in the chromosome; loss of resistance to tetracycline indicated loss of pRT291. Clones producing translucent colonies were subcultured, and stable translucent isolates were saved for further analysis.

To ascertain the number of *TnphoA* insertions within each mutant, chromosomal DNA was isolated and digested with *Bam*HI and *Sal*I (data not shown), which cut within *TnphoA*. The digested DNA was electrophoresed in a 0.7% agarose gel and transferred to MagnaGraph membranes (MSI, Westboro, Mass.). A ca. 900-bp *Pst*I kanamycin resistance gene fragment was digoxigenin labeled (Genius system; Boehringer, Mannheim, Germany), hybridized with the blot under stringent conditions at 42°C overnight, and then washed and processed according to the manufacturer's recommendations. This probe will hybridize with chromosomal pieces containing the 5-kb DNA fragment at the 5' end of *TnphoA* and a portion of DNA from the vibrio chromosome. The number of reactive bands for each mutant corresponds to the number of *TnphoA* insertions.

* Corresponding author. Mailing address: VAMC Baltimore, 4D-148, 10 N. Greene St., Baltimore, MD 21201. Phone: (410) 605-7000, ext. 5338. Fax: (410) 605-7911.

Serum killing. To determine the resistance of strains to serum killing, 0.065 ml of pooled human serum from at least three donors with added guinea pig complement was mixed with 0.035 ml of 10^7 bacteria per ml as previously described (11). After 30 min of incubation at 37°C, killing was determined by plate counts of serial dilutions. For control tubes, the serum was heat inactivated at 60°C for 30 min before addition of bacteria.

Electron microscopy. Bacteria were fixed and stained with polycationic ferritin, and thin sections were examined by electron microscopy as previously described (11).

Capsule and LPS extractions. LPS was isolated from cells with hot aqueous phenol and further purified as described previously (25). Purified LPS was lyophilized and resuspended in 0.5% triethylamine. Capsule was isolated as previously reported (11). Briefly, cells were shaken with $0.5\times$ phosphate-buffered saline (PBS), followed by enzyme treatment (DNase, RNase, and pronase) of the supernatant, phenol-chloroform extraction, and ultracentrifugation. Capsule was further purified on a Bio-Gel P-100 column (Bio-Rad Laboratories, Richmond, Calif.). Quantitation of capsular polysaccharide was by phenol-sulfuric assay as previously described (11).

SDS-polyacrylamide gel electrophoresis (PAGE), Western blotting (immunoblotting), and dot blotting. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels were prepared, electrophoresed as described by Laemmli (16), and silver stained (Bio-Rad) according to the manufacturer's recommendations. Approximately 20 μ g of LPS was loaded per well. For Western blots, whole-cell lysates were run on SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, Mass.), using standard transfer buffer with a 200-mA current. For each strain, 10^7 cells were loaded. Blots were blocked in PBS containing 3% nonfat dry milk (PBS-milk) and then incubated for 1 h in 1:1,000 rabbit antiserum specific for O139 (a kind gift of T. Takeda) in PBS-milk. The blots were washed five times with PBS and incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) at 1:10,000 in PBS for 1 h. The blot was washed five times with PBS, and Western Blue colorimetric detection solution (Promega, Madison, Wis.) was added until bands developed. Dot blots were prepared by spotting 5 μ l of an overnight culture grown in LB broth or purified capsular polysaccharide from the void volume of the P-100 column on Immobilon membranes. The membranes were air dried and processed as for Western blots.

Cloning of the *TnphoA* insertion junctions. The DNA comprising the junction between the 5' end of *TnphoA* and the vibrio genome was cloned by using either *SalI* or *BamHI*, both of which cleave just downstream of the kanamycin resistance gene of *TnphoA*. Chromosomal DNA from each mutant was digested with the appropriate enzyme and cloned into the *BamHI* or *SalI* site of pBR322. The DNA was ligated overnight at 15°C and transformed into *E. coli* DH5 α , using standard techniques. Desired transformants were selected on LB agar plates containing ampicillin and kanamycin. Plasmid DNA was extracted from the resulting colonies and digested with either *SalI* or *BamHI* and electrophoresed in agarose gels to confirm the expected insert size.

Generation of probes from *TnphoA* insertion junctions. A short segment of vibrio DNA corresponding to the *TnphoA* insertion region cloned above was sequenced by automated sequencing, using the primer K36 (5'CCAGCCT TCATAATACGTAGGC), which recognizes the 5' end of the IS50 region of *TnphoA*. The resulting sequence was used to generate primers in the opposite orientation approximately 300 to 350 bp from the K36 primer. Vibrio DNA was amplified by 30 cycles of PCR from the corresponding plasmids. The cycle times were as follows: 48°C for 1 min, 72°C for 1 min, and 95°C for 1 min. The PCR product was subjected to electrophoresis in a 1.2% agarose gel, and the fragment of the correct size was gel purified by electroelution.

Hybridization of probes with *Vibrio* strains. Purified PCR products generated as described above were labeled with [α - 32 P]dATP, using a random priming kit (Gibco/BRL). Colony blots were prepared on Whatman 541 filters as described previously (28), hybridized at 37°C in buffer (40% formamide, $5\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS, 1 mM EDTA, $1\times$ Denhardt's solution, 100 μ g of sonicated salmon sperm DNA per ml) containing the labeled probe, and then washed at 65°C.

RESULTS

Transposon mutagenesis. We have previously shown that heavily encapsulated *V. cholerae* non-O1 strain NRT36S forms naturally opaque colonies which can undergo phase variation to become translucent upon loss of the polysaccharide capsule (11). Like NRT36S, *V. cholerae* O139 Bengal forms opaque colonies and undergoes phase variation at a rate of ca. 1 in 10^4 to 10^5 . N16961, like other O1 *V. cholerae* strains, had a translucent colony morphology; no opaque colonies were seen even after exposure to serum or animal passage.

This difference in colony morphology was used to select for acapsular mutants. Only stably translucent mutants showing no reversion to the opaque phenotype were analyzed. Three

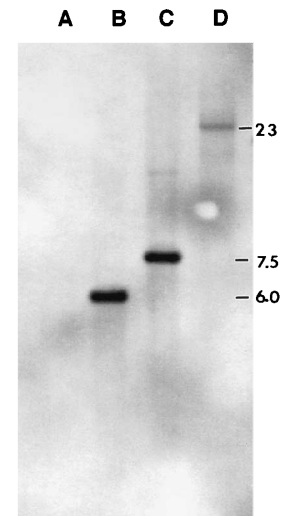


FIG. 1. Southern hybridization analysis of *BamHI* digests of chromosomal DNA from parent and mutant strains hybridized under stringent conditions with a 900-bp probe for the kanamycin resistance gene of *TnphoA*. Lanes: A, *V. cholerae* O139 Bengal AI1837 (parent); B, *TnphoA* mutant 1E; C, *TnphoA* mutant 3B; D, *TnphoA* mutant 6A. Molecular sizes in kilobases are shown at the right.

TnphoA insertion mutants generated from strain AI1837, designated 1E, 3B, and 6A, were studied. Southern blot analysis demonstrated that each mutant contained only a single *TnphoA* insertion (Fig. 1).

Capsule and O-antigen expression. Encapsulation has previously been shown to correlate with an opaque colony morphology, resistance to serum killing, and the amount of extractable surface polysaccharide expressed (11). Therefore, we examined AI1837 and the mutants for these characteristics (Table 1). AI1837 is quite resistant to the bactericidal action of normal human serum, but the translucent mutants have lost this resistance, showing a drop in CFU per milliliter more than 3 logs greater than for the wild type (Table 1). Like the unencapsulated O1 El Tor strain N16961, no colonies were recovered from serum inoculated with mutant 1E or 6A. Only a few translucent colonies were recovered from mutant 3B. Capsular material was quantitated from both the parent and mutant strains. The data in Table 1 demonstrate that less capsular material was isolated from mutant strains than from the encapsulated wild-type strain. Loss of capsule in mutants 6A and 3B was confirmed by electron microscopic examination (Fig. 2).

To determine if capsule represents all or part of the O139 antigen, we examined the ability of the mutants to react with *V. cholerae* O139 typing serum (Table 1). Dot blots of the parent strain and purified capsule, but not the mutants, reacted with the O139 typing serum. However, unlike the parent strain, the mutants reacted slightly with O1 typing serum by slide agglutination (Table 1).

To determine whether LPS was altered in the mutants, LPS was extracted from both wild-type and mutant *V. cholerae* O139 Bengal strains and analyzed by SDS-PAGE. As shown in Fig. 3, the wild-type strain AI1837 produced a low-molecular-weight band and a short ladder as described by Manning et al. (17) as well as some higher-molecular-weight material. In contrast, the mutants produced only the lowest-molecular-weight material.

Western immunoblot analysis of whole-cell lysates shows three clusters of bands, representing low-, medium-, and high-

TABLE 1. Strain characteristics

<i>V. cholerae</i> strain	Colony morphology	O139 ^a	O1 ^b	Serum killing ^c	Avg capsule content (ng of saccharide/10 ⁸ cells) ± SE ^d
O139 Bengal AI1837 (parent)	Opaque	++++	–	1.3	8.2 ± 2.3
<i>TnphoA</i> mutants					
1E	Translucent	–	+	>5	2.0 ± 2.4
3B	Translucent	–	+	4.8	5.1 ± 2.6
6A	Translucent	–	+	>5	1.6 ± 1.5
N16961	Translucent	–	++++	>5	2.6 ± 1.8

^a Dot blots of whole cells from an overnight culture incubated with the *V. cholerae* O139 typing serum. +++++, strongly positive, –, no reaction.

^b Slide agglutination of whole cells with O1 typing serum. +++++, strongly positive, within 1 min; +, weak reaction, >5 min; –, no reaction.

^c Log₁₀ decline in CFU per milliliter during a 30-min incubation compared with the control with heat-inactivated (55°C for 30 min) serum.

^d Determined by phenol sulfuric assay of small-scale extractions. Results are averages of three samples.

molecular-weight material, that are recognized by the O139-specific antiserum (Fig. 4). The three *TnphoA* mutants lack all but the lowest-molecular-weight material. The specificity of the O139 typing serum is confirmed by the lack of reaction with the El Tor strain (Fig. 4).

Mapping of *TnphoA* insertion sites. Figure 1 shows that *Bam*HI digestion of chromosomal DNA from mutant 3B yields a *TnphoA*-vibrio junctional fragment of approximately 7.5 kb. Cleavage of chromosomal DNA from mutant 6A with *Sal*II produce a 5-kb junctional fragment. The *Bam*HI and *Sal*II fragments from mutants, 3B and 6A, respectively, were successfully cloned into pBR322, resulting in plasmids pLC51 and pLC52. Plasmids pLC51 and pLC52 contained approximately 2.5 kb and 500 bp, respectively, of vibrio DNA. Repeated attempts using either enzyme to clone the junctional fragment from mutant 1E into pBR322 were unsuccessful. Additional attempts to clone this fragment into the low-copy-number vector pACYC184 were equally unsuccessful. Lastly, cloning the 3' end of *TnphoA* with the adjoining vibrio DNA from mutant 1E was attempted. Digestion of chromosomal DNA from mutant 1E with *Dra*I cleaves just 5' of the kanamycin resistance gene of *TnphoA* and into the vibrio DNA, yielding a fragment of approximately 7.5 kb. This DNA was ligated into the *Eco*RV site of pACYC184. Once again, after repeated attempts, no transformants containing the correct size insert resulted.

Generation of probes from pLC51 and pLC52 and analysis of region. The junctional sequences contained on plasmids pLC51 and pLC52 were sequenced by using primer K36 from *TnphoA*, and new primers corresponding to vibrio sequences 300 to 350 bp from the junction were created. For mutant 6A, the primer was 5'GGTCGGAAGTATTATGCATCCG, and for mutant 3B, the primer was 5'CCGTTCCTGTTGCAAGGCTCAT. These primers, in conjunction with primer K36, were used to amplify the vibrio DNA by PCR. The bands corresponding to the anticipated size fragments were gel purified, labeled, and referred to as 3BP and 6AP.

Next, experiments were conducted to determine if the *TnphoA* insertions of mutants 1E, 3B, and 6A were all contained in the same region of the *V. cholerae* O139 chromosome. Chromosomal DNA from wild-type strain AI1837 was digested with various enzymes that cut within *TnphoA*. Southern blots of these digestions were hybridized with labeled probe 6AP, and the sizes of the resulting reactive bands were determined. Digestion of chromosomal DNA from AI1837 with *Eco*RI yielded a large 6AP-reactive fragment of approximately 21 kb (Fig. 5a, lane A). Therefore, if the *TnphoA* insertions of mutants 1E and 3B were contained on this large *Eco*RI fragment, there should be a shift in the 6AP-reactive band when chromosomal DNA from these mutants is digested with *Eco*RI.

Figure 5 shows that the *TnphoA* insertions of mutants 1E and 3B were also contained within this large *Eco*RI fragment. Analysis of the resulting fragment sizes allows for a map of this *Eco*RI fragment showing the *TnphoA* insertion sites of each mutant (Fig. 5b). The three mutants mapped to a region of at least 11 kb.

Hybridization of probes 3BP and 6AP with various *Vibrio* strains. Because this region of DNA is involved in the synthesis of capsule and the modified LPS O side chain which are unique antigens to strain O139, hybridization analysis was performed with probes 3BP and 6AP to determine if this DNA is unique to strain O139 or is present but unexpressed in other *V. cholerae* isolates. Both probes hybridized with all of the O139 strains tested, but neither probe hybridized with any of 103 strains of O1 *V. cholerae* (predominantly El Tor) (Table 2). Probe 3BP did react, however, with about 70% of non-O1 strains as well as some of the isolates from five other *Vibrio* species but not with *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, or *Pseudomonas aeruginosa*. Probe 6AP hybridized only with *V. cholerae* O139 and *Vibrio damsela*.

DISCUSSION

It has previously been shown that *V. cholerae* O139 Bengal is closely related to seventh-pandemic strains of *V. cholerae* O1 El Tor, possessing virtually all of the recognized virulence factors for cholera except the O1 LPS and producing clinically indistinguishable disease. In spite of the high degree of similarity, epidemiologic studies (9, 22) and studies in animals (1, 2) indicate that preexisting immunity to *V. cholerae* O1 El Tor does not protect against O139 Bengal infection. This finding suggests that *V. cholerae* O139 Bengal has been able to spread so rapidly not because of an inherent increase in virulence for humans but because it has undergone modifications in the nature or expression of critical antigens that allow it to cause disease in populations that have high levels of immunity to *V. cholerae* O1 strains. To date, the two major dissimilarities between El Tor and O139 strains that might account for this difference in immunogenicity are that O139 strains produce a capsule and have an altered LPS.

Our data confirm that *V. cholerae* O139 Bengal is encapsulated and, like other strains of non-O1 *V. cholerae*, can shift between an encapsulated form with an opaque colony morphology and an unencapsulated form that exhibits a translucent morphology. We have isolated three transposon mutants that have lost the ability to produce the capsular polysaccharide, as shown by electron microscopy, and which have insertion sites that map within an 11-kb region. As in other strains of *V. cholerae*, loss of capsule was associated with reduction in

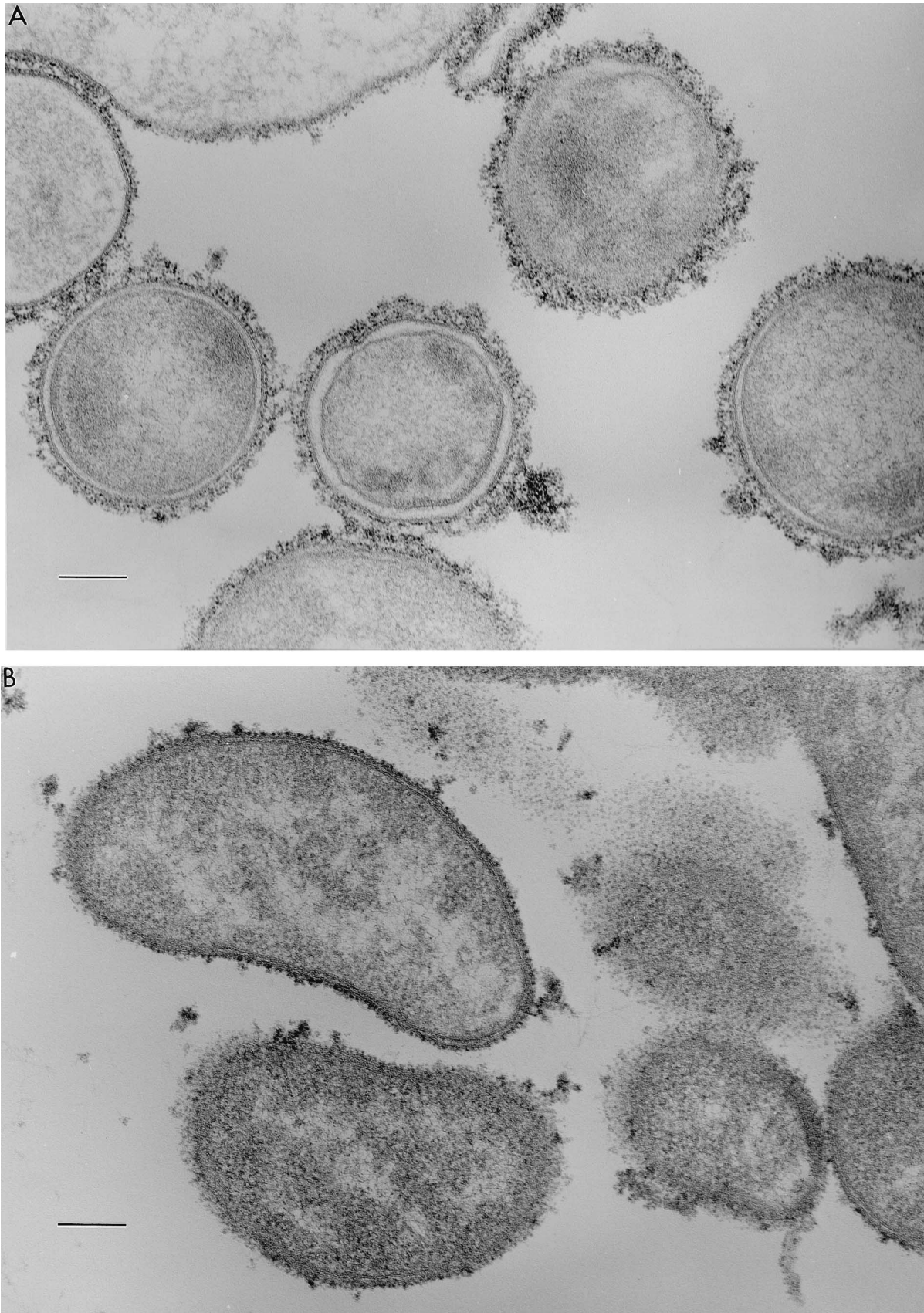


FIG. 2. Thin sections of *V. cholerae* O139 Bengal and *TnphoA* mutants stained with polycationic ferritin. (A) *V. cholerae* O139 Bengal AI1837 (parent); bar = 200 nm. (B) *TnphoA* mutant 3B; bar = 200 nm. (C) *TnphoA* mutant 6A; bar = 200 nm.

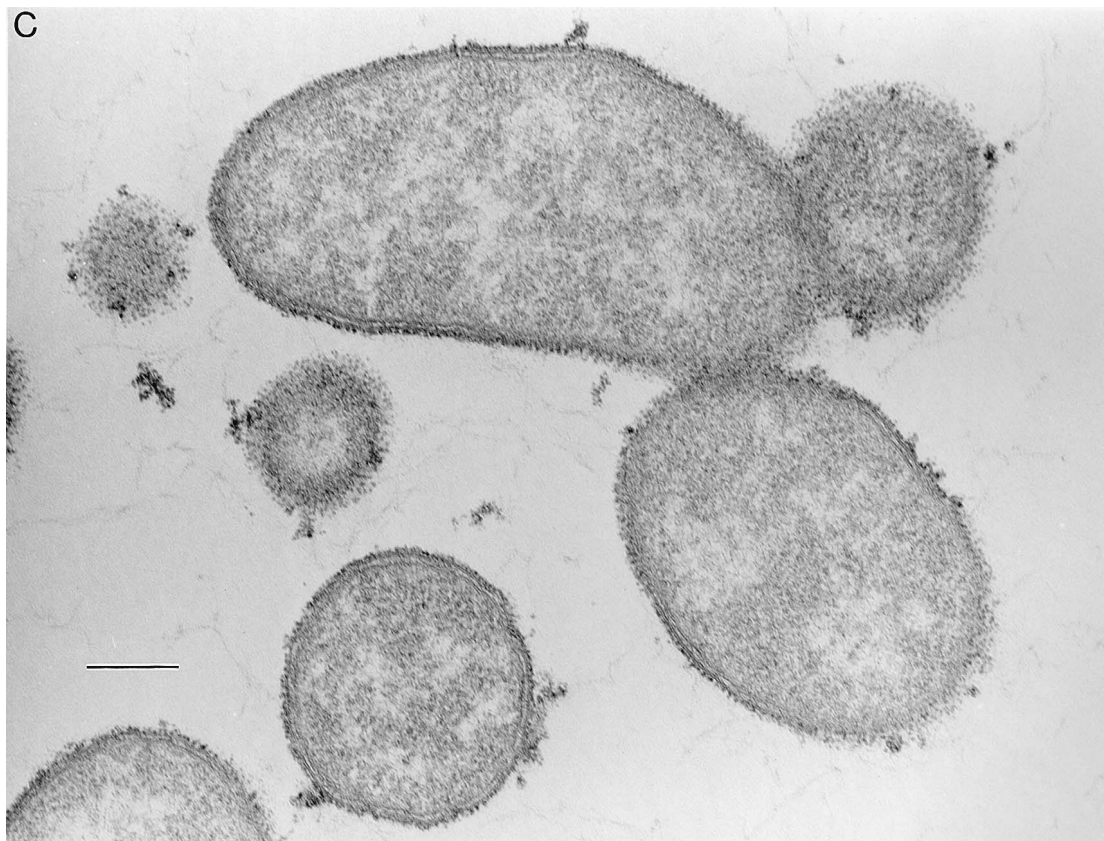


FIG. 2—Continued.

extractable surface polysaccharide and loss of resistance to killing by normal human serum.

In addition to expressing the capsule, O139 strains have alterations in the LPS compared with O1 strains. As shown by Manning et al. (17), *V. cholerae* O139 Bengal strains lack *rfbA*, *-B*, *-D*, *-E*, *-G*, *-H*, *-I*, *-K*, *-L*, *-M*, *-N*, *-O*, *-P*, and *-T* genes but retain *rfaD* (involved in core oligosaccharide biosynthesis) and slightly modified locus for *rfbQ*, *-R*, and *-S*. Changes in *rfbR* and

-S are also suggested by the failure of oligonucleotide probes specific for these genes to bind to *V. cholerae* O139 Bengal DNA (12). By SDS-PAGE analysis, O139 strains lack the typical LPS ladder produced by O1 strains but instead appear to have a truncated LPS (semirough) consisting of lipid A plus a core oligosaccharide which is modified (17). Hisatsune et al.

TABLE 2. Hybridization of colony blots with O139 polysaccharide-associated gene probes 3BP and 6AP

Strain	Hybridization with probe			
	3BP		6AP	
	+ ^a	- ^b	+ ^a	- ^b
<i>V. cholerae</i>				
O139 Bengal	195	0	195	0
O1	0	103	0	103
Non-O1, non-O139	90	36	0	126
<i>V. parahaemolyticus</i>	0	17	0	17
<i>V. vulnificus</i>	12	25	0	37
<i>V. fluvialis</i>	2	4	0	6
<i>V. mimicus</i>	4	3	0	7
<i>V. hollisae</i>	3	0	0	3
<i>V. damsela</i>	2	0	2	0
<i>A. hydrophila</i>	0	5	0	5
<i>P. aeruginosa</i>	0	9	0	9

^a Number of strains hybridizing with probe.

^b Number of strains not hybridizing with probe.

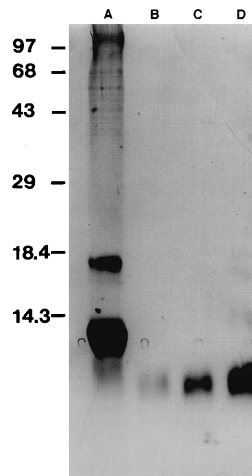


FIG. 3. Silver-stained SDS-polyacrylamide gel of LPS from *V. cholerae* O139 Bengal and acapsular mutants. Lanes: A, *V. cholerae* O139 Bengal AI1837 (parent); B, *TnphoA* mutant 1E; C, *TnphoA* mutant 3B; D, *TnphoA* mutant 6A. Molecular masses in kilodaltons are shown at the left.

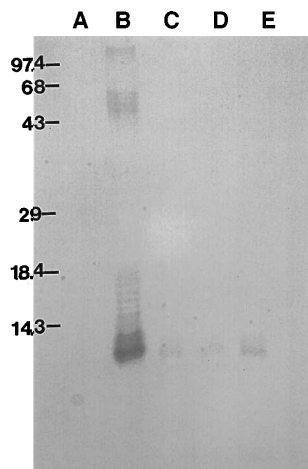


FIG. 4. Western blot of SDS-polyacrylamide gel of whole-cell lysates probed with *V. cholerae* O139 typing serum. Lanes: A, *V. cholerae* O1 E7946; B, *V. cholerae* O139 Bengal AI1837 (parent); C, *TnphoA* mutant 1E; D, *TnphoA* mutant 3B; E, *TnphoA* mutant 6A. Molecular masses in kilodaltons are shown at the left.

(7) have examined the structure of the polysaccharide portion of O139 LPS and reported a structure very similar to that of semirough LPS but with the possible addition of colitose. Weintraub et al. (27), on the other hand, show the same sugars as in the core polysaccharide with the addition of glucosamine, with the colitose (or abequose) being found exclusively in the capsule.

Our acapsular transposon mutants have lost the higher-molecular-weight polysaccharide representing the lipid A containing the modified core and have also lost the very high molecular mass (>90-kDa) fraction that we believe represents the capsular polysaccharide. The mutants retain only a band of molecular weight corresponding to lipid A plus the core oligosaccharide. A similar mutant has been described by Waldor and Mekalanos (26). Their mutant, like the mutants reported here, does not react with O139 typing serum and is located on a DNA fragment that is present in O139 but not O1 strains. However, their mutant maps to a 1.4-kb *EcoRI* fragment, suggesting that the O139 antigen-associated region extends beyond our ca. 21-kb *EcoRI* fragment or that there is an additional locus affecting expression of the phenotype. Because no mutants that lost solely the O antigen or capsule were isolated, the same genes may be responsible for both expression of the capsular polysaccharide and addition of the short O antigen. Alternatively, these mutations may affect common regulatory components or have pleiotropic polar effects. Since mutants were screened for a translucent phenotype, it is possible that the translucent phenotype in this strain is not due to loss of capsule alone but also requires the loss of the LPS modification. Spontaneous translucent phase variants still react with O139 typing serum (27), suggesting that they retain some surface polysaccharide that the mutants lack as is seen in NRT-36S (12). At this time, it is not clear which biosynthetic pathways or sugar moieties are common to *V. cholerae* O139 O antigen and capsule.

The serological typing system for *V. cholerae* was developed before the discovery that many strains of *V. cholerae* produce polysaccharide capsules. Therefore the 139 "O" antigens recognized by these typing sera probably represent a combination of true O polysaccharide antigens and capsular antigens. Purified, high-molecular-weight *V. cholerae* O139 Bengal capsular

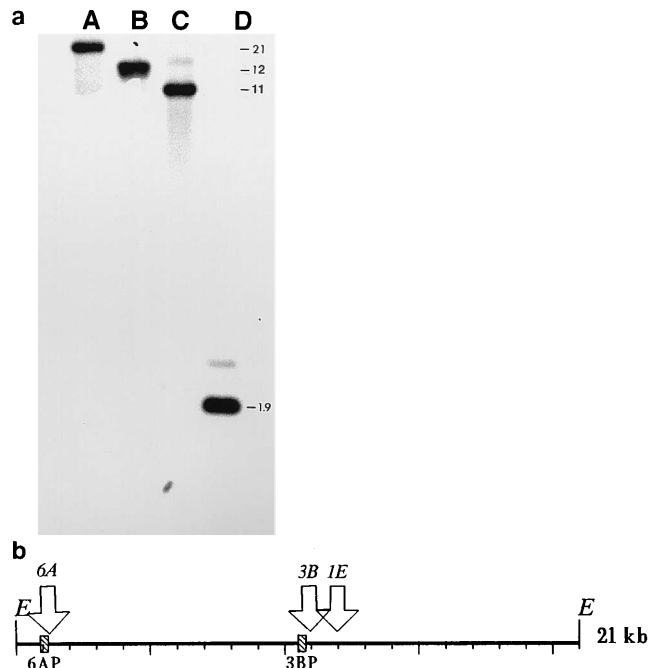


FIG. 5. (a) Southern hybridization analysis of *EcoRI* digests of chromosomal DNA from parent and mutants hybridized under stringent conditions with probe 6AP. Lanes: A, *V. cholerae* O139 Bengal AI1837 (parent); B, *TnphoA* mutant 1E; C, *TnphoA* mutant 3B; D, *TnphoA* mutant 6A. Molecular sizes in kilobases are shown at the right. (b) Physical map of the *TnphoA* insertion sites and binding sites of the 6AP and 3BP probes.

polysaccharide reacted with O139 typing serum in immunoblots. Western blot analysis demonstrated three molecular weight fractions that were recognized by the O139 typing sera. The most strongly reacting band was found at the position of the lipid A containing the modified core. Reactive bands of ca. 50 to 60 kDa that may be composed of long O-antigen polymers or capsule, and a fraction of >90 kDa that presumably represents the capsule, were also seen. These data strongly suggest that both the modified LPS and the capsule contribute to the O139 antigen. Loss of the O139 antigen may expose a molecule that is common to both O1 and O139 strains, accounting for the weak agglutination of the mutants with O1 typing serum.

The biosynthesis of capsules and O side chains is not always clearly distinct. In *E. coli*, the K9 polysaccharide and O104 LPS side chain have identical structures, as is also seen with K87 and O32 antigens (13). In addition to having analogous structures, the genes needed for expressing polysaccharide capsules and O antigens may have similar arrangements and functions. Biosynthesis genes for both capsule and O antigens are generally arranged in regions of approximately 15 to 20 kb (15, 23, 29), although there may be genes located in other chromosomal regions as occurs in the *Salmonella typhi* *viaA/viaB* system (8, 14). Our three mutants all map to a single stretch of at least 11 kb, defining a *V. cholerae* O139 Bengal polysaccharide-associated region.

In light of these findings, we wished to determine whether the capsule of *V. cholerae* O139 Bengal is encoded on newly acquired genes or is due to expression of genes already present, but not expressed, in O1 strains. Therefore, we probed a large collection of vibrios with DNA fragments 3BP and 6AP from this region. Under conditions of high stringency, 6AP hybridized with only O139 strains and two isolates of *V. dam-*

sela, a marine vibrio. Probe 3BP appears to recognize a more conserved region; this probe hybridized with 71% of non-O1 *V. cholerae* strains and with about 30% of other vibrios. In spite of the close phylogenetic relationship between O139 and O1 strains, neither 3BP nor 6AP hybridized with any of 103 O1 strains. These data imply that the O139 polysaccharide-associated region was recently acquired by a *V. cholerae* O1 isolate during evolution to become the O139 strain. The ability of 6AP to hybridize to *V. damsela* suggests that this organism is a possible source of the capsule-associated region. It is interesting that the 3B region appears in non-O1 *V. cholerae* and other vibrios that are less closely related to the O139 strain than O1 *V. cholerae* but that are known to express polysaccharide capsules. This finding suggests that the 3B region is common to a variety of capsular types but the 6A region comprises a gene specific to expression of the O139 capsular type.

We were unable to determine the extent of conservation of the 1E sequences, as repeated attempts to clone this region were unsuccessful. It may be that this region contains a gene that is lethal when cloned at a higher copy number or in the absence of regulatory elements.

In summary, this report describes the identification of a region of the chromosome of *V. cholerae* O139 Bengal that is required for expression of capsule and full-length LPS. These two elements together represent the so-called O139 antigen. This region is present in all O139 strains examined to date but is not found in O1 strains.

ACKNOWLEDGMENTS

This work was supported by PHS grants R29-AI35729 (J. A. Johnson), AI19716 (J. B. Kaper), and R01-AI29471 (M. M. Levine), PHS contract N01-AI15096 (M. M. Levine), and the Department of Veterans Affairs (J. A. Johnson).

REFERENCES

- Albert, M. J., K. Alam, M. Ansaruzzaman, F. Qadri, and R. B. Sack. 1994. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O139 (Bengal strain) after oral immunization of rabbits with *V. cholerae* O1 vaccine strain CVD103-HgR. *J. Infect. Dis.* **169**:230-231. (Letter.)
- Albert, M. J., K. Alam, A. S. M. Hamidur Rahman, S. Huda, and R. B. Sack. 1994. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O1 after oral immunization of rabbits with *V. cholerae* O139 Bengal. *J. Infect. Dis.* **169**:709-710.
- Bhattacharya, S. K., M. K. Bhattacharya, G. B. Nair, D. Dutta, A. Deb, T. Ramamurthy, S. Garg, P. K. Saha, P. Dutta, A. Moitra, B. K. Mandal, T. Shimada, Y. Takeda, and B. C. Deb. 1993. Clinical profile of acute diarrhoea cases infected with the new epidemic strain of *Vibrio cholerae* O139: designation of the disease as cholera. *J. Infect.* **27**:11-15.
- Chongsa-Nguan, M., W. Chaicumpa, P. Moolasart, P. Kandhasingha, T. Shimada, H. Kurazono, and Y. Takeda. 1993. *Vibrio cholerae* O139 Bengal in Bangkok. *Lancet* **342**:430-431. (Letter.)
- Das, B., R. K. Ghosh, L. Sharma, N. Vasin, and A. Ghosh. 1993. Tandem repeats of cholera toxin gene in *Vibrio cholerae* O139. *Lancet* **342**:1173-1174.
- Hall, R. H., F. M. Khambaty, M. Kothary, and S. P. Keasler. 1993. Non-O1 *Vibrio cholerae*. *Lancet* **342**:430. (Letter.)
- Hisatsune, K., S. Kondo, Y. Isshiki, T. Iguchi, Y. Kawamat, and T. Shimada. 1993. O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. *Biochem. Biophys. Res. Commun.* **196**:1309-1315.
- Houng, H.-S. H., K. F. Noon, J. T. Ou, and L. S. Baron. 1992. Expression of Vi antigen in *Escherichia coli* K-12: characterization of ViaB from *Citrobacter freundii* and identity of ViaA with RcsB. *J. Bacteriol.* **174**:5910-5915.
- International Center for Diarrheal Disease Research, Bangladesh, Cholera Working Group. 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. *Lancet* **342**:387-390.
- Johnson, J. A., A. Joseph, P. Panigrahi, and J. G. Morris, Jr. 1992. Frequency of encapsulated versus unencapsulated strains of non-O1 *Vibrio cholerae* from patients with septicemia or diarrhea, or from environmental sources. In Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
- Johnson, J. A., P. Panigrahi, and J. G. Morris, Jr. 1992. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance, and virulence in mice. *Infect. Immun.* **60**:864-869.
- Johnson, J. A., C. A. Salles, P. Panigrahi, M. J. Albert, R. J. Johnson, and J. G. Morris, Jr. 1994. *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* El Tor but has important differences. *Infect. Immun.* **62**:2108-2110.
- Kogan, G., B. Jann, and K. Jann. 1992. Structure of the *Escherichia coli* O104 polysaccharide and its identity with the capsular K9 polysaccharide. *FEMS Microbiol. Lett.* **91**:135-140.
- Kolyva, S., H. Waxin, and M. Y. Popoff. 1992. The Vi antigen of *Salmonella typhi*: molecular analysis of the *viaB* locus. *J. Gen. Microbiol.* **138**:297-304.
- Kroll, J. S., S. Zamze, B. Loynds, and E. R. Moxon. 1989. Common organization of chromosomal loci for production of different capsular polysaccharides in *Haemophilus influenzae*. *J. Bacteriol.* **171**:3343-3347.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Manning, P. A., U. H. Strocher, and R. Morona. 1994. Molecular basis for O-antigen biosynthesis in *Vibrio cholerae* O1: Ogawa-Inaba switching, p. 77-94. In I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera. American Society for Microbiology, Washington, D.C.
- Manoil, C., and J. Beckwith. 1985. *TnpA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* **82**:8129-8133.
- Morris, J. G., Jr. 1994. Non-O group 1 *Vibrio cholerae* strains not associated with epidemic disease, p. 103-115. In I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera. American Society for Microbiology, Washington, D.C.
- Morris, J. G., Jr., and Cholera Laboratory Task Force. 1994. *Vibrio cholerae* O139 Bengal, p. 95-102. In I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera. American Society for Microbiology, Washington, D.C.
- Nair, G. B., T. Ramamurthy, S. K. Bhattacharya, A. K. Mukhopadhyay, S. Garg, M. K. Bhattacharya, T. Takeda, T. Shimada, Y. Takeda, and B. C. Deb. 1994. Spread of *Vibrio cholerae* O139 Bengal in India. *J. Infect. Dis.* **169**:1029-1034.
- Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazono, A. Pal, and Y. Takeda. 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* **341**:703-704.
- Roberts, I., R. Mountford, N. High, D. Bitter-Suermann, K. Jann, K. Timmis, and G. Boulnois. 1986. Molecular cloning and analysis of genes for production of K5, K7, K12, and K92 capsular polysaccharides in *Escherichia coli*. *J. Bacteriol.* **168**:1228-1233.
- Safrin, S., J. G. Morris, M. Adams, V. Pons, R. Jacobs, and J. E. Conte. 1987. Non-O1 *Vibrio cholerae* bacteremia: a case report and review. *Rev. Infect. Dis.* **10**:1012-1017.
- Thomashow, L. S., and S. C. Rittenberg. 1985. Isolation and composition of sheathed flagella from *Bdellovibrio bacteriovorus* 109J. *J. Bacteriol.* **163**:1047-1054.
- Waldor, M. K., and J. J. Mekalanos. 1994. *Vibrio cholerae* O139 specific gene sequences. *Lancet* **343**:1366. (Letter.)
- Weintraub, A., G. Widmalm, P.-E. Jansson, M. Jansson, K. Hultenby, and M. J. Albert. 1994. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. *Microb. Pathog.* **16**:235-241.
- Wright, A. C., Y. Guo, J. Johnson, J. P. Nataro, and J. G. Morris, Jr. 1992. Development and testing of a non-radioactive DNA oligonucleotide probe that is specific for *Vibrio cholerae* cholera toxin. *J. Clin. Microbiol.* **30**:2302-2306.
- Zhang, L., A. Al-Hendy, P. Toivanen, and M. Skurnik. 1993. Genetic organization and sequence of the *rjb* gene cluster of *Yersinia enterocolitica* serotype O:3: similarities to the dTDP-L-rhamnose biosynthesis pathway of *Salmonella* and to the bacterial polysaccharide transport systems. *Mol. Microbiol.* **9**:309-321.