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Genetic rearrangements in the *rfb* regions of *Vibrio cholerae* O1 and O139

(chromosomal rearrangements/serotype/lipopolysaccharide/H-repeat/insertion sequences)

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ABSTRACT **The recent emergence of a pathogenic new non-O1 serotype (O139) of *Vibrio cholerae* has led to numerous studies in an attempt to identify the origins of this new strain. Our studies indicate that O139 strains have clear differences in the surface polysaccharides when compared with O1 strains: the lipopolysaccharide can be described as semi-rough. Southern hybridization with the O1 *rfb* region demonstrates that O139 strains no longer contain any of the *rfb* genes required for the synthesis of the O1 O-antigen or its modification and also lack at least 6 kb of additional contiguous DNA. However, O139 strains have retained *rfaD* and have a single open reading frame closely related to three small open reading frames of the O1 *rfb* region. This region is closely related to the H-repeat of *Escherichia coli* and to the transposases of a number of insertion sequence elements and has all the features of an insertion sequence element that has been designated VcIS*1*. Transposon insertion mutants defective in O139 O-antigen (and capsule) biosynthesis map to the same fragment as VcIS*1*. Preliminary sequence data of complementing clones indicate that this DNA encodes a galactosyltransferase and other enzymes for the utilization of galactose in polysaccharide biosynthesis. We propose a mechanism by which both the Ogawa serotype of O1 strains and the O139 serotype strains may have evolved.**

Cholera in humans has traditionally been associated with *Vibrio cholerae* serotype O1. However, in 1992, apparently for the first time in recorded history, a non-O1 strain of *V. cholerae* was responsible for an epidemic of cholera (1, 2). Previously, non-O1 strains caused only sporadic cases of diarrhea but not epidemics of cholera (3). This strain did not react with antibodies to any of the known serotypes and was designated O139 synonym Bengal (4) for its first site of isolation. By June 1993, this serotype had reached Karachi and was displacing O1 as the epidemic serotype. A similar O139 strain may have emerged in Argentina (5).

Over 100,000 cases of O139 cholera have been reported, and the fear exists that O139 could initiate the next, eighth pandemic. How has this new serotype arisen? It has been proposed that the O139 (Bengal) serotype is due to a mutation in the O-antigen (*rfb*) locus (6). Our data do not support such a simple hypothesis.

Previous studies have sought to distinguish O139 from O1 strains by means other than serotyping. Basically these studies suggested that O139 (Bengal) is very closely related to the El Tor biotype O1 strains (7–10). However, unlike O1 strains, O139 strains are encapsulated (8, 11).

Using oligodeoxynucleotides as probes for the O1 *rfbR* and *rfbS* genes, Johnson *et al.* (11) suggested that these genes are absent in O139 strains. However, in this study we find that both O1 and O139 strains have a region defined by the *rfbQ*, *-R*, and *-S* genes (12) that is related to an open reading frame (ORF) called the H-repeat of RHS elements in *Escherichia coli*. RHS elements were first recognized as chromosomal rearrangement hotspots (13, 14). This region appears to represent an insertion sequence (IS).‖

MATERIALS AND METHODS

Bacterial Strains. The *V. cholerae* O1 strains used included O17 (El Tor, Ogawa); 569B (classical, Inaba); AA14073 (El Tor, Ogawa); BM69 (El Tor, Inaba); 64 (El Tor, Ogawa); and H-1 (El Tor, Ogawa.) Strains 1074 and 1196 are nontoxigenic El Tor O1 environmental isolates. AI-1837, AI-1838, AI-4450, AI-1841, AI-1852, AI-1854, AI-1855, and AI-4260 are O139 isolates from the Indian subcontinent. Arg-3 is an O139 isolate from Argentina (5). The rough strains V663 (*rfb*::Tn*5*) and V665 (*rfb*::Tn*5*) are derived from 569B (15).

Southern Hybridization. DNA from *V. cholerae* O1 and O139 strains was prepared as described (16). Transfer of DNA from agarose gels to nitrocellulose filters (Schleicher & Schuell) was performed as described by Southern (17) with modifications (18). Detection was by enhanced chemiluminescence (ECL; Amersham) and autoradiography.

Polymerase Chain Reaction (PCR). Amplification using Amplitaq DNA polymerase (Hoffmann–La Roche) was carried out by standard protocols with the oligodeoxynucleotide primers described.

Antibodies. The initial polyclonal antiserum (antiserum 1) and monoclonal antibody (mAb) 1CL-12 have been described (19). Antiserum 2 to O139 was prepared from a rabbit immunized with heat-killed AI-1837 and subsequently was absorbed to remove crossreactive antibodies.

Lipopolysaccharide (LPS) Silver Staining and Immunoblotting. LPS from 1-ml overnight (18-hr) cultures (20) was analyzed by SDS/PAGE followed by silver staining or immunoblotting (21).

Sequencing Procedures. The nucleotide sequence of the *rfb* regions of O17 and 569B was determined as described (12). Sequencing of the O139 region was performed with an Applied Biosystems 373A automated DNA sequencer. Sequences can be obtained under the accession numbers X59554 (Ogawa) and X91246 (O139).

T7 Expression System. The temperature-inducible, T7 RNA polymerase/promoter system (22) was used to express

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Abbreviations: IS, insertion sequence; LPS, lipopolysaccharide; mAb, monoclonal antibody; ORF, open reading frame.

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‖ The sequences reported in this paper have been deposited in the GenBank database (accession nos. X91246).

L-[^{35}S]methionine (Amersham)-labeled proteins. Proteins were visualized by autoradiography following SDS/PAGE. Molecular size markers were from Pharmacia.

Tn*phoA* Mutagenesis. The suicide plasmid pRT733 (23) was used for isolation of Tn*phoA* insertions in strain AI-1837 by selecting kanamycin- and polymyxin B-resistant exconjugants.

RESULTS

SDS/PAGE of the LPS of O139 Strains. We examined a number of *V. cholerae* O139 (Bengal) strains used in other studies (8, 24). Analysis of LPS from these strains by SDS/PAGE and silver staining showed that O139 strains do not contain the typical ladder of LPS bands detected in O1 strains, nor do they resemble typical rough O1 mutants. However, O139 strains do have a distinct pattern resembling that of a semirough LPS: a region corresponding to the lipid A-core oligosaccharide of O1 is observed, as well as a more heavily staining, slower-migrating region (Fig. 1 *Left*). From the apparent molecular size, the difference between the two O139 bands is likely to be only one or two sugars which presumably cap the lipid A-core. The LPS from the O139 Argentinian isolate (Arg-3) differs again and is slightly smaller.

Colony blotting using O139 mAb 1CL-12 (19) revealed strong crossreactivity with rough O1 strains (V663 and V665), indicating that the mAb reacts with epitopes present in the cores of both O1 and O139 LPS (data not shown). Western blotting of proteinase K-treated whole cells with antiserum 2 showed crossreactivity to the slower-migrating material of both Bengal and Argentinian O139 strains (Fig. 1 *Right*).

Analysis of the *rfb* Region of O139 by Southern Hybridization. The unusual LPS of O139 strains led us to initially investigate the *rfb* region in the O139 serotype by probing with four plasmids which span the O1 *rfb* region (Fig. 2). Probes pPM4202 and pPM4203 did not hybridize with genomic DNA from the O139 strains, but pPM4201 and pPM4204 hybridized weakly. Probes derived from *rfaD* hybridized strongly to all O139 strains, including Arg-3 (data not shown). The use of the *rfaD* probe on *Eco*RI-digested chromosomal DNA revealed a restriction polymorphism within the *rfaD* genes of O139 strains

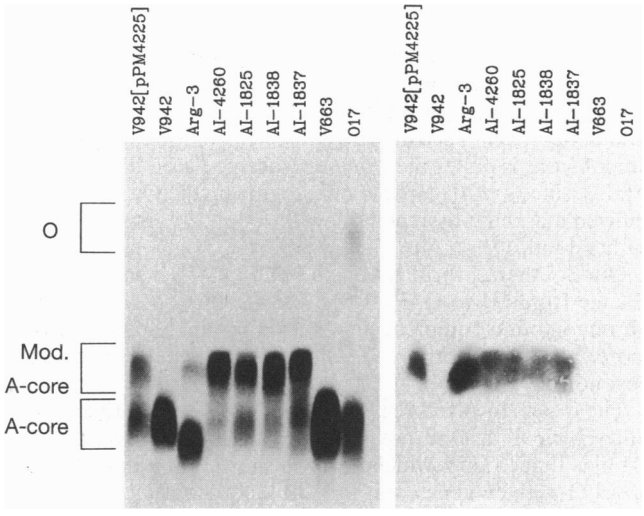

FIG. 1. (*Left*) Analysis of LPS from *V. cholerae* O1 and O139 by SDS/15% PAGE with silver staining. Strains are indicated above the lanes. V663 is an *rfb*::Tn*5* mutant of *V. cholerae* O1 (569B) and lacks any O antigen (15). V942[pPM4225] is the complemented V942 strain. The O139 transposon mutant is V942. The positions of the lipid A-core and O-antigen linked to lipid A-core are indicated, as is the position of the modified lipid A-core in O139 strains. (*Right*) Western blot of an identical gel, probed with the O139-specific antiserum 2. The antibodies react exclusively with the substituted lipid A core.

distinguishing the Bengal isolates from the Argentinian strain. In addition, PCR analysis revealed that Arg-3 has a defective *rfaD* gene. These data correlate with the altered LPS pattern seen in Arg-3 (Fig. 1). By PCR using primers within *rfaD* and *rfbA*, we showed that one end of the deletion in the O139 Bengal strains is close to *rfaD* but that the deletion endpoint in Arg-3 is within *rfaD* (data not shown).

Southern hybridization analysis with the probe derived from the PCR product generated with primer 773 (corresponding to the 17-bp repeat sequences flanking the *rfbQRS* DNA; Fig. 3) revealed only a single copy of this region in O1 strains. However, two distinct copies on separate *Sac* I fragments of 21.5 and 6.8 kb were detected in the O139 strains. Only one of these copies contains a distinctive *Eco*RI restriction polymorphism and can be PCR amplified, indicating that the second copy is incomplete or is only a related region of DNA.

Plasmid pEVX12 (25), which contains DNA outside of the *Sac* I fragment harboring the O1 *rfb* region, was used to probe O139 chromosomal DNA. The probe hybridized only weakly, probably with the *rfbQRS* region, suggesting that more than just the O1 *rfbA–P* and *rfbT* genes are absent in O139 strains. pEVX12 contains two ORFs (ORFI and ORFII) not previously described which show homology to O-antigen biosynthesis proteins (P.A.M., A. Fallerino, and C. Mavrangelos, unpublished work).

Given the complete nucleotide sequences of the El Tor Ogawa and classical Inaba O1 *rfb* regions, we assessed the extent of homologous DNA in O139 strains. Only the *rfaD* and *rfbQRS* regions were detected. Together with the data from the subclones and pEVX12 (Fig. 2), this indicates that >20 kb of O1 DNA is not present in O139 (Bengal).

***rfbQ, -R,* and *-S* Are Homologous to *E. coli* H-Repeats.** The predicted amino acid sequences of the O1 *rfbQ, -R,* and *-S* ORFs share similarity to the single ORF found within the H-repeat of RHS elements of *E. coli* (14). These H-repeats are also bounded by inverted repeats as is the *rfbQRS* region. Immediately adjacent to the 3′ end of the repeat is the promoter for the Ogawa determining gene, *rfbT* (12). As will be seen below, *rfbQ, -R,* and *-S* form a single ORF in O139 that is homologous not only to the H-repeat of *E. coli* but also to the transposases of a number of IS elements. Thus, the entire region found in O139 is organized like an IS element, which we have designated IS*1358*; the defective version found in O1 strains is IS*1358d1*.

Sequence of IS*1358*. The PCR product obtained from O139 strain AI-1837 by using primer 773 has been sequenced (Fig. 3) and found to comprise a single ORF and not three separate ORFs as are found in the O17 O1 strain. Thus, IS*1358* of O139 resembles the H-repeat of the *E. coli* RHS *B* and *E* elements, which are the H-repeats in *E. coli* containing a single ORF (14). The IS*1358* ORF also most closely resembles the IS element (AsIS*1*) found in *Aeromonas salmonicida* (26). This is of particular interest since AsIS*1* is the first of these elements which has been shown to actually transpose. The transposases of *E. coli* IS elements show a lower level of identity (≈18–20%) than AsIS*1* (27.5%). Thus, it appears likely that the ORF within IS*1358* in O139 encodes a transposase and that together with the inverted repeats the whole region represents an IS element.

Restriction Fragment Polymorphisms Within IS*1358*. The *rfbQRS* region (IS*1358* element) was PCR amplified from all available O139 strains and from several O1 strains. Restriction enzyme analysis revealed an *Eco*RI polymorphism: all O139 strains have an *Eco*RI site ≈400 bp from one end of the inverted repeat, but this is not present in any of the O1 strains tested (Figs. 3 and 4). This region of the chromosome has been a site of genetic rearrangement activity during the evolution of O139.

The New LPS Genes in O139 Are Linked to IS*1358*. Transposon mutagenesis of the O139 strain AI-1837 using Tn*phoA*

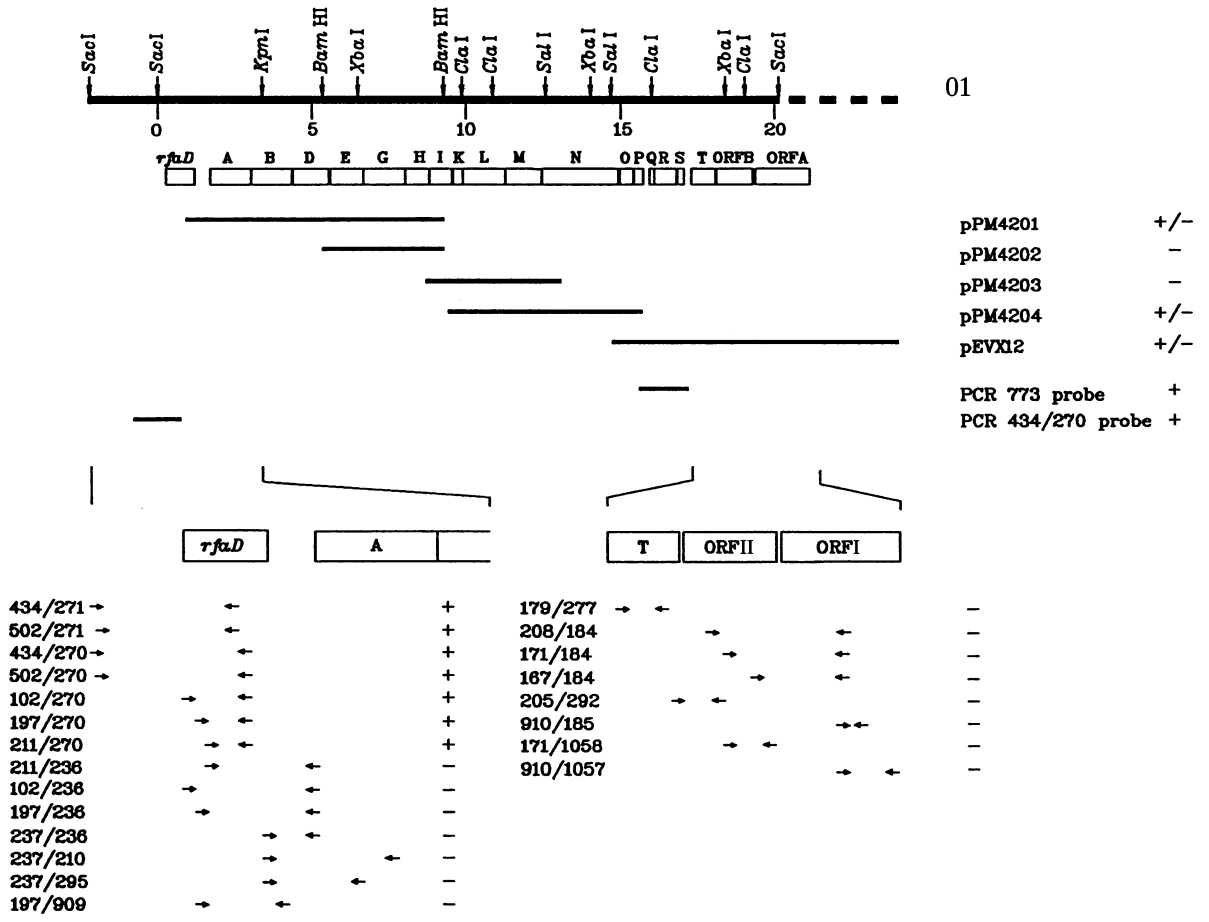

FIG. 2. The *rfb* region of *V. cholerae* O1. The *rfb* genes are indicated by boxes *rfbA–T*. ORFI and ORFII encode proteins homologous to other polysaccharide biosynthetic genes. Plasmids pPM4201, pPM4202, pPM4203, and pPM4204 were used as probes. The PCR probe 773 was obtained by using a single oligodeoxynucleotide primer, no. 773, to the inverted repeat flanking VcIS*1*. PCR probe 434/270 was derived from oligodeoxynucleotide primers 434 (outside of *rfaD*) and 270 (within *rfaD*). PCR amplification was carried out with the indicated primer pairs. Positive hybridization with O139 genomic DNA by either Southern hybridization or PCR amplification is indicated by +. Negative reactions are indicated by −.

(23) yielded two mutants, V942 and V946, which no longer reacted with antiserum 2. Mutant V942 was selected for further analysis by silver staining and shown to lack the slower-migrating O139 LPS band and to be defective in capsule biosynthesis (data not shown) (Fig. 1). Southern hybridization using the PCR product from primer 773 as a probe for IS*1358* revealed that the transposon had inserted within the same *Sac* I fragment (Figs. 4 and 5), indicating that the new LPS genes are linked to IS*1358*. V942 can be complemented to wild type by a cosmid clone (pPM4225) derived from the O139 strain AI-1837 (Fig. 5). Sequencing of this cosmid has shown that it contains genes homologous to known LPS genes associated with incorporation of galactose, including a galactosyltransferase gene (U.H.S. and P.A.M., unpublished work).

Expression of the IS*1358* ORF. The IS*1358* ORF is predicted to encode a 42-kDa protein. We have used a T7 RNA polymerase/promoter system (22) to express this region of DNA from both O1 and O139 strains. A protein which migrates at ≈42 kDa (Fig. 6) is encoded within the O139 region, but no product is seen with O1. Thus, IS*1358* appears to contain an intact transposase gene, but this is defective in O1 strains.

DISCUSSION

Our SDS/PAGE analysis shows that strains of the O139 serotype have LPS which differs from LPS from O1 strains. The LPS of the O139 strains does not appear to have typical long O-antigen chains, unlike O1 strains, where the core is substituted with an average of 17 repeat units. The O139 LPS has the appearance of an efficiently substituted core oligosaccharide, albeit with only one or two additional sugar molecules. This is reminiscent of the semirough LPS or lipooligosaccharide of organisms such as *Neisseria*. We propose that the O139 lipid A-core is either the same or closely related to that of O1 strains, since a mAb capable of recognizing the O139 LPS (19) showed marked crossreactivity with rough O1 strains, but not with smooth O1 strains.

Our Southern hybridization and PCR analyses show that the change from O1 to O139 is not just a simple mutation in the *rfb* region but a complete substitution of this region. Furthermore, Southern hybridization shows that the deletion extends beyond the *rfb* region.

The events involved in the change from O1 to O139 are not fully elucidated. One possible scenario for the evolution of O139 is that IS*1358*, which we have shown to be linked to the novel O-antigen genes, was able to facilitate their incorporation by recombination into the *V. cholerae* O1 chromosome via homology to *rfbQRS* (IS*1358d1*). IS*1358* and IS*1358d1* are 96% homologous, sufficient to permit homologous recombination, and identical in size. Following the acquisition of the novel LPS genes, one might expect incompatibility with the original O1 *rfb* genes that, with immune pressure, would select for the O139 genes. Similarly, the loss of ORFI and ORFII may also have been due to incompatibility with the incoming O139 capsular polysaccharide genes and thus have led to their

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CAGGGAAACCGCATGAAGGTCGTCGATATATAAGGGCTGCGGTGTTGCTTAGAATTAGT    59

TACCAATGCTTCCTTAATCTCAGTTTTTTGATGTAACCCGATGAAATTTAAGATCACCTT   119
                                        RBS
GTAGATCATTTCAGTTGTTTTGTAATAGCTCCTAGACAATATAGGAGCCTAAATATGAGC   179
                                          M  S                   2

GAGTTAATCAACCCATTTATGCATTTCCAAATCATTAAAGACTATCGACAAGAAAGCAAA   239
 E  L  I  N  P  F  M  H  F  Q  I  I  K  D  Y  R  Q  E  S  K    22

GTAGAACACAAATTATCAGACATTATTTTGCTGACAATTTGTGGTGTTTTGTCGTGTCAC   299
 V  E  H  K  L  S  D  I  I  L  L  T  I  C  G  V  L  S  C  H    42

GATGGCTGGGATGGCATTATCGATTTTGGTAATGCTCGCTTAGATTTCCTTAAACGATAT   359
 D  G  W  D  G  I  I  D  F  G  N  A  R  L  D  F  L  K  R  Y    62
          EcoRI
GGTCACTTTGAAGCTGGAATTCCTTCTGCGGATACGCTGTCTCGCGTGATGGGTATGATT   419
 G  H  F  E  A  G  I  P  S  A  D  T  L  S  R  V  M  G  M  I    82
                HindIII
AATCCTGTCGCTTTGCAAAGAAGCTTCATTGCCTGGATGAAGAACTGCCATACACTGACG   479
 N  P  V  A  L  Q  R  S  F  I  A  W  M  K  N  C  H  T  L  T   102

GATGGTGAAGTGATTGCCATCGACGGTAAAACATTACGCGGCTCTTACGACCGCTCGAAA   539
 D  G  E  V  I  A  I  D  G  K  T  L  R  G  S  Y  D  R  S  K   122

GGCAAAGGAACGATCCATATGGTGAACGCTTTTGCTACAGCAAATGGAATGAGCATTGGG   599
 G  K  G  T  I  H  M  V  N  A  F  A  T  A  N  G  M  S  I  G   142

CAACTGAAGGTTGATTCTAAGAGTAACGAGATTACCGCGATCCCCAAGCTACTTGACTTG   659
 Q  L  K  V  D  S  K  S  N  E  I  T  A  I  P  K  L  L  D  L   162

CTAGATGTAAAAGGCTGCTTGATTACGATTGATGCCATGGGCTGTCAAAAGAAAATAGCG   719
 L  D  V  K  G  C  L  I  T  I  D  A  M  G  C  Q  K  K  I  A   182

CAGAAAATCCGTGATAAAGAAGCGGATTATTTATTGGCGGTCAAAGGCAATCAGGGAATG   779
 Q  K  I  R  D  K  E  A  D  Y  L  L  A  V  K  G  N  Q  G  M   202

CTTGAGCAAGCCTTTGATGATTATTTTCGAATGGACATGCTTCAAGACTTCGACGGTAGT   839
 L  E  Q  A  F  D  D  Y  F  R  M  D  M  L  Q  D  F  D  G  S   222

TCTTATAGTACTCAAGAAAAAAGTCACGGAAGAATAGAAACGAGAGTGGCTTTAGTGAAT   899
 S  Y  S  T  Q  E  K  S  H  G  R  I  E  T  R  V  A  L  V  N   242

CGCGATTTGTCGGTTTTGGGTGATATTGAACATGAATGGCCTGGGCTTAAATCAATGGGC   959
 R  D  L  S  V  L  G  D  I  E  H  E  W  P  G  L  K  S  M  G   262

ATCGTGGCTTCAATTCGACAAGAATCGGCAGTAGCAACAGAGCAAGATGTGAGTATTCGT  1019
 I  V  A  S  I  R  Q  E  S  A  V  A  T  E  Q  D  V  S  I  R   282

TACTACATATGCTCTAAAGAATTGGAAGCTCAAACGTTACTTGAAGCGACACGTTCTCAC  1079
 Y  Y  I  C  S  K  E  L  E  A  Q  T  L  L  E  A  T  R  S  H   302

TGGGGTGTAGAGGTCATGCATTGGTCACTTGATACTGCATTTTGTGAGGACAATTCGCGT  1139
 W  G  V  E  V  M  H  W  S  L  D  T  A  F  C  E  D  N  S  R   322

ATTAGAGCGGACGATCGAGCAGAGGCTTTTGCAAGGATCAGGCAGATATGTTTGAACCTA  1199
 I  R  A  D  D  R  A  E  A  F  A  R  I  R  Q  I  C  L  N  L   342

TTAAAGAGCGAAACCACGTTTAAAGGTGGTATCAAACGTAAACGGATGAACTGCGCAATG  1259
 L  K  S  E  T  T  F  K  G  G  I  K  R  K  R  M  N  C  A  M   362

GACGAAAAGTACCTAAGTAAGGTTCTTGAAAGCCTTACGTGACGGTAATGTTCATGCGGT  1319
 D  E  K  Y  L  S  K  V  L  E  S  L  T  *                     376

TTCCGTG                                                        1326
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FIG. 3. Nucleotide sequence of IS*1358* from O139 strain AI-1837. The sequence corresponds to IS*1358* within the 17-bp inverted repeats at the ends (solid arrows). Smaller inverted repeats (dashed arrows) cover the ribosome binding site (RBS), which is shown in boldface type. The stop codon is indicated by an asterisk. Relevant restriction sites are shown and underlined. The *Eco*RI site corresponding to a polymorphism between O1 and O139 strains is indicated.

elimination. If IS*1358* mediated the event, the novel DNA could be expected to be flanked by two copies of IS*1358*, and indeed there appear to be two elements located on adjacent *Sac* I fragments of 21.5 kb and 6.8 kb in O139 strains (data not shown). Since the second copy can no longer be amplified by PCR and does not contain the *Eco*RI site, it may well be the remains of the O1 *rfbQ*, *-R*, and *-S* genes after the recombination and deletion events.

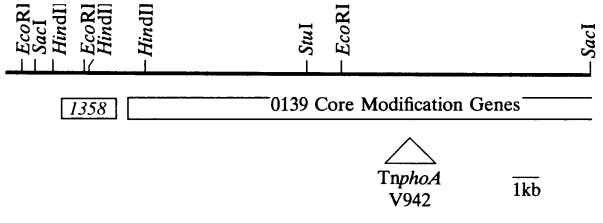

FIG. 4. Model for the organization of the chromosomal region in the vicinity of IS*1358* in O139. The O139 physical map of the 21-kb *Sac* I fragments containing IS*1358* is based upon Southern hybridization data obtained from the strain AI-1837. Locations of the new core modification genes were determined by transposon insertion analysis with Tn*phoA* and nucleotide sequencing.

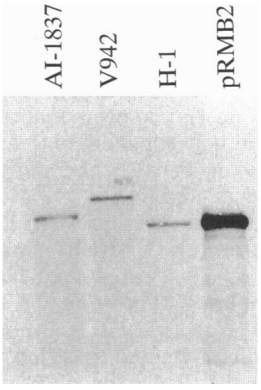

FIG. 5. Southern hybridization of *Sac* I-digested O1 (H-1) and O139 (AI-1837) genomic DNA with probe 773. Plasmid pRMB2 contains the 20-kb fragment harboring the O1 *rfb* region and was used as a positive control for IS*1358d1*. DNA fragments were electrophoresed in 0.8% agarose, transferred to nitrocellulose, and probed with PCR probe 773, which spans IS*1358d1*. The IS*1358d1* genes lie on a 20-kb *Sac* I fragment in the O1 strain (H-1), whereas in the O139 isolate (AI-1837) homologous DNA lies on a 21.5-kb *Sac* I fragment. This fragment has an increased size in the Tn*phoA* mutant of AI-1837 (V942), which lacks O antigen. This increase corresponds to the size of the transposon insert.

An alternative explanation for the origin of the O139 region is that the segment of O139-specific DNA may have been recombined via flanking homologous DNA such as *rfaD* at the proximal end and whatever the corresponding region is at the distal end. This could be a simple transduction event, not as mechanistically complex as that above.

Since IS*1358* shows strong homology to *Aeromonas* AsIS*1*, a known mobile genetic element (26), it is possible that IS*1358* is also an IS element. Further evidence, relating to organizational features, suggests that IS*1358* is an IS element: IS*1358* is flanked by inverted repeats (16 of 17 nt are identical) at its ends and has an inverted repeat encompassing the ribosome binding site. A similar genetic structure is seen in IS*10* and IS*50*, where these inverted repeats have been shown to stop readthrough transcription from external promoters and thus reduce the expression of the transposase (27, 28). If IS*1358* is

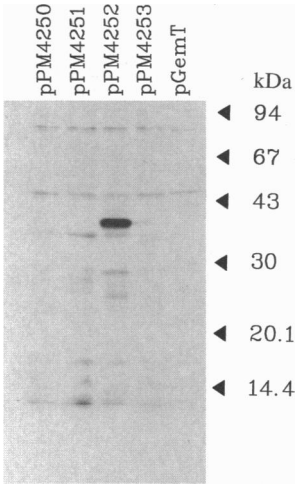

FIG. 6. Expression of the IS*1358*-encoded protein. Whole cells harboring the indicated plasmids were induced to overexpress the protein and solubilized in sample buffer. After SDS/15% PAGE, proteins were stained with Coomassie blue G250. pPM4250 and pPM4251 contain IS*1358d1* cloned in opposite orientations into pGEM-T (Promega). pPM4252 contains the IS*1358* element cloned into pGEM-T under the control of the T7 promoter; pPM4253 has the same clone in the opposite orientation.

confirmed to contain an IS, it will represent the first IS element detected in *V. cholerae* and only the second mobile genetic element, after RS*1* (29).

From a map of the O139 chromosome around IS*1358* based on Southern data (Fig. 4), it appears that the change from O1 to O139 has involved not only the replacement of the original *rfb* region with a new one but also the removal and replacement of ≥6 kb of additional contiguous DNA.

Given that IS*1358d1* of O1 represents a defective form of IS*1358*, the Ogawa form of O1 strains may have derived from an Inaba ancestor by IS*1358*-mediated introduction of *rfbT* (the Ogawa determining gene) and degenerated due to a lack of selection. In support of this notion, variation in the IS*1358d1* ORF can be detected in different O1 strains (ref. 12; unpublished data). The role of H-repeat-related elements in generating new O-antigen or LPS specificities has been proposed by Xiang *et al.* (30) and may represent a common mechanism. Further studies of these elements may provide insights into horizontal gene transfer and genetic evolution of O-antigen specificities in Gram-negative bacteria.

The polymorphisms located in the vicinity of *rfaD* can be used to readily distinguish the Argentinian strain from the Bengal O139 strains. Indeed, there are still further differences in Arg-3 to both O1 and O139 Bengal strains which suggest that this isolate has arisen independently and from a different progenitor.

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1. Albert, M. J., Siddique, A. K., Islam, M. S., Faruque, A. S. G., Ansaruzzaman, M., Faruque, S. M. & Sack, R. B. (1993) *Lancet* **341,** 704.
2. Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Nair, G. B., Shimada, T., Takeda, T., Karasawa, T., Kurazono, H., Pal, A. & Takeda, Y. (1993) *Lancet* **341,** 703–704.
3. Morris, J. G., Jr. (1990) *Epidemiol. Rev.* **12,** 179–191.
4. Shimada, T., Nair, G. B., Deb, B. C., Albert, M. J., Sack, R. B. & Takeda, Y. (1993) *Lancet* **341,** 1347.
5. Rivas, M., Toma, C., Miliwebsky, E., Caffer, M. I., Galas, M., Varela, P., Tous, M., Bru, A. M. & Binsztein, N. (1993) *Lancet* **342,** 926–927.
6. Hall, R. H., Khambaty, F. M., Kothary, M. & Keasler, S. P. (1993) *Lancet* **342,** 430.
7. Das, B., Gosh, R. K., Sharma, C., Vasin, N. & Gosh, A. (1993) *Lancet* **342,** 1173–1174.
8. Johnson, J. A., Albert, M. J., Panigrahi, P., Wright, A. C., Joseph, A., Comstock, L., Truksis, M., Michalski, J., Johnson, R. J., Kaper, J. B. & Morris, J. G., Jr. (1993) in *Proceedings of the 26th Joint Conference, U.S.–Japan Co-operative Medical Science Program, Cholera and Related Diarrheal Diseases* (National Institutes of Health, Bethesda, MD), pp. 35–38.
9. Calia, K. E., Murtagh, M., Ferraro, M. J. & Calderwood, S. B. (1994) *Infect. Immun.* **62,** 1504–1506.
10. Waldor, M. K. & Mekalanos, J. J. (1994) *Infect. Immun.* **62,** 72–78.
11. Johnson, J. A., Salles, C. A., Panigrahi, P., Albert, M. J., Wright, A. C., Johnson, R. J. & Morris, J. G., Jr. (1994) *Infect. Immun.* **62,** 2108–2110.
12. Stroeher, U. H., Karageorgos, L. E., Morona, R. & Manning, P. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2566–2570.
13. Lin, R.-J., Capage, M. & Hill, C. W. (1984) *J. Mol. Biol.* **177,** 1–18.
14. Zhao, S., Sandt, C. H., Feulner, G., Vlazny, D. A., Gray, J. A. & Hill, C. W. (1993) *J. Bacteriol.* **175,** 2799–2808.
15. Ward, H. M. & Manning, P. A. (1988) *Mol. Gen. Genet.* **218,** 367–370.
16. Manning, P. A., Heuzenroeder, M. W., Yeadon, J., Leavesley, D. I., Reeves, P. R. & Rowley, D. (1986) *Infect. Immun.* **53,** 272–277.
17. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), Vols. 1–3, 2nd Ed.
19. Qadri, F., Chowdhury, A., Hossain, J., Chowdhury, K., Azim, T., Shimada, T., Islam, K. M. N., Sack, R. B. & Albert, M. J. (1994) *J. Clin. Microbiol.* **32,** 1589–1590.
20. Hitchcock, P. J. & Brown, T. M. (1983) *J. Bacteriol.* **154,** 269–277.
21. Macpherson, D. F., Morona, R., Beger, D. W., Cheah, K.-C. & Manning, P. A. (1991) *Mol. Microbiol.* **5,** 1491–1499.
22. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1074–1078.
23. Taylor, R. K., Manoil, C. & Mekalanos, J. J. (1989) *J. Bacteriol.* **171,** 1870–1878.
24. Nakasone, N., Yamashiro, T., Albert, M. J. & Iwanaga, M. (1993) in *Proceedings of the 26th Joint Conference, U.S.–Japan Co-operative Medical Science Program, Cholera and Related Diarrheal Diseases* (National Institutes of Health, Bethesda, MD), pp. 52–54.
25. Ward, H. M., Morelli, G., Kamke, M., Morona, R., Yeadon, J., Hackett, J. A. & Manning, P. A. (1987) *Gene* **55,** 197–204.
26. Gustafson, C. E., Chun, S. & Trust, T. J. (1994) *J. Mol. Biol.* **237,** 452–463.
27. Davis, M. A., Simons, R. W. & Kleckner, N. (1985) *Cell* **43,** 379–387.
28. Schulz, V. P. & Reznikoff, W. S. (1991) *J. Mol. Biol.* **221,** 65–80.
29. Pearson, G. D., Woods, A., Chiang, S. L. & Mekalanos, J. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3750–3754.
30. Xiang, S.-H., Hobbs, M. & Reeves, P. R. (1994) *J. Bacteriol.* **176,** 4357–4365.