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Novel *Vibrio cholerae* O139 Genes Involved in Lipopolysaccharide Biosynthesis

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The sequence of part of the *rfb* region of *Vibrio cholerae* serogroup O139 and the physical map of a 35-kb region of the O139 chromosome have been determined. The O139 *rfb* region presented contains a number of open reading frames which show similarities to other *rfb* and capsular biosynthesis genes found in members of the *Enterobacteriaceae* family and in *V. cholerae* O1. The cloned and sequenced region can complement the defects in O139 antigen biosynthesis in transposon insertions within the O139 *rfb* cluster. Linkage is demonstrated among IS*1358* of *V. cholerae* O139, the *rfb* region, and the recently reported *otnA* and *otnB* genes (E. M. Bik, A. E. Bunschoten, R. D. Gouw, and F. R. Mooi, EMBO J. 14:209–216, 1995). In addition, the whole of this region has been linked to the *rfaD* gene. Furthermore, determination of the sequence flanking IS*1358* has revealed homology to other *rfb*-like genes. The exact site of insertion with respect to *rfaD* is defined for the novel DNAs of both the Bengal and the Argentinian O139 isolates.

Until recently only *Vibrio cholerae* strains of the O1 serogroup have been known to cause widespread epidemics of cholera, but with the emergence of the O139 serogroup, this is no longer true (22, 36, 38, 43). Extensive studies have shown that *V. cholerae* O139 has most probably been derived from an El Tor O1 strain by the acquisition of new polysaccharide genes and the subsequent or simultaneous deletion of the *V. cholerae* O1 *rfb* genes (26, 33). It is the *rfb* gene cluster which determines the serogroup in *V. cholerae*. Southern hybridization and PCR analysis of the O139 chromosome has revealed that there exist several regions of homology linked to the O1 *rfb* region (33, 45). The *rfaD* gene thought to be responsible in part for core biosynthesis (47) remains as do regions 3′ of *rfaD*. The other homologous region comprises the three open reading frames (ORFs) known as *rfbQ*, *-R*, and *-S* (IS*1358dl*) in *V. cholerae* O1, which correspond to a defective form of IS*1358* which shows homology to the *Hin*c repeat (H-rpt) of the rearrangement hot spot (*RHS*) element found in *Escherichia coli* (6, 18, 45).

In *E. coli* K-12 there appear to be five *RHS* elements, four of which have been localized to regions on the chromosome. Not all *RHS* elements contain the H-rpt, but when present it is found downstream of the core region. The H-rpt is separated from the core by a region which is unique for each *RHS* element. In most cases the H-rpts are very similar and only diverge by 2.4% between *RhsB* and *RhsE*. These H-rpts are 1,291 bp in length and contain a 1,134-bp ORF when not interrupted. Not all H-rpts in *E. coli* are exactly the same. For example, in *RhsC* there are three tandem copies of the H-rpt, all of which are defective due to interruptions in the ORF. The H-rpt has also been found as a 291-bp fragment in *E. coli* which is not associated with a *RHS* element (58).

The H-rpt has certain features which resemble insertion sequences. These include an 11-bp inverted repeat at the end of the H-rpt; similar inverted repetitions are found associated with some insertion sequences. The multiple occurrence of discrete homologous sequences also points towards a mobile genetic element (14). Furthermore, overlapping the ribosome binding site is an inverted repeat whose function is as yet unknown but which may serve to decrease the level of expression of the following ORF. A similar genetic organization has been seen in IS*10* and IS*50* where these inverted repeats have been shown to stop read-through transcription and thus dramatically reduce the expression of the transposase (10, 41). Unlike many insertion sequences, there are no repeated target sequences flanking the H-rpts. As yet no transposition activity has been associated with these H-rpts, even at a frequency as low as 10^{-9} (58). The H-rpt has been found linked to the *rfb* operon which is found in *Salmonella enterica* (56). Here it has been proposed that the H-rpt was involved in intraspecific recombination between the *rfb* regions of *S. enterica* D1 and E1, giving rise to the new *rfb* region and subsequent serotype D2 (56).

The lipopolysaccharide (LPS) of *V. cholerae* O139 differs from that of O1 in that the LPS can be described as semirough, i.e., it does not appear to possess long O antigen chains but instead has a heavily substituted core (33). Furthermore, the LPS of O139 is biochemically distinct from that of O1 (19, 27, 37, 54). A distinguishing feature of *V. cholerae* O139 is that the cells are encapsulated, which is thought to increase resistance to killing by serum (25, 54). It has been demonstrated that two types of O antigen and capsular mutants can be generated in O139: those which are independent and those that lead to the loss of both the capsule and O antigen (9, 53). This would indicate that there are genes which are common to both biosynthetic paths as well as specific capsule and O antigen genes. There have been several previous reports on parts of the *V. cholerae* O139 surface polysaccharide genes. The initial report demonstrated that between IS*1358* and *rfaD* lies 13 kb of novel O139 DNA within which two genes, termed *otnA* and *otnB*, were identified (3). Since then the rest of the *otn* region has been described, and mutations in *otnA* and *otnB* have been shown to eliminate capsule but not O antigen biosynthesis (4). An additional ORF, termed *rfbD* due to its homology to RfbD of the *V. cholerae* O1 *rfb* operon, was described and is located downstream of IS*1358* (4). Thus in *V. cholerae* O139, IS*1358* is flanked by genes involved in surface polysaccharide biosynthesis. Recently an additional 12 kb of novel O139 DNA has been

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reported, and this region also contains a number of ORFs which show homology to surface polysaccharide biosynthetic proteins (8).

In this study it is demonstrated that the novel region of DNA found in O139 which differs from O1 is approximately 40 kb in length and contains several genes which are likely to be involved in polysaccharide biosynthesis. Furthermore, this completes the sequence of the *rfb*-capsular region of *V. cholerae* O139. Like the H-rpt element found in *S. enterica* serovar Typhimurium which is linked to the *rfb* region (56, 57), IS*1358* is linked to the *rfb* operon of *V. cholerae* O1 and O139.

MATERIALS AND METHODS

Bacterial strains. The *V. cholerae* O1 strain O17 (El Tor, Ogawa) was used. Strains AI-1837, AI-1838, AI-4450, AI-1841, AI-1852, AI-1854, AI-1855, AI-4260 are O139 isolates from the Indian subcontinent. Arg-3 is an O139 isolate from Argentina (39). V941 and V946 represent the Tn*phoA* insertions which eliminate O antigen biosynthesis in AI-1837 (45).

Media. *V. cholerae* strains were grown in either brain heart infusion broth (Difco) or Luria broth (34). Cultures were grown with shaking at 37°C.

Routine DNA procedures. Restriction enzymes were used with the buffers supplied by the manufacturers (Amersham, Little Chalfont, United Kingdom; Boehringer GmbH, Mannheim, Germany; and New England Biolabs, Beverly, Mass.). Procedures, including agarose gel electrophoresis, were performed as previously described (40).

Extraction of whole chromosomal DNA. Whole genomic DNA of *V. cholerae* O1 and O139 strains was prepared according to the method of Manning et al. (32). The method was modified for the extraction of DNA from O139 strains. Proteinase K-treated cells were incubated in 0.1 M Tris-HCl (pH 8.0)-saturated phenol for 1 h at 4°C prior to three sequential phenol extractions. Genomic DNA was precipitated by the addition of 4 volumes of ice-cold 100% ethanol.

Cosmid bank construction. Genomic DNA from the O139 strain AI-1837 was partially digested with *Sau*3AI. Fragments of approximately 40 kb were then cloned into the cosmid vector pPM2101 (42). This material was packaged into λ phage heads by using the Packagene system (Promega Corp., Madison, Wis.) and transfected into *E. coli* DH5α cells. The cosmid bank was subsequently conjugated into V946 and screened by using O139-specific antiserum.

Southern transfer and detection. Unidirectional transfer of DNA from agarose gels to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) was performed as described by Southern (44) and modified according to Sambrook et al. (40). Detection was carried out using an enhanced chemiluminescence kit (Amersham) and autoradiography.

PCR. PCR amplification was carried out by using standard protocols with the oligodeoxynucleotides described in the text below. *Taq* polymerase from Perkin-Elmer was used in all PCRs.

Antibodies. Antiserum to O139 was prepared from a rabbit immunized with heat-killed AI-1837 and subsequently absorbed four times overnight with 10^{10} *E. coli* DH5α and *V. cholerae* O1 strain O17 to remove any cross-reactive antibodies.

LPS silver staining and immunoblotting. LPS was prepared from 1 ml of overnight (18 h) cultures (20). LPS was electrophoresed and detected by silver staining or by immunoblotting as previously described (31). The LPS samples were treated at 100°C for 5 min prior to electrophoresis in sodium dodecyl sulfate (SDS) on 15% polyacrylamide gels (1.5 by 20 cm). Whole-cell immunoblotting was performed as previously described (34).

Sequencing procedures. Sequencing of the O139 region was performed with an AB 373A Automated DNA Sequencer with a Stretch upgrade by using dye terminator and dye primer sequencing protocols (Applied Biosystems, Foster City, Calif.). The O139 sequences can be obtained under accession number Y07786.

T7 expression system. The temperature-inducible, T7 RNA polymerase expression system of Tabor and Richardson (50) was used for the expression of proteins. L-[^{35}S]methionine (37 TBq/mmol) (Amersham) was incorporated into the reactions for labelling protein. Proteins were visualized by autoradiography following SDS-polyacrylamide gel electrophoresis.

Tn*phoA* mutagenesis. The suicide plasmid pRT773 (51) harboring Tn*phoA* was used for generating Tn*phoA* insertion mutants in strain AI-1837 by selecting kanamycin- and polymyxin-B-resistant exconjugants. pRT773 requires the presence of the *pir* protein for replication, and this is provided in the chromosome of the *E. coli* donor strain.

RESULTS

Cloning of the O139 *rfb* region and complementation of V941. A cosmid bank of the *V. cholerae* O139 strain AI-1837 in *E. coli* SM10 was constructed by using the cosmid cloning vector pPM2101 (42). DNA from AI-1837 was prepared by partial *Sau*3AI digestion and cloned into the *Bam*HI site of pPM2101. The ligated material was subsequently packaged into bacteriophage λ and used to transfect *E. coli* SM10. Plasmid pPM2101 contains the origin of transfer from the conjugative plasmid RP4, so that the cosmid bank prepared from *V. cholerae* O139 strain AI-1837 could be conjugated into the O139 *rfb* mutant (V941) where colonies were screened for complementation with the antiserum to O139 O antigen. The DNA was subsequently extracted from the colonies corresponding to the *E. coli* cosmid clones and analyzed by restriction endonuclease cleavage. One clone, designated pPM4225, contained a complementing region corresponding to 6.7 kb of O139 DNA and had presumably undergone an internal deletion (45). A second rough mutant (V946) also derived by transposon insertion could not be complemented. This second insertion lies outside the region described here. Furthermore, these two transposon insertions show differing patterns upon silver staining, indicating that they probably do not affect the same biosynthetic pathway. The insertion in V941 appears to be an O-antigen-defective mutant, whereas V946 probably corresponds to a deep rough mutant since the core appears to migrate with a lower molecular weight, i.e., this strain contains an insertion in an *rfa* gene (Fig. 1). A second region encompassing IS*1358*, termed pPM4279, and which was derived from pPM4225, is described below. The gap between pPM4225 and pPM4279 was also cloned and sequenced and is designated pPM4280.

Sequence determination and analysis of O139-specific DNA. Plasmid pPM4279 is a 4.7-kb *Eco*RV subclone which was derived from pPM4225 and which contains IS*1358*. The sequence to the left end of IS*1358* has been reported and contains several genes thought to be involved in O antigen chain length determination and export (3). Consequently, only the sequence to the right of IS*1358* has been determined here. Based upon the homologies of the ORFs, it is suggested that the region to the right of IS*1358* is involved in O antigen biosynthesis. Upon sequencing from the right-hand end of the inverted repeat in IS*1358*, a direct repeat of the last 31 bp of IS*1358* was identified, and this was followed by an 8-bp repeat of part of the 17-bp inverted repeat (Fig. 2). The significance of this region is as yet unknown but its existence may be due to an imprecise recombination event perhaps upon the introduction of the O139 polysaccharide region. Another region approximately 2 kb upstream of IS*1358* has been identified by PCR analysis and contains at least one copy of the 31 bp in an inverted orientation. The region from the end of pPM4279 to pPM4225 was cloned by PCR amplification into pGem-T (Promega) and corresponds to plasmid pPM4280 (Fig. 2).

Plasmid pPM4279 contains one complete ORF and a partial ORF designated ORF41x9 and ORF34x6, respectively. The product encoded by ORF41x9 is closely related to RfbD (81.4% identity) of *V. cholerae* O1, which is thought to be an oxido-reductase involved in perosomine biosynthesis (4, 46), and to YefA (79.3% identity) of *E. coli* K-12 (Table 1). In fact, ORF41x9 is identical to the *rfbD* of *V. cholerae* O139 described by Bik et al. (4). ORF34x6 shows homology to ORF6.7 of the *E. coli* O111 colitose biosynthetic region (1), to NolK (49.7% identity), which is involved in nodulation of *Azorhizobium caulinodans* and in bacteria-plant interactions (15) (Table 1), and to YefB, an ORF associated with a capsule biosynthesis operon and which has been identified as an ORF in the *E. coli* K-12 sequencing project. Interestingly, ORF34x6 has a motif of GXXGXXG, which is also found in epimerases and dehydrogenases (55). Sequencing of the region adjacent to pPM4279 and contained on pPM4280 revealed two additional genes which show homology to ORFs found in the *E. coli* O111

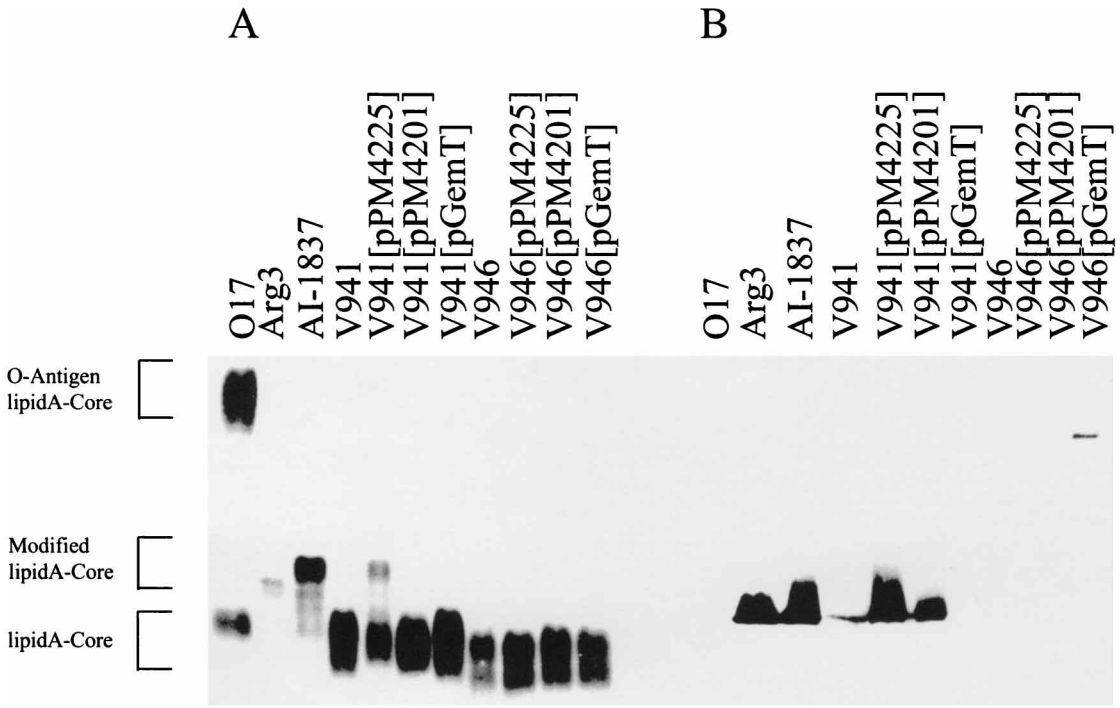

FIG. 1. (A) Analysis of LPSs of *Vibrio cholerae* O1 and O139. Samples of LPS prepared by proteinase K treatment of whole-cell lysates were electrophoresed in SDS on 20% polyacrylamide gels and silver stained. The LPS in each lane is from the strains indicated. Strain O17 is a smooth *V. cholerae* O1 which produces the Ogawa O antigen. Arg-3 is the Argentinian O139 isolate. The O139 transposon mutants, V941 and V946, are indicated. V941[pPM4225] and V946[pPM4225] represent the complemented AI-1837(Tn*phoA*) strains V941 and V946, respectively. V941[pPM4201] and V946[pPM4201] represent V941 and V946 complemented with the *V. cholerae* O1 *rfbA* gene. The positions of the lipid A-core and O antigen linked to the lipid A-core are indicated, as is the position of the modified lipid A-core in O139 strains. (B) Identical samples to those for panel A were electrophoresed, transferred to nitrocellulose, and analyzed by Western blotting. The filter was immunoblotted with the O139-specific antiserum. The reaction shown is exclusively related to the modified lipid A-core.

colitose biosynthetic operon (Table 1) (1). These ORFs, termed ORF43x9 and ORF18x8, are 73.8 and 42.1% identical to ORF7.7 and ORF3.4, respectively (1). Furthermore, ORF43x9 also shows homology to the RfbH protein of *Salmonella typhimurium*, which uses CDP-4-keto-6-deoxy-D-glucose as a substrate and is a dehydratase. Thus, it is conceivable that ORF43x9 has a similar function, using GDP-4-keto-6-deoxy-D-mannose as a substrate, which would make ORF43x9 a GDP-4-keto-6-deoxy-D-mannose dehydratase. From the ORFs described so far we have been able to ascribe a putative function, and these ORFs appear to be translationally coupled in that there are only a few base pairs between them. However, this region is followed by a gap of 57 bp after which there are four ORFs for which no homolog was found by searching the databases. We do, however, have a mutation in one of these, termed ORF54x4, which affects the production of the *V. cholerae* O139 surface polysaccharide (Table 1) (12). The last ORF on plasmid pPM4280 is ORF39x2, and it shows good homology to a number of glycosyl transferases from *Neisseria gonorrheae* (30.5%) and *Yersinia enterocolitica* (39.4%). As yet we have no exact role for this ORF.

The next region to be sequenced was the 6.7-kb region of O139 DNA on pPM4225. This region was shown to contain five large ORFs (Table 1). Four of these could be assigned a putative function based on sequence homologies, but ORF41x8 showed no homology to any known protein (Table 1). ORF35x9 also appears to be a glycosyl transferase and shows homology to AmsB of *Erwinia amylovora* (24.6% identity) (5), TrsC of *Yersinia pseudotuberculosis* (19.1% identity), and RfbN of *S. typhimurium* (18.5% identity) (24) (Table 1). Again, we have not been able to ascribe a role to this ORF. ORF41x8 is very AT rich (27.9%) and shows no homology to any known proteins but may be involved in O antigen transfer, i.e., it is *rfc*-like, since the *rfc* genes of a number of *Enterobacteriaceae* show unusually high AT content (35). Furthermore, this ORF is very hydrophobic and contains a number of putative transmembrane domains. These properties are also found in other Rfc proteins such as those of *Shigella flexneri* (35) and *S. enterica* serovar Typhimurium (7) (Fig. 3). A large ORF (ORF71x8) shows homology to asparagine synthetase from a number of species (Table 1). It seems very unusual to find this ORF within what appears to be an *rfb* operon. However, insertions in this ORF have been reported to eliminate O antigen biosynthesis in *V. cholerae* O139 (9), but it is uncertain whether ORF71x8 plays a role in O antigen production or whether the insertion is polar on downstream genes. ORF41x3 is likely to be a galactosyl transferase since it shows good homology to RfbF of *Klebsiella pneumoniae* (30.1% identity) (49) and RfbF of *Serratia marcescens* (31.9% identity) (49), both of which are involved in O antigen biosynthesis (Table 1). These homologies are consistent with what is known about the sugar composition of the O139 capsule and LPS, which contain galactose (possibly in the core) and colitose (19, 27, 37, 54). The last ORF, ORF50x8, is most likely a mannose-1-phosphate guanyltransferase due to the marked homology to CpsB and RfbM from *E. coli* and *Salmonella choleraesuis*, respectively (29), and to the lesser homology to RfbA of *V. cholerae* O1 (46) (Table 1). ORF41x8, ORF71x8, ORF41x3, and ORF50x8 described above correspond to ORF1 to -4 published previously (8), although ORF41x8 was not identified as a putative Rfc nor was ORF71x8 identified as an asparagine synthetase.

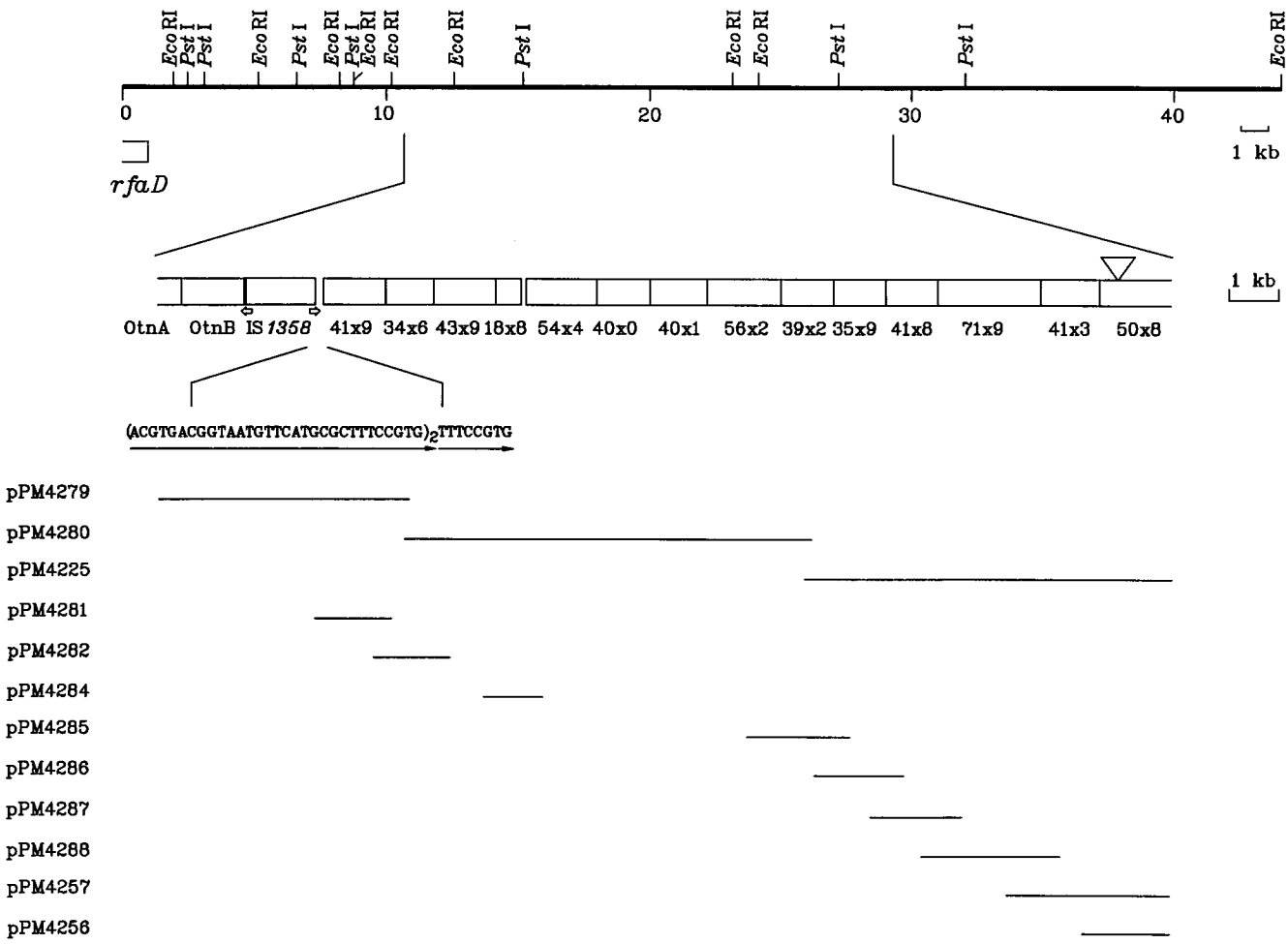

FIG. 2. Map of the *rfb*/*cps* region of *V. cholerae* O139. The genes and ORFs are shown in boxes. The putative insertion element IS*1358* is indicated (45). The position in ORF50x8 of the Tn*phoA* insertion in strain V941 is indicated by the triangle. The region containing the various ORFs is shown in more detail, as is the region surrounding IS*1358*. Arrows indicate the 17-bp inverted repeats flanking and the 31-bp repeats to the right of IS*1358*. The plasmids pPM4281, pPM4282, pPM4284, pPM4285, pPM4286, pPM4287, pPM4288, pPM4256, and pPM4257 shown on the map are derived from the PCR cloning of the various ORFs into pGemT (Promega).

Once the sequence of ORF50x8 had been obtained and homology to RfbA of *V. cholerae* O1 was found, attempts were made to complement the transposon insertions with this gene from *V. cholerae* O1. Surprisingly, the transposon insertion in V941 could be complemented to restore phage sensitivity and O antigen biosynthesis by the *rfbA* gene from *V. cholerae* O1, which is 52.1% identical to ORF50x8; the latter contains a Tn*phoA* insertion in the putative mannose-1-phosphate guanyltransferase (46). However, this complementation is poor, which may be a consequence of plasmid instability or possibly due to differences in the exact enzymatic function. We know that upon overnight culture there is a loss of approximately 20% of pBR322-based plasmids; however, in our experiments we have used exponential-phase and not stationary-phase cultures, thus plasmid instability is unlikely to be the cause. One would perhaps expect better complementation with a protein more closely related to O139 ORF50x8, such as CpsB or RfbM. It is likely that the *V. cholerae* RfbA protein does not interact as efficiently with the resident *V. cholerae* Rfb proteins, thereby causing the complementation to be poor. This is especially the case if the Rfb proteins form a metabolon where the substrate is transferred directly from one enzyme to the next without a free intermediate. It is also possible that the *V. cholerae* O1 RfbA protein has a lower activity than that of the host enzyme. This is suggested by the fact that pPM4225 can complement very efficiently.

To determine the exact point of insertion in V941, Tn*phoA* was PCR amplified out of the chromosome and the sequence was determined. The insertion had occurred in the amino-terminal coding region of ORF50x8, adjacent to codon 71, clearly identifying ORF50x8 as being involved in O antigen biosynthesis in *V. cholerae* O139 Bengal.

It is clear from these data that the ORFs described by us are alone insufficient to encode all the functions for the biosynthesis of not only the O antigen but also the capsule. Thus, it seems likely that the O139 *rfb* operon starts just to the right of IS*1358* and continues through pPM4279 and pPM4280 and out beyond the sequence of pPM4225. Evidence for this comes from the work of Comstock et al. (9) who have reported a further 6 kb of sequence involved in O139 polysaccharide biosynthesis downstream of pPM4225. This would indicate that the O139 polysaccharide operon is at least 17 kb in length. This is not unusual for *rfb* operons, which are commonly in the vicinity of 15 to 20 kb in size.

The complete sequence of the *V. cholerae* O139 *rfb*/capsule region to the right of IS*1358* does not contain a just upstream

TABLE 1. Properties of ORFs of *V. cholerae* O139 *rfb*/capsule region

ORF[a]	No. of amino acids	Molecular mass (Da)	Nucleotide position in sequence	G+C content (%)	Isoelectric point	Predicted function	Homology to[b]:	% Identity (no. of amino acids)[c]	Accession no.
ORF41x9/RfbD	372	41,989	190–1308	46.6	5.59	Oxidoreductase	*V. cholerae* O1 RfbD	81.4 (371)	S28470
							E. coli YefA	79.3 (323)	P23054
ORF34x6	308	34,653	1313–2239	43.4	5.51	Colitose biosynthesis	*E. coli* O111 *rfb* ORF6.7	55.0 (302)	U13629
							A. caulinodans NolK	50.5 (300)	P33217
ORF43X9	390	43,942	2232–3404	41.4	4.72	Colitose biosynthesis	*E. coli* O111 *rfb* ORF7.7	73.8 (389)	U13629
							S. typhimurium RfbH	32.9 (356)	P26398
ORF18X8	161	18,857	3404–3889	35.2	5.36	Colitose biosynthesis	*E. coli* O111 *rfb* ORF3.4	42.1 (133)	U13629
ORF54X4	478	54,489	3946–5382	34.4	9.72	Unknown	No homolog		
ORF40X0	338	40,010	5379–6395	30.2	8.97	Unknown	No homolog		
ORF40X1	337	40,130	6395–7408	29.3	8.92	Unknown	No homolog		
ORF56X5	485	56,529	7398–8855	30.2	7.95	Unknown	No homolog		
ORF39X2	337	39,234	8852–9865	29.7	7.26	Glycosyl transferase	*N. gonorrheae* LgtD	30.5 (255)	U14554
							Y. enterocolitica ORF10.9	39.4 (208)	U46859
ORF35X9	310	35,914	9868–10800	33.9	8.29	Glycosyl transferase	*E. amylovora* AmsB	24.6 (285)	X77921
							Y. enterocolitica TrsC	19.1 (304)	S51262
ORF41X8/ORF1	354	41,862	10797–11861	28	9.8	Rfc-like	No homolog		
ORF71X9/ORF2	636	71,949	11849–13759	43.1		Asparagine synthetase	Large number of species		
ORF41X3/ORF3	377	41,340	13756–14889	41.3	7.82	Galactosyl transferase	*Proteus mirabilis* CpsF	31.3 (374)	L36823
							S. marcescens RfbF	31.9 (373)	L34167
ORF50X8/ORF4	460	50,837	14885–16265	46.8	5.12	PMI/GMP activity	*V. cholerae* O1 RfbA	52.1 (461)	Q07024
							E. coli CpsB	56.3 (469)	P24174
							S. choleraesuis RfbM	55.4 (464)	Q01410

[a] RfbD and ORF1 to -4 are from Bik et al. (4) and Comstock et al. (8), respectively. See Addendum in Proof.
[b] Only some of the homologies are shown.
[c] Number of amino acids in the protein with which ORFs of the *V. cholerae* O139 *rfb* region share homology.

of many polysaccharides (JUMP)-start sequence but one could be detected upstream of *otnA* (4, 21). The precise function of these sequences is not known but the single JUMP-start sequence may be sufficient for the proposed role in transcription of the whole region.

Expression of the ORF-encoded proteins. The regions encompassing the genes for which we have ascribed a function have been cloned via PCR amplification into the vector pGemT (Promega), giving rise to plasmids pPM4281, pPM4282, pPM4284, pPM4285, pPM4286, pPM4287, pPM4288, pPM4256, and pPM4257 (Fig. 2). We were not able to clone ORF43x9 in pGEMT, and although the reasons for this are unknown, it is possible that the gene product is deleterious to the cell. The T7 RNA polymerase protein overexpression system of Tabor and Richardson (50) was subsequently used to overproduce these proteins to confirm the sizes as predicted from the sequence (Table 1). We were able to detect a product of the appropriate size for ORF41x9, ORF18x8, ORF39x2, ORF35x9, ORF71x8, ORF41x3, and ORF50x8. However, we could not detect any products for ORF34x6 and ORF41x8. If ORF41x8 corresponds to an Rfc homolog then it is not surprising that no product could be detected since as yet no known Rfc protein has been overexpressed at a high level. These results would indicate that most of the ORFs described are expressed in *V. cholerae* O139 and are of the expected sizes.

Chromosomal map of the O139 *rfb* region. It has been shown that none of the O1 *rfb* genes are present in O139 (33), although *rfaD*, which is thought to be essential for core biosynthesis, is present as is a region defined as IS*1358* (45, 47). Southern hybridization of the chromosome and restriction endonuclease digestion of PCR products have facilitated mapping of this region. Since both IS*1358* and *rfaD* are virtually conserved between O1 and O139 it has been possible to determine the distance by long-range PCR to be approximately 13 kb between *rfaD* and IS*1358*. By using PCR and sequencing, the exact end point of the deletion in O1 and the overlap into novel O139 DNA at the *rfaD* end was determined (Fig. 4). This 13-kb region is thought to also contain the second very degenerative copy of IS*1358* reported previously (45) and is located approximately 7 kb away from the intact IS*1358*. Overall, this would give this region the structure of a compound transposon with one intact IS element and a degenerative element.

With the oligodeoxynucleotide primers 2099 (5′CCTTACG TGACGGTAATGTTCATGCGGTTTCC3′), and 2115 (5′CA TTGTTACGCGCAGCCGATACACCTTGATTC3) to IS*1358* and to the sequenced region of the complementing *rfb* clone pPM4225, it has been possible to show that these regions are 10.5 kb apart and that the sequenced region corresponds to pPM4280. We have shown by sequencing that this region contains other *rfb*-like genes. Using our data and the data from Comstock et al. (8) and Bik et al. (4), a map has been determined for the O139 chromosome containing *rfaD*, IS*1358*, and a number of genes involved in polysaccharide biosynthesis of the O139 antigens (Fig. 2).

Junction at *rfaD* between *V. cholerae* O1 and Bengal and Argentinian O139 isolates. From previous studies it has been shown that there is an *Eco*RI restriction-length polymorphism which permits the differentiation of the Argentinian and Bengal O139 isolates in the vicinity of RfaD (45). Furthermore, the region downstream of *rfaD* appears to be identical in both the O139 isolates and in the O1 strains. By PCR it was possible to generate products between *rfaD* and IS*1358* for both the Bengal and Argentinian O139 isolates. Both of these products appear to be of the same length (~13 kb). Sequencing of these products revealed that the sequences for the two O139 isolates were different preceding the coding region for the RfaD protein (Fig. 4). This could explain the polymorphism seen with the different O139 isolates. If there has been a deletion or insertion one would expect upon further sequencing to come to

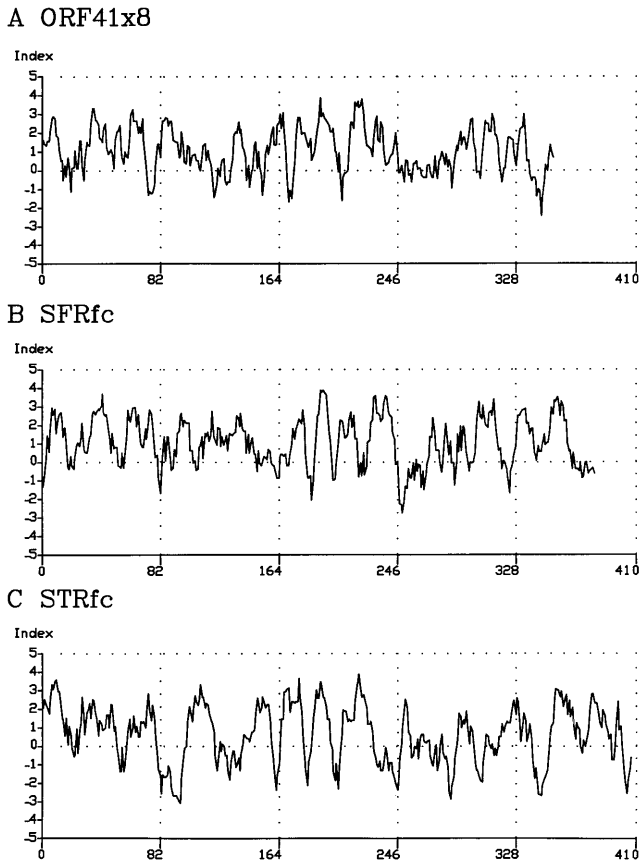

FIG. 3. Comparison of hydropathy plots. The hydropathy plots were generated by using the algorithm of Kyte and Doolittle (PROSIS, Hitachi) (28) for three O antigen polymerases shown here as ORF41x8 (*V. cholerae* O139 putative Rfc) (A), SFRfc (*S. flexneri* Rfc) (B), and STRfc (*S. enterica* serovar Typhimurium Rfc) (C). The polymerases were aligned in AUTOCAD and a window size of 10 was used. The numbers on the *x* axis correspond to the amino acid position, and the *y* axis values correspond to the relative hydrophobicity indices.

a region of homology between the O139 isolates. Why there is such a difference and why *rfaD* is relatively conserved are unknown, but perhaps the fact that the isolate from O139 Argentina (Arg-3) is not derived from a *V. cholerae* O1 parent (39) is significant.

DISCUSSION

In the *V. cholerae* O139 chromosome, as is the case for *V. cholerae* O1, the *rfaD* gene, thought to be involved in core biosynthesis, as well as a putative insertion element designated IS*1358* are linked to the genes for the biosynthesis of the O antigen (45, 47).

IS*1358* is homologous to the H-rpts of *E. coli*, which are almost always detected as a complete RHS element (6, 18). However, sequencing out of both ends of IS*1358* has revealed that this is not the case in either *V. cholerae* O139 or O1. This is also true for the H-rpt-like element found in *S. enterica* (56) and for a similar element, IS*As1*, in *Aeromonas salmonicida* (17). Thus, it would appear that H-rpt homologs are not necessarily part of an RHS element. The linkage of these IS elements to polysaccharide genes in *V. cholerae* does not appear to be unusual in *Vibrio* species. Indeed, it has been found that IS*1358* is commonly linked to LPS-related biosynthetic genes in other *Vibrio* spp. (23).

Initial investigations have shown that IS*1358* is flanked by a 17-bp inverted repeat (45). Subsequent sequencing out of the ends of IS*1358* in *V. cholerae* O139 has shown that the right-hand inverted repeat has in fact two 31-bp direct repeats and an additional 8 bp which have the same sequence as the last 8 bp of the 31-bp repeats. The roles of the direct 31-bp repeats at the end of IS*1358* and of the last 8-bp repeat following the two 31-bp repeats have as yet not been elucidated. However, there appear to be other copies of this 31-bp repeat in the vicinity of IS*1358*, since by using 17 bp of this repeat as a PCR primer, it is possible to amplify products between 50 and 200 bp without the need for a second primer, indicating that these repeats are in an inverted orientation.

It has been proposed that the H-rpt homolog found in *Salmonella* is involved in O antigen variation due to the ability of this region to recombine with other H-rpt sequences (56). IS*1358dl* in *V. cholerae* O1 is flanked on one side by the *rfbA-O* operon and on the other by *rfbT* and further *rfb* genes (11, 34, 48). The *rfbT* gene is responsible for conversion from the Ogawa to the Inaba serotypes in *V. cholerae* (48). Why are genes which are involved in modifying the O antigen and essential to O antigen biosynthesis separated by IS*1358dl* yet so closely linked to the *rfb* operon? It is possible that IS*1358dl* was involved in the insertion of these genes, allowing *V. cholerae* O1 to express different serotypes, i.e., either Inaba or Ogawa. This scenario may also exist for O139 where on the *rfaD* side of IS*1358* there are the *otn* genes (3, 4) and on the other side are the *rfb* genes described here and by Comstock et al. (8). This is consistent with the proposal by Reeves and coworkers for the acquisition of new O antigens by *Salmonella* (56). Thus, the 31-bp direct repeats may have arisen by insertion and deletion events or imprecise recombination involving IS*1358* during the evolution of the new O139 *rfb* region.

The LPS of *V. cholerae* O139 contains a number of sugars which include colitose, galactose, glucose, glucosamine, and heptose (27, 37, 54), so it is not surprising to find genes involved in galactose utilization such as the galactosyl transferase (ORF41x3). It has been reported that unlike O1 strains, the *V.*

FIG. 4. Sequence of the region where O139-specific DNA joins *rfaD* DNA found in *V. cholerae* O1. The top line represents the sequence determined from O139 Bengal strain AI-1837. The middle line represents the *V. cholerae* O1 sequence (47). The bottom line shows the sequence derived from the Argentinian (Arg-3) O139 isolate. The arrows indicate the directions of transcription of *rfaD*. The shading indicates regions of sequence identity between *V. cholerae* O1 and the two O139 strains.

cholerae O139 strains are encapsulated and that this capsule appears to be made essentially of the 3,6-dideoxyhexose colitose (27, 37, 54). This sugar has not previously been reported in *V. cholerae*; however, it is found in *Salmonella*, *Yersinia*, and a limited number of *E. coli* serotypes such as O111 and O55 (1, 2, 16, 30, 52). Colitose is similar to perosamine, the primary sugar found in the O antigen of *V. cholerae* O1 strains. The pathway leading to the synthesis of colitose in *E. coli* only diverges from that for perosamine at the final steps, such that in O139 it is probably made from fructose-6-phosphate which is converted to mannose-6-phosphate (13). It is thus not surprising to find both RfbA and RfbD homologs in *V. cholerae* O139, i.e., ORF50x8 and ORF41x9, respectively. Further investigation has also revealed a homolog for RfbB which is thought to be a phosphomannose mutase (46); this is ORF5, which has been described previously (8). RfbA protein belongs to a group of bifunctional proteins in that they are phosphomannose isomerases and guanosine-diphosphomannose-pyrophosphorylases. One would thus suspect that ORF50x8 in *V. cholerae* O139 probably has the same functions. ORF41x9, which shows considerable homology to a protein from the *rfb* region of *V. cholerae* O1, RfbD, is an oxido-reductase and would be expected to convert GDP-mannose synthesized by ORF50x8 and ORF5 (described above) to GDP-4-keto-6-dideoxymannose. The ORF designated ORF43x9 may subsequently use GDP-4-keto-6-dideoxymannose as a substrate and carry out a pyridoamine 5-phosphate-dependent dehydratase reaction similar to what is thought to occur in *E. coli* O111 with ORF7.7 and RfbH of *S. typhimurium* (1, 24). At least three additional steps are then required to synthesize the 3,6 dideoxyhexose, i.e., colitose. This would be undertaken by as yet unidentified ORFs, possibly an epimerase (ORF34x9) and the colitose synthetase. Thus, it seems highly likely that the genes which have been described here are involved in both capsule and O antigen biosynthesis, and indeed, we have shown that the Tn*phoA* insertion in ORF50x8 in V941 eliminates both functions. ORF18x8, ORF54x4, ORF40x1, ORF40x0, and ORF56x5 have not as yet been ascribed functions; however, we do know that mutations in ORF54x4 affect surface polysaccharide production in *V. cholerae* O139 (12). ORF39x2 and ORF35x9 show homology to glycosyl transferases and are obviously sugar transferases, but it is not possible to predict at this stage which sugar. The last four ORFs described by us are identical to ORF1 to ORF4 described previously (8).

The Bengal and Argentinian O139 strains have clearly evolved independently. Many studies have presented evidence that the O139 Bengal strain probably arose from an O1 *V. cholerae* progenitor, which does not appear to have been the case for the O139 Argentinian isolate (39). Not only are the *rfaD* genes different between the two strains, but the South American isolate does not produce cholera toxin. Furthermore, the Argentinian isolate agglutinates chicken erythrocytes and produces a heat-stable enterotoxin which the Bengal isolates do not (39). Since the *rfaD* genes differ it is most likely that the Argentinian strain did not arise by horizontal gene transfer from the Bengal O139 strains. This only leaves the possibility that the Argentine and Bengal O139 strains have evolved independently. The sequence downstream from *rfaD* leading to novel O139 DNA also differs significantly between these two isolates.

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ADDENDUM IN PROOF

Since the entire *rfb* region of *Vibrio cholerae* O139 has now been determined, it has been possible to agree on a uniform nomenclature based upon the system of Reeves et al. (Trends Microbiol. **12:**495–503, 1996). This nomenclature is to be published elsewhere (U. H. Stroeher and P. A. Manning, Trends Microbiol., in press).

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