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Serotype conversion in *Vibrio cholerae* O1

(lipopolysaccharide/cell wall/endotoxin/O antigen)

UWE H. STROEHER, LITSA E. KARAGEORGOS, RENATO MORONA, AND PAUL A. MANNING*

Department of Microbiology and Immunology, The University of Adelaide, G.P.O. Box 498, Adelaide, South Australia, 5001, Australia

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ABSTRACT ***Vibrio cholerae* O1 exists as two major serotypes, Inaba and Ogawa, which are associated with the O antigen of the lipopolysaccharide and are capable of unequal reciprocal interconversion. The 20-kilobase *rfb* regions encoding O-antigen biosynthesis in strains 569B (Inaba) and O17 (Ogawa) have been cloned in *Escherichia coli* K-12 and the nucleotide sequences have been determined. Besides several base substitutions and a small deletion in the 569B sequence relative to O17, there is a single nucleotide change resulting in a TGA stop codon within the gene for the 32-kDa RfbT protein. We have demonstrated that *rfbT* is responsible for serotype conversion (Inaba to Ogawa). The construction of a specific *rfbT* mutation in the Ogawa strain O17, and the ability of the gene from O17 to complement Inaba strains to Ogawa, confirmed *rfbT* as the gene required for the Ogawa serotype. By Southern hybridization and sequencing of PCR products of a number of strains, we have shown that the changes observed in one Inaba strain (569B) are not conserved in other Inaba strains. This may explain why some Inaba strains are able to convert to Ogawa whereas others are not. The protein encoded by *rfbT* has been identified and expressed in *E. coli* K-12 using a phage T7 expression system. Amino-terminal analysis of partially purified protein has identified the translational start of the protein. Primer extension studies have enabled the 5′ end of the mRNA to be defined. It exists as a separate transcript from the rest of the *rfb* region, and the distinctive G+C content of *rfbT* suggests that it has been acquired from a non-*Vibrio* source.**

The lipopolysaccharides (LPSs) of Gram-negative organisms are the most abundant molecules on the cell surface and provide a protective barrier to hydrophobic agents and detergents. LPS consists of three distinct regions: the lipid A region, which forms part of the lipid bilayer of the outer membrane; the core oligosaccharide; and the serotype-specific O antigen. This outermost region, the O antigen, provides the major antigenic variability of the cell surface, and on the basis of this heat-stable polysaccharide the species *Vibrio cholerae* is divided into more than 30 O-serotypic groups (1). Only *V. cholerae* of the O1 serotype is associated with cholera in humans, and the other serotypes are usually referred to as non-O1 or noncholera vibrios. The O1 serotype exists as two biotypes, Classical and El Tor, and both can be subdivided further into serotypes designated Ogawa and Inaba (2). Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen (3, 4), whereas Inaba strains express only the A and C antigens. A third serotype, termed Hikojima, exists; it is rare and unstable. Hikojima strains possess all three antigens, A, B, and C (3, 4). Since it is possible to get interconversion between the serotypes, Hikojima has been suggested to represent strains that undergo conversion at an elevated frequency (3).

Analysis of the chemical structure of the O antigen has shown it to be composed of a homopolymer containing the amino sugar D-perosamine substituted with 3-deoxy-L-glycerotetronic acid (5, 6). This structure is present in both Inaba and Ogawa serotypes and thus may be the A antigen. However, the nature of the B and C antigens is unknown.

V. cholerae O1 strains have been demonstrated to interconvert between the Ogawa and Inaba forms (7–11). However, the reciprocal nature of these conversions has been the subject of contention. Initial reports demonstrated the conversion of Ogawa cells to Inaba by growth in the presence of anti-Ogawa serum; the reverse switch could not be shown (8). In 1967, Nobechi and Nakano (12) showed the switch from Inaba to Ogawa and vice versa, and since that time a number of other groups have reported similar findings (7, 11). The frequency of conversion of Ogawa to Inaba is approximately 10^{-5} (10), whereas the conversion from Inaba to Ogawa appears to be less frequent and may be strain dependent.

The seroconversion *in vivo* correlates well with the host immune response. This is supported by observations with germ-free mice (7) and a clinical study carried out by Sheehy *et al.* (11). It has been proposed that the seroconversion from Inaba to Ogawa occurs only *in vivo*, since only the Ogawa to Inaba conversion has been observed *in vitro*.

The gene clusters that determine the biosynthesis of both the Ogawa and Inaba O antigens have been cloned and expressed in *Escherichia coli* K-12 (13). These genes, referred to as *rfb*, are located on a 20-kilobase (kb) *Sac* I (*Sst* I) restriction fragment (14), but only about 18 kb of this region is required for biosynthesis of *V. cholerae* O1 O antigen in *E. coli* K-12.

In this paper we report the sequence† differences between the Ogawa and Inaba serotypes, which have led us to determine the molecular basis for serotype variation in *V. cholerae* O1.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Plasmids were maintained in *E. coli* K-12 strain DH5. *E. coli* strain SM10 was obtained from A. Pühler, as were plasmids for mobilization of cloned genes into *V. cholerae* (15). Plasmid pGP1-2 was obtained from S. Tabor (16). *V. cholerae* strains were from laboratory stocks and their sources have been described elsewhere (17, 18).

PCR Amplification. PCR amplification was carried out by using standard protocols with oligodeoxyribonucleotides ("oligos") no. 292 (5′-CCAAACACAATCTTGAAA-3′) and no. 293 (5′-TTTGCTGACAATATGTGG-3′).

Cloning and Sequencing. PCR products were cloned after gel purification and cleavage with *Hin*dII and *Hin*dIII (Boehringer Mannheim). The complete sequences of the *Sac* I fragment harboring *rfb* from O17 and 569B were determined by using deoxyadenosine 5′-[α-[35S]thio]triphosphate and

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Abbreviations: Km^R, kanamycin resistance; LPS, lipopolysaccharide; ORF, open reading frame.
*To whom reprint requests should be addressed.
†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X59553).

Sequenase (United States Biochemical). Cloned PCR products were sequenced on an Applied Biosystems 373A automated sequencer using both dye primers and terminators.

Primer Extension and RNA Extraction. Total cellular RNA was extracted by using the hot-phenol method as described (19). Transcripts were extended from oligo no. 381 (5'-AGACTTTGACTGAATAG-3') radioactively labeled with [γ-^{32}P]-ATP at the 5' end by T4 polynucleotide kinase (Boehringer Mannheim). The primer was hybridized to RNA by denaturing at 80°C for 3 min and then incubating at 42°C for 60–90 min. Extensions were carried out in 24 μl of extension mix (20) with 10 units of avian myeloblastosis virus reverse transcriptase (Pharmacia) and incubating at 42°C for 60 min. Products were visualized on a 6% acrylamide/urea sequencing gel.

T7 Expression and N-Terminal Analysis. T7 overexpression was carried out by using the method of Tabor and Richardson (16). Partially purified protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad) and the N-terminal sequence was determined on a model 470 Applied Biosystems protein sequencer.

Haemagglutination Inhibition Assays (HIAs) and Antisera. HIAs were carried out as previously described (13). Typing sera were purchased from Wellcome Diagnostics. The monoclonal antibodies α-A (H4) and α-B (H8) were obtained from T. Holme, and α-C (13B) will be described elsewhere (U.H.S., H. M. Ward, C. J. Thomas, and P.A.M., unpublished results).

RESULTS

Identification of the Ogawa Gene. Comparison of the nucleotide sequence of the 20-kb *Sac* I fragment including the *rfb* gene cluster from strain 569B (Classical, Inaba) with that of O17 (El Tor, Ogawa) has revealed a remarkably high degree of identity, with only very minor differences (Table 1). This was surprising, since these strains differ not only in serotype but also in biotype and site and date of isolation. The only major changes detected were in *rfbR* and *rfbT* at the 3' end of the *rfb* region. The 3-kb *Cla* I fragment (Fig. 1) corresponding to the region of variability was subsequently cloned from the O17 *rfb* gene cluster in pPM2101 (18) to generate plasmid pPM2122, which was then mobilized by conjugation into the Inaba strains 569B and BM29. Both strains were converted to the Ogawa serotype, indicating that the changes detected outside of this region had no effect on serotype specificity (Table 2). When the homologous region from 569B, present in plasmid pPM2123, was introduced, no effect on the expression of the A, B, and C antigens could be detected, by either slide agglutination or hemagglutination inhibition assays, in strains O17 and CA411 (Table 2).

To determine the genetic basis for serotype specificity, and to gain an insight into the mechanism of serotype conversion, the following experiments were undertaken.

The most obvious sequence variation from the O17 sequence was an 8-bp deletion in *rfbR* in 569B. Using an oligo (no. 286: 5'-GATGTAAAAGGCTGCT-3') spanning this region, we probed 40 strains of both serotypes by dot-hybridizations to determine if this deletion correlated with the serotype of the strain. Although this deletion was detected in other, but not all, Inaba strains, it rarely occurred in Ogawa strains. This suggested that this change alone was

Table 1. Nucleotide sequence changes in the *rfb* region of different *V. cholerae* O1 strains relative to strain O17 (Ogawa)

Strain (biotype/serotype)*	Changes	Consequence
569B (C/I)	G-6654 to A	No effect, lies in an intergenic region
	T-16378 to C-16385 deleted	Frameshift in RfbR, gives a truncated protein
	C-17676 to A	RfbT Gln^{163} to Lys
	G-17844 to T	RfbT Gly^{219} to Stop
	C-18086 to G	
	T-19631 to G	ORF2 Tyr^{47} to Asp
H-1 (E/O)	A-17729 to G	No change to RfbT Arg^{180}
64 (C/O)	None	
BM69 (E/I)	C inserted after T-17976	Frameshift: RfbT-Ser^{263}-Ala-Glu to Phe^{263}-Arg-Stop
CA401 (C/I)	G-17327 to T	RfbT Leu^{46} to Trp
	TACA-17495 to ACAC	RfbT Thr^{102} to His
	C-17676 deleted	Frameshift: RfbT Gln^{163}-Lys-Asn-Thr-Asp-Ile to Lys^{163}-Ile-Gln-Thr-Stop
Z17561 (C/I)	G-17327 to T	RfbT Leu^{46} to Trp
	TACA-17495 to ACAC	RfbT Thr^{102} to His
	C-17676 deleted	Frameshift: RfbT Gln^{163}-Lys-Asn-Thr-Asp-Ile to Lys^{163}-Ile-Gln-Thr-Stop

The entire sequence of the 20.1-kb *Sac* I fragment harboring the *rfb* region from O17 will appear in the European Molecular Biology Laboratory/GenBank/DNA Data Base in Japan Nucleotide Sequence Data Libraries under the accession number X59553. ORF, open reading frame.

*The biotypes and serotypes are abbreviated C, Classical; E, El Tor; I, Inaba; and O, Ogawa.

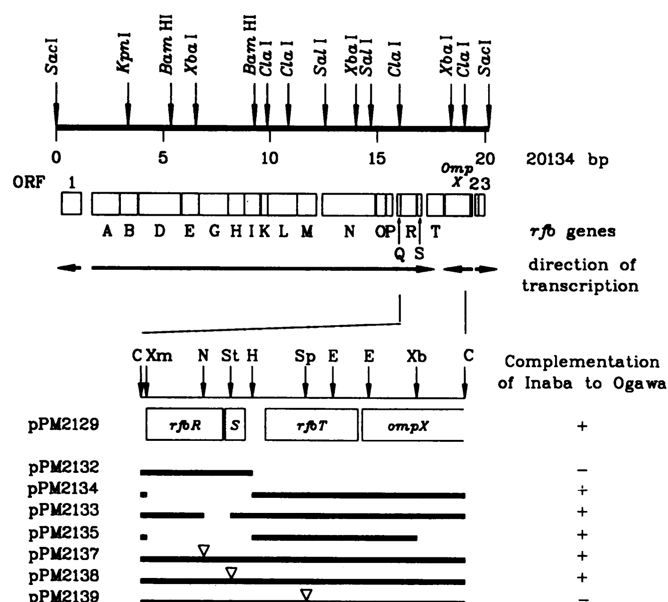

FIG. 1. Physical map of pPM2129, showing the deletions and insertions used to determine that *rfbT* is the gene required for seroconversion. bp, Base pairs. pPM2129 contains the 3-kb *Cla* I fragment shown cloned in pUC18. Plasmid pPM2132 is a *Hin*dII cutdown leaving *rfbR* and *rfbS* intact but removing *rfbT*. Plasmids pPM2133 and pPM2134 represent *Nru* I/*Stu* I and *Hin*dII/*Xmn* I deletions, respectively. These plasmids interrupt or remove *rfbR* and *rfbS*, but leave *rfbT* and the C-terminal coding region of *ompX*. The plasmid pPM2135 is a deletion clearly showing that only *rfbT* is required for serotype conversion. Plasmids pPM2137, pPM2138, and pPM2139 contain the kanamycin resistance (Km^R) cartridge inserted into the *Nru* I, *Stu* I, and *Spe* I sites of pPM2129, respectively, as indicated by the triangles. The insertion of a Km^R gene into *rfbR*, *rfbS*, and *rfbT* shows that if *rfbT* is interrupted it no longer seroconverts. The seroconverting status is indicated and was determined by agglutination on slides in the presence of typing sera (Wellcome). ORF1, -2, and -3 and OmpX correspond to non-*rfb* potential open reading frames. C, *Cla* I; E, *Eco*RI; H, *Hin*dII; N, *Nru* I; Sp, *Spe* I; St, *Stu* I; Xb, *Xba* I; Xm, *Xmn* I.

Table 2. Hemagglutination inhibitions for quantitation of antigens

Strain*	Plasmid	Hemagglutination inhibition with monoclonal antibody, μg of LPS†		
		α-A	α-B	α-C
569B (I)	None	0.156	—	0.078
	pPM2101	0.156	—	0.078
	pPM2122	0.078	0.018	0.078
	pPM2123	0.078	—	0.078
CA411 (O)	None	0.039	0.009	0.039
	pPM2101	0.039	0.005	0.039
	pPM2122	0.039	0.009	0.039
	pPM2123	0.078	0.005	0.039
O17 (O)	None	0.039	0.018	0.078
	pPM2101	0.156	0.018	0.078
	pPM2122	0.078	0.018	0.078
	pPM2123	0.156	0.009	0.078
BM69 (I)	None	0.078	—	0.078
	pPM2101	0.156	—	0.078
	pPM2122	0.078	0.039	0.078
	pPM2123	0.156	—	0.078

*The serotypes of the strains are indicated in parentheses; I, Inaba; O, Ogawa.

†Hemagglutination inhibition is measured as the lowest amount of LPS (μg) required to inhibit the agglutination of sheep erythrocytes coated with O162 (Ogawa) LPS. LPS was purified from 100-ml cultures and used as previously described (13). A — indicates no inhibition was observed, implying the absence of the antigen corresponding to the particular antibody.

not sufficient for generating an Inaba strain. In addition (see below), complementation of the *rfbT* mutation but not the *rfbR* mutation in 569B restored Ogawa specificity, implying that *rfbR* is required for neither O-antigen synthesis nor serotype specificity.

Strains were also screened for the sequence change leading to a *Dra* I site (at nucleotide 17676 in the Ogawa sequence; Fig. 1) in *rfbT* of 569B by Southern hybridization using pPM2122 as a probe. This site results in the loss of an 860-bp fragment and the generation of two new fragments, 300 and 560 bp. This change was not conserved between Inaba and Ogawa strains, and it was detected in Inaba as well as some Ogawa strains. Thus, there was no correlation between the presence of the specific *rfbR* and *rfbT* differences and serotype.

Derivatives of pPM2129 have been constructed which demonstrate that *rfbT* is the only gene required for serotype specificity in *V. cholerae* O1 (Fig. 1). Only plasmids that contained an intact *rfbT* gene from O17 were capable of converting strain 569B from Inaba to Ogawa.

In further support, a mutation was constructed in *rfbT* of strain O17 by introducing a Km^R cartridge into the *Spe* I site in *rfbT* and recombining this mutation into the O17 chromosome by allelic exchange. Agglutination of strain O17 (*rfbT*::Km^R) with specific typing sera indicated that it had been converted from Ogawa to Inaba. This mutant strain could be complemented to Ogawa by pPM2122 but not by pPM2123 or pPM2101.

Characterization of RfbT. The 3-kb *Cla* I fragment (Fig. 1) was inserted in both orientations into pBluescript (K/S) (Stratagene) to give pPM2127 and pPM2128, of which the former was confirmed, as expected, to place *rfbT* under the control of the T7 promoter. In the presence of pGP1-2 (16) in *E. coli* K-12 strain DH5, a 32-kDa protein, presumed to be RfbT, was overproduced (Fig. 2). Pulse–chase experiments have shown this protein to be stably expressed and not subject to processing. Thus, it seems likely that although the various Rfb proteins are normally produced in very low levels (unpublished data), they probably have long half-lives and are not turned over at a significant rate. RfbT from these experiments provided a source of protein for N-terminal sequence determination and for generating a specific antiserum. The N-terminal sequence of the 32-kDa protein was determined to be Met-Lys-His-Leu-Ile-Lys-Asn-Tyr-Val-Glu-Lys-Leu-Ile-Lys.

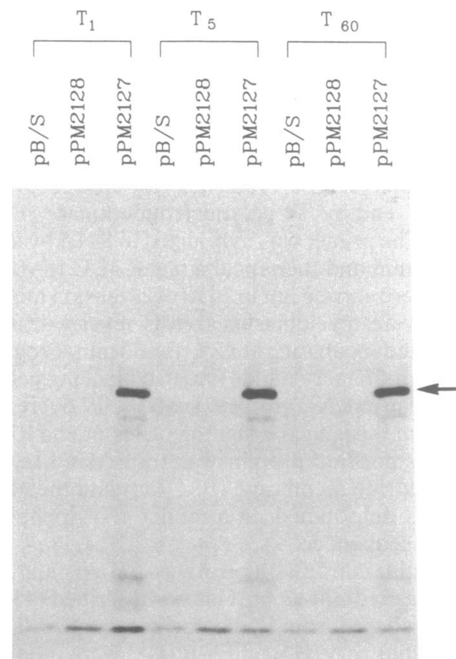

FIG. 2. Production of RfbT from plasmids pPM2127 and pPM2128. DH5 cells carrying pGP1-2 and one of the indicated plasmids were labeled with [^{35}S]methionine for 5 min and then incubated with unlabeled methionine. Samples were taken at 0, 1, 2, 5, and 60 min. Lanes 1, 2, and 3 represent pBluescript (pB/S), pPM2128, and pPM2127, respectively, at 1 min; lanes 4–6 are at 5 min; and lanes 7–9 are at 60 min. Arrow indicates the 32-kDa protein.

Nucleotide Sequence of *rfbT*. The nucleotide sequence of *rfbT* reveals an ORF of 286 residues, corresponding to 32,917 Da, with the first 14 amino acids corresponding to the N-terminal sequence of the 32-kDa protein identified by using the T7 expression system (Fig. 3). Analyzing hydropathy plots of the protein and scanning the sequence, we conclude that RfbT is a relatively hydrophilic protein lacking an N-terminal signal sequence.

Since pPM2127 and pPM2128 are able to mediate a phenotypic serotype conversion, and all deletions and insertions in *rfbR* and *rfbS* have no effect on expression of *rfbT* (Fig. 1), this implied the presence of a promoter immediately prior to *rfbT*. Primer extension analysis performed on mRNA isolated from *V. cholerae* and *E. coli* with or without the cloned gene clearly confirmed the start of the mRNA from a promoter in front of *rfbT* (Fig. 4). As this was observed in both *V. cholerae* and subclones in *E. coli*, it is unlikely that the predicted transcriptional start site is the result of processing from a larger mRNA. The transcriptional start site is localized to nucleotide 17091, indicating the presence of a 99-nucleotide 5′ untranslated region on the mRNA (Fig. 3).

The G+C content of *rfbT* (31.7%) is quite low compared with the rest of the *rfb* region (39.1%) and with other *V. cholerae* genes in general (about 48% for most genes, but about 40% or lower for virulence-associated genes; P.A.M., unpublished data). Since *rfbT* is a nonessential gene with unusual G+C composition and its product only contributes to antigenic variability, it seems likely that it has been acquired from a non-*Vibrio* source.

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         T TGA CA                          TA TAA T              ∇
17046  TTT GGA TGA AAT TCC TTC TAA ATG TCA ATA AAA TGG CAA ATC CAT TAT  17093
           HincII
17094  TGG TCA ACA ATG CCC TTT CAG GTC CTC AAA CCT GCA TCT GCA AGT TGA  17141

17142  TTC TGT ATG TTA TTT TTT ACG CTA ATA TTA TTT AAA ATT GAG GTA GTA  17189

17190  ATG AAA CAT CTA ATA AAA AAC TAT GTA CAA AAA TTA ATT AAA ACA GAG  17237
    1  Met Lys His Leu Ile Lys Asn Tyr Val Gln Lys Leu Ile Lys Thr Glu     16

17238  CTT GAT GCT ATT CAG TCA AAG TCT GTT CAT GAT AAT CGA AAC TTC ATT  17285
   17  Leu Asp Ala Ile Gln Ser Lys Ser Val His Asp Asn Arg Asn Phe Ile     32

17286  TAC AAT GGA GAG TTT TTA ATT CTT GAA AGC GAA TTT GGA TTG CAT TGT  17333
   33  Tyr Asn Gly Glu Phe Leu Ile Leu Glu Ser Glu Phe Gly Leu His Cys     48

17334  TTT CCC AGA GTG CAG TTG AAC CAT GCT TTA AGC TAC AAA AAC CCA AAC  17381
   49  Phe Pro Arg Val Gln Leu Asn His Ala Leu Ser Tyr Lys Asn Pro Asn     64

17382  TTT GAT TTA GGT ATG CGT CAC TGG ATT GTT AAT CAT TGT AAG CAT GAC  17429
   65  Phe Asp Leu Gly Met Arg His Trp Ile Val Asn His Cys Lys His Asp     80

17430  ACC ACT TAT ATT GAT ATC GGT GCA AAC GTT GGA ACT TTC TGT GGA ATC  17477
   81  Thr Thr Tyr Ile Asp Ile Gly Ala Asn Val Gly Thr Phe Cys Gly Ile     96

17478  GCT GCT CGT CAT ATT ACA CAA GGA AAA ATT ATA GCG ATA GAA CCA CTC  17525
   97  Ala Ala Arg His Ile Thr Gln Gly Lys Ile Ile Ala Ile Glu Pro Leu    112

17526  ACA GAA ATG GAA AAT AGT ATT AGG ATG AAT GTT CAA TTA AAT AAT CCA  17573
  113  Thr Glu Met Glu Asn Ser Ile Arg Met Asn Val Gln Leu Asn Asn Pro    128

17574  CTA GTT GAG TTT CAT CAT TTT GGC TGT GCA ATA GGT GAG AAT GAA GGG  17621
  129  Leu Val Glu Phe His His Phe Gly Cys Ala Ile Gly Glu Asn Glu Gly    144

17622  GAA AAT ATT TTC GAA GTT TAT GAG TTT GAT AAT AGG GTG TCA TCA TTA  17669
  145  Glu Asn Ile Phe Glu Val Tyr Glu Phe Asp Asn Arg Val Ser Ser Leu    160

17670  TAT TTT CAA AAA AAT ACA GAC ATA GCA GAT AAG GTT AAA AAT AGC CAA  17717
  161  Tyr Phe Gln Lys Asn Thr Asp Ile Ala Asp Lys Val Lys Asn Ser Gln    176

17718  GTT CTG GTT AGA AAG TTA AGT AGT TTA GAT ATA TCG CCT ACT AAC TCT  17765
  177  Val Leu Val Arg Lys Leu Ser Ser Leu Asp Ile Ser Pro Thr Asn Ser    192

17766  GTA GTT ATA AAA ATT GAT GCT GAA GGC GCA GAA ATA GAG ATA TTA AAC  17813
  193  Val Val Ile Lys Ile Asp Ala Glu Gly Ala Glu Ile Glu Ile Leu Asn    208
                   EcoRI
17814  CAG ATT TAC GAA TTC ACA GAA AAG CAT AAT GGA ATT GAA TAT TAT ATT  17861
  209  Gln Ile Tyr Glu Phe Thr Glu Lys His Asn Gly Ile Glu Tyr Tyr Ile    224

17862  TGC TTT GAA TTT GCA ATG GGT CAT ATA CAG AGG TCT AAT AGA ACT TTT  17909
  225  Cys Phe Glu Phe Ala Met Gly His Ile Gln Arg Ser Asn Arg Thr Phe    240

17910  GAT GAG ATT TTT AAC ATA ATA AAC TCA AAA TTC GGA AGT AAG GCA TAT  17957
  241  Asp Glu Ile Phe Asn Ile Ile Asn Ser Lys Phe Gly Ser Lys Ala Tyr    256

17958  TTT ATT CAT CCA TTA TCA TCC GCT GAA CAT CCT GAG TTT AAT AAA GCA  18005
  257  Phe Ile His Pro Leu Ser Ser Ala Glu His Pro Glu Phe Asn Lys Ala    272

18006  ACG CAG GAT ATT AAT GGG AAT ATC TGT TTT AAA TAT GTA TCA TAA AAT  18053
  273  Thr Gln Asp Ile Asn Gly Asn Ile Cys Phe Lys Tyr Val Ser ***        286

18054  AAT TTA ATA TAT TCC GTA TGT CAT TGC AAG TTC AAC AGA CAT TTC CGA  18101

18102  AGA GTT CAC TAT ACA GTT TAG TAT AGC TTT GTG CAT AGC GAT GTG CTG  18149
        EcoRI
18150  TGA ATT C                                                          18156
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FIG. 3. Nucleotide sequence of *rfbT*. The nucleotide sequence shown corresponds to *rfbT* within the 20.1-kb *Sac* I fragment, which contains the entire *rfb* region from *V. cholerae* O1 strain O17 (Ogawa). The transcriptional start site determined by primer extension is indicated ∇. The putative −35 and −10 regions are underlined, with the corresponding *E. coli* consensus sequences (TTGACA and TATAAT, respectively) above. G-17844, which is replaced by T in 569B, leading to chain termination, is indicated in large type. Relevant restriction sites are also shown and underlined. The underlined amino acid sequence was obtained by N-terminal sequencing of the partially purified 32-kDa protein. The overlined nucleotide sequence is complementary to the oligo used for primer extension analysis (Fig. 4). These sequence data will appear in the European Molecular Biology Laboratory/GenBank/DNA Data Base in Japan Nucleotide Sequence Data Libraries under the accession number X59553.

Cellular Localization of RfbT. We have used an antiserum specific for the 32-kDa RfbT protein to probe its cellular location. Whole *V. cholerae* cells of both O17 and 569B were fractionated as previously described (21, 22) and the fractions were subjected to SDS/PAGE and immunoblotting (23). The results (Fig. 5) clearly show that both RfbT from O17 and the truncated form from 569B (see below) are located in the cytoplasmic membrane. This suggests that the hydrophobic domains detected near the N terminus (amino acids 86–98 and 121–130) are sufficient for membrane localization.

Sequence Variation in Other Strains. To determine if the sequence change leading to a stop codon in 569B *rfbT* was

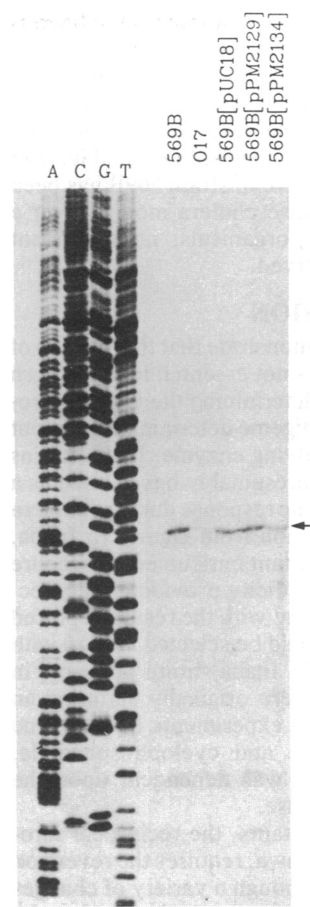

FIG. 4. Primer extension analysis. (*Right*) Total cellular mRNA was isolated from the various strains shown. Primer extensions were performed by using oligo no. 381 hybridized to the mRNA and extended with reverse transcriptase from avian myeloblastosis virus. The position of the extension product is indicated by the arrow. (*Left*) Corresponding sequencing ladder obtained by using the same oligo as primer with pPM2129 as a template.

conserved in other Inaba strains, PCR using synthetic primers (nos. 292 and 293) was used to amplify the region of interest, which was cloned as a *Hin*dII/*Hin*dIII fragment in pBluescript (K/S). The *rfbT* genes of three Inaba strains (CA401, Z17561, and BM69) and two Ogawa strains (H-1 and 64) were each sequenced, using two independent clones to eliminate any sequence ambiguities due to PCR amplification. The Ogawa strains revealed no significant sequence alterations (Table 1). All of the Inaba strains showed marked sequence changes leading to truncated RfbT proteins of various sizes due to reading frameshifts. The change reported above in 569B led to a 25.1-kDa truncated product. Strains CA401 and Z17561 can produce RfbT proteins of 19.3 kDa, whereas BM69 has a protein of 30.5 kDa. Thus, none of the Inaba strains encoded a full-length RfbT protein of 32 kDa.

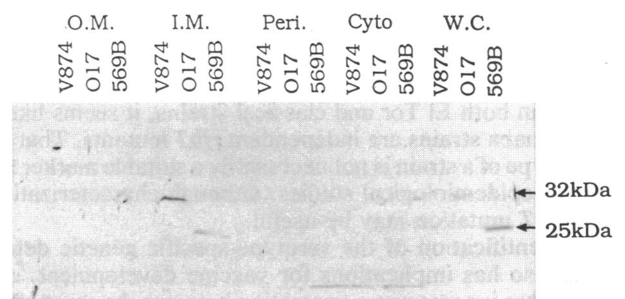

FIG. 5. Cell fractionations. Whole cells of *V. cholerae* O17, 569B, and the *rfbT*::Km^R mutant of O17 were fractionated and the various fractions were subjected to SDS/PAGE followed by immunoblotting using a rabbit antiserum against the SDS/PAGE-purified 32-kDa RfbT protein from O17. The cell fractions are O.M., outer membrane; I.M., inner membrane; Peri., periplasmic shock fluid; Cyto., cytoplasm; W.C., whole unfractionated cells.

Virulence of Seroconverted Strains. An intact O antigen is essential for the virulence of *V. cholerae* in the infant mouse cholera model (U.H.S., H. M. Ward, C. J. Thomas, and P.A.M., unpublished results). However, no data are available on isogenic strains to assess whether serotype specificity plays a role. Consequently, the virulence of O17 and its *rfbT* mutant and also an Ogawa derivative of strain 569B has been assessed by using the infant mouse cholera model. Over a range of doses from 10^5 to 10^8 organisms, no significant difference in virulence was observed.

DISCUSSION

The data reported here clearly demonstrate that the product of the *rfbT* gene of *V. cholerae* O1 is not essential for O-antigen biosynthesis but is required for determining the Ogawa serotype specificity. It is not the B-antigenic determinant itself but presumably is an O-antigen-modifying enzyme. Inaba strains are effectively *rfbT* mutants and presumably have arisen as a result of selection due to the immune response during a cholera infection. Thus, serotype conversion from Ogawa to Inaba, during an infection, is simply a mutant enrichment procedure with antibodies to the Ogawa specificity providing the selection. This interpretation is consistent with the results reported in the literature. Inaba variants could be selected *in vitro* with anti-Ogawa or anti-B sera (3, 4, 7). Inaba strains appeared in stools of isolated patients who were originally suffering an Ogawa infection (9, 11). Also the experiments of Sack and Miller (7), using germ-free mice and cyclophosphamide, showed that serotype conversion was dependent upon the development of an immune response.

Since Inaba strains are *rfbT* mutants, the reciprocal serotype conversion, from Inaba to Ogawa, requires the reversion of the original mutation. Thus, although a variety of changes in *rfbT* appear possible to produce an Inaba strain, the correction of that specific change is necessarily going to be a rare event. Whereas Ogawa to Inaba may be detectable *in vitro*, the reciprocal change may not. This would account for the paradox as to the reciprocal nature of serotype conversion in *V. cholerae* O1.

The sequence changes observed in the *rfb* region of 569B compared with O17 and the deletion and insertion data (Fig. 1) demonstrate that *rfbR* is not required for O-antigen biosynthesis or specificity. The region encoding *rfbR*, *rfbS*, and *rfbT* shows a marked divergence from the other *rfb* genes in that it has a low G+C content (31–32%), compared with about 40%. These observations suggest that this segment of *rfb* is recently acquired as a luxury function (antigenic variability) and that the ancestral *V. cholerae* strain was an Inaba. In this regard it would be of interest to examine the *rfb* regions of *Vibrio* strains with different alternative antigenic determinants (24). Perhaps they represent further variants of the ancestral strain, which have acquired alternative gene(s) for an additional serotype specificity.

These data also have implications on the clonal nature of *V. cholerae* O1. Although Ogawa and Inaba serotypes are detected in both El Tor and classical strains, it seems likely that the Inaba strains are independent *rfbT* mutants. That is, the serotype of a strain is not necessarily a suitable marker for clonal or epidemiological studies, although characterization of the *rfbT* mutation may be useful.

The identification of the serotype-specific genetic determinant also has implications for vaccine development. Although there is some cross-protection between the serotypes, primarily due to the A antigen (13), it is still highly desirable to immunize against both serotypes. Thus, attenuated candidate vaccine strains that have already undergone extensive testing in humans could be simply converted, by allelic exchange, to the alternative serotype. Since it has also been shown that serotype specificity has no effect on virulence, then this should not require extensive retesting of the strains to gain approval for use in humans.

The chemical nature of the A, B, and C antigenic determinants is unknown. Preliminary NMR studies on isogenic serotype-converted strains suggest that this change is subtle (N. Packer, M. Batley, J. W. Redmond, and P.A.M., unpublished data). However, with new extraction procedures, and the ability to specifically cleave the LPS molecule, the use of such strains should greatly enhance the likelihood of identifying the chemical structures involved.

Further characterization of RfbT and its membrane topology could also aid in the study of LPS and O-antigen biosynthesis. For example, by constructing strains in which the expression of *rfbT* is tightly controllable, it may be possible to follow the incorporation of the Ogawa-specific determinant into the LPS molecule and study its translocation to the cell surface, a phenomenon that is still poorly understood.

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