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Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis

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Only *Vibrio cholerae* strains of serotype O1 are known to cause epidemics, while non-O1 strains are associated with sporadic cases of cholera. It was therefore unexpected that the recent cholera epidemic in Asia was caused by a non-O1 strain with the serotype O139. We provide evidence that O139 arose from a strain closely related to the causative agent of the present cholera pandemic, *V.cholerae* O1 El Tor, by acquisition of novel DNA which was inserted into, and replaced part of, the O antigen gene cluster of the recipient strain. Part of the novel DNA was sequenced and two open reading frames (*otnA* and *otnB*) were observed, the products of which showed homology to proteins involved in capsule and O antigen synthesis, respectively. This suggests that the *otnAB* DNA determines the distinct antigenic properties of the O139 cell surface. The *otnAB* DNA was not detected in O1 strains, but was present in two non-O1 *V.cholerae* strains with serotypes O69 and O141. In the O69 and O139 strains the *otnAB* genes were located proximate to the putative insertion sequence (IS) element *rfbQRS*, which is associated with O antigen synthesis genes in O1 strains, and may have played a role in the insertion of the *otnAB* DNA in the recipient chromosome. Our results suggest that the O139 strain arose by horizontal gene transfer between a non-O1 and an O1 strain. The acquired DNA has altered the antigenic properties of the recipient O1 strain, providing a selective advantage in a region where a large part of the population is immune to O1 strains. This genetic event may have contributed to the present cholera epidemic in Asia.

Key words: evolution/gene transfer/O139/polysaccharide synthesis/*Vibrio cholerae*

Introduction

Vibrio cholerae is the causative agent of cholera which, in its most severe form, is characterized by profuse diarrhoea, vomiting and muscle cramps. The loss of fluids and salts caused by cholera can cause vascular collapse and death in hours. *Vibrio cholerae* strains are natural inhabitants of brackish water and estuarine systems (Colwell and Huq, 1994) and have been divided into two groups, O1 and non-O1, based on their ability to cause cholera epidemics. All three cholera pandemics since 1881 have been caused by *V.cholerae* strains of serotype O1 (Barua, 1992) and it was therefore assumed that only this serotype has epidemic potential. *Vibrio cholerae* non-O1 strains are a heterogeneous group, comprising >130 serotypes, and are generally only isolated from sporadic cases of cholera, indicating that they do not have the potential to cause epidemics. It was unprecedented, therefore that the recent cholera epidemic, which started in 1992 in Madras and soon spread to other parts of Asia, is due to a non-O1 strain (Albert *et al.*, 1993a; Bhattacharya *et al.*, 1993; Ramamurthy *et al.*, 1993). Since its emergence, the O139 strain has affected hundreds of thousands of people and has replaced *V.cholerae* O1 strains as the predominant cause of cholera on the Indian subcontinent (Garg *et al.*, 1993; Jesudason and John, 1993). As this strain did not belong to any of the 138 known *V.cholerae* serogroups, it was assigned to the new serogroup O139 with the synonym Bengal (Shimida *et al.*, 1993).

At first, it was not clear whether the new strain was a derivative of a non-O1 serotype that has acquired the potential to cause epidemic cholera, or whether its origin was an O1 strain with an altered O antigen. There is now abundant evidence for the latter hypothesis (Morris, 1994). The O139 strain contains a number of virulence genes which are not, or only infrequently, found in non-O1 strains but which are typical for O1 strains (Albert *et al.*, 1993a; Hall *et al.*, 1993; Ramamurthy *et al.*, 1993; Calia *et al.*, 1994). The *V.cholerae* O1 serogroup can be further differentiated into two biotypes, designated classical and El Tor. Recent studies on biochemical and DNA characteristics of the O139 serotype have shown that it is more similar to the El Tor biotype than to the classical biotype (Hall *et al.*, 1993; Calia *et al.*, 1994; Faruque *et al.*, 1994; Waldor and Mekalanos, 1994a). Not withstanding these similarities, important differences have been observed between the O139 and O1 strains. The cell wall of the O139 strain contains colitose, a sugar not hitherto reported as a constituent of *V.cholerae* (Hisatsune *et al.*, 1993). Further, like other non-O1 strains, the O139 strain expresses a capsule (Johnson *et al.*, 1994). Thus it seemed likely that the O139 strain arose from an El Tor-like strain by acquisition of exogenous DNA. Here we confirm this assumption and show that the genesis of the O139 strain involved replacement of O1 antigen synthesis DNA by exogenous DNA involved in polysaccharide synthesis. We present evidence that the exogenous DNA is derived from non-O1 *V.cholerae* strains. Finally, the possible involvement of IS elements in the genesis of the O139 strain is discussed.

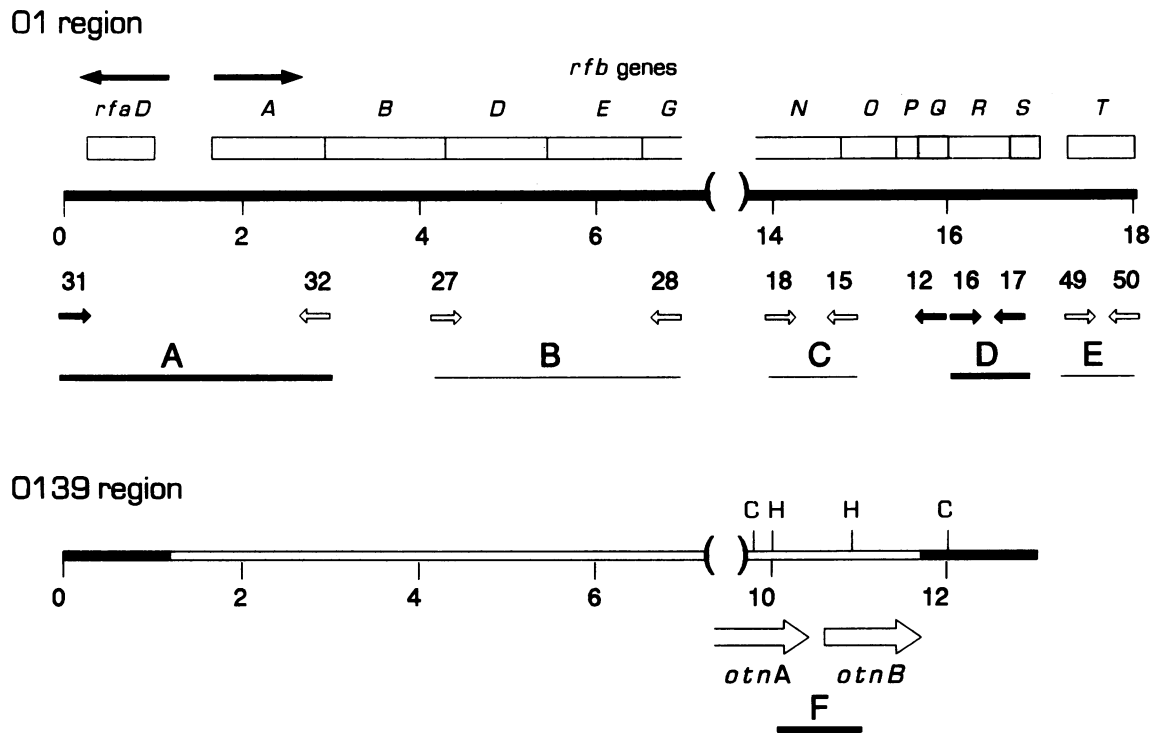

Fig. 1. Comparison of *rfb* regions in *V.cholerae* O1 and O139. The representation of the O1 *rfb* region is based on the sequence submitted to GenBank (accession No. X59554) and Stroeher *et al.* (1992). The large black arrows indicate the direction of transcription of the O1 genes. O139 regions homologous or non-homologous to O1 DNA are indicated by closed or open boxes, respectively. The position of the junction of homologous and non-homologous DNA in the left region of the O139 DNA is tentative. Open arrows mark the position and transcriptional orientation of the *otnA* and *otnB* genes. Numbered black and open arrows mark positions of PCR primers which were and were not able to amplify O139 DNA, respectively. The probes A–F used for Southern blotting are indicated by horizontal lines. Probes which hybridized with O139 DNA are indicated by thick lines, whereas probes which failed to hybridize are indicated by thin lines. Brackets delineate DNA regions omitted for brevity. The scales are indicated in kbp. Abbreviations; C, *Cla*I; H, *Hin*dIII.

Results

Analysis of an O139 DNA region homologous to the rfb locus of V.cholerae O1

Since the O1 and O139 strains express distinct O antigens, we investigated whether differences could be observed between these strains in the chromosomal region coding for O antigen synthesis. A chromosomal DNA region, designated *rfb* and involved in O1 antigen synthesis, has been isolated from *V.cholerae* O1 strains and sequenced (Stroeher *et al.*, 1992). Primers were derived from the O1 sequence and used to amplify O1 or O139 DNA by means of the polymerase chain reaction (PCR) (Figure 1). The absence of a PCR product does not exclude the presence of homologous DNA, since it may be caused by a few mutations in the primer target region. Therefore, O139 DNA was also analysed with Southern blotting using probes generated by amplification of O1 *rfb* DNA (Figure 1). Both the PCR and Southern blot analysis of O1 DNA revealed DNA fragments which were predicted by the sequence of the O1 *rfb* DNA (not shown). When O139 DNA was analysed, no PCR products were detected with the primer pairs 27/28 and 18/15, which were derived from the central region of the *rfb* DNA (Figure 1). O1 probes generated by these primers (respectively, probe B and probe C) did not hybridize to O139 DNA, confirming that the central region of the *rfb* cluster was missing in the O139 strain. Primers 31/32, derived from the left end of the *rfb* cluster, also failed to amplify O139 DNA. However, the corresponding PCR probe (probe A) hybridized to a 2200 bp *Dra*I fragment from the O139 strain. With the O1 strain two *Dra*I fragments, with sizes of 800 and 2400 bp, were detected with probe A. Primers 16/17, derived from *rfbR* which is located on the right end of the *rfb* DNA, gave a 400 bp PCR product with O139 DNA. A product of identical size was found with O1 DNA in accordance with the DNA sequence of this region. The corresponding probe (probe D) hybridized to a 2000 bp *Hpa*II fragment from the O139 strain, whereas a 4500 bp *Hpa*II fragment was detected with the O1 strain. The primers 49 and 50, derived from *rfbT*, did not amplify O139 DNA, and the corresponding DNA probe (probe E) failed to hybridize with O139 DNA, indicating that a *rfbT* homologue is not present in the O139 strain.

These results indicated that a large part of the *rfb* gene cluster was absent from the O139 strain. To determine whether the deleted *rfb* sequences were replaced by other DNA, the size of the O139 DNA located between the two regions homologous to the O1 *rfb* DNA was amplified with primers 31 and 12 (Figure 1) and KlenTaq-LA which allows the amplification of very large DNA fragments (Barnes, 1994). Amplification of O1 DNA revealed a fragment of 16 000 bp in accordance with the DNA sequence of this region, whereas a 12 000 bp fragment was detected with O139 DNA (Figure 1). DNA sequencing and hybridization with the O1-specific probe B and the O139-specific probe F (see below; Figure 1) confirmed the specificity of the PCR (data not shown). Taken together, these results indicate that, compared with the O1 *rfb* sequence, the O139 DNA has a mosaic structure, being

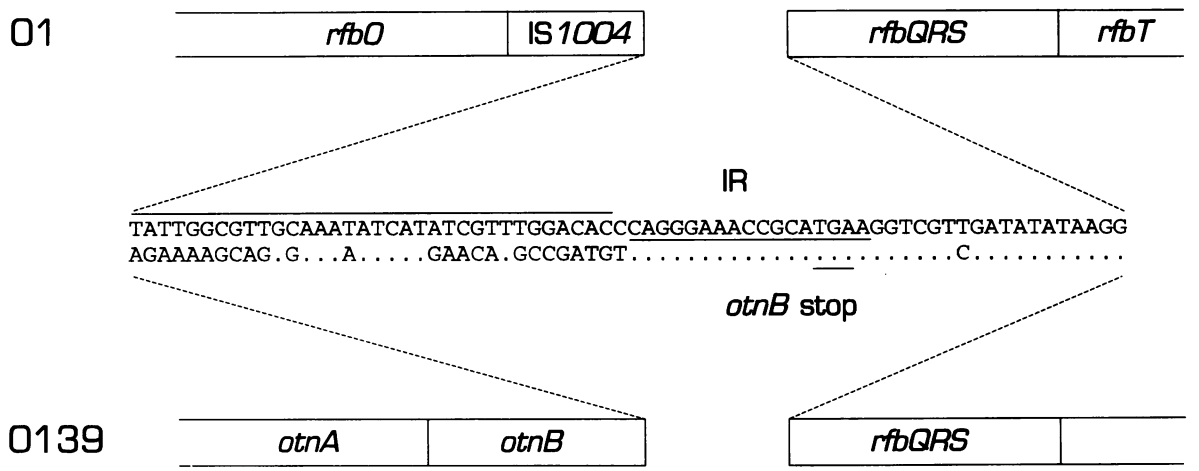

Fig. 2. Comparison of the O1 and O139 *rfbQRS* DNA regions. The sequence of the junction between the *rfbQRS* element and, respectively, the IS*1004* fragment and *otnB* is indicated. Dots indicate sequence identity. The stop codon of *otnB* and the left inverted repeat (IR) of the *rfbQRS* element are underlined. The sequence of the IS*1004* fragment is overlined. The sequence to the right of the O139 *rfbQRS* element has not been determined, but this region does not hybridize to O1 *rfbT* DNA. The O139 sequence is available from the EMBL database under the accession number X79822.

composed of as yet undefined DNA, interspersed by DNA homologous to the O1 *rfb* cluster (Figure 1).

Our data are consistent with those of Manning *et al.* (1994) who showed that the genes *rfbA-P* and *rfbT* are deleted in the O139 strain, while *rfaD*, *rfbQ*, *rfbR* and *rfbS* are still present, and extend these data by showing that the deleted sequences were replaced by O139-specific DNA (see below).

Sequence analysis of the O139 DNA proximate to conserved rfbR sequences: homology with genes involved in polysaccharide synthesis

It was interesting that the right-hand boundary of the O139-specific DNA contained sequences homologous to *rfbR* (Figure 1). In the O1 strains, the *rfbR* gene is part of a putative IS element, comprising the genes *rfbQ*, *rfbR* and *rfbS*, which are flanked by two inverted repeats (Zhao *et al.*, 1993; Manning *et al.*, 1994). It is conceivable that the *rfbQRS* element was involved in the evolution of this DNA region. To investigate this possibility and to characterize the O139-specific DNA , a region composed of *rfbR* and sequences upstream of this gene, was cloned from the O139 strain and sequenced. The DNA sequence revealed the presence of the *rfbQRS* element, which showed very little sequence divergence (4%) from its O1 homologue. Immediately to the left of the *rfbQRS* element, the sequences of the O1 and O139 strains diverged (Figure 2). In this region two large ORFs were observed in the O139 DNA, comprising 732 and 336 codons and tentatively designated *otnA* and *otnB*, respectively (otn, for one-three-nine). The stop codon of *otnB* is located within the left inverted repeat of the *rfbQRS* element (Figure 2). Homology searches indicated that the *otnA* product showed sequence similarity with KpsD (Figure 3), an *Escherichia coli* protein which is involved in transport of capsular precursors across the periplasmic space (Silver *et al.*, 1987; Bronner *et al.*, 1993). The *otnB* product showed similarity with a family of proteins, found in *E.coli* and *Salmonella enterica*, involved in regulation of O antigen chain length (Figure 3; Batchelor *et al.*, 1992; Meier-Dieter *et al.*, 1992; Bastin *et al.*, 1993). The closest similarity was observed with the *rol* product of *S.enterica* (Figure 3). No significant similarity was observed between the *otnAB* products and the products of the O1 *rfb* cluster. The observed homologies suggest that this DNA region is involved in synthesis of cell wall polysaccharides.

DNA homologous to the O139 otnAB region is absent from O1 strains, but found in non-O1 V.cholerae strains with serotype O69 and O141

On the basis of the O139 DNA sequence a probe (probe F, Figure 1) was devised which contained O139 DNA but was devoid of O1 *rfb* sequences. Southern blots revealed that probe F hybridized to O139 DNA, but not to O1 DNA (Figure 4A). These results show that the O139 strain not only lacks O1 DNA, but that this has been replaced by genes which are not present in *V.cholerae* O1 strains.

To determine whether genes homologous to *otnAB* could be detected in other non-O1 *V.cholerae* strains, 60 different non-O1 isolates were screened with probe F. DNA from two non-O1 serotypes was found to hybridize with probe F; serotype O69, an Indian patient isolate, and several O141 strains, isolated from both patients and the environment in different parts of the USA (Figure 4A). Significantly, DNA fragments of identical size were detected in the O139, O69 and O141 strains, suggesting sequence conservation in this DNA region.

The otnAB region is associated with the rfbQRS element in O139 and O69 strains, but not in O141 strains

To determine whether the *otnAB* genes were also located adjacent to the *rfbQRS* element in the O69 and O141 strains, a PCR analysis was performed using primers 33 and 12, derived from the *otnA* and *rfbQ* sequence, respectively (Figure 2). Amplification of O139 DNA gave a 2000 bp DNA fragment, in accordance with the DNA sequence. No fragment was detected with O141 DNA, while a 2500 bp fragment was detected with O69 DNA. To confirm the identity of the 2500 bp DNA fragment, it was partially sequenced (results not shown). As expected,

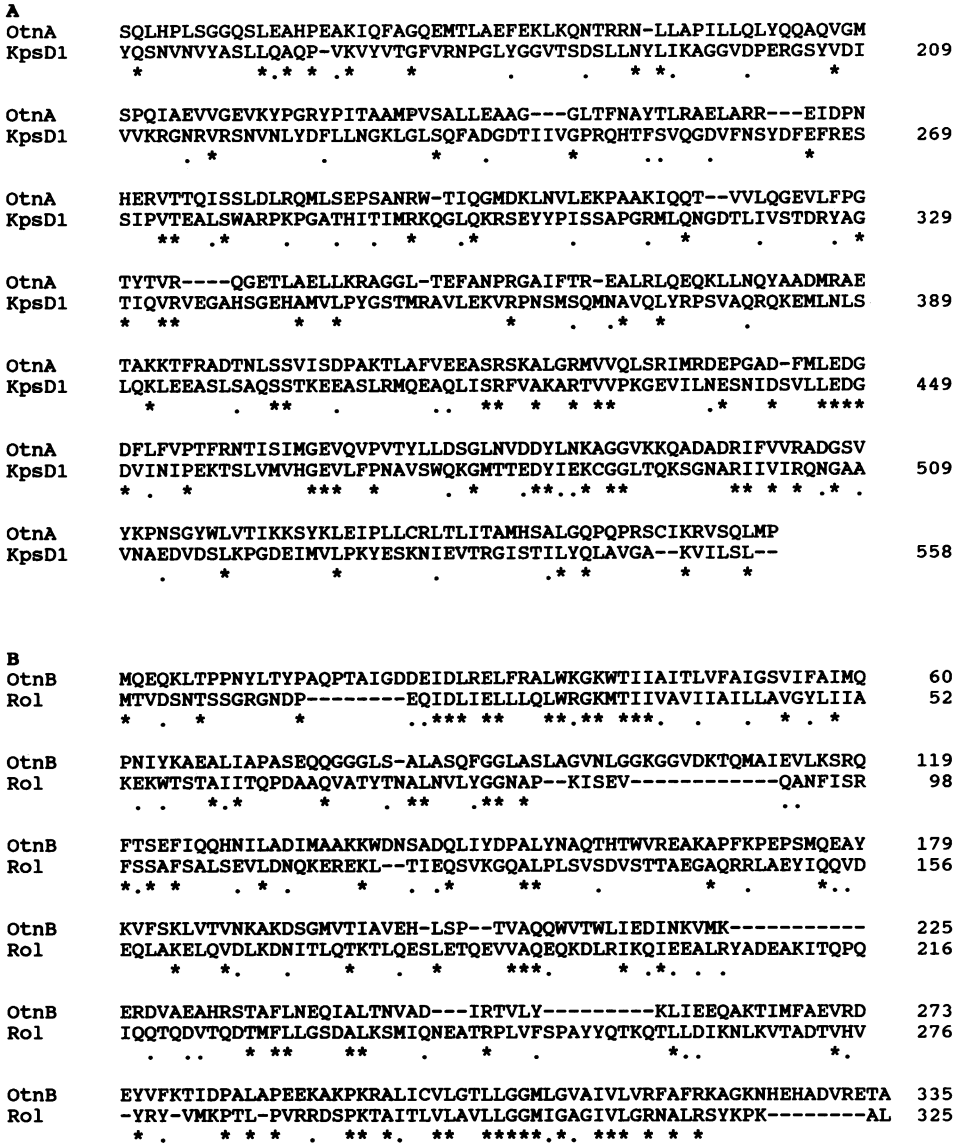

Fig. 3. Sequence similarity (**A**) between the C-termini of OtnA and KpsD1 and (**B**) between OtnB and Rol. KpsD1 is an *E.coli* protein involved in transport of K1 capsular precursors, whereas Rol is a *S.enterica* protein involved in regulation of O antigen chain length. Positions with similar or identical amino acid residues are indicated by · and *, respectively. Dashes have been introduced to increase the number of matches. The alignment scores between OtnA/KpsD1 and OtnB/Rol were both highly significant, being 8.1 and 11.4 SD above the mean score obtained with random sequence comparisons, respectively.

one end of the fragment was homologous to *otnA*, while the other end was homologous to *rfbQ*. However, small but significant differences were observed between the O139 and O69 sequences (results not shown). Thus it is unlikely that the O139 *otnAB–rfbQRS* region was derived from an O69 strain.

The absence of an amplification product in the O141 strains suggested that the *rfbQRS* element was missing. To confirm this possibility, Southern blots were prepared using a *rfbQRS* PCR fragment as a probe (probe D, Figure 1). *Hpa*II digested DNA revealed a single band with the O1 and O139 strains (Figure 5, lanes 1–3), while two bands were observed with the O69 DNA (Figure 5, lane 10). The presence of two hybridizing bands in strain O69 suggests that it contains two copies of the *rfbQRS* element. Alternatively, the two bands may be due to the fact that the O69 *rfbQRS* element contains a *Hpa*II site. No hybridization was detected with chromosomal DNA from the O141 strains (Figure 5, lane 5), demonstrating that the *rfbQRS* element is indeed absent from this strain. When other non-O1 strains were probed with the *rfbQRS* probe multiple hybridizing bands were occasionally detected (Figure 5). This suggests that *rfbQRS* may indeed be a mobile element.

Phylogenetic relationships between O1, O69, O141 and O139 strains

Recently, we identified a 623 bp IS element, designated IS*1004*, in *V.cholerae* O1 (unpublished data). IS*1004* is present in multiple copies in the chromosome of most *V.cholerae* strains, and revealed restriction fragment length polymorphism (RFLP) when used as a probe on *Hpa*II digested chromosomal DNA. We made use of the fact that the genetic relatedness between strains can be derived from such polymorphism (Lawrence *et al.*, 1989). The RFLP patterns generated by the IS*1004* probe indicated

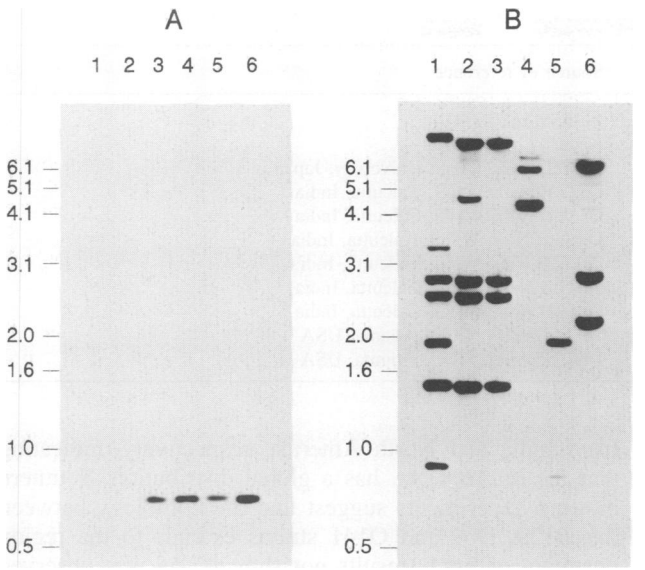

Fig. 4. (**A**) Occurrence of *otnAB* DNA among *V.cholerae* O1 and non-O1 strains. Chromosomal DNA was digested with *Hpa*II and hybridized with probe F (Figure 1). (**B**) RFLP patterns of *V.cholerae* O1 and non-O1 strains. The membrane used in A was reprobed with IS*1004*. Lane 1, O1 classical; lane 2, O1 El Tor; lane 3, O139; lane 4, O69; lane 5, O141 (patient isolate); lane 6, O141 (environmental isolate). Mol. wt markers are indicated on the left (in kbp).

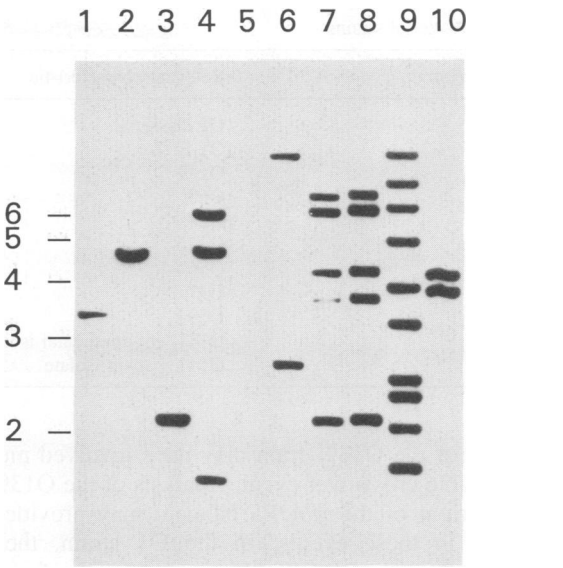

Fig. 5. Occurrence of the *rfbQRS* element in *V.cholerae* strains. Chromosomal DNA was digested with *Hpa*II and probed with a PCR fragment derived from the O1 *rfbQRS* element (probe D, Figure 1). Lane 1, O1 classical; lane 2, O1 El Tor; lane 3, O139; lane 4, O60; lane 5, O141; lane 6, O2; lane 7, O45; lane 8, O56; lane 9, O37; lane 10, O69. Mol. wt markers are indicated on the left (in kbp).

that the classical, El Tor and O139 strains were very closely related (Figure 4B). The closest similarity was found between the patterns of the O139 and El Tor strains, which were identical with the exception of a 4500 bp *Hpa*II fragment that was absent from the O139 strain. The O69 and O141 strains showed a banding pattern completely different from that of the O1 and O139 strains. These results are consistent with the hypothesis that the O139 strain did not arise by modification of O69 or O141 strains but was derived from an El Tor or El Tor-like strain.

Since the 4500 bp *Hpa*II fragment constituted the only difference between the O1 and the O139 strain, we surmised that this fragment was derived from a part of the O1 *rfb* cluster which contained a copy of IS*1004* and which was absent from the O139 strain. A homology search confirmed these assumptions and a fragment of IS*1004*, comprising 185 bp, was found in a region of the *rfb* DNA that was absent from the O139 strain. Interestingly, the IS*1004* fragment was located immediately to the left of the putative IS element comprised of *rfbQRS* (Figure 2).

Discussion

Our findings are consistent with the hypothesis that the novel *V.cholerae* O139 strain arose from an O1 El Tor strain by horizontal gene transfer of a DNA region, designated *otn*, involved in polysaccharide synthesis. The *otn* DNA probably originated from a non-O1 *V.cholerae* strain, and replaced a large part of the O1 *rfb* genes by (homologous) recombination, a process mediated by the *rfbQRS* element. This genetic event may have contributed to the present cholera epidemic occurring in Asia.

The *otn* DNA is flanked by regions homologous to O1 *rfb* DNA, and these regions may have mediated the insertion of *otn* DNA into the O1 chromosome. One of these homologous regions is composed of the *rfbQRS* genes, and a number of observations suggests that these genes comprise an IS element which mediates DNA rearrangements. First, like most IS elements, the *rfbQRS* genes are flanked by inverted repeats. Further, the *rfbQRS* element is homologous to the H-rpt (*Hin*c repeat), associated with the *rhs* elements of *E.coli* (Zhao *et al.*, 1993; Manning *et al.*, 1994). The *rhs* elements were discovered as hot spots for DNA rearrangements in *E.coli*, and provide homology for *recA*-dependent intrachromosomal recombination (Zhao *et al.*, 1993). Recently, an element homologous to the H-rpt has also been found in *S.enterica* (Xiang *et al.*, 1994). As is the case for *V.cholerae*, the *S.enterica* H-rpt was not associated with a *rhs*-like element and was located within a gene cluster involved in O antigen synthesis. It was proposed that the *S.enterica* H-rpt had been involved in intraspecific recombination between *rfb* sequences (Xiang *et al.*, 1994). We have detected the *rfbQRS* element in most *V.cholerae* strains analysed, generally in one copy, although some strains contained multiple copies. The fact that these elements are found in varying copy numbers in three species (but not within all members of those species) suggests that they indeed represent IS elements. Taken together these observations strongly argue for a role of the *rfbQRS* element in the genesis of the O139 strain. It has been suggested that the *rfbQRS* element has also played a role in the evolution of O1 antigen genes by placing *rfbT* in juxtaposition to the O1 antigen synthesis genes (Manning *et al.*, 1994). There is sequence divergence between the O1 and O139 *rfbQRS* element, and this suggests that the O139 element was not part of the recipient strain, but was co-transferred with the *otn* DNA. In the O139 strain, O1 *rfb* sequences have been deleted on both sides of the *rfbQRS* element suggesting that the

Table I. Bacterial strains

V.cholerae strain	Relevant characteristic	Source or reference
395	O1 classical	Cash *et al.* (1974)
JBK70	O1 El Tor	Kaper *et al.* (1984)
MO45	O139	Dr Y.Takeda, Kyoto University, Japan
SG2	O60	Dr G.B.Nair, WHO, Calcutta, India
SG4	O2	Dr G.B.Nair, WHO, Calcutta, India
SG6	O45	Dr. G.B.Nair, WHO, Calcutta, India
SG7	O56	Dr. G.B.Nair, WHO, Calcutta, India
SG8	O37	Dr G.B.Nair, WHO, Calcutta, India
SG10	O69	Dr G.B.Nair, WHO, Calcutta, India
3176–78	O141, environmental isolate	Dr J.C.Feeley, CDC, Atlanta, USA
609–84	O141, patient isolate	Dr J.C.Feeley, CDC, Atlanta, USA

genesis of the O139 strain may have involved more than one double cross-over event. Analysis of the O139 region to the right of the *rfbQRS* element may provide further insight in these events. In the O1 strain, the region proximate to the *rfbQRS* element contains a fragment of IS*1004* (Figure 2). Involvement of IS*1004* in the insertion of the *otn* DNA seems less likely at present because, in contrast to the *rfbQRS* element, IS*1004* is not associated with *otn* DNA in the O139 and O69 strains (results not shown).

A comparison of cell wall polysaccharides from O1 and O139 strains has revealed important differences. First, the O antigen of the O139 strain has a much shorter chain length compared with O1 strains, suggesting that genes required for O antigen synthesis may be missing or inactive in the O139 strain (Manning *et al.*, 1994). Further, the cell wall of the O139 strain contains colitose, a sugar not hitherto reported as constituent of *V.cholerae* (Hisatsune *et al.*, 1993). Conversely, perosamine, a characteristic component sugar of the O1 cell wall, is absent from O139 strains (Hisatsune *et al.*, 1993). Another important difference between the two strains is that, in contrast to O1 strains, the O139 strain is capsulated (Johnson *et al.*, 1994). In this respect, the O139 strain is similar to other non-O1 strains which are able to produce a polysaccharide capsule (Johnson *et al.*, 1992). Thus it is plausible that the genesis of strain O139 involved acquisition of DNA involved in capsule synthesis. This assumption is confirmed by sequence analysis of the *otn* DNA, which revealed two open reading frames, *otnA* and *otnB*, the predicted products of which show homology with proteins involved in synthesis of capsule and O antigen synthesis, respectively. The presence of a capsule may permit the O139 to cause invasive disease, as has been observed with other capsulated *V.cholerae* strains (Safrin *et al.*, 1987). In fact, the O139 strain has been isolated from the blood stream (Jesudason *et al.*, 1993). Using transposon analysis Waldor and Mekalanos (1994b) have identified a locus in the O139 strain involved in O139 antigen synthesis. No sequence data of this region were presented, but the region was shown to be absent from O1 strains. It is possible that the identified locus is located in the *otn* region. Further studies are required to determine whether the O1 and O139 strains differ in other chromosomal regions than the O1 gene cluster.

DNA homologous to *otnAB* DNA was found in two of 60 non-O1 *V.cholerae* strains analysed. The two strains expressed the serotypes O69 and O141 and were isolated from India and North America, respectively, indicating that the *otnAB* DNA has a global distribution. Southern blotting experiments suggest that the homology between the O139, O69 and O141 strains extends to the region upstream of *otnA* (results not shown). As was observed for the O139 strain, the *otnAB* DNA was associated with the *rfbQRS* element in the O69 strain. The O141 strain did not contain the *rfbQRS* element. Thus with respect to the *otnAB–rfbQRS* junction, the O139 strain is more similar to the O69 strain than to the O141 strain. However, both PCR analysis and sequence analysis indicated that the *otnAB–rfbQRS* regions are not identical in the O139 and O69 strains. Thus it seems unlikely that the O139 *otnAB* DNA originated from an O69 strain, although our results strongly suggest that another non-O1 *V.cholerae* strain was the donor.

Antibodies directed against the O1 antigen confer protective immunity against cholera (Guinée *et al.*, 1988), and in endemic areas most adults have acquired a degree of natural immunity against *V.cholerae* O1 strains. Such conditions may facilitate the emergence of novel strains, like O139, which combine the epidemic potential of the O1 serotypes with the ability to circumvent acquired immunity by expressing new cell surface polysaccharides. Indeed, field observations (Albert *et al.*, 1993b) and studies with rabbits (Albert *et al.*, 1994) have indicated that immunity to *V.cholerae* O1 does not protect against *V.cholerae* O139. Cholera has been endemic in the Ganges delta since recorded history and it seems likely that the emergence of strains with altered cell surface polysaccharides has also occurred in the past. The displacement of one type of strain by an other type is not without precedent. The causative agent of the fifth and sixth cholera pandemics, the classical biotype of *V.cholerae* O1, has been replaced by the El Tor biotype of *V.cholerae* O1 which is causing the current, seventh, pandemic (Barua, 1992). The El Tor strain is sturdier than the classical strain, and causes more inapparent infections. Both factors have probably contributed to the dissemination of this strain. The O139 strain may have retained these features, and increased its fitness further by its ability to infect humans immune to *V.cholerae* O1, and possibly also by its ability to invade the blood stream. *Vibrio cholerae* O139 is replacing O1 strains in some areas, and it has been suggested that the O139 strain may cause the eighth cholera pandemic (Garg *et al.*, 1993; Jesudason and John, 1993). Effective vaccines against O1 strains have been developed and are being tested in field trials (Holmgren

et al., 1994; Levine *et al.*, 1994). The study of genes involved in O antigen and capsule synthesis in O1 and O139 strains may facilitate the development of vaccines effective against both O1 and O139 *V.cholerae*.

Materials and methods

Strains and culture conditions

Bacterial strains used are listed in Table I. *Vibrio cholerae* strains were cultured on 5% sheep blood agar or in nutrient broth (Difco).

DNA techniques

Unless stated otherwise, DNA techniques were performed following the protocols of Ausubel *et al.* (1987).

Amplification of DNA

Chromosomal DNA (10 ng/μl) of *V.cholerae* strains O1, biotype El Tor and O139 was used for PCR amplification with different primer pairs. Twenty-five cycles of PCR amplification (1 min 94°C, 1 min 60°C and 1.5 min 72°C) were performed with a ThermoCycler (Perkin Elmer-Cetus). Amplified fragments were analysed by electrophoresis on a 0.8% agarose gel. A schematic representation of primer positions is depicted in Figure 1. The primers were derived from the DNA sequence of the *rfb* cluster of *V.cholerae* strain O17, serotype O1, biotype El Tor (Stroeher *et al.*, 1992). The sequences of the primers correspond to the following positions in the *rfb* DNA sequence (the gene harbouring the sequence is indicated in parentheses): primer 12, 15908–15887 (*rfbQ*); primer 15, 14915–14895 (region between *rfbN* and *rfbO*); primer 16, 16373–16393 (*rfbR*); primer 17, 16767–16747 (*rfbR*); primer 18, 14013–14033 (*rfbN*); primer 27, 4273–4291 (*rfbB*); primer 28, 7072–7054 (*rfbG*); primer 31, 55–74 (left of *rfaD*); primer 32, 3011–2992 (*rfbA*); primer 49, 17231–17252 (*rfbT*); primer 50, 18028–18007 (*rfbT*). Primer 33 (5′-TGCAAGGGGAAGTGTTATTT-3′) was derived from the O139 *otnA* sequence.

PCR conditions using KlenTaq-LA were as described by Barnes (1994). Products obtained were analysed on a 0.5 % agarose gel. DNA sequencing and hybridization with the O1-specific probe B and the O139-specific probe F (Figure 1) confirmed the specificity of the PCR.

Cloning and sequencing of O139 DNA

O139 DNA was cloned using inverse PCR (Rich and Willis, 1990). Chromosomal DNA of *V.cholerae* O139 was digested with restriction enzyme *Dra*I and self-ligated. Subsequently, the ligation mixture was used for inverse PCR with primers 20 and 26, based on base pair positions 16393–16373 and 16405–16425, respectively, of the O1 *rfb* sequence. Twenty-five cycles of PCR amplification (30 s 94°C, 1 min 60°C, 3 min 72°C) yielded a 1400 bp product. This fragment was blunted, cloned and sequenced. Based on this sequence, two new primers, 29 (5′-CCATTGTGTTGGTCAGGTT-3′) and 30 (5′-GTTTGGGTTTGGCTTTTTC-3′) were chosen for further inverse amplification. Chromosomal DNA of *V.cholerae* O139 was digested with *Cla*I, self-ligated and amplified with primers 29 and 30, as described above. A 2200 bp product was obtained and used as a probe to screen cloned chromosomal *Cla*I fragments from the O139 strain. A positive clone with a 2200 bp *Cla*I insert was obtained, which was sequenced on both strands. The sequence of other DNA regions was determined on cloned PCR fragments or directly on amplified chromosomal DNA.

DNA sequencing was performed with an ABI 373A DNA sequencer according to the instructions provided by the manufacturer (Applied Biosystems, CA, USA). The sequences reported in this study are available from the EMBL database under the accession number X79822.

Preparation of probes

When used as a probe, PCR fragments were purified by gel electrophoresis and labelled with horseradish peroxidase using the ECL system (Amersham, UK). The IS*1004*-specific probe was obtained by amplifying an internal IS*1004* fragment with the primers 5′-ATAAAAATCCGCCTTCTTAG-3′ and 5′-ATTGTCATCCCTAAACCACC-3′. Probe F consisted of a 889 bp *Hin*dIII fragment derived from the cloned *Cla*I fragment (Figure 1). The *Hin*dIII fragment was purified by gel electrophoresis and labelled with horseradish peroxidase.

Southern blotting

Chromosomal DNA from *V.cholerae* strains was digested with *Hpa*II or *Dra*I, fractionated on a 1% agarose gel and transferred to a Hybond N^+ membrane (Amersham, UK). Hybridization with horseradish peroxidase-labelled probe and detection of hybridizing bands was carried out according to the procedure provided by the manufacturer of the ECL system (Amersham, UK).

Homology searches

Searches and statistical significance of alignments scores were performed with the FASTA16 package (Pearson and Lipman, 1988). GenBank release 37 was used for the homology searches.

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