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Molecular characterisation of rough variants of *Vibrio cholerae* isolated from hospitalised patients with diarrhoea

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Seven rough isolates of *Vibrio cholerae* isolated as the sole infecting agent from patients with cholera-like diarrhoea were examined for the presence of the regulatory element *toxR* and certain virulence-associated genes of the CTX genetic element and *V. cholerae* pathogenicity island (VPI). Multiplex PCR analysis with *wb*-specific genes of either O1 or O139 origin showed that six of the seven isolates produced an O1 *wb*-specific amplicon and the remaining isolate produced an O139-specific amplicon. Analysis of lipopolysaccharide profiles of smooth variants of *V. cholerae* revealed the presence of long repeated units of 'O' polysaccharide side chains but all the rough variants appeared to be devoid of the latter and possessed only core oligosaccharide. PCR amplification with primers specific to the *ctxA*, *ctxB*, *tcpA*, *tagA*, *int*, *aldA*, *toxT*, *LJ*, *RJ* and *toxR* genes revealed that six of the seven rough isolates were positive for these genes. One isolate was found to be negative for *tagA* and *RJ*, indicating the presence of an altered VPI. Each of these isolates showed media-dependent expression of cholera toxin (CT) and produced more toxin than the reference *V. cholerae* O1 El Tor strain VC20 or O139 strain SG24 under comparable conditions. Studies on the clonality of these isolates by the analysis of rRNA genes indicated their relatedness to strains of *V. cholerae* O1 El Tor or O139, isolated during the same time period.

Introduction

Vibrio cholerae, the aetiological agent of cholera, is classified on the basis of the heat-stable somatic 'O' antigen into more than 193 serogroups [1]. The antigenic specificity of the repetitive units of the polysaccharides ('O' antigen) of the lipopolysaccharide (LPS) forms the basis of the *V. cholerae* serotyping scheme [2]. The use of flagellar (H) antigen has no practical applicability in this scheme, as all strains of a given *Vibrio* species share an identical H antigen [3]. Traditionally, strains belonging to serotype O1 were responsible for epidemics and pandemics of cholera [4, 5]. In late 1992, *V. cholerae* strains which did not agglutinate with the 'O' specific antisera then available emerged as the causative agent of epidemic cholera and were later designated as O139 Bengal [6]. *V. cholerae* O139 Bengal strains possess virulence factors common

to O1 El Tor and cause diarrhoea which is clinically indistinguishable from serotype O1 cholera [7, 8]. Molecular epidemiological analyses such as zymovar analysis, ribotyping and pulsed-field gel electrophoresis (PFGE) showed that *V. cholerae* O139 Bengal resembles the seventh pandemic O1 El Tor biotype [9, 10]. In contrast to serotype O1, *V. cholerae*, O139 Bengal strains possess a short truncated form of LPS and are characterised by the presence of a capsular polysaccharide material [11].

To establish infection in the gut, vibrios express a number of co-ordinately regulated virulence factors, the most important of which are cholera toxin (CT), toxin co-regulated pilus (TCP) and the global regulatory element ToxR. Genes for CT (*ctxAB*) have been shown to be located on a 4.5-kb DNA segment called 'core region', flanked by one or more copies of a direct repeat sequence (RS) of 2.4–2.7 kb [12]. Recently, it has been demonstrated that a region of *c.* 7 kb, comprising the core region and RS2, is not an integral part of the *V. cholerae* genome but is acquired by the site-specific lysogenic conversion by a filamentous bacteriophage CTX ϕ [13]. The genes for the expression of TCP, the important pilus colonisation factor,

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have been shown to be present along with other putative virulence genes on a 39.5-kb DNA segment collectively known as *V. cholerae* pathogenicity island (VPI) [14]. Recent studies have also shown that *V. cholerae* acquired VPI by lysogenic conversion by another filamentous phage VPI ϕ [15].

V. cholerae, non-O1, non-O139 represent heterogeneous serogroups, which so far have not been recognised as having epidemic potential and are generally devoid of most of the virulence genes associated with the epidemic-causing serogroups (O1 and O139) of *V. cholerae*. However, the association of non-O1, non-O139 *V. cholerae* with sporadic cases of diarrhoea has been reported [16]. By biochemical tests, *V. cholerae* strains belonging to O1 and O139 serogroups are indistinguishable from their non-O1, non-O139 counterparts except for their ability to be agglutinated by specific antisera and their sensitivity to specific lytic phages [3, 16]. Smooth variants of a given species in the genus *Vibrio* are characterised by the presence of both smooth (S; O antigens) and rough (R) antigens, while rough variants express only the R antigens. The exact genetic basis for the generation of a rough variant from a corresponding smooth variant or *vice versa* is not clearly understood at present. Serological studies have shown that *V. cholerae* strains possess identical R antigens irrespective of their O antigenic types [17] which need to be absorbed in the preparation of O-specific antiserum. Organisms expressing only the R antigen are usually indistinguishable from the corresponding S-form by colony morphology and biochemical tests, but may be differentiated by agglutination with rough antiserum [3].

Many studies have focused on the biochemical constituents of R antigens of *V. cholerae* [18–21]. However, very limited information is available on the characterisation of the virulence genes of the rough variants of *V. cholerae*, particularly those isolated from clinical cases. Existing data on rough mutants of wild-type smooth virulent strains of *V. cholerae* show that these mutants are comparatively less virulent when tested in animal models [22], which has been attributed to their severe defect in small bowel colonisation properties when tested in the infant mouse model [23, 24]. This study describes the extensive molecular characterisation of seven rough variants of *V. cholerae* isolated as the sole infecting pathogen from cholera-like cases.

Materials and methods

Bacterial strains

Seven rough isolates of *V. cholerae* from the stools of hospitalised patients with cholera-like disease were included in this study. They were isolated as the sole pathogen from patients from different parts of India, as shown in Table 1. These isolates were indistinguishable

from smooth variants of *V. cholerae* by biochemical tests, but agglutinated only with specific rough antiserum. Reference *V. cholerae* O1 classical biotype strain O395. El Tor biotype strains VC20 and MO1 and the O139 strain SG24 were included for comparison. All the isolates and reference strains were maintained in nutrient agar (NA) as stabs at room temperature.

Analysis of bacterial LPS

LPS profiles of the rough isolates and the reference strains of *V. cholerae* were analysed by SDS-PAGE of proteinase K-treated whole-cell lysates [25]. After electrophoresis, polysaccharide materials were fixed in the gel by treatment with ethanol-acetic acid fixative and silver stained [26] to visualise the LPS profiles.

Detection of virulence genes by PCR

Purified genomic DNA (c. 50 ng) were used as the template for PCR experiments with 0.75 units of Taq DNA polymerase (Takara, Kyoto, Japan) in a final reaction volume of 25 μ l. In all PCR assays except for multiplex PCR with *ctxA*- and *wb*-specific primers, each primer and each of four dNTPs were used at concentrations of 1 μ M and 200 μ M, respectively, and 30 amplification cycles were performed. Nucleotide sequences of all primers and conditions for PCR are presented in Table 2 [27–32]. Multiplex PCR experiments for the detection of *ctxA*- and *wb*-specific genes of either O1 or O139 origin were performed with specific primers to these genes as described previously [27]. The presence of *ctxA* and *tcpA* in *V. cholerae* strains was also detected by multiplex PCR assay with primers specific to these genes. In separate sets of PCR experiments, specific primer pairs were used for the detection of *ctxB*, the regulatory gene *toxR* and genes comprising the *V. cholerae* pathogenicity island (VPI) including *tagA*, *int*, *aldA*, *toxT*, *LJ* and *RJ*. Amplicons obtained with each set of PCR experiments were resolved with agarose gels, UV irradiated and documented with the Gel-Doc 2000 system (BioRad Laboratories, Hercules, CA, USA).

CT assay

The ability of *V. cholerae* strains to produce CT and the amount produced were determined by the GM₁-ELISA assay with cultures grown in LB (pH 6.5) or AK1 (pH 7.4) broth at 30°C for 16 h [33, 34]. Cell-free culture supernates were added to wells of a microtitration plate (Nunc, Kamstrup, Roskilde, Denmark) coated with GM₁ (monosialoganglioside) (Sigma). Plates were sequentially treated with appropriately diluted (1 in 100) rabbit anti-CT serum, anti-rabbit Ig peroxidase conjugate (Sigma) and developed with the substrate solution containing O-phenylenediamine dihydrochloride 1 mg/ml and H₂O₂ 0.12%. For each set of experiments, known amounts of purified CT (Sigma) were used to generate a standard curve from which the

Table 1. Characteristics of clinical isolates of *V. cholerae* used in this study

			Detection of virulence genes*												
			wb of		ctxφ		VPI							Regulatory element	
							tagA	int	aldA	tcpA [†]	LJ	RJ			
Isolates	Place/date of isolation	Serogroup	O1	O139	ctxA	ctxB	tagA	int	aldA	tcpA [†]	LJ	RJ	toxT	toxR	
ALO45	Allepy 06.08.96	Rough	+	—	+	+	+	+	+	+	+	+	+	+	
ALO46	Allepy 06.08.96	Rough	+	—	+	+	+	+	+	+	+	+	+	+	
MO77	Madras 05.11.92	Rough	—	+	+	+	+	+	+	+	+	+	+	+	
MO99	Madras 05.11.92	Rough	+	—	+	+	+	+	+	+	+	+	+	+	
DO66	Delhi 19.03.97	Rough	+	—	+	+	+	+	+	+	+	+	+	+	
DO70	Delhi 19.03.97	Rough	+	—	+	+	+	+	+	+	+	+	+	+	
SG15	Calcutta 20.11.92	Rough	+	—	+	+	—	+	+	+	+	—	+	+	
MO1 [‡]	Madras 05.11.92	O1	+	—	+	+	+	+	+	+	+	+	+	+	
SG24	Calcutta 14.12.92	O139	—	+	+	+	+	+	+	+	+	+	+	+	
VC20 [‡]	Calcutta 07.05.92	O1	+	—	+	+	+	+	+	+	+	+	+	+	

+, amplicon of similar size to that of the control; —, no amplicon.

*Tested by PCR analysis with specific primers.

[†]All these strains produced *tcpA* amplicons similar to that of O1 El Tor strain.

[‡]El Tor biotype.

Table 2 Nucleotide sequence of primers and the PCR conditions used in this study

Gene	Primer	Primer sequence (5'–3')	PCR conditions			Ref. no.
			Denaturing	Annealing	Extension	
<i>wb</i> O1	O1F2-1	GTTCCTACTGAACAGATGGG	94°C, 60 s	55°C, 60 s	72°C, 60 s	27
	O1R2-2	GGTCATCTGTAAGTACAAC				
<i>wb</i> O139	O139F2	AGCCTCTTTATTACGGGTGG	94°C, 60 s	55°C, 60 s	72°C, 60 s	27
	O139R2	GTCAAACCCGATCGTAAAGG				
<i>ctxB</i>	<i>ctxB</i> -F	GGTTGCTTCTCATCATCGAACCAC	94°C, 60 s	55°C, 90 s	72°C, 90 s	28
	<i>ctxB</i> -R	GATACACATAATAGAATTAAGGAT				
<i>ctxA</i>	<i>ctxA</i> 1	CTCAGACGGGATTTGTTAGGCACG	94°C, 90 s	60°C, 90 s	72°C, 90 s	29
	<i>ctxA</i> 2	TCTATCTCTGTAGCCCCCTATTA CG				
<i>tcpA</i> classical	<i>tcpA</i> -F	CACGATAAGAAAACCGGTCAA GAG	94°C, 90 s	60°C, 90 s	72°C, 90 s	29
	<i>tcpA</i> -R	ACCAAATGCAACGCCGAATGGAGC				
<i>tcpA</i> El Tor	<i>tcpA</i> -F	GAAGAAGTTGTAAAAGAAGA ACAC	94°C, 90 s	60°C, 90 s	72°C, 90 s	29
	<i>tcpA</i> -R	GAAAGGACCTTCTTTACGTTG				
<i>toxR</i>	<i>toxR</i> -F	CGGGATCCATGTTCCGATTAGGACAC	94°C, 30 s	64°C, 30 s	72°C, 30 s	30
	<i>toxR</i> -R	CGGGATCCTACTCACACACTTTGATGGC				
<i>toxT</i>	<i>toxT</i> -F	ACTGTCGACGCAAAGCATATTCAGAGA	94°C, 40 s	55°C, 40 s	72°C, 90 s	31
	<i>toxT</i> -R	CGCGGATCCATAACAATCGAAAATAGGA				
<i>tagA</i>	<i>tagA</i> -F	GGTGGTAAGATATTCAGTTAATG	94°C, 40 s	55°C, 40 s	72°C, 40 s	14
	<i>tagA</i> -R	GAGACATCTATAGAATACTGG CTG				
<i>int</i>	<i>int</i> -F	GAAGTAATGAAACCGATAAGTGG	94°C, 40 s	55°C, 40 s	72°C, 40 s	32
	<i>int</i> -R	TGCTTTGTACCAAGTCACAGATAG				
<i>aldA</i>	<i>aldA</i> -F	GCCTCTGATTATGACCAAGAATAGG	94°C, 40 s	55°C, 40 s	72°C, 90 s	This study
	<i>aldA</i> -R	GTCAATGGATGAAGCCACACAGTG-				
<i>LJ</i>	<i>LJ</i> -F	GTGAATCTTGATGAGACGCTC TG	94°C, 40 s	55°C, 40 s	72°C, 60 s	This study
	<i>LJ</i> -R	GGTGAGCCAGGCTTATTGGG				
<i>RJ</i>	<i>RJ</i> -F	TCGTTAGCGTGTCTCGGTTTCGAGG	94°C, 40 s	55°C, 40 s	72°C, 90 s	This study
	<i>RJ</i> -R	TGCTTTGTACCAAGTCACAGATAG				

amount of CT in the test samples was interpolated. Results were expressed as μg of CT produced/ml of supernate/opacity unit (at 540 nm) of the bacterial cell suspension.

Southern hybridisation analysis of rrn genes (ribotyping)

Genomic DNA from *V. cholerae* strains was prepared by the cetyltrimethylammonium bromide-phenol extraction method [35] from 3 ml of overnight cultures grown in LB broth at 37°C. Purified DNA thus obtained was subjected to enzymic digestion with the restriction endonuclease *Bgl*I and electrophoresed on an agarose 0.8% (w/v) gel. Electrophoresed DNA fragments were transferred to a nylon membrane (Hybond-N⁺; Amersham Life Science, Little Chalfont, Bucks) and allowed to hybridised with the DNA probe specific for *rrn* genes. The probe for *rrn* genes consisted of a 7.5-kb *Bam*HI fragment of the recombinant plasmid pKK3535 containing the r-RNA operon of *Escherichia coli* [36]. The probe was labelled with a chemiluminescent dye (ECL Labelling Kit, Amersham) and the probe-treated membrane was developed according to the manufacturer's protocol (ECL Detection Kit, Amersham).

Computer-assisted analysis of patterns

Quantity One software, version 4 (BioRad) was used to compare the hybridisation patterns obtained by *Bgl*I ribotyping of *V. cholerae* strains. The software clustered strains with similar ribotypes. Defined-similarity dendrograms were generated and used to join the clusters

by the neighbour-joining method. The pattern for each strain was checked by visual observation.

Results

LPS profiles of rough variants of V. cholerae

LPS profiles of the rough variants of *V. cholerae* along with strains belonging to O1 and O139 serogroups are presented in Fig. 1. The LPS profiles of rough variants (Fig. 1, lanes 1–7) were remarkably similar to each other, but differed considerably from those of the smooth variants belonging to either the O1 and O139 serogroups. LPS profiles of rough variants were found to be devoid of repetitive units of polysaccharides and were characterised by the presence of core oligosaccharides alone (indicated in Fig. 1). On the other hand, *V. cholerae* O1 strains belonging to El Tor biotype (Fig. 1, lanes 8 and 9) were characterised by the presence of long repetitive units of polysaccharides along with core oligosaccharides migrating to the same region as seen for the rough variants. The LPS profile of O139 strain SG24 appeared to be somewhat truncated in nature, having short repetitive units of polysaccharides with core oligosaccharides (Fig. 1, lane 10).

Presence of virulence genes in rough variants of V. cholerae

Purified genomic DNA isolated from rough variants of *V. cholerae* strains were analysed by PCR for the presence of virulence genes found in toxigenic *V. cholerae*. Results with specific pairs of primers for the virulence genes of *V. cholerae* are summarised in Table

Fig. 1. LPS profiles of proteinase K-treated whole cells of rough variants (lanes 1–7), O1 (lanes 8, 9) and O139 (lane 10) *V. cholerae* isolates: lane 1, ALO45; 2, ALO46; 3, MO77; 4, MO99; 5, DO66; 6, DO70; 7, SG15; 8, MO1; 9, VC20; 10, SG24. Position of core oligosaccharide is indicated by arrow.

1. Interestingly, six of the seven rough variants of *V. cholerae* (ALO45, ALO46, MO99, DO66, DO70 and SG15) produced an O1 *wb* gene-specific amplicon size of 192 bp (Fig. 2, lanes 1,2,4–7) similar to that obtained with reference O1 El Tor strains MO1 and VC20 (Fig. 2, lanes 8 and 9). Strain MO77, on the other hand, produced a 449-bp O139 *wb*-specific amplicon (Fig. 2, lane 3) similar to that obtained with reference O139 strain SG24 (Fig. 2, lane 10). Each of these strains produced a 301-bp *ctxA* amplicon. Multiplex PCR with *ctxA* and *tcpA* primers revealed that all seven rough variants were positive for these genes and the *tcpA* amplicons of these strains were of El Tor type (Table 1). Amplicon sizes of 350, 344, 1115, 1101, 1010, 1499, 460 and 900 bp were produced by all the rough variants of *V. cholerae* except SG15, when tested with the respective primers specific for *tagA*, *int*, *aldA*, *toxT*, *LJ*, *RJ*, *ctxB* and *toxR*. Strain SG15 was found to possess all these genes, but failed to produce an amplicon with primers specific to either *tagA* or *RJ* (Table 1), indicating that this strain has an altered VPI.

Production of CT by the rough variants of *V. cholerae*

CT production by the rough variants of *V. cholerae* was studied by growing the strains in either LB, or AKI

broth at 30°C for 16 h and measuring the amount of CT produced in the culture supernates by a GM₁-ELISA. The summarised results are presented in Table 3. The rough variants of *V. cholerae* produced CT in liquid culture in amounts that varied from isolate to isolate. Five of seven rough variants (ALO45, ALO46, MO77, MO99 and SG15) produced more CT in AKI medium than in LB medium. On the other hand, two strains (DO66 and DO70) produced more toxin in LB medium than AKI medium (Table 3). As expected, LB culture conditions favoured CT production by the O1 classical strain (O395) and CT production by El Tor (VC20 and MO1) and O139 (SG24) strains was favoured in AKI culture conditions.

Organisation of *rrn* genes in rough variants of *V. cholerae*

The organisation of *rrn* genes (ribotype) in the chromosome of the *V. cholerae* isolates was analysed by Southern hybridisation experiments with *Bgl*I-digested chromosomal DNA and the rRNA-specific DNA probe. Results generated in Southern blot experiments with the rough variants of *V. cholerae* are presented in Fig. 3. Reference *V. cholerae* O1 El Tor strains VC20 and MO1, and O139 strain SG24 showed ribotypes previously designated as RI, RII [37]

Fig. 2. Agarose gel electrophoresis of PCR amplicons obtained with primers specific for *ctxA* and *wb* of O1 and O139 *V. cholerae* isolates: lane 1, ALO45; 2, ALO46; 3, MO77; 4, MO99; 5, DO66; 6, DO70; 7, SG15; 8, MO1; 9, VC20; 10, SG24; 11, *E. coli* strain DH5 α as a negative control. Amplicon sizes of *ctxA* (301 bp), O1 *wb* (192 bp) and O139 *wb* (449 bp) are indicated.

Table 3. CT production by the rough isolates and reference strains of *V. cholerae* grown in different media

Strain or isolate no.	Serogroup	Amount of CT* produced in	
		AKI [†]	LB [‡]
ALO45	Rough	0.15	0.02
ALO46	Rough	0.14	0.02
MO77	Rough	0.2	0.04
MO99	Rough	0.24	0.03
DO66	Rough	0.15	0.56
DO70	Rough	5.80	8.6
SG15	Rough	0.21	0.02
MO1 [§]	O1	0.03	0.01
VC20 [§]	O1	0.08	0.06
O395	O1	0.04	0.6
SG24	O139	0.04	0.01
<i>E. coli</i> DH5 α	—	< 0.001	< 0.001

*Measured by GM₁-ELISA and expressed as μ g of CT produced/ml of supernate/unit opacity (540 nm) of bacterial suspension.

[†]Grown in AKI medium (pH 7.4) [34] at 30°C for 16 h.

[‡]Grown in LB medium (pH 6.5) at 30°C for 16 h.

[§]El Tor biotype.

^{||}Classical biotype.

and BI [38], respectively, and were included in Fig. 3 for comparison. The DNA probe specific to *rrn* genes hybridised with eight-to-ten bands ranging in size from 15.0 to 2.1 kb in each *V. cholerae* isolate and the patterns could be classified into six different ribotypes. It is evident from Fig. 3 that rough variant *V. cholerae* strain SG15 (Fig. 3, lane 7) produced ribotype identical to that of reference O1 strain VC20 (Fig. 3, lane 9; RI

Isolates ALO45 (Fig. 3, lane 1), ALO46 (Fig. 3, lane 2), DO66 (Fig. 3, lane 5) and DO70 (Fig. 3, lane 6) produced ribotypes identical to each other and belonged to the RIII ribotype of O1 El Tor strains which emerged after the 1992–1993 O139 epidemics [37]. On the other hand, isolate MO77 produced a ribotype pattern (Fig. 3, lane 3) (BII) [38] similar to the BI pattern obtained with reference O139 strain SG24 (Fig. 3, lane 10). Interestingly, the ribotype of isolate MO99 (Fig. 3, lane 4) appeared to be unique in nature as compared with the other *V. cholerae* isolates tested in this study.

Discussion

This study was initiated to understand the molecular characteristics of seven rough variants of *V. cholerae* isolated from hospitalised cases of cholera-like diarrhoea. Serogrouping of *V. cholerae* has been shown to be very useful in differentiating between epidemic and non-epidemic strains, with currently recognised epidemic strains belonging to either the O1 or O139 serogroups. However, the use and scope of serogrouping only applicable to the smooth variants of pathogenic bacteria including *V. cholerae* and has no, or very little, applicability to the rough variants. While much attention has been directed to study of the smooth variants of *V. cholerae*, very limited information is available regarding the virulence genes and their

Fig. 3. Southern hybridisation analysis of *Bgl*I-digested genomic DNA from *V. cholerae* strains with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. *V. cholerae* strains: lane 1, ALO45; 2, ALO46; 3, MO77; 4, MO99; 5, DO66; 6, DO70; 7, SG15; 8, MO1; 9, VC20; 10, SG24. Positions of λ HindIII molecular size markers are indicated.

expression in rough variants of *V. cholerae*, particularly those isolated as the sole pathogen from clinical cases.

LPS profiles of rough variants of *V. cholerae* (Fig. 1) as documented in this study, clearly established that these strains are devoid of any repetitive units of polysaccharides and the LPS profiles of rough variants are identical despite being different 'O' lineages, as shown from the *wb* analysis. Although the long repetitive units of polysaccharide form the basis of O antigenic specificity [2], an earlier study [39] demonstrated that with only the LPS core in the LPS profile, a non-O1, non-O139 strain (V315-1) was untypable but not rough [30]. Thus, the presence of only the core region in the LPS profile should not be construed as an exclusive phenotype of the rough characteristic. Interestingly, all these rough variants were found to be PCR positive with either O1 or O139 *wb* (Table 1) whereas environmental isolates of rough variants were shown to be negative in both O1 and O139 *wb* PCR (unpublished data). Earlier studies conducted in an animal mode with *wb* mutants demonstrated the role of LPS in the pathogenesis of cholera [23, 24]. Despite the loss of the functional expression of the O antigenic marker of epidemic *V. cholerae*, these strains appeared to retain gene(s) responsible for the expression of epidemic-related O antigen. Detection of either O1 or O139 *wb*-specific genes in these clinical isolates as compared with the environmental rough variants has immense significance from the epidemiological point of view.

All seven rough variants of *V. cholerae* were found to possess *ctxA* and *ctxB* and expressed CT in culture supernates (Table 3). The presence of *toxR* in these strains was also established by PCR (Table 1). *toxR* in these strains appeared to be functional, as evidence by the media-dependent regulation of CT expression observed in these isolates. However, the amount of CT produced by these isolates varied from strain to strain. While classical strain O395 produced more CT in LB, El Tor, O139 and five of the rough variants produced more CT in AKI medium. Interestingly, two rough strains DO66 and DO70, isolated from Delhi, India, produced more CT in LB than in AKI. All these strains produced less CT in nutrient broth than in either LB or AKI medium (data not shown). Interestingly, the amount of CT produced by the rough variants was much greater than that by O1 El Tor and O139 strains.

Epidemic isolates of *V. cholerae* O1 and O139 have been shown to possess the cluster of genes which comprise the VPI. As expected, clinical isolates of the rough variants studied here also possessed all the genes of the VPI with one exception, as evidenced by PCR with specific primers (Table 1). One of the seven rough strains (SG15) was found to be negative by PCR for *tagA* and *RJ*, whereas the others were positive for all the genes sought in this study. Results obtained in multiplex PCR with *tcpA* primers established that these

rough variants are related to either O1 El Tor or O139 strains. These data are consistent with the findings that regulation of CT expression by these isolates, except for two Delhi isolates, was similar to that of El Tor or O139 strains.

*Bgl*I-generated ribotypes obtained in this study also indicate that these isolates possess at least nine copies of the *rrn* operon in their genomes. However, this needs further investigation to establish clearly the exact copy number of the *rrn* operon. *rrn*-mediated recombination may be one of the mechanisms by which pathogenic bacteria can maintain their genomic plasticity or diversity, and the possibility of genome rearrangements is greater when there are more *rrn* operons in an organism [40]. In this context, the significant finding from the ribotyping analysis was that these rough variants could be grouped with smooth variants of *V. cholerae* (Fig. 4) and these results are in agreement with that of the *wb* PCR analysis. Dendrogram analysis, generated from ribotype patterns, clustered O1 El Tor strain VC20 with one rough variant SG15 which was isolated before the onset of the O139 epidemics in 1992. Strains isolated after the

Fig. 4. Dendrogram generated by the average percentage of matched bands summarising the degree of similarity of *Bgl*I-ribotypes of *V. cholerae* isolates.

onset of O139 epidemics, e.g., ALO45, ALO46, DO66 and DO77, clustered in another group closely related to O1 strain VC20 and had ribotype pattern (RIII) identical to those of the post-O139 El Tor O1 isolates [37]. These results are consistent with earlier findings which indicated that pre- and post-O139 O1 El Tor strains can be grouped on the basis of ribotype patterns [37]. As expected, dendrogram analysis placed SG24 and MO77 in the same lineage (Fig. 4). Furthermore, both the strains were positive with O139 *wb*-specific PCR (Fig. 2) and had similar but not identical ribotypes. The significance of these findings is yet to be elucidated. Perhaps horizontal transfer of genes including genes for O antigen biosynthesis may be facilitated in a strain with a 'rough' background. Interestingly, ribotypes of MO1 and MO99 which were O1 *wb* PCR-positive, appeared to be more closely related to the O139 strains and were grouped with the O139 cluster. This is consistent with an earlier report which described MO1 as the progenitor strain of *V. cholerae* O139 [41]. Whether or not these rough variants are derived from the prevailing epidemic strains or act as a potential source for generation of newer variants with increased virulence, or both, is an area which needs to be addressed.

The significance of the occurrence of the rough strains of *V. cholerae* is still not properly understood. Epidemiological and surveillance studies have shown that the appearance of the rough strains of *V. cholerae* normally precedes the period when a shift of serotype from either Inaba to Ogawa or *vice versa* is observed. One of the rough strains (SG15) included in this study was isolated just before the first isolation of *V. cholerae* O139 in Calcutta in Nov. 1992. It is tempting to speculate that reversion to a rough variant is a state that makes the organism more amenable to change, perhaps by becoming more permeable to intake of foreign DNA than its smooth counterpart.

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