Structural organization of the transfer RNA operon I of *Vibrio cholerae*: Differences between classical and El Tor strains

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Nine major transfer RNA (tRNA) gene clusters were analysed in various *Vibrio cholerae* strains. Of these, only the tRNA operon I was found to differ significantly in *V. cholerae* classical (sixth pandemic) and El Tor (seventh pandemic) strains. Amongst the sixteen tRNA genes contained in this operon, genes for tRNA Gln3 (CAA) and tRNA Leu6 (CUA) were absent in classical strains as compared to El Tor strains. The observation strongly supported the view that the above two pandemic strains constitute two different clones.

1. Introduction

Vibrio cholerae, a Gram-negative bacterium is the causative agent of the disease cholera. Nearly 200 serogroups (serovars) of this organism have been distinguished on the basis of epitopic variation in the cell surface lipopolysaccharide (LPS) (Yamai *et al* 1997). From an epidemiological standpoint, the species have been divided into serogroup O1 and serogroup non-O1 strains, which were long believed to differ in ability to cause epidemic cholera (Blake *et al* 1980; Colwell 1996). Historically, serogroup O1 strains, consisting of two biotypes classical and El Tor, have been responsible for all major epidemics, including seven pandemics (Karaolis *et al* 1995). The first six pandemics of cholera were caused by classical biotype strains (Pollitzer 1959); the seventh pandemic started in 1960s when the El Tor biotype replaced the classical strains from the global endemic scene (Faruque *et al* 1998). But in 1992 an epidemic clone of serogroup O139 (Bengal) also emerged as a new causative agent replacing the existing serogroup O1 El Tor strains (Ramamurthy *et al* 1993). However, the El Tor biotype reappeared in Calcutta replacing the O139 serogroup (Jesudason *et al* 1994; Nair *et al* 1994). The disappearance and reappearance of the El Tor biotype and its sub-

sequent predominance in areas in which O139 dominated in the preceding year are difficult to explain. Resurgence of O139 serogroup for a short period in 1996 in Calcutta added a new dimension to this problem (Sharma *et al* 1997b). Cholera thus is categorized as one of the "emerging and reemerging" infections (Satcher 1995). This novel feature of the disease raises a question regarding the genetic similarity or diversity of the different toxigenic clones. It was reported from this laboratory that *V. cholerae* El Tor strains isolated 'before' the serogroup O139 epidemic had different restriction fragment length polymorphism for ribosomal RNA genes (ribotype) than those isolated 'after' the O139 outbreak. Structural organization and chromosomal locations of the CTX genetic elements of these 'before' and 'after' strains were also different (Sharma *et al* 1997a). A similar analysis for the *V. cholerae* serogroup O139 strains revealed that the resurgent serogroup O139 strains of 1996 had wider drug resistance compared to the serogroup O139 strains of 1992–1993 and an unconventional structural organization of the CTX genetic element (Sharma *et al* 1997b). In the present investigation, an attempt has been made to reveal and to analyse the intra-biotype and inter-biotype variations amongst O1 classical, O1 El Tor (isolated before, during and after the serogroup O139 outbreak) and O139 (isolated

in 1993 and 1996) strains. With the availability of the complete nucleotide sequence of the genome of the *V. cholerae* El Tor strain N16961 (Heidelberg *et al* 2000) rapid and reliable determination of intra- and inter-genomic sequence heterogeneity have become easier. Here we have analysed the sequence variation in transfer RNA (tRNA) gene clusters that are present as independent operons. Data presented here have revealed that there is a significant difference in one tRNA operon (tRNA operon I) present in serogroup O1 classical, El Tor and serogroup O139 strains.

2. Materials and methods

2.1 *Bacterial strains, media and growth conditions*

V. cholerae strains used in this study are given in table 1 and were obtained from National Institute of Cholera and Enteric Diseases, Kolkata. All the strains were clinical isolates. The strains were routinely grown and maintained as described before (Sharma *et al* 1997a).

2.2 *DNA isolation and polymerase chain reaction amplification*

Chromosomal DNA was isolated from bacterial cultures as described before (Kar *et al* 1996). Polymerase chain reactions (PCRs) were carried out using the Perkin Elmer GenAmp2400 system. A pair of oligonucleotides, the forward primer P1 (5′ CGCCTCTTTGTTTAAAAAAAA CCCG 3′) and the reverse primer P2 (5′ GCTTAGCTT CCCGCCGGAAAGCG 3′) was used to amplify the tRNA-operon I. Chromosomal DNAs isolated from the different *V. cholerae* strains (table 1) were used as templates. The reaction was performed in a total volume of 50 μl containing 40 pmol of each primer, 200 μM (each) deoxynucleoside triphosphate (dNTP), and 2 units of Taq polymerase (Roche Applied Science) in 1X reaction buffer. PCR was carried out using the following programme: (i) initial extensive denaturation step of 3 min at 94°C; (ii) 30 reaction cycles of denaturation for 30 s at 94°C, annealing for 45 s at 60°C, and extension for 2 min at 72°C and (iii) a final extension step of 7 min at 72°C. Amplified products were electrophoresed on 1% agarose gels and stained with ethidium bromide.

2.3 *Restriction digestion*

PCR amplified DNAs were purified using the QIAquick PCR purification kit (Qiagen) and were digested with the restriction enzyme *Eco*RI at 37°C according to the instructions of the manufacturer (New England Biolabs).

Prior to loading on the gel, the digests were heated at 65°C for 10 min and quenched in ice to melt the hydrogen-bonded overlaps. Restriction fragments were separated by electrophoresis on 1% agarose horizontal slab gel formed in 90 mM Tris, 90 mM Boric acid (pH 8·2) and 2 mM EDTA. Bands were visualized by staining with ethidium bromide.

2.4 *DNA sequencing*

PCR products purified as mentioned above were subjected to automated DNA sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, Conn., USA) and an ABI 377 automated DNA sequencer using three primers, forward (P1), reverse (P2) as given above and the primer P3 (5′ CGCTCG AAAAGATGGCTACGTAGCT 3′) designed from sequence internal to the N16961 tRNA operon I.

3. Results and discussion

3.1 *Analysis of the* V. cholerae *genome database for rRNA and tRNA operons*

Over the years ribotyping has proved to be a very valuable tool in the molecular epidemiological surveillance of *V. cholerae* (Sharma *et al* 1997a). While working with tRNA genes in our laboratory we have observed that tRNA typing (Southern blot analysis of *V. cholerae* chromosomal DNA using [^{32}P]-labelled bulk tRNA as probe) profile of *V. cholerae* strains, especially between classical and El Tor strains, also produces subtle but significant variations (data not shown). This prompted us to carry out a detailed investigation of the tRNA operons among different *V. cholerae* strains, with special emphasis on the observed variation between classical and El Tor strains, to identify the microheterogeneity in the structural organization of the tRNA operons amongst different strains of this organism. Availability of the genome sequence has provided an opportunity to analyse diversity amongst the genome of related organisms and microdiversity amongst the various biotypes of the same organism. The published genome sequence of *V. cholerae* was analysed for the various stable RNA operons. All stable RNA genes were present only in chromosome I, the largest of the two chromosomes (Waldor and RayChaudhuri 2000). Incidentally the *V. cholerae* strain N16961 used for genome sequencing was a serogroup O1, biotype El Tor strain (Levine *et al* 1981) and thus more useful in determining sequence variation between classical and El Tor strains. Apart from the tRNA genes present in the intergenic spacer region (ISR) between 16S–23S rRNA genes in the eight ribosomal RNA (*rrn*) operons, nine

major tRNA operons containing sixteen to four tRNA genes were identified on the published genome sequence (Heidelberg *et al* 2000). Our present investigation was restricted only to the major tRNA operons independent of *rrn* operons. For comparative analysis of these major tRNA operons in the different *V. cholerae* strains, PCR products were generated using primers designed from the published sequence as described under methods. The products were then subjected to size as well as restriction analysis. Eight of these tRNA operons produced PCR products of identical size as well as restriction profile of the PCR product for any particular tRNA operon examined for all the strains tested (data not shown). Variation in the

length of the PCR product as well as restriction profile could be established for only the largest tRNA operon (see below) designated henceforth as the tRNA operon I; subsequent discussion would be restricted to this operon only.

3.2 *Analysis of the transfer RNA operon I*

This operon consists of 2·028 kb sequence and is located between base positions 2330763 to 2332790 of the chromosome I of *V. cholerae* N16961. The fragment carries 16 tRNA sequences (figure 1b). To analyse the sequence variation in this operon in the genome of the classical

Table 1. *V. cholerae* strains used in this study.

Strains	Serogroup, serotype, biotype	Place and year of isolation	Reference
Strain used for whole genome sequencing by Heidelberg *et al* (2000)			
1. N16961	O1, Inaba, El Tor	Bangladesh 1975	Kaper *et al* 1984
Classical biotype strains			
2. 569B	O1, Inaba, Classical	Calcutta, India 1948	Mekalanos 1983
3. 154	O1, Ogawa, Classical	Calcutta, India 1956	Ghosh and Mukherjee 1960
4. O395	O1, Ogawa, Classical	Calcutta, India 1964	Mekalanos 1983
Serogroup O139 strains			
5. BO4	O139	Bangladesh 1993	Mitra *et al* 1995
6. BO2	O139	Bangladesh 1993	Mitra *et al* 1995
7. PO7	O139	Maharashtra, India 1993	Mitra *et al* 1995
El Tor biotype strains isolated before the O139 outbreak			
8. VC1	O1, El Tor	Calcutta, India, March 1992	Sharma *et al* 1997a
9. VC3	O1, El Tor	Calcutta, India, April 1992	Sharma *et al* 1997a
10. VC5	O1, El Tor	Calcutta, India, April 1992	Sharma *et al* 1997a
11. VC44	O1, El Tor	Calcutta, India, May 1992	Sharma *et al* 1997a
12. VC106	O1, El Tor	Calcutta, India, December 1992	Sharma *et al* 1997a
El Tor biotype strains isolated during the O139 outbreak			
13. CO327	O1, El Tor	Calcutta, India, September 1993	Sharma *et al* 1997a
14. CO371	O1, El Tor	Calcutta, India, October 1993	Sharma *et al* 1997a
15. CO427	O1, El Tor	Calcutta, India, November 1993	Sharma *et al* 1997a
El Tor biotype strains isolated after the O139 outbreak			
16. CO458	O1, El Tor	Calcutta, India, March 1994	Sharma *et al* 1997a
17. CO460	O1, El Tor	Calcutta, India, March 1994	Sharma *et al* 1997a
18. CO461	O1, El Tor	Calcutta, India, March 1994	Sharma *et al* 1997a
19. CO650	O1, El Tor	Calcutta, India, July 1994	Sharma *et al* 1997a
20. CO970	O1, El Tor	Calcutta, India, June 1995	Sharma *et al* 1997a
Resurgent O139 strains			
21. AS212	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b
22. AS213	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b
23. AS231	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b
24. AS258	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b
25. AS259	O139	Calcutta, India, (August–September) 1996	Sharma *et al* 1997b

strains, and in the strains belonging to El Tor biotype isolated during different time frames, PCR reactions were carried out using the primers P1 and P2 and the chromosomal DNA of various strains mentioned in table 1.

All El Tor and O139 strains produced bands identical in size to the reference strain N16961. However, the classical strains gave bands that were smaller by about 250 bp. As the length of the PCR products for all the El Tor, O139 and the resurgent O139 strains were identical, the one observed for N16961 is shown as a representative (figure 1a, lane 1); two classical strains, 569B and 154 are also included (lanes 2 and 3 respectively).

In order to resolve this difference in size, the PCR products were digested with *Eco*RI. The *Eco*RI digestion profile for the serogroup O1 El Tor strains including N16961 (figure 1a, lanes 4, 9–11) and serogroup O139 strains (lanes 7, 8) were identical and consisted of three fragments of about 880 bp, 655 bp and 567 bp. The *Eco*RI digestion for the classical strains (figure 1a, lanes 5 and 6) also showed three fragments of 880 bp, 567 bp and 400 bp suggesting that the 655 bp internal fragment was reduced by about 250 bp (see figure 1b) and this exhibited a distinct variation from that of O1 El Tor and O139 strains. The relative order of these fragments as deduced
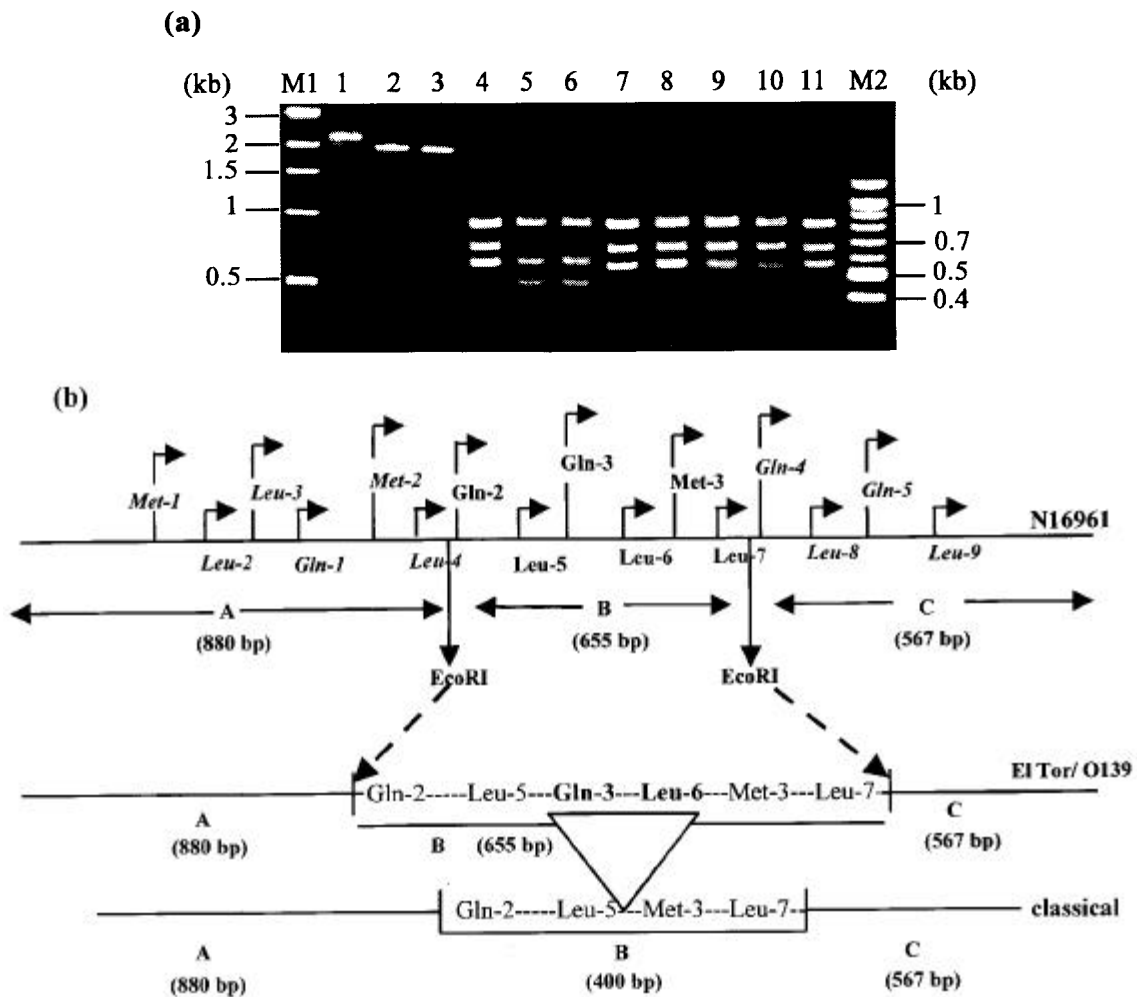

Figure 1. Comparison of the structural organization of the tRNA operon I in *V. cholerae* strains. (**a**) PCR analysis of tRNA operon I in different *V. cholerae* strains (lanes 1 to 3) and the *Eco*RI restriction digestion profile of tRNA operon I in different *V. cholerae* strains (lanes 4 to 11). Lane M1, 1 kb ladder; lane 1, N16961, sequenced El Tor strain; lane 2, 569B, classical strain; lane 3, VC154, classical strain; lane 4, N16961, sequenced El Tor strain; lane 5, 569B, classical; lane 6, VC154, classical; lane 7, BO4, O139 strain of 1992; lane 8, As231, resurgent O139 of 1996; lane 9, CO458, El Tor strain after O139 era; lane 10, CO327, El Tor strain during O139 era; lane 11, VC1, El Tor strain before O139 era; lane M2, 100 bp DNA ladder. (**b**) Schematic representation of the *Eco*RI restriction map and the structural organization of tRNA operon I in *V. cholerae* El Tor/O139 and classical strains.

from the restriction sites in the published sequence is given in figure 1b.

To confirm that the tRNA operon I present in classical strains had an internal deletion the PCR products generated using the same set of primers P1 and P2 using chromosomal DNA from classical strains as template were sequenced. The sequences (GenBank accession number for the tRNA operon I from *V. cholerae* classical strains is AY847461) were aligned with the *V. cholerae* El Tor N16961 genome sequence using BLAST2 (Altschul *et al* 1990) which revealed that the tRNA operon I sequence in classical strains had an internal deletion of 268 bp, resulting in the absence of two tRNA genes, tRNA Gln3 (CAA) and tRNA Leu6 (CUA) (figure 1b). All El Tor and O139 strains, however, had tRNA operon I sequences identical to that of the *V. cholerae* El Tor strain N16961.

One of the major problems in cholera research is the emergence of new biotypes and new clones of the same serotypes and biotypes. From the epidemiological standpoint identification of these strains and establishment of clonal relationship are of utmost importance. Much research has been conducted over the years towards understanding the evolution of newer pathogenic clones of the cholera *Vibrio*. Yet the clonal relationship even between the sixth and seventh pandemic strains remain undecided; there are evidences both in favour of their evolution from the same origin as also their clonal diversity. Sequence analysis of the four house keeping genes namely *mdh* (malate dehydrogenase), *hly*A (hemolysin A), *rec*A and *dna*E suggested that the sixth and seventh pandemic strains are closely related (Byun *et al* 1999). On the other hand studies involving ribotyping, pulse-field gel electrophoresis and multi-locus enzyme electrophoresis of various *V. cholerae* strains together have revealed that the sixth and seventh pandemic strains constitute two different clones (Karaolis *et al* 1995). Nucleic acid sequence analysis of the *asd* gene, present in various *V. cholerae* strains, also revealed that the sixth pandemic classical biotype strains are not closely related to the seventh pandemic El Tor strains and have evolved independently from a lineage separate from the latter (Karaolis *et al* 1995). Biological significance of the result presented here regarding the differences in one of the tRNA operons present in *V. cholerae* serogroup O1 classical and El Tor strains is difficult to ascertain at this moment but this observation provides an additional support towards the clonal diversity of these strains. Much more information on the sequence similarity or divergence would be necessary to clearly resolve the relationship of the pathogenic clones.

Acknowledgements

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