

Rearrangements in the Genomes of *Vibrio cholerae* Strains Belonging to Different Serovars and Biovars

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The intron-encoded enzyme I-CeuI provides an excellent tool for rapidly examining the organization of genomes of related species of bacteria. *Vibrio cholerae* strains belonging to serovars O1 and O139 have 9 I-CeuI sites in their genomes, and *V. cholerae* strains belonging to serovars non-O1 and non-O139 have 10 I-CeuI sites in their genomes. This information can be used as a criterion to differentiate O1 strains from non-O1 and non-O139 strains. To our knowledge, intraspecies variation in the number of *rrn* operons has not been reported in any other organism. Our data revealed extensive restriction fragment length polymorphism based on a comparison of the I-CeuI digestion profiles of strains belonging to different serovars and biovars. From the analysis of partial digestion products, I-CeuI macrorestriction maps of several classical, El Tor, and O139 strains were constructed. While the linkage maps are conserved within biovars, linkage maps vary substantially between biovars.

Vibrio cholerae, a noninvasive gram-negative bacterium, is the causative agent of the diarrheal disease cholera, which still is a public health problem in most developing countries. The specificity of the somatic O antigen of *V. cholerae* resides in the polysaccharide moiety of the lipopolysaccharide present in the outer membrane, which forms the basis for serological classification of this organism (16). Strains belonging to serovar O group 1 (serovar O1) are responsible for cholera. The strains of serovar O1 belong to two biovars, biovars classical and El Tor. Strains other than O1 strains are often called non-O1 vibrios, and these organisms can cause only sporadic infections and do not have the potential to cause epidemics (10). Only recently, an outbreak of cholera in the Indian subcontinent was caused by a novel non-O1 strain, *V. cholerae* O139 Bengal (1, 12). However, several lines of evidence have suggested that this strain very closely resembles biovar El Tor of serovar O1 (2, 3, 18). By digesting the intact genomic DNA with enzymes *NotI* and *SfiI*, which produced fragments of suitable sizes that could be resolved by pulsed-field gel electrophoresis (PFGE), the genome size of the classical O1 strains of *V. cholerae* was estimated to be about 3.2 Mb (14). A physical map of the genome of strain 569B was constructed by using restriction endonucleases *NotI*, *SfiI*, and I-CeuI, and on this map 20 cloned homologous and heterologous genes and seven *rrn* operons were positioned (8).

I-CeuI is encoded by a class I mobile intron (9). I-CeuI digestion followed by PFGE can be a useful tool for examining intra- and interspecies genome rearrangements (5). This enzyme cleaves genomic DNA only in a 19-bp sequence in the 23S rRNA gene of the *rrn* operon in all of the organisms examined so far (6). The number of I-CeuI sites in the genome of an organism is considered a measure of the number of *rrn* operons present in that organism. The number and location of *rrn* operons in enteric bacteria appear to be highly conserved. The I-CeuI restriction maps reported for a number of *Escherichia coli* and *Salmonella typhimurium* strains are almost identical (5, 6). However, the I-CeuI maps of *Salmonella typhi* Ty2

and *S. typhimurium* LT2 revealed chromosomal rearrangements in these two species (7). In the present report we describe strains of *V. cholerae* belonging to serovars O1 and O139 that have 9 I-CeuI sites in their genomes and *V. cholerae* strains belonging to serovar non-O1 that have 10 I-CeuI sites in their genomes. Although the number of *rrn* operons in strains belonging to serovar O1 is conserved, the positions of the operons in the chromosome are different, suggesting that there have been major genomic rearrangements in the classical and El Tor biovars.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *V. cholerae* strains used in this study are listed in Table 1. *V. cholerae* cells were grown in a gyratory shaker at 37°C in nutrient broth containing 0.1 M NaCl (pH 8.0) and were maintained as described previously (13). *E. coli* cells were grown in Luria-Bertani medium with shaking at 37°C.

Preparation of high-molecular-weight genomic DNA and enzyme digestion. Agarose plugs of cells in the logarithmic phase of growth were prepared by mixing equal volumes of cell suspension and molten 1.4% low-melting-point agarose as described previously (8, 14). The plugs were suspended in lysis solution (8) and incubated at 37°C for 16 h with gentle shaking. After the incubation period, the lysis solution was replaced by a solution containing 0.5 M EDTA (pH 9.0), 1% Sarkosyl, and 1 mg of proteinase K per ml, and the preparation was incubated for 48 h at 50°C, following which the plugs were stored in the same solution at 4°C. The agarose plugs containing the genomic DNA were digested with I-CeuI as described previously (8). For partial digestion with I-CeuI, six agarose pieces were digested with 1 U of the enzyme in 100 µl of the reaction mixture for 2 h at 37°C.

PFGE and hybridization experiments. Electrophoresis was carried out with a Pulsaphor Plus System with a hexagonal electrode array (Pharmacia, Uppsala, Sweden) as described previously (8, 14). FastLane agarose (FMC Corp., Philadelphia, Pa.) was used when DNA was to be transferred to membranes for Southern blotting. The enzyme-digested DNA was separated on a 1% agarose gel by using 20 mM Tris-acetate (pH 8.3)–0.5 mM EDTA (TAE) as the running buffer at 5 to 10 V/cm (depending on the size of the fragment that needed to be resolved) and various pulse times (depending on the experimental conditions). Phage λ multimeric DNA and yeast chromosomal DNA were used as molecular mass markers. Preparation of ³²P-labeled DNA probes, *rpoH* (encoding σ³² of *V. cholerae* [15]), *mutL*, and *NotI*-linking clone NLH20 of *V. cholerae* genomic DNA (8) and Southern blot hybridizations were carried out by using previously described procedures (8). Details concerning the cloned DNA probes used in this study have been described previously (8). End labeling of DNA fragments following I-CeuI digestion was done by incubating the agarose blocks in a buffer containing Klenow enzyme and [α-³²P]dCTP, and the preparations were subjected to PFGE followed by autoradiography.

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TABLE 1. *V. cholerae* strains used

Strain designation as received	Serovar	Biovar	CT gene ^a	Origin			Sender
				Source	Place	Year	
569B (= ATCC 25870)	O1	Classical	CT ⁺	Clinical	India	1948	NICED, Calcutta, India ^b
O395 (= ATCC 39541)	O1	Classical	CT ⁺	Clinical	India	1964	J. J. Mekalanos, United States
VC44	O1	El Tor	CT ⁺	Clinical	India	1992	G. B. Nair, India
CO457	O1	El Tor	CT ⁺	Clinical	India	1994	G. B. Nair, India
C6709	O1	El Tor	CT ⁺	Clinical	Peru	1991	J. J. Mekalanos, United States
MAK757 (= ATCC 51352)	O1	El Tor	CT ⁺	Clinical	Indonesia	1961	NICED, Calcutta, India
MO45 (= ATCC 51394)	O139		CT ⁺	Clinical	India	1992	G. B. Nair, India
SG24	O139		CT ⁺	Clinical	India	1992	G. B. Nair, India
V18	O11		CT ⁻	Clinical	India	1989	G. B. Nair, India
RB	Non-O1, non-O139		CT ⁻	Clinical	India	1991	G. B. Nair, India
V254	Non-O1, non-O139		CT ⁻	Clinical	India	1991	G. B. Nair, India
NRT36	Non-O1, non-O139		CT ⁻	Clinical	Japan	1986	G. B. Nair, India
VCE232	Non-O1, non-O139		CT ⁺	Environmental	India	1985	G. B. Nair, India
VCE309	Non-O1, non-O139		CT ⁻	Environmental	India	1985	G. B. Nair, India

^a CT, Cholera toxin gene.

^b NICED, National Institute of Cholera and Enteric Diseases.

RESULTS AND DISCUSSION

I-CeuI map of strain 569B. It has been reported that I-CeuI digestion of *V. cholerae* 569B and O395 produced seven fragments ranging in size from 1,420 to 70 kb (8). In the course of our studies on the construction of a macrorestriction map of the *V. cholerae* genome and for better resolution of DNA fragments following enzyme digestion, refinement of PFGE methods by altering various parameters, such as the nature of pulsing, pulse time, and time of electrophoresis, was necessary. One of the modified procedures revealed that previously described I-CeuI fragment C7 is a doublet comprising 85- and 80-kb fragments. After *V. cholerae* 569B genomic DNA in an agarose block was digested with I-CeuI and end labeled with [α -³²P]dCTP, after the fragments were separated by PFGE,

and after the dried gel was autoradiographed, we confirmed that I-CeuI fragment C7 is a doublet and detected another small, 6-kb fragment which was not visible in the ethidium bromide-stained gels (Fig. 1a). Under identical conditions, I-CeuI digestion of the *E. coli* W3110 genome produced only seven fragments, as reported previously (5). Thus, the *V. cholerae* 569B genome has nine I-CeuI sites instead of the seven sites reported previously (8). To examine whether, as in other organisms, the I-CeuI sites in the *V. cholerae* genome are also located only in the *rm* operons, a 450-bp region of the 23S rRNA gene of *V. cholerae* (characterization of this gene will be described in a separate report) was used as a probe for Southern blot hybridization of the I-CeuI-digested *V. cholerae* 569B genome. All nine fragments hybridized with the probe (data not shown), confirming that there are nine *rm* operons in the *V. cholerae* 569B genome and that I-CeuI does not have sites that are not in the *rm* operon. The other classical strain examined in this study, O395, also has nine I-CeuI sites in its genome.

To position the two additional fragments on the I-CeuI macrorestriction map of the *V. cholerae* 569B genome (8), a restriction map was reconstructed from the results of the analysis of partial digestion products. Eleven partial fragments (Table 2) obtained by using different pulse times during PFGE were analyzed to assemble the I-CeuI map. From partial fragments P10 and P11 the linkage C2-C1-C3 was established. These partial fragments, along with partial fragments P5 and P7, gave the linkage C2-C1-C3-C7-C5. When partial fragments P1, P2, P3, and P4 were combined, the linkages between I-CeuI frag-

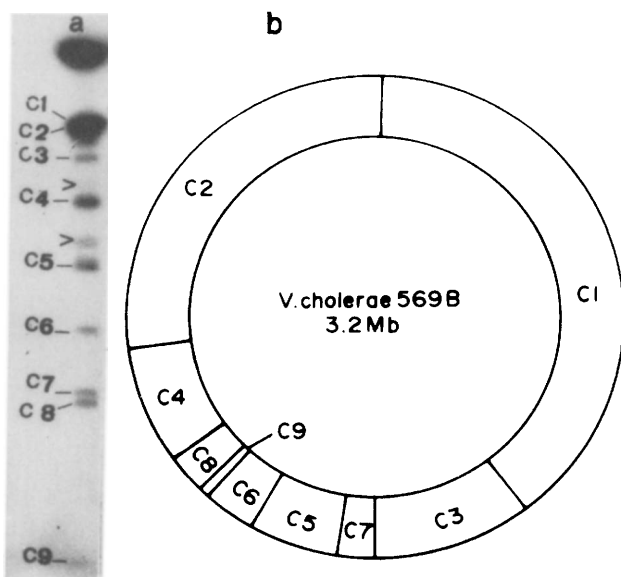


FIG. 1. (a) Autoradiogram of PFGE-separated *V. cholerae* 569B genome digested with I-CeuI either partially or to completion. Digested DNAs in agarose blocks were end labeled with [α -³²P]dCTP and separated in 1% FastLane agarose (FMC) containing 0.5 \times TAE, with the pulse times ramping between 5 and 25 s for 22 h at 10 V/cm. Fragment numbers are indicated on the left. The arrowheads indicate the partial digestion products. (b) I-CeuI map of *V. cholerae* 569B genome.

TABLE 2. I-CeuI partial digestion products of the 569B genome

Partial fragment (kb)	Possible linkage (kb)
P1 (88)	C8 and C9 (86)
P2 (200)	C6, C8, and C9 (206)
P3 (270)	C5 and C6 (290)
P4 (375)	C5, C6, C8, and C9 (376)
P5 (412)	C3 and C7 (410)
P6 (500)	C4, C6, C8, and C9 (451)
P7 (588)	C3, C5, and C7 (575)
P8 (1,350)	C2, C4, C6, C8, and C9 (1,351)
P9 (1,515)	C2, C4, C5, C6, C8, and C9 (1,521)
P10 (1,835)	C1 and C3 (1,725)
P11 (2,450)	C1 and C2 (2,300)

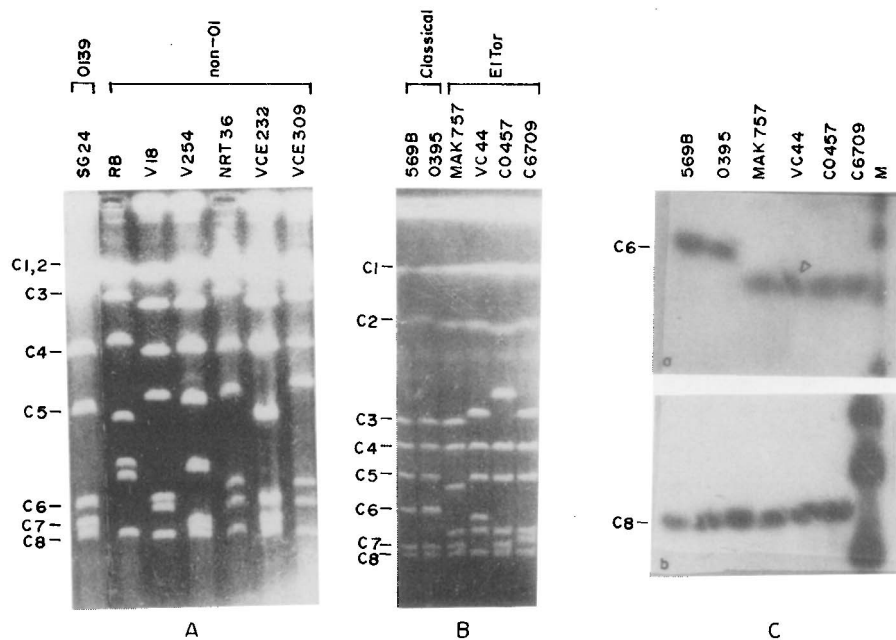


FIG. 2. (A and B) *I-CeuI* digestion profiles of the genomes of *V. cholerae* strains belonging to different serovars and biovars. Enzyme-digested DNAs were separated by using 1% FastLane agarose (FMC) and $0.5\times$ TAE, with pulse times ramping from 5 to 25 s for 22 h at 10 V/cm (A) or with pulse times of 5 s (for 4 h), 10 s (for 4 h), 20 s (for 4 h), and 100 s (for 12 h) (B). (C) Southern blot hybridization of the gel in panel B by using *rpoH* (arrowhead indicates fragment C7 of the *I-CeuI*-digested VC44 genome) (a) and NLH20 (b) as the probes. The fragment numbers are indicated on the left. Lane M contained λ concatameric DNA used as a marker.

ments were found to be C2-C1-C3-C7-C5-C6-C9-C8. Finally, by using partial fragments P6, P8, and P9, the *I-CeuI* linkage map of strain 569B was constructed (Fig. 1b). The linkages were confirmed by using *I-CeuI* fragment-specific DNA probes. For example, when a C6-specific probe, *mutL* (8), was hybridized to a Southern blot of the *V. cholerae* 569B genome partially digested with *I-CeuI*, it hybridized with partial fragments P2, P3, P4, P6, P8, and P9. The *rpoH* gene of *V. cholerae* 569B (15), which is also a C6-specific probe, hybridized with partial fragments P2, P3, and P4. Similarly, when the C8-specific probe, linking clone NLH 20 (8), which links N11 and N25 fragments of the *NotI*-digested 569B genome (8), was used as a probe in the hybridization experiment, it hybridized with partial fragments P2, P4, P6, P8, and P9. These results show that C6, C9, and C8 are linked. The *I-CeuI* linkage maps of strains 569B and O395 were identical.

Comparison of the *I-CeuI* digestion profiles of genomes of *V. cholerae* strains. Since *I-CeuI* can serve as a tool to examine the

genome organization of related strains, the *I-CeuI* digestion profiles of *V. cholerae* cells belonging to different serovars and biovars were compared (Fig. 2A and B). While the genomes of classical and El Tor strains have 9 *I-CeuI* sites, all non-O1 and non-O139 strains examined have 10 *I-CeuI* sites in their genomes (Table 3 and Fig. 3). The difference in the number of *I-CeuI* sites in the genomes of strains belonging to the O1 serovar and the non-O1 and non-O139 serovars can thus be used as a criterion to differentiate them. Interestingly, strain SG24 belonging to serogroup O139 has 9 *I-CeuI* sites in its genome, while the other non-O1 strains have 10 sites. The *I-CeuI* digestion profile of the SG24 genome resembles that of O1 El Tor strain CO457 (Fig. 3, lanes a and g), a strain isolated immediately after the O139 epidemic. To our knowledge, intraspecies variation in the number of *rm* operons has not been reported in any other organism. Some laboratory-maintained strains of *Bacillus subtilis*, a gram-positive bacterium, have been reported to have 9 *rm* operons rather than the more

TABLE 3. *I-CeuI* fragment sizes for the genomes of different *V. cholerae* strains

Strain	Fragment sizes (kb)									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
569B	1,400	900	325	245	170	120	85	80	6	
O395	1,400	900	325	245	170	120	85	80	6	
MAK757	1,400	790	325	245	150	92	85	80	6	
VC44	1,400	790	364	245	170	92	90	80	6	
CO457	1,400	750	460	245	170	92	84	80	6	
C6709	1,400	820	364	245	170	94	88	80	6	
SG24	1,400	750	460	245	170	92	84	80	6	
RB	1,400	950	350	254	164	116	106	80	6	5
V18	1,400	820	340	245	192	98	92	80	6	5
V254	1,400	820	340	245	180	116	84	80	6	5
NRT36	1,400	750	364	245	196	106	92	80	6	5
VCE232	1,400	950	340	245	168	94	84	80	6	5

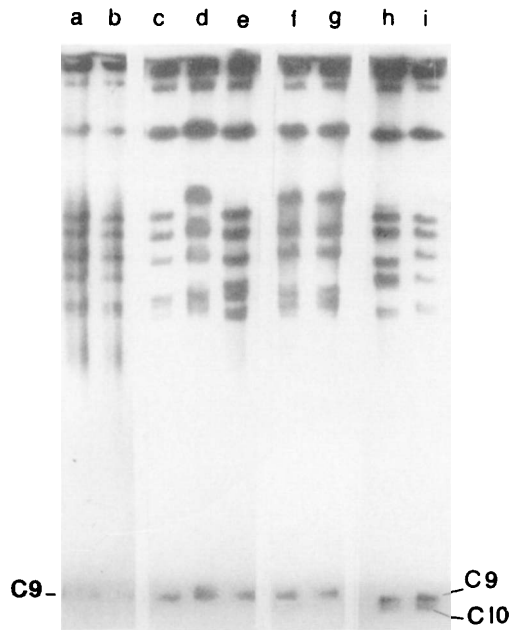


FIG. 3. Autoradiograph of I-CeuI-digested genomes of *V. cholerae* O1 El Tor, non-O1 and non-O139 strains and O139 strains following end labeling with [α - 32 P]dCTP of the digestion products and PFGE. Lane a, 569B; lane b, O395; lane c, C6709; lane d, CO457; lane e, VC44; lane f, MO45; lane g, SG24; lane h, RB; lane i, V254. Electrophoresis was carried out by using pulse times of 5 s (for 4 h), 10 s (for 4 h), 25 s (for 4 h), and 100 s (for 11 h) at 10 V/cm, followed by pulse times of 10 s (for 7 h) and 150 s (for 6 h) at 5 V/cm at 3°C. C9 and C10 indicate the positions of I-CeuI fragments 9 and 10, respectively.

common 10 *rm* operons (4, 17). It was proposed that homologous recombination between closely linked rRNA genes caused deletion of one of the loci (19). The number of *rm* operons in the other organisms examined so far is seven or less (4).

No detectable restriction fragment length polymorphism (RFLP) was observed in the two O1 classical strains examined (Fig. 2B). The four El Tor strains, on the other hand, exhibited a high degree of RFLP. Strains VC44 and CO457 exhibited extensive RFLP, which is surprising in view of the fact that these two strains were isolated within a span of 1.5 years immediately before and after the O139 epidemic (11). Strain C6709, which was isolated during the Peru epidemic in 1991, is different from other El Tor strains. The I-CeuI digestion profiles of the non-O1 and non-O139 strains examined showed extensive RFLP, which were more pronounced among the low-molecular-weight fragments (Fig. 2A).

When I-CeuI fragment-specific probes were used in Southern blot hybridization experiments with I-CeuI-digested genomic DNAs from a number of O1 classical and El Tor strains, the extent of RFLP could be detected in greater detail. When the *rpoH* gene of *V. cholerae* 569B (15), which hybridizes with the C6 fragment of the 569B genome, was used as a probe, it hybridized with the 120-kb C6 fragment of 569B and O395 and the 92-kb C6 fragment of all of the El Tor strains except VC44 (Table 3). Thus, there is an ~30-kb deletion in the C6 fragment of the O1 El Tor strains compared to the C6 fragment of O1 classical strains. The deletion or insertion of DNA fragments presumably occurred via mobile elements and resulted in the observed RFLP differences between strains. In the genomic digest of VC44, the *rpoH* probe hybridized with 92-kb fragment C7 (Fig. 2Ca). Fragment C7 of VC44 comigrated with fragment C6 of other El Tor strains (Fig. 2B).

O1 Classical	
569B	-C1- C2- C4- C8- C9- C6- C5- C7- C3- C1-
O395	-C1- C2- C4- C8- C9- C6- C5- C7- C3- C1-
O1 El Tor	
VC 44	-C1- C3- C5- C8- C9- C6- C7- C4- C2- C1-
CO 457	-C1- C3- C5- C8- C9- C6- C7- C4- C2- C1-
O139 Bengal	
SG 24	-C1- C8- C9- C6- C4- C7- C3- C5- C2- C1-

FIG. 4. I-CeuI linkage map of genomes of *V. cholerae* 569B, O395, VC44, CO457, and SG24.

Linking clone NLH 20 (8), a C8-specific probe, hybridized to 80-kb fragment C8 of all classical and El Tor strains (Fig. 2Cb). The *secY* gene of *V. cholerae*, located in fragment C4 (8), hybridized to 245-kb fragment C4 of all of the strains belonging to the classical and El Tor biovars.

The I-CeuI macrorestriction maps of several classical, El Tor, and O139 strains were constructed by analyzing the I-CeuI partial digestion products (Fig. 4). The linkage of the I-CeuI fragments of the genomes of all of the classical strains and the linkage of all of the El Tor strains examined are conserved. However, the linkages are different between the strains belonging to the two biovars. These results suggest that although the number of *rm* operons is conserved among strains belonging to serovar O1, the positions of the operons in the chromosome have changed, suggesting that there have been major genomic rearrangements in the classical and El Tor biovars. The I-CeuI linkage map of the genome of strain SG24 is different from the I-CeuI linkage maps of the genomes of the classical and El Tor strains (Fig. 4), although the I-CeuI digestion profile of strain SG24 is similar to that of El Tor strain CO457.

ACKNOWLEDGMENTS

We thank G. B. Nair, National Institute of Cholera and Enteric Diseases, Calcutta, India, for providing the *V. cholerae* strains and J. J. Mekalanos, Harvard Medical School, Boston, Mass., for strain C6709. We are grateful to all members of the Biophysics Division, Indian Institute of Chemical Biology, for their kind cooperation and encouragement during this study, particularly to Rajat Banerjee and Amit Chakraborty.

This work was supported by grants BT/TF/15/03/91 and BT/MB/05/012/94 from the Department of Biotechnology, Government of India. S.N. and R.M. are grateful to the Council of Scientific & Industrial Research, New Delhi, India, for predoctoral fellowships, and S.K. is grateful for a postdoctoral fellowship.

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