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Resurgent *Vibrio cholerae* O139: Rearrangement of Cholera Toxin Genetic Elements and Amplification of *rrn* Operon†

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The unprecedented genesis of a novel non-O1 *Vibrio cholerae* strain belonging to serogroup O139, which caused an epidemic in late 1992 in the Indian subcontinent, and its subsequent displacement by El Tor O1 vibrios after 18 months initiated a renewed investigation of the aspects of the organism that are related to pathogenesis. The reappearance of *V. cholerae* O139 with altered antibiotic sensitivity compared to O139 Bengal (O139B) in late 1996 has complicated the epidemiological scenario of *V. cholerae* and has necessitated an examination of possible rearrangements in the genome underlying such rapid changes in the phenotypic traits. With a view to investigating whether the phenotypic changes that have occurred are associated with alteration in the genome, the genome of the resurgent *V. cholerae* O139 (O139R) strains were examined. Pulsed-field gel electrophoresis analysis of *Not*I- and *Sfi*I-digested genomic DNA of O139R isolates showed restriction fragment length polymorphism including in the cholera toxin (CTX) genetic element locus and with O139B isolates. Analyses of the organization of the CTX genetic elements in O139R strains showed that in contrast to two copies of the elements connected by two direct-repeat sequences (RS) in most of the genomes of O139B isolates, the genomes of all O139R strains examined, except strain AS192, have three such elements connected by a single RS. While the RS present in the upstream of the CTX genetic elements in the genome of O139R is of O139B origin, the RS connecting the cores of the elements has several new restriction sites and has lost the *Bgl*II site which is supposed to be conserved in all O1 strains and O139B. The endonuclease I-*Ceu*I, which has sites only in the *rrn* operons in the genomes of all organisms examined so far, has 10 sites in the genomes of O139R strains, compared to 9 in the genomes of O139B strains. The recent isolates of *V. cholerae* O139 have thus gained one *rrn* operon. This variation in the number of *rrn* operons within a serogroup has not been reported for any other organism. The results presented in this report suggest that like the pathogenic El Tor O1 strains, the genomes of O139 strains are undergoing rapid alterations.

Vibrio cholerae, a noninvasive gram-negative bacterium, is the causative agent of the diarrheal disease cholera. The specificity of the somatic O antigen of *V. cholerae* resides in the polysaccharide moiety of the lipopolysaccharide in the outer membrane, which forms the basis of the serological classification of this organism (36). The *V. cholerae* strains causing cholera epidemics have, until recently, been confined to serogroup O1, which consists of two biotypes, classical and El Tor. The classical biotype was responsible for cholera epidemics until 1961, when the El Tor biotype displaced it. *V. cholerae* strains other than O1, which are collectively called non-O1 vibrios, cause only sporadic infections and are believed to lack the potential to cause epidemics (26). In late 1992, for the first time in the history of cholera, a non-O1 strain, *V. cholerae* O139 Bengal (henceforth designated O139B), caused an epidemic in the Indian subcontinent (1, 32) which persisted for about 1 year (28). Strains isolated from different parts of India and Bangladesh during the epidemic were found to be of clonal origin, and several lines of evidence have suggested that strain O139B arose from the El Tor biotype (5–7, 9, 10) by the acquisition of a 35-kb novel DNA segment which replaced most of the O1-antigen-encoding gene cluster, the *rfb* locus, of the recipient strain (8, 11). Thus, serogroup O139 combines the virulent properties of epidemic strains (*V. cholerae* O1) with the outer appearance of nonepidemic strains (*V. cholerae* non-O1). In subsequent outbreaks O139B was again replaced by El Tor O1 strains (27, 28). Surprisingly, the genomes of El Tor strains isolated immediately before and after the O139B outbreak showed extensive restriction fragment length polymorphism (RFLP) among themselves and with the genome of O139B (30, 42). Alterations in a well-characterized clonal strain within such a short period are unexpected.

Recently, a resurgence in Calcutta, India (25), of *V. cholerae* O139 (henceforth designated O139R) having altered antibiotic sensitivity compared to O139B was recorded. The genomes of O139R strains were examined in the present study to investigate whether the observed phenotypic changes relative to O139B are associated with alterations in the genome. Gross plasticity in the organization of the genomes of *V. cholerae* strains belonging to different serovars and biovars has recently been found by using the intron-encoded enzyme I-*Ceu*I, which provides an excellent tool for the rapid examination of the organization of genomes of related species of bacteria. *V. cholerae* strains belonging to serovars O1 and O139 have 9 I-*Ceu*I sites in their genomes, and those belonging to serovars non-O1 and non-O139 have 10 I-*Ceu*I sites in their genomes (30). The results presented here show that the clonality of O139 is not retained and that an amplification of *rrn* operon has occurred in the genomes of O139R strains relative to those of O139B strains. Moreover, there are three copies of the cholera toxin (CTX) genetic element in the genomes of O139R strains, and

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

Strain	Time of isolation	Serotype, biotype, and CT gene	Antibiotic sensitivity[a] to:	
			Streptomycin	SXT
AS192	Aug. 1996	O139, CT^+	R	S
AS197	Aug. 1996	O139, CT^+	R	S
AS199	Aug. 1996	O139, CT^+	R	S
AS207	Aug. 1996	O139, CT^+	R	S
AS209	Aug. 1996	O139, CT^+	R	S
AS210	Aug. 1996	O139, CT^+	R	S
AS212	Aug. 1996	O139, CT^+	R	S
AS213	Aug. 1996	O139, CT^+	R	S
SG24	Dec. 1992	O139, CT^+	R	R
VC44	May 1992	O1, El Tor, CT^+	S	S
CO457	Mar. 1995	O1, El Tor, CT^+	R	R

[a] Assayed by the disc diffusion method (28). Streptomycin (10 μg) and sulfamethoxazole (23.75 μg)-trimethoprim (1.25 μg) were used. R, resistant; S, sensitive.

the organization of these elements and the direct-repeat sequence (RS) connecting the core regions are considerably different from those of O139B strains.

MATERIALS AND METHODS

Bacterial strains and growth condition. The *V. cholerae* strains used in this study are described in Table 1. All strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. The cells were grown in a gyratory shaker at 37°C in Luria-Bertani broth and maintained as described previously (33). Bacterial strains were tested for their susceptibility to streptomycin (10 μg) and sulfamethoxazole (23.75 μg)-trimethoprim (1.25 μg) (SXT) by the disc diffusion method (28).

Preparation of high-molecular-weight genomic DNA and enzyme digestion. Intact bacterial chromosomal DNA was prepared as described previously (22, 34). Briefly, *V. cholerae* cells in the logarithmic phase of growth were suspended in a 10 mM Tris-HCl (pH 7.6) buffer containing 1 M NaCl. Agarose plugs were prepared by mixing equal volumes of bacterial cells and molten 1% low-melting-point agarose (Pharmacia, Uppsala, Sweden). Bacterial cells embedded in agarose were lysed in the presence of RNase, treated with proteinase K, and stored in 0.5 M EDTA (pH 9.0) at 4°C. The agarose plugs containing intact genomic DNA were treated with phenylmethylsulfonyl fluoride (final concentration, 1 mM) to inactivate proteinase K and washed with 10 volumes of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) to remove the phenylmethylsulfonyl fluoride. The agarose slice containing intact genomic DNA was digested with *Not*I, *Sfi*I, or I-*Ceu*I (New England Biolabs, Beverly, Mass.) as directed by the manufacturer and subjected to pulsed-field gel electrophoresis (PFGE).

PFGE. Electrophoresis was carried out in a Pulsaphor Plus system with a hexagonal electrode array (Pharmacia). The enzyme-digested DNA was separated on a 1% FastLane agarose gel (FMC, Rockland, Maine) with 20 mM Tris-acetate (pH 8.3)–0.5 mM EDTA as the running buffer at 5 to 10 V/cm (depending upon the size of the fragment that needed to be well resolved) and various pulse times (depending upon experimental conditions). Phage λ multimeric DNA and yeast chromosomal DNA were used as molecular mass markers. The gels were stained with ethidium bromide.

Molecular genetic methods. About 1 μg of a 780-bp *Nde*I fragment from the *ctxA* gene or 2.3-kb *Bgl*II-*Pst*I DNA segment containing the RS, *cep*, and part of *orfU* in plasmid pCVD15 (12) or cosmid pSXT1 (40) containing SXT resistance genes was nick translated with [α-^{32}P]dCTP (Amersham, Little Chalfont, United Kingdom) and used as probes in Southern blot hybridization. The restriction fragment of DNA excised from the gel was directly labeled with [α-^{32}P]dCTP by the random-priming method with a kit from New England Biolabs. End labeling of DNA fragments following I-*Ceu*I or *Not*I digestion was done by incubating the agarose blocks in a buffer containing Klenow enzyme and [α-^{32}P]dCTP (for I-*Ceu*I) or [α-^{32}P]dCTP plus dGTP (for *Not*I) and subjected to PFGE followed by autoradiography. For Southern blot hybridization, genomic DNA was digested with restriction endonucleases, separated by electrophoresis, transferred to Nytran membranes, and hybridized with labeled DNA probe at 60°C. The filters were washed under high-stringency conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 60°C), air dried, and exposed to Kodak X-OMAT AR5 films as described previously (7).

RESULTS

Antibiotic sensitivity of O139R. One of the features that differentiates O139B from strains belonging to the El Tor biotype of O1 vibrios, from which it is believed to have originated, is its sensitivity to SXT (29). A self-transmissible, site-specific 62-kb conjugative transposon encodes the functions that confer SXT and streptomycin resistance to O139B strains (40). From an examination of the *Sfi*I-digested, PFGE-separated profiles of the genomes of *Escherichia coli* and *V. cholerae* El Tor strains, it was concluded that SXT-sensitive strains have two closely migrating genomic DNA fragments of about 400 kb and that SXT-resistant strains have only one of these fragments (40). The *Sfi*I digestion profile of the genomes of SXT-resistant (CO457) and -sensitive (VC44) El Tor O1 strains (Fig. 1A) used in the present study confirmed this conclusion. However, the *Sfi*I digestion profiles of the genomes of O139R strains have a single DNA fragment in the 400-kb region (Fig. 1A), which, according to the above criterion, should indicate that like O139B, O139R is resistant to SXT. While the resurgent O139 strains retained their streptomycin-resistant phenotype, they were sensitive to SXT (Table 1). Since the *Sfi*I digestion profiles of the genomes of the O139R strains and of the O139B strains are similar, and since the streptomycin-resistant phenotype is retained in O139R isolates, the possibility of deletion of the 62-kb genetic element from the genomes of the resurgent strains is unlikely. This was confirmed by Southern blot hybridization of *Pst*I-digested genomic DNA of O139 strains with the gene encoding SXT resistance as a probe. The SXT probe hybridized with seven fragments of the *Pst*I-digested plasmid pSXT1 (Fig. 1C, lane d) and with six fragments (>23, 15, 12, 5, 2, and 1 kb) of the genomic DNA of SXT-resistant O139B (lane c), as reported previously (40). However, the *Pst*I-digested genome of representative O139R strains hybridized with the first four fragments only (lanes a and b). Thus, a 3-kb (2 kb plus 1 kb) deletion from the 62-kb element in the resurgent strains makes them sensitive to SXT while retaining the streptomycin resistance. The SXT probe did not hybridize with *Pst*I-digested genomic DNA of the SXT-sensitive biotype El Tor strain VC44, which does not possess the 62-kb element (data not shown).

***Not*I digestion profiles of the genomes of O139R strains.** When the intact genomic DNAs of O139R and O139B strains were digested with *Not*I, end labeled, and analyzed by PFGE, RFLP was recorded between the resurgent strains and O139B strains (Fig. 1A). It has been reported (7) that the *ctxA* gene hybridized with two *Not*I fragments of 84 and 7 kb (Fig. 1B, lane a) of the O139B genome. However, only one 97-kb *Not*I fragment hybridized with the O139R genome (Fig. 1B, lane d, and Fig. 2). The 7-kb fragment is generated due to the presence of a *Not*I site in the RS flanking the core region of the CTX genetic element (Fig. 2B) of O139B (6, 7). The absence of this fragment suggests that the *Not*I site in the RS is lost in O139R. Although the genome of resurgent strain AS192 showed distinct RFLP with that of other O139R and O139B strains (Fig. 1A and B), the *ctxA* gene hybridized with the 84- and 7-kb *Not*I fragments like O139B (Fig. 2). Thus, unlike other resurgent clones, the organization and location of CTX genetic element in the genome of AS192 have not changed from that of O139B (see Fig. 4).

***rrn* operons in O139R.** In all the bacterial genomes examined so far (19–21, 23), including the classical and El Tor biotypes of *V. cholerae* (17, 30), the I-*Ceu*I sites are located only in the *rrn* operons (19–21, 23, 30). The number of I-*Ceu*I sites in the genome is taken as the measure of the number of

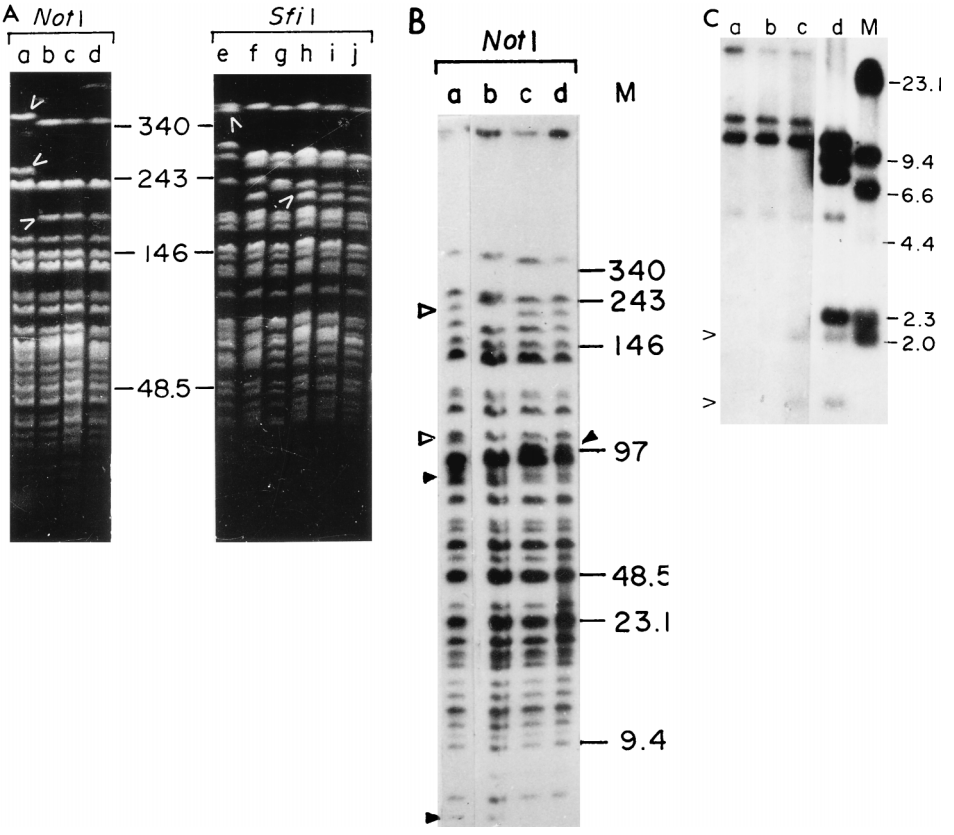

FIG. 1. (A) PFGE of *Not*I- and *Sfi*I-digested genomic DNA of *V. cholerae* strains. The enzyme-digested DNA was electrophoresed on 1% FastLane agarose with a Pulsaphor Plus System with pulse time interpolated between 5 and 25 s for 22 h at 10 V/cm at 3°C. The gels were stained with ethidium bromide. Lanes: a and g, AS192 (O139R); b and h, AS197 (O139R); c and i, AS199 (O139R); d and j, SG24 (O139B); e, VC44 (El Tor, O1); f, CO457 (El Tor, O1). Numbers indicate the molecular size markers in kilobases. Arrowheads indicate RFLP between AS192 and O139R and O139B strains. The arrowhead in lane e indicates two *Sfi*I fragments in the 400-kb region (see the text for details). (B) Autoradiogram of *Not*I-digested, end-labeled, PFGE-separated *V. cholerae* O139 genomic DNA. The conditions of electrophoresis were as described for panel A. Lanes: a, SG24 (O139B); b, AS192 (O139R); c, AS197 (O139R); d, AS199 (O139R). The open arrowheads indicate RFLP between O139B and O139R genomes, and the solid arrowheads indicate the CTX genetic element locus (see the text). The *ctxAB* genes are located in 84- and 7-kb *Not*I fragments in the genomes of strains SG24 (O139B) and AS192 (O139R) (lanes a and b, solid arrowheads) and in a 97-kb *Not*I fragment of O139R genomes (lanes c and d, solid arrowheads). Numbers in the margin indicate the molecular size markers in kilobases. (C) Southern blot analysis of the hybridization of SXT-encoding genes (pSXT1) with SXT-resistant or -sensitive O139 strains. The cosmid pSXT1 (as control) and the chromosomal DNA of O139 strains were digested with *Pst*I, electrophoresed, and analyzed by Southern blot hybridization with pSXT1 as a probe. Lanes: a, AS197 (O139R); b, AS210 (O139R); c, SG24 (O139B); d, pSXT1. M denotes molecular size markers in kilobases. The open arrowheads indicate the deletion of 2- and 1-kb *Pst*I fragments of the SXT element from the genomes of O139R strains. The *Pst*I fingerprint of O139B strain SG24 is identical to that of different O139B strains, as reported previously (40).

rrn operons present in the genome. There are nine I-*Ceu*I sites and hence nine *rrn* operons in the genome of *V. cholerae* O1 and O139B strains (30). When genomic DNA of O139R strains was digested with I-*Ceu*I and size fractionated by PFGE, eight restriction fragments of about 1,400, 1,000, 460, 245, 170, 103, 87, and 80 kb, with no detectable RFLP with O139B or themselves, could be resolved in ethidium bromide-stained gels (Fig. 3a). Two additional fragments of 6 and 5.5 kb in O139R and one of 6 kb in O139B were resolved in the autoradiogram of end-labeled DNA (Fig. 3b). This result indicates that in contrast to 9 *rrn* operons in O139B (30), the genome of O139R has 10 *rrn* operons. Since the number and location of *rrn* operons in any bacterium appear to be highly conserved, the presence of an additional copy of the *rrn* operon in the genome of O139R is unexpected.

Organization of the CTX genetic element in O139R. The genes encoding the A and B subunits of CTX (*ctxAB*), along with other virulence genes encoding core pilin (*cep*), accessory cholera enterotoxin (*ace*), and zonula occludence toxin (*zot*), as well as an unknown open reading frame (*orfU*), are located on a 4.5-kb central core region of the CTX genetic element (Fig. 4) (4, 31, 37). The core region is flanked by an RS of about 2.4 to 2.8 kb, having one *Bgl*II site and one *Eco*RV site that are conserved in all O1 and O139B strains examined (7, 31). The RS of O139B strains has, in addition to the conserved *Bgl*II and *Eco*RV sites, a *Not*I site (6, 7). The hybridization of *ctxA* with only one 97-kb *Not*I fragment of O139R (Fig. 2A) indicated either that its genome has only one copy of the CTX genetic element or that multiple copies from which the *Not*I site in the RS is lost are present in tandem. There is no *Hin*dIII or *Eco*RI site in the CTX genetic element of the *V. cholerae* genome (6, 15, 16, 24, 31, 38), and so these enzymes were used to ascertain whether multiple copies of the element are separated or arranged in tandem (38). In a Southern blot hybridization with the *ctxA* gene as probe and the *Hin*dIII- or *Eco*RI-digested genome of O139B, a 23-kb *Hin*dIII fragment (Fig. 2) and a 23-kb *Eco*RI fragment (data not shown) lit up in the autoradiogram as expected. On the other hand, the *ctxA* gene hybridized with three *Hin*dIII fragments of 13.5, 8.6, and 7.3 kb (Fig. 2B) and with three *Eco*RI fragments of 23, 17, and 7.3 kb (data not shown) of the O139R genome. These observations confirm that there are three copies of the CTX genetic element in the

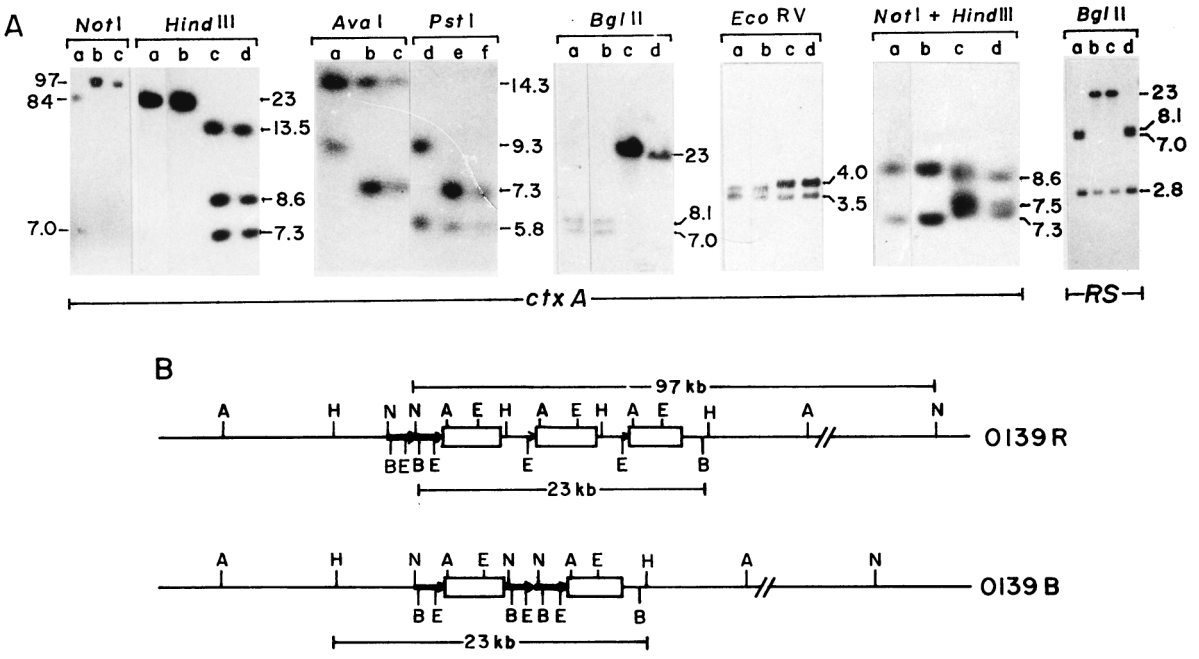

FIG. 2. (A) Southern blot hybridization of different restriction enzyme-digested genomes of *V. cholerae* O139 strains with *ctxA* or *RS* as a probe. With the *ctxA* probe, *Not*I, lanes: a, AS192; b, AS197; c, AS199; *Hin*dIII, lanes: a, SG24; b, AS192; c, AS197; d, AS199; *Ava*I, lanes: a, AS192; b, AS197; c, AS199; *Pst*I, lanes: d, AS192; e, AS197; f, AS199; *Bgl*II or *Eco*RV or *Not*I-*Hin*dIII, lanes: a, SG24; b, AS192; c, AS197; d, AS199. *RS* probe: *Bgl*II, lanes: a, AS192; b, AS197; c, AS199, d, SG24. Hybridization was carried out at 60°C, and the filter was washed under high-stringency conditions (0.1× SSC at 60°C). (B) Schematic representation of the origin of the 97-kb *Not*I and 23-kb *Bgl*II fragments in O139R and the 23-kb *Hin*dIII fragment of O139B and AS192 genomes in Southern hybridization with the *ctxA* gene probe (panel A). The open boxes represent core regions of the CTX genetic element. Thick and thin arrows indicate RS of O139B and O139R origin, respectively. Thin lines represent chromosomal DNA. A, *Ava*I; B, *Bgl*II; E, *Eco*RV; H, *Hin*dIII; N, *Not*I.

genomes of O139R strains, and the presence of the 7.3-kb common *Hin*dIII and *Eco*RI fragment, comprising one core region (4.5 kb) and one RS segment (2.8 kb), shows that these elements are present in tandem. Southern blot hybridization with the *ctxA* gene probe of *Ava*I- or *Pst*I-digested genomic DNA (Fig. 2A) also confirmed this possibility.

Single *Ava*I and *Pst*I sites are present in the core region of the CTX genetic element of O1 and O139B, and no site is present in the RS (7, 24). The probe hybridized with 14.3- and 7.3-kb *Ava*I and 5.8- and 7.3-kb *Pst*I fragments. The intensity of the common 7.3-kb DNA fragment is double that of other fragment (Fig. 2A). Hence, the 7.3-kb fragment comes from two comigrating copies of the CTX genetic element. The 14.3-kb *Ava*I and 5.8-kb *Pst*I fragments originated downstream of the CTX genetic elements and are similar in both O139R and O139B strains. Thus, the chromosomal location of the CTX genetic elements in the genomes of resurgent strains is probably same as in O139B strains (Fig. 2A and 4). Since two *Ava*I and *Pst*I (Fig. 2A) and three *Hin*dIII (Fig. 2A) and *Eco*RI fragments hybridized in Southern blot hybridizations with *ctxA* as probe, the *Hin*dIII and *Eco*RI sites must be located in the RS region of the CTX element in the O139R genome and not in the core. This was confirmed from the nucleotide sequence of the RS connecting the core regions of the CTX element (unpublished observation). Thus, two RS, as opposed to one in O139B (Fig. 4), link three copies of the CTX genetic elements in the genomes of O139R strains. The RS of the resurgent strains has *Hin*dIII and *Eco*RI sites, which are not present in the RS of other vibrios. This has prompted us to examine the RS upstream and downstream of the core in greater detail.

*Bgl*II has a single site in the RS of the CTX genetic element of O1 and O139B strains (7, 31). If RS flanks the three copies of the CTX genetic elements of O139R on both sides and if the *Bgl*II site is present in the RS, only one fragment of about 7.3 kb should hybridize with the *ctxA* probe. In contrast, if RS does not flank one of the CTX genetic elements on both sides, two DNA fragments would hybridize. The results presented here show that only one 23-kb *Bgl*II fragment of the O139R genome hybridized with the *ctxA* probe, except for the genome of O139R strain AS192, which hybridized with two *Bgl*II fragments of 7.0 and 8.1 kb, similar to the case for O139B strains (Fig. 2A).

The 23-kb *Bgl*II fragment originates from three of core regions ($4.5 \times 3 = 13.5$ kb), two RS segments ($2.8 \times 2 = 5.6$ kb), the chromosomal *Bgl*II site downstream of the elements (1.1 kb), and 2.3 kb of the upstream RS (Fig. 2B). Thus, the RS present upstream of the element contains a *Bgl*II site (Fig. 2 and 4). Similar arguments from Southern blot hybridization of *Not*I-*Hin*dIII-digested genomic DNA of O139R strains with the *ctxA* gene as a probe showed that the upstream RS contains a *Not*I site but no *Hin*dIII site (Fig. 2A). Hence, while the RS segments connecting the core regions of the CTX genetic elements in the O139R genome have undergone alterations, the RS present in upstream of the elements is of O139B origin. The status of the RS downstream of the CTX elements was examined by digesting genomic DNA of O139R strains with *Eco*RV, which has one site in the RS and one in the core region of the CTX genetic element (7, 31, 37), and hybridizing with the *ctxA* gene probe. The results presented here showed that two fragments of 4.0 and 3.5 kb hybridized (Fig. 2A). If RS were present downstream of the third element, only one fragment, of 4.0 kb, would have hybridized. The intensity of the 4.0-kb *Eco*RV fragment of the O139 genome is double that of other fragment (Fig. 2A). Hence, the 4.0-kb fragment comes from two comigrating copies of the CTX genetic element. This result also indicates that there are three copies of the CTX

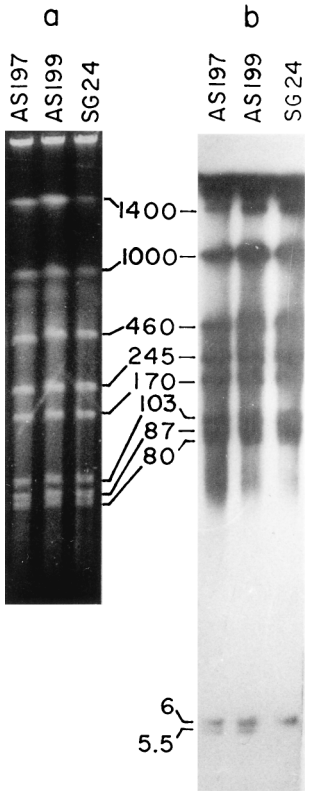

FIG. 3. PFGE of I-*Ceu*I-digested genomes of *V. cholerae* O139R and O139B strains. (a) The genomes of representative O139R and O139B strains were digested with I-*Ceu*I and electrophoresed with a pulse time interpolated between 5 and 100 s for 24 h at 10 V/cm at 3°C, and the gels were stained with ethidium bromide. (b) Autoradiogram of I-*Ceu*I-digested, end-labeled, PFGE-separated genomic DNA of *V. cholerae* O139R and O139B. The pulse time described in panel a was followed by ramping between 10 and 150 s for another 12 h at 5 V/cm. Two more I-*Ceu*I fragments in the O139R genome and one more in the O139B genome could be resolved under these pulse conditions by autoradiography. The numbers represent the I-*Ceu*I fragment sizes in kilobases.

genetic element in the O139R genome (Fig. 2B). Thus, as in the genomes of O139B strains, there is no RS downstream of the CTX element in the resurgent strains and the *Eco*RV sites are retained. Similar analyses of the genome of resurgent strain AS192 showed that the organization of its CTX genetic element is same as that in O139B strains (Fig. 4). It is worth mentioning that the organization of the CTX genetic element in different O139B strains isolated during the initial outbreak showed genetic heterogeneity in the population (7, 39). While most of the O139B isolates had two copies of the CTX genetic element, in some strains the number of elements was either amplified (three copies [39]) or reduced (one copy [7]).

The number of RS present upstream of the CTX elements in the genomes of O139R strains was determined by hybridizing *Bgl*II-digested genomic DNA with RS as a probe. The probe hybridized with two fragments of 23 and 2.8 kb (Fig. 2A). Since the cores of the CTX genetic elements in the genome of O139R are connected by one RS with no *Bgl*II site (Fig. 2A and 4), the 2.8-kb signal can arise only if there are two copies of RS of O139B origin present in tandem. The RS probe hybridized with three fragments of 8.1, 7.0, and 2.8 kb of O139B genome (Fig. 2A) as reported previously (7). The results of hybridization of genomic DNA of O139R strains with RS as a probe complement the results obtained with the *ctxA* gene as a probe. The presence of *Ava*I, *Pst*I, *Bgl*II, *Hin*dIII, and *Eco*RV in identical positions in the chromosomal DNA downstream of the CTX genetic element suggests that the location of these elements in the genome of O139R strains, including AS192, is same as that in the genomes of O139B strains (Fig. 4).

DISCUSSION

Resurgence of *V. cholerae* O139 with altered sensitivity to a number of antibiotics compared to O139B within a short period is unexpected in well-characterized clonal strains. The present study shows that in O139R strains clonality has not been retained and that the organization of the CTX genetic elements in the resurgent strains is different from that in O139B. Furthermore, an amplification of *rrn* operons has taken place in the genomes of O139R strains, including strain AS192, which showed extensive RFLP with other O139 isolates. This strain is sensitive to SXT and resistant to streptomycin and, unlike other resurgent strains, has not undergone any alteration in the organization of the CTX genetic element compared to that in O139B strains. Strain AS192 perhaps represents a different clone from those belonging to the O139B and O139R variants.

Resistance to SXT is used as a presumptive diagnosis of O139B strains (41), and all cells resistant to SXT possess a 62-kb self-transmissible, site-specific, and chromosomally integrating genetic element (40). The results presented in this report showed that the SXT-sensitive resurgent O139 strains, including AS192, still possess the 62-kb element, from which a 3-kb segment has been deleted. From the fact that all O139B strains are resistant and the resurgent isolates are sensitive to SXT, it is tempting to speculate that the sensitive phenotype might be of selective advantage to the organism for survival in the environment, maintaining its epidemic potential.

The gene encoding CT (*ctxAB*) is located in a 7.0- to 9.7-kb CTX genetic element in the genome of toxigenic *V. cholerae* strains, frequently in multiple copies arranged in tandem, but is absent in nontoxinogenic vibrios (31). Attachment sites that flank this element promote its integration into the genome by a site-specific mechanism. Several studies have indicated that even in strains of clonal origin, the CTX genetic elements can undergo rapid rearrangement in the form of amplification or deletion (7, 39). The O139B genome has two copies of CTX genetic elements in tandem, with the core regions being connected by two RS segments. In agreement with a recent report (35), the present study demonstrates that the genome of O139R strains has three copies of the element present in tandem. The present study also demonstrates that the RS connecting core regions of the elements is different from that in O139B strains (Fig. 4); it has gained *Hin*dIII and *Eco*RI and lost *Not*I and *Bgl*II sites. In all strains where the elements are present in tandem, only one *Hin*dIII or *Eco*RI fragment hybridizes, since there is no *Hin*dIII or *Eco*RI site either in the core or in the RS element of all O1 and O139B strains examined (6, 7, 24). Since the RS in the CTX genetic elements of O139R strains contains *Hin*dIII and *Eco*RI sites (Fig. 4), these enzymes can no longer be considered diagnostic for differentiating biotypes. Besides, the *Bgl*II site, which is conserved in the RS of all O1 and O139B strains, is lost in the RS of O139R strains. Surprisingly, RS of O139B origin is retained upstream of the three CTX genetic elements of O139R strains and two copies of it are present in tandem compared to one in O139B strains.

The number and location of *rrn* operons in enteric bacteria appear to be highly conserved. In contrast to seven *rrn* operons in the genomes of different enteric gram-negative organisms

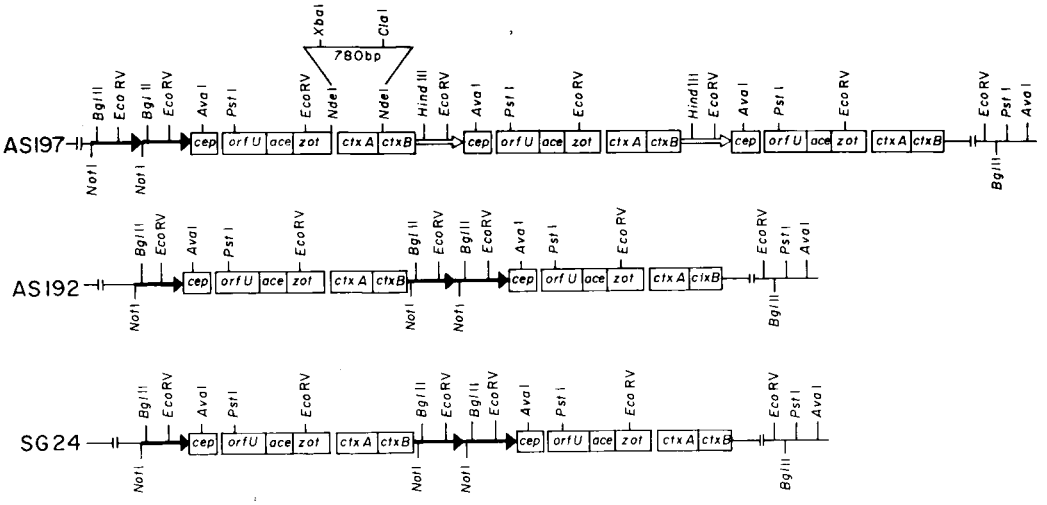

FIG. 4. Schematic representation of the organization of the CTX genetic element in *V. cholerae* O139R and O139B strains (not drawn to scale). Open boxes are coding regions of the corresponding genes comprising the core region of the element; thick arrows indicate RS of O139B origin; hollow arrows indicate RS of O139R origin; and thin lines represent chromosomal DNA. The 780-bp *Nde*I fragment of the *ctxAB* gene was used as a probe.

(20), the genomes of *V. cholerae* O1 and O139B have nine (17, 30). *V. cholerae* strains belonging to non-O1 and non-O139 serogroups, which are autochthonous organisms in the aquatic environment, have 10 *rrn* operons in their genome (30), as in the O139R genome investigated in the present study. Intraspecies variation in the number of *rrn* operons has recently been found in *V. cholerae* (30). The present study shows variation in the number of *rrn* operons even within the same serovar. While Sharma et al. (35) could not detect any difference in the ribotype between O139R and O139B strains, Faruque et al. (13) have shown that a 3-kb *Bgl*I fragment containing the rRNA gene is unique to reemerging *V. cholerae* O139 strains isolated in Bangladesh during 1995 and 1996. The 3-kb *Bgl*I fragment cannot originate from the generation of a *Bgl*I site in one of the *rrn*-containing fragments in the genomes of O139 strains isolated during 1992 and 1993, because the rRNA gene restriction patterns of two clones, apart from the 3-kb *Bgl*I fragment, are identical (13). It was also reported that the strains belonging to a new ribotype also belonged to a new *ctx* genotype (13). It appears that the reemergent O139 strains described by Faruque et al. (13) are probably of the same genotype as the O139R isolates of Indian origin. Thus, an amplification of *rrn* operons in the genomes of O139R strains has occurred. However, Basu et al. (3) have shown that very recent Bangladesh O139 isolates have similar ribotype and *ctx* genotype to O139B but that all these strains are sensitive to streptomycin. Thus, the Bangladesh O139 strains studied by Basu et al. are different from O139B and O139R strains and the strains described by Faruque et al. (13).

Spontaneous duplication of *rrn* operons by homologous recombination has been found in *Salmonella typhimurium* during laboratory maintenance (2, 14). This has not happened in O139R strains since the cells were minimally subcultured. Besides, the genomes of all the O139R isolates produced identical I-*Ceu*I cleavage profiles (Fig. 3). No change in the number of *rrn* operons was detected in the genomes of O139B strains, which were repeatedly subcultured in the laboratory since 1992. The number of *rrn* operons is directly proportional to the selective advantage of the survival of the organisms in a continually fluctuating environment (18). Many different factors in the aquatic environment affect the growth and survival of *V. cholerae* cells. Furthermore, in the course of the transition from a typical environmental source such as water to the human intestine, these bacteria are exposed to a series of environmental changes. The observed modulation in the number of *rrn* operons might be necessary for the survival of the organism in the human intestine as well as for maintaining the genomic plasticity through *rrn*-mediated rearrangements which have been shown to be characteristic of *V. cholerae* (30). It has been predicted that the possibility of genome rearrangements is greater when there are more repeats of the *rrn* operons (18).

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REFERENCES

1. **Albert, M. J., A. K. Siddique, M. S. Islam, A. S. J. Faruque, M. Ansaruzzaman, S. M. Faruque, and R. B. Sack.** 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet **341:**704.
2. **Anderson, R. P., and J. R. Roth.** 1978. Gene duplication in bacteria: alteration of gene dosage by sister chromosome exchange. Cold Spring Harbor Symp. Quant. Biol. **43:**1083–1087.
3. **Basu, A., A. K. Mukhopadhyay, C. Sharma, J. Jyot, N. Gupta, A. Ghosh, S. K. Bhattacharya, Y. Takeda, A. S. G. Faruque, M. J. Albert, and G. B. Nair.** 1998. Heterogeneity in the organization of the CTX genetic element in strains of *Vibrio cholerae* O139 Bengal isolated from Calcutta, India and Bangladesh and its possible link to the dissimilar incidence of O139 cholera on the two locales. Microb. Pathog. **24:**175–183.
4. **Baudry, B., A. Fasano, J. Ketley, and J. B. Kaper.** 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. **60:**428–434.
5. **Berche, P., C. Poyart, E. Abachin, H. Lelivre, J. Vandepitte, A. Dodin, and J.-M. Fournier.** 1994. The novel epidemic strain O139 is closely related to the pandemic strain O1 of *Vibrio cholerae*. J. Infect. Dis. **170:**701–704.
6. **Bhadra, R. K., S. Roychowdhury, and J. Das.** 1994. *Vibrio cholerae* O139 El Tor biotype. Lancet **343:**728.
7. **Bhadra, R. K., S. Roychoudhury, R. K. Banerjee, S. Kar, R. Majumdar, S. Sengupta, S. Chatterjee, G. Khetawat, and J. Das.** 1995. Cholera toxin (CTX) genetic element in *Vibrio cholerae* O139. Microbiology **141:**1977–1983.
8. **Bik, E. M., A. E. Bunschoten, R. D. Gouw, and F. R. Mooi.** 1995. Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal

transfer of genes involved in polysaccharide synthesis. EMBO J. **14:**209–216.
9. **Calia, K. E., M. Muratagh, M. J. Ferraro, and S. B. Calderwood.** 1994. Comparison of *Vibrio cholerae* O139 with *V. cholerae* O1 classical and El Tor biotypes. Infect. Immun. **62:**1504–1506.
10. **Cholera Working Group.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342:** 387–390.
11. **Comstock, L. E., J. A. Johnson, J. M. Michalski, J. G. Morris, Jr., and J. B. Kaper.** 1996. Cloning and sequence of a region encoding a surface polysaccharide of *Vibrio cholerae* O139 and characterization of the insertion site in the chromosome of *Vibrio cholerae* O1. Mol. Microbiol. **19:**815–826.
12. **Dasgupta, U., R. K. Bhadra, D. K. Panda, A. Deb, and J. Das.** 1994. Recombinant derivative of a naturally occurring non-toxinogenic *Vibrio cholerae* O1 expressing the B subunit of cholera toxin: a potential oral vaccine strain. Vaccine **12:**359–364.
13. **Faruque, S. M., K. M. Ahmed, A. K. Siddique, K. Zaman, A. R. M. Abdul Alim, and M. J. Albert.** 1997. Molecular analysis of toxigenic *Vibrio cholerae* O139 Bengal strains isolated in Bangladesh between 1993 and 1996: evidence for emergence of a new clone of the Bengal vibrios. J. Clin. Microbiol. **35:**2299–2306.
14. **Hill, C. W., and G. Combriato.** 1973. Genetic duplications produced at very high frequency by ultraviolet irradiation in *Escherichia coli*. Mol. Gen. Genet. **127:**197–214.
15. **Kaper, J. B., S. L. Moseley, and S. Falkow.** 1981. Molecular characterization of environmental and nontoxinogenic strains *Vibrio cholerae*. Infect. Immun. **32:**661–667.
16. **Kaper, J. B., H. B. Bradford, N. C. Roberts, and S. Falkow.** 1982. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. J. Clin. Microbiol. **16:**129–134.
17. **Koblavi, S., F. Grimont, and P. A. D. Grimont.** 1990. Clonal diversity of *Vibrio cholerae* O1 evidenced by rRNA gene restriction patterns. Res. Microbiol. **141:**645–657.
18. **Krawiec, S., and M. Riley.** 1990. Organization of the bacterial genome. Microbiol. Rev. **54:**502–539.
19. **Liu, S.-L., A. Hessel, and K. E. Sanderson.** 1993. Genomic mapping with I-*Ceu*I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli* and other bacteria. Proc. Natl. Acad. Sci. USA **90:**6874–6878.
20. **Liu, S.-L., and K. E. Sanderson.** 1995. Rearrangements in the genome of the bacterium *Salmonella typhi*. J. Bacteriol. **177:**3355–3357.
21. **Liu, S.-L., and K. E. Sanderson.** 1996. Highly plastic chromosomal organization in *Salmonella typhi*. Proc. Natl. Acad. Sci. USA **93:**10303–10308.
22. **Majumder, R., S. Sengupta, G. Khetawat, R. K. Bhadra, S. Roychoudhury, and J. Das.** 1996. Physical map of the genome of *Vibrio cholerae* 569B and localization of genetic markers. J. Bacteriol. **178:**1105–1112.
23. **Marshall, P., and C. Lemieux.** 1991. Cleavage pattern of the homing endonuclease encoded by the fifth intron in the chloroplast large subunit rRNA-encoding gene of *Chlamydomonas eugametos*. Gene **104:**241–245.
24. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35:**253–263.
25. **Mitra, R., A. Basu, D. Dutta, G. B. Nair, and Y. Takeda.** 1996. Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. Lancet **348:**1181.
26. **Morris, J. G.** 1990. Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. Epidemiol. Rev. **12:**179–191.
27. **Mukhopadhyay, A. K., S. Garg, G. B. Nair, S. Kar, R. K. Ghosh, S. Pajni, A. Ghosh, T. Shimada, T. Takeda, and Y. Takeda.** 1995. Biotype traits and antibiotic susceptibility of *Vibrio cholerae* serogroup O1 before, during and after the emergence of the O139 serogroup. Epidemiol. Infect. **115:**427–434.
28. **Mukhopadhyay, A. K., S. Garg, R. Mitra, A. Basu, K. Rajendran, D. Dutta, S. K. Bhattacharya, T. Shimada, T. Takeda, Y. Takeda, and G. B. Nair.** 1996. Temporal shifts in traits of *Vibrio cholerae* strains isolated from hospitalized patients in Calcutta: a 3-year (1993–1995) analysis. J. Clin. Microbiol. **34:** 2537–2543.
29. **Nair, G. B., T. Shimada, H. Kurazono, J. Okuda, A. Pal, T. Karasawa, T. Mihara, Y. Uesaka, H. Shirai, S. Garg, P. Saha, A. Mukhopadhyay, T. Ohashi, J. Tada, T. Tada, T. Nakayama, S. Fukushima, T. Takeda, and Y. Takeda.** 1994. Characterization of phenotypic, serological, and toxigenic traits of *Vibrio cholerae* O139B. J. Clin. Microbiol. **32:**2775–2779.
30. **Nandi, S., G. Khetawat, S. Sengupta, R. Majumder, S. Kar, R. K. Bhadra, S. Roychoudhury, and J. Das.** 1997. Rearrangements in the genomes of *Vibrio cholerae* strains belonging to different serovars and biovars. Int. J. Sys. Bacteriol. **47:**858–862.
31. **Pearson, G. D. N., A. Woods, S. L. Chiang, and J. J. Mekalanos.** 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. USA **90:**3750–3754.
32. **Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazono, A. Pal, and Y. Takeda.** 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341:**703–704.
33. **Roy, N. K., G. Das, T. S. Balganesh, S. N. Dey, R. K. Ghosh, and J. Das.** 1982. Enterotoxin production, DNA repair and alkaline phosphatase of *Vibrio cholerae* before and after animal passage. J. Gen. Microbiol. **128:**1927–1932.
34. **Roychoudhury, S., R. K. Bhadra, and J. Das.** 1994. Genome size and restriction fragment length polymorphism analysis of *Vibrio cholerae* strains belonging to different serovars and biotypes. FEMS Microbiol. Lett. **115:** 329–334.
35. **Sharma, C., S. Maiti, A. K. Mukhopadhyay, A. Basu, I. Basu, G. B. Nair, R. Mukhopadhyaya, B. Das, S. Kar, R. K. Ghosh, and A. Ghosh.** 1997. Unique organization of the CTX genetic element in *Vibrio cholerae* O139 strains which reemerged in Calcutta, India, in September 1996. J. Clin. Microbiol. **35:**3348–3350.
36. **Shimada, T., E. Arakawa, K. Itoh, T. Okitsu, A. Matsushima, Y. Asai, S. Yamai, T. Nakazato, G. B. Nair, M. J. Albert, and Y. Takeda.** 1994. Extended serotyping scheme for *Vibrio cholerae*. Curr. Microbiol. **28:**175–178.
37. **Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, and J. B. Kaper.** 1993. Accessory cholera enterotoxin (*Ace*), the third toxin of a *Vibrio cholerae* virulence cassette. Proc. Natl. Acad. Sci. USA **90:**5267–5271.
38. **Wachsmuth, K., O. Olsvik, G. M. Evins, and T. Popovic.** 1994. Molecular epidemiology of cholera, p. 357–370. *In* I. K. Wachsmuth, P. A. Blake, and F. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. American Society for Microbiology, Washington, D.C.
39. **Waldor, M. K., and J. J. Mekalanos.** 1994. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in *Vibrio cholerae* O139 and development of a live vaccine prototype. J. Infect. Dis. **170:**278–283.
40. **Waldor, M. K., H. Tschape, and J. J. Mekalanos.** 1996. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J. Bacteriol. **178:**4157–4167.
41. **Yam, W.-C., K.-Y. Yuen, S.-S. Wong, and T.-L. Que.** 1994. *Vibrio cholerae* O139 susceptible to vibriostatic agent O/129 and co-trimoxazole. Lancet **344:**404–405.
42. **Yamasaki, S., G. B. Nair, S. K. Bhattacharya, S. Yamamoto, H. Kurazono, and Y. Takeda.** 1997. Cryptic appearance of a new clone of *Vibrio cholerae* serogroup O1 biotype El Tor in Calcutta, India. Microbiol. Immunol. **41:**1–6.

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