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Genetic Mapping of *Vibrio cholerae* Enterotoxin Structural Genes

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The structural genes which constitute the cholera toxin operon, *ctxAB*, were genetically mapped in the *Vibrio cholerae* El Tor strain RV79. This strain of *V. cholerae* contains two copies of the *ctx* operon located on a 7-kilobase-pair tandemly duplicated region. We began by isolating a vibriophage VcA1 insertion mutation in one of the two *ctxA* genes located in this region. The mutant carrying this *ctxA*::VcA1 insertion, DC24, was converted to a VcA1-facilitated donor by introduction of the conjugal plasmid pSJ15, which carries an inserted copy of a defective VcA1-like prophage. The donor characteristics of DC24(pSJ15) indicated that the *ctxA*::VcA1 insertion mutation was near the *trp* region of the *V. cholerae* chromosome. Subsequent RV79 three-factor crosses were performed between VcA1-facilitated donors and recipient strains carrying one of two structural gene mutations in *ctx*, either Δ*ctxA23P* Km^r or Δ*ctx-7922*. The former was constructed by an in vivo marker exchange procedure and could be scored either by its kanamycin resistance phenotype or by its lack of DNA sequences homologous to the *ctxA* region. The Δ*ctx-7922* mutation is a total deletion of both *ctx* copies of strain RV79. The three-factor cross data strongly suggest that the two *ctx* loci of RV79 map between the *nal* and *his* genes of *V. cholerae* in the *trp nal his* linkage group. Physical analysis and heterologous crosses between an RV79 El Tor donor and a 569B classical recipient indicates that one of the two 569B *ctx* operon copies maps in the same region as the RV79 *ctx* loci (i.e., linked to *nal*). Together with previously published observations, these data show that the *ctx* structural genes are not closely linked to other genes known to affect toxin production in *V. cholerae*.

Toxinogenic strains of *Vibrio cholerae* produce an extracellular heat-labile protein that is primarily responsible for the diarrheal syndrome observed in Asiatic cholera (8). Cholera toxin is now recognized as the prototype for a growing family of protein enterotoxins that produce their toxic effects via the activation of adenylate cyclase in eucaryotic cells (4, 5, 9). The heat-labile enterotoxin of *Escherichia coli* also belongs to this family, and recent DNA sequence analysis has shown the heat-labile enterotoxin genes to be 78% homologous to the cholera toxin genes (5; J. J. Mekalanos, D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. deWilde, Nature [London], in press). However, the LT operon *eltAB* appears to be exclusively located on plasmids (7, 28), while the data suggest that the cholera toxin operon *ctxAB* is located on the bacterial chromosome. This conclusion has been based primarily on the absence of demonstrable plasmids in toxinogenic *V. cholerae* strains of either the classical or El Tor biotypes (15, 22).

Although a variety of laboratories have isolated mutants and genetically mapped mutations that affect toxin production in the highly toxinogenic classical strain 569B, all of these mutations have turned out to be regulatory mutations (2, 10, 12, 17, 19, 20). The explanation for this failure to obtain structural gene mutations in *ctx* in this particular *V. cholerae* strain is presumably related to the fact that the *ctx* operon is duplicated in 569B (22, 25). Although the precise physical structure of the *ctx* duplication of strain 569B is not yet known, the same *ctx* duplication is present in all *V. cholerae* strains of the classical biotype. This is indicated by the fact that all classical strains examined show the same two bands as 569B when their DNA is analyzed by Southern blot hybridization with probes derived from cloned *ctxAB* genes (J. J. Mekalanos, Cell, in press). Other studies also demonstrated that some *V. cholerae* strains of the El Tor biotype contain multiple copies of the *ctx* operon which are organized on tandemly repeated DNA. For example, the El Tor strain RV79 was shown to contain two copies of the *ctx* operon located on a 7-kilobase-pair (kb) tandemly duplicated region. These studies also suggested that the DNA duplicated in strain RV79 might define a genetic element that is responsible for possible *ctx* transposition and amplification events.

In this paper, we report our results concerning the genetic mapping of the *ctx* loci of strains RV79 and 569B. The tandem duplication carrying the *ctx* loci of strain RV79 was shown to map between the *nal* and *his* genes of *V. cholerae*. Although one of the two *ctx* copies of strain 569B appears to have a similar physical and genetic location, the other *ctx* copy is genetically separable from the first *ctx* copy and the *nal* region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *V. cholerae* and the plasmids are listed in Table 1. The plasmid content of a strain is given in parentheses after its name in the text.

Media. LB and CYE media, BHI broth, and M63 minimal medium have been described previously (20, 21). Adenine and thiamine were always added to M63 minimal medium to a final concentration of 20 and 2.5 μg/ml, respectively. TYCC medium contained 10 g of tryptone, 1 g of yeast extract, 1 g of dextrose, 8 g of NaCl, and 0.5 g of $CaCl_2$ per liter; the pH was adjusted to 7.8. Trimethoprim medium consisted of M63 medium containing 50 μg of thymine, 10 μg of trimethoprim, and 20 μg of methionine per ml. Plates contained 15 g of Bacto-Agar (Difco Laboratories) per liter. Soft agar for overlays consisted of TYCC medium and 5 g of Bacto-Agar per liter. When Thy$^-$ strains were used, all media were supplemented with 100 μg of thymine per ml. Amino acids and nucleotide bases were added as needed at a concentration of 100 μg/ml. Unless otherwise stated, antibiotics were present at the following concentrations: 100 μg of

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TABLE 1. *V. cholerae* strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Other information (reference)
RV79	Prototrophic	El Tor biotype (same as RJ1 [14])
RV79 derivatives		
JM7943	*str-79*	Spontaneous Sm^r mutant of RV79
DC24	*str-79 ctxA*::VcA1	From JM7943 by lysogenization with VcA1
SM101	*met-101 spc-101* (VcA1)	From RV79 by NTG[b] mutagenesis followed by selection for spontaneous resistance to spectinomycin
RV79110	*met-14*[c] *trp-1 rif-79110 str-79110*	Johnson, Ph.D. thesis
SM102	*met-14 trp-1 rif-79110 str-79110* (VcA1)	From RV79110
SM103	*met-14 trp-1 rif-79110 str-79110 nal-103* (VcA1)	Spontaneous Nal^r mutant of SM102
RV792	*his-1 str-792*	Johnson, Ph.D. thesis
SM104	*his-1 str-792 rif-104 nal-104* (VcA1)	Spontaneous Rif^r Nal^r mutant of RV792, lysogenized with VcA1
M7922	Δ*ctx-7922*	(18)
SM19	Δ*ctx-7922 met*::VcA1	From M7922 by lysogenization with VcA1
SM31	Δ*ctx-7922 his*::VcA1	From M7922 by lysogenization with VcA1
SM201	Δ*ctxA23P* Km^r *str-79*	From JM7943 by marker exchange with pJM290.14 (this work)
SM798	*his*::VcA1 *rif-798*	Spontaneous Rif^r mutant of RV79 lysogenized with VcA1
SM204	Δ*ctxA23P* Km^r *str-79 nal-204 thy*::VcA1	From SM201 by random lysogenization with VcA1 and selection on trimethoprim and thymine
SM231	Δ*ctxA23P* Km^r *his*::VcA1	From SM31 by transduction of Δ*ctxA23P* Km^r from SM201 with vibriophage CP-T1 (23)
569B	Prototrophic	Classical biotype
569B derivatives[d]		
RV503	*arg-1 spc-503*	(20)
RV504	*arg-1 ilv-1 rif-504 spc-503*	From RV503
RV505	*arg-1 trp-505 rif-505 spc-503*	(20)
RV508	*arg-1 met-2 rif-508 spc-503*	From RV503 by NTG-induced *rif* comutagenesis
SM601	*arg-1 trp-505 rif-505 spc-503 thy-601*	From RV505 by selection with trimethoprim and thymine
JM32	*cys-1 his-3 nal-32 rif-32 spc-32 tox-101*	From MN1 (19) by NTG mutagenesis and selection for spontaneous resistance to spectinomycin
Plasmids		
pSJ15	P::Tn*1*(::VcA1 defective) Ap^r	(13)
pJM290.14	IncP Δ*ctxA23P* Km^r Tc^r Km^r	This work
pPH1JI	IncP Gm^r Sp^r	(27)

[a] Designations correspond to those of Johnson and Romig (14). Laboratory strains of *V. cholerae* often acquire a spontaneous purine auxotrophy that is not listed in the genotype. All minimal media used therefore contained 20 μg of adenine per ml. Lysogens containing a random insertion of VcA1 are designated by (VcA1) after the genotype.

[b] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

[c] The *met-14* allele was not used in this study because it was too leaky to score Met$^+$ recombinants reliably.

[d] All 569B derivatives are lysogenic for a defective VcA1-like prophage.

streptomycin (SM) per ml, 50 μg of spectinomycin (Sp) per ml in M63, 75 μg of spectinomycin per ml in rich media, 15 μg of rifampin (Rif) per ml in M63, 30 μg of rifampin per ml in rich media, 30 μg of kanamycin (Km) per ml in M63, 45 μg of kanamycin per ml in rich media, 15 μg of tetracycline (Tc) per ml, 30 μg of gentamicin (Gm) per ml, and 50 μg of ampicillin (Ap) per ml. Nalidixic acid (Nal) was used at a concentration of 2 μg/ml to select spontaneous single-step resistant mutants. This low concentration of nalidixic acid was used to avoid possible linkage ambiguity associated with two-step, high-level resistance (24).

Construction of VcA1 lysogens. RV79 derivatives were infected with vibriophage VcA1 (13) by the agar overlay method (1) so as to obtain 10^3 to 10^4 plaques per plate. Plates were incubated at 30°C overnight. A sample of the overlay was suspended in LB broth, grown at 37°C with gentle shaking for 2 h, and streaked out for single colonies on TYCC plates. The colonies were purified by restreaking on TYCC plates and were confirmed to be VcA1 lysogens by stab inoculation into a lawn of phage-sensitive RV79 indicator cells. A zone of clearing around an individual stab indicated phage release and therefore lysogeny.

Isolation of VcA1-induced auxotrophs of RV79. A lysogen population was prepared from plates containing about 10^4 plaques as described above. After growth in LB broth at 37°C for 2 h, the cells were pelleted by centrifugation at 4,500 rpm for 5 min in a Sorvall SS34 rotor. The cells were suspended in an equal volume of M63 salts and plated onto M63 plates supplemented with 200 μg of nutrient broth (Difco) to give isolated colonies (13, 33). After incubation at 37°C for 2 days, tiny colonies were transferred with toothpicks onto TYCC plates. After incubation, the colonies were transferred to M63 plates. Colonies that were unable to grow on M63 plates were tested for their ability to grow on M63 plates supplemented vith various amino acids and were confirmed to be lysogens by the phage-release test.

A *thy*::VcA1 insertion mutant of strain SM201 was constructed by making a VcA1 lysogen population on an LB-kanamycin plate containing 100 μg of thymine per ml. The plate was incubated at 30°C overnight, and plaques were scraped off and suspended in 5 ml of LB. One milliliter of this suspension was incubated in 9 ml of LB-kanamycin-thymine for 3 h at 37°C. The culture was then plated onto

trimethoprim plates prepared as described above, except that the trimethoprim plates contained kanamycin. Trimethoprim-resistant colonies were transferred with toothpicks onto M63 plates and scored for their inability to grow in the absence of thymine and lack of reversion to the Thy^+ phenotype. Lysogeny was confirmed by the phage-release test.

Construction of the *ctxA*::VcA1 mutant strain DC24. A VcA1 lysogen population derived from strain JM7943 was prepared as described above. The lysogen population was grown at 37°C in TYCC broth for several hours, and colonies derived from this culture were screened by the ganglioside filter assay (17) for reduction or loss of toxin production. Mutant strain DC24 was confirmed to be a VcA1 lysogen, and analysis by Southern blots showed that it carries a VcA1 prophage inserted in the *ctx* operon (see below).

Mating procedures. Overnight cultures (2 ml) of each donor and recipient were grown at 37°C in BHI-ampicillin and BHI broth, respectively. The donor culture was pelleted and suspended in an equal volume of BHI, and both donor and recipient cultures were separately diluted 1:20 into 2 ml of BHI. After 1 h of incubation at 37°C, 0.2 ml of the donor culture was added to the recipient culture. The mating mixture was incubated at 37°C with gentle shaking for 2 h. A 1.5-ml volume of the mating mixture was then transferred to a sterile 1.5-ml microfuge tube, centrifuged for 15 s, suspended in 0.1 ml of BHI, and plated onto selective medium. Donors were always counterselected with an antibiotic and, whenever possible, by the absence of an amino acid essential for their growth. M63 plates were incubated for 2 to 3 days, and LB and TYCC plates were incubated for 24 h at 37°C. All recipients were made lysogenic for VcA1 to avoid zygotic induction upon entry of pSJ15.

Where quantitative matings were performed, the mating mixtures were diluted in M63 liquid medium before plating on selective media. Donor input was quantitated by determining the number of viable donor cells added to the mating mixture. Transfer frequencies are expressed as number of recombinants obtained per input donor cell.

Construction of strain SM201. Strain SM201 is a derivative of strain RV79 that has had its two resident *ctx* copies replaced by the *ctx* construction present on plasmid pJM290.14. Details on the construction of pJM290.14 and strain SM201 will be presented elsewhere (Mekalanos, in press), but a summary is given here. Plasmid pJM290.14 contains a cloned DNA fragment from strain 569B that carries a *ctx* operon copy, together with adjacent sequences homologous to the DNA that flanks the two *ctx* copies of strain RV79. The *ctx* operon copy of pJM290.14 also contains a 450-base-pair (bp), in vitro-generated internal deletion of the *ctxA* cistron which makes this *ctx* construction unreactive with LT-A1 probe (see below). About 2.7 kb upstream of this *ctxA* deletion is inserted a 1.4-kb *Pst*I fragment encoding resistance to kanamycin. The entire construction contained on the cloned *Eco*RI fragment of pJM290.14 is referred to as the *ΔctxA23P* Km^r allele. Construction of strain SM201 involved an in vivo marker exchange procedure similar to that of Ruvkun and Ausubel (26) and is schematically shown in Fig. 3. Plasmid pJM290.14 was first mobilized into an Sm^r RV79 derivative, JM7943. JM7943(pJM290.14) was then superinfected with plasmid pPH1JI (30), and Km^r Gm^r Sm^r colonies were selected. Since pJM290.14 and pPH1JI are incompatible, these triply resistant colonies represented recombinants that had recombined the *ΔctxA23P* Km^r allele onto the RV79 chromosome via crossover events between homologous DNA flanking the *ctx* regions. One particular recombinant, which was recognized by Southern blot analysis, had both of its wild-type *ctx* copies replaced by the *ΔctxA23P* Km^r allele as shown in Fig. 3. This recombinant was cured of pPH1JI to give strain SM201.

Isolation of Km^r transductants. Stocks of vibriophage CP-T1 (23) were grown on SM201 by the method of Adams (1). Titers were found to be approximately 10^{11} pfu/ml. A culture of the strain to be transduced was grown in LB to an optical density at 590 nm of 0.45. The phage stock was diluted 1:100 in 10 ml of mM Tris-chloride (pH 7.2)–10 mM $MgCl_2$ and gently swirled in a petri dish for 30 s under a Sylvania 615T8 germicidal lamp, the output of which was calibrated to be 8 ergs/s-mm^2 at the surface of the dish. This irradiation reduced the plaque-forming activity of the phage suspension by a factor of about 10^3. Samples (2 ml) of a fresh bacterial culture were prepared and 1.0, 0.1 and 0.01 ml of the UV-irradiated phage suspension were added. The phage was absorbed at 37°C for 10 min with gentle shaking. The infected cells were then centrifuged in a Sorvall SS-34 rotor at 4,500 rpm for 5 min and washed twice in 0.85% saline. The cells were concentrated 10-fold in saline and spread onto LB-kanamycin plates. Km^r colonies were purified, and their DNA was analyzed by Southern blots with a CT-1 probe (see below) to confirm that these transductants had the *ΔctxA23P* Km^r allele recombined in place of their wild-type *ctx* sequences.

Southern blot analysis. Chromosomal DNA was prepared as described by Brenner et al. (3). Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) Genomic digests were fractionated by electrophoresis on 0.7% agarose gels and transferred to nitrocellulose sheets as described by Southern (31). Hybridization of radioactive probes to the nitrocellulose was performed as described by Moseley and Falkow (22). The LT-A1 probe is a 475-bp *Xba*I-*Hin*dIII fragment from EWD299 (6), whereas the LT-B probe is a 490-bp *Eco*RI-*Hin*dIII fragment from the same plasmid. The CT-1 probe is a 918-bp *Xba*I-*Hin*cII fragment from plasmid pJM17 (25). The LT-A1 and LT-B probes are homologous to *ctxA* and *ctxB*, respectively, whereas the CT-1 probe is homologous to both *ctxA* and *ctxB*. Probes were purified from polyacrylamide gel slices by electroelution and labeled with [α^{32}P]dCTP (7,000 Ci/mmole; New England Nuclear Corp., Boson, Mass.) by nick translation (16).

***In situ* colony hybridization.** The method of Grunstein and Hogness (11) was used to score colonies for the presence of sequences homologous to the LT-A1 probe. Colonies were grown in patches on TYCC plates at 30°C overnight. They were replica plated onto TYCC plates on which a sterile nitrocellulose filter (BA85; Schleicher & Schuell Co., Keene, N.H.) had been laid. After incubation at 30°C overnight, the filters were sequentially placed onto five sets of four stacked Whatman no. 1 filter papers containing 0.5 M NaOH, 1 M Tris (pH 7.0), 1 M Tris (pH 7.0), 1 M Tris (pH 7.0), and 1 M Tris (pH 7.0) with 1.5 M NaCl, respectively. The nitrocellulose filters were exposed to these solutions for 10, 1, 1, 1, and 10 min, respectively. The filters were then dried at 37°C and baked at 85°C for 5 to 10 min. Hybridization with the LT-A1 probe was performed as described by Moseley and Falkow (22). Positive and negative control colonies were included on each filter in this analysis.

RESULTS

Isolation and characterization of a VcA1 insertion mutation in *ctxA*. VcA1 is a temperate vibriophage with biological

properties resembling those of phage Mu in *E. coli* (13). Johnson et al. (13) have recently described a gene transfer system that uses inserted VcA1 prophages as portable regions of homology between the *V. cholerae* conjugal sex factor P and the *V. cholerae* chromosome. In this system, the chromosomal location of a VcA1 prophage can be inferred from the donor characteristics of the corresponding lysogenic strain when it carries the plasmid pSJ15. Since pSJ15 contains an inserted copy of a defective VcA1-like prophage, transient integration of pSJ15 into the *V. cholerae* chromosome presumably occurs, directed by homologous crossover events between the chromosomal and plasmid copies of VcA1. Thus, the location and orientation of the VcA1 prophage on the chromosome determines the origin and polarity of transfer of genetic markers by one of these donors (13). Such donor strains containing a chromosomal VcA1 prophage and plasmid pSJ15 are referred to as VcA1-facilitated donors.

We began our genetic mapping of *ctx* by analyzing the VcA1-facilitated donor properties of a toxin mutant that carried a VcA1 insertion in *ctx*. This mutant was isolated from a pool of VcA1 lysogens of strain JM7943 that was screened for a reduction or loss of toxin production in the ganglioside filter assay (17). Out of approximately 17,000 colonies screened, we obtained 24 VcA1-induced hypotoxinogenic mutants, of which one, strain DC24, was shown by Southern blot analysis to carry a VcA1 insertion in the *ctx* locus (Fig. 1). The structural analysis of this mutant was

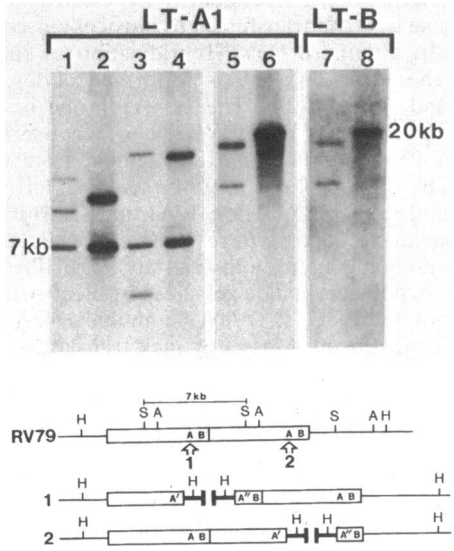

FIG. 1. (Top) Southern blot analysis of RV79 and DC24. DNA (about 1 μg) from either RV79 (even-numbered lanes) or DC24 (odd-numbered lanes) was digested with restriction enzymes and fractionated by electrophoresis on a 0.7% agarose gel. The DNA fragments were transferred to nitrocellulose sheets and hybridized to either radioactive LT-A1 or LT-B probe at the appropriate level of stringency (22). The DNA was digested as follows: lanes 1 and 2, *Sph*I; lanes 3 and 4, *Ava*I; lanes 5 through 8, *Hin*dIII. (Bottom) Schematic model of the RV79 chromosome showing two possible *ctxA* insertion sites for the VcA1 prophage (arrows) in DC24 and the predicted *Hin*dIII fragments generated in the Southern blot analysis. The open boxes represent the 7 kb of DNA tandemly duplicated in RV79 which each carry a *ctxAB* operon. Above these are shown the approximate locations of sites for the enzymes *Hin*dIII (H), *Sph*I (S), and *Ava*I (A). The heavy bars represent VcA1 sequences that carry sites for *Hin*dIII as indicated.

TABLE 2. Transfer frequencies of selected markers by donor DC24(pSJ15) into RV79- and 569B-derived recipients

Recipient	Selected phenotype	Transfer frequency[a]
RV79 derivatives		
SM101	Met^+	8.5×10^{-6}
SM103	Trp^+	4.6×10^{-3}
SM104	His^+	8.0×10^{-6}
569B derivatives		
JM32	His^+	8.6×10^{-4}
SM601	Trp^+	4.2×10^{-3}
	Thy^+	1.8×10^{-4}
RV504	Ilv^+	1.0×10^{-4}
RV508	Arg^+	2.3×10^{-4}
	Met^+	4.8×10^{-3}

[a] Recombination frequencies are reported as recombinants per input donor. The donor was counterselected with either rifampin or spectinomycin.

complicated by the fact that strain RV79 carries two copies of the *ctx* locus, located on a tandemly duplicated 7-kb region of the chromosome (Mekalanos, in press).

Southern blot analysis of RV79 DNA with LT-A1 or LT-B probes gives two bands with either *Sph*I or *Ava*I, enzymes that are known to cut once within the tandem repeat at positions upstream from *ctx* (Mekalanos, in press) (Fig. 1). One of these fragments spans the novel joint of the tandem duplication and is 7 kb in size in both enzyme digests. The VcA1 insertion in DC24 does not affect the mobility of the 7-kb band in either the *Ava*I or *Sph*I genomic digests but does split the other band into two new fragments that both hybridize to the LT-A1 probe (Fig. 1).

This observation indicates that the prophage is inserted in *ctx* sequences homologous to the LT-A1 probe. The enzyme *Hin*dIII, which cuts outside the tandemly duplicated region, produced in this blot analysis a single 20-kb band for RV79, but gave two new bands for DC24, both of which hybridized to both the LT-A1 and LT-B probes. Moreover, when this same blot was probed with radioactively labeled VcA1 DNA, we found that these two unique *Hin*dIII fragments of DC24 hybridized with VcA1 sequences as well (data not shown). Together these observations support the conclusion that a VcA1 prophage is inserted in the second or downstream *ctxA* gene copy in mutant DC24 as indicated in model 2 of Fig. 1.

The approximate chromosomal location of the *ctxA*::VcA1 insertion carried by DC24 was determined by characterizing the donor properties of this lysogen after introduction of the plasmid pSJ15. The properties of such a VcA1-facilitated donor (13) should include high-frequency transfer of genetic markers physically close to the *ctxA*::VcA1 insertion. Accordingly, the transfer frequencies for a variety of chromosomal markers were determined in quantitative matings between DC24(pSJ15) and auxotrophic recipients derived from either the parental strain RV79 or classical strain 569B. Table 2 shows that DC24(pSJ15) transferred trp^+ at high frequency into both RV79- and 569B-derived recipients. Elevated transfer of met^+ was observed in a 569B recipient, but not in RV79. These data suggest that the chromosomal *ctxA*::VcA1 insertion of DC24 and thus the *ctx* operons of strain RV79 are located in the vicinity of the *met trp* region on the *V. cholerae* chromosome (Fig. 2).

Genetic mapping of *ctx* in RV79 by homologous three-factor crosses. To determine a more precise chromosomal location

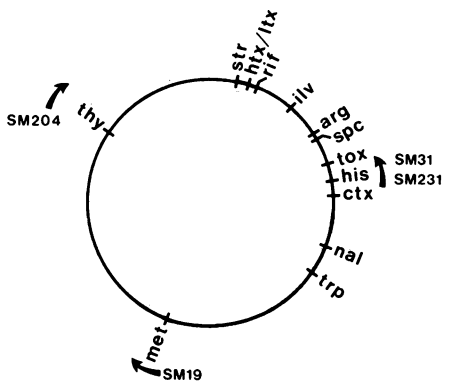

FIG. 2. Circular genetic map of *V. cholerae*. This generalized map is a summary of data obtained with strains RV79 (13, 14; Johnson, Ph.D. thesis), 569B (19, 20), and 162 (32). The arrows show the polarity of transfer for some of the VcA1-facilitated donors used in this study.

for the *ctx* sequences of RV79, we performed three-factor crosses between RV79 derivatives that had one of two identifiable structural alterations in *ctx*. The first *ctx* structural mutation analyzed in these crosses was Δ*ctx-7922*, a toxin deletion mutation carried by the RV79 mutant M7922 (18). Two VcA1-facilitated donors, SM19(pSJ15) and SM31(pSJ15), were constructed from M7922 by isolating appropriately oriented VcA1 insertion mutations in the *met* and *his* genes, respectively, that allowed high-frequency transfer of trp^+ after the introduction of pSJ15. Both donors transferred trp^+ to SM103 at a frequency of about 10^{-3}. This indicated that the orientation of the chromosomal VcA1 prophage in SM19 was allowing polar transfer originating at *met* and proceeding counterclockwise on the *V. cholerae* map, whereas SM31(pSJ15) was donating from *his* in the clockwise direction (Fig. 2).

SM103 Trp^+ Rif^r recombinants selected in crosses with these two donors were scored for the unselected donor markers Nal^s and Δ*ctx7922*. The Δ*ctx7922* allele was scored by in situ colony hybridization with the CT-1 probe. Linkage of both nal^s and Δ*ctx-7922* to trp^+ was detected (Table 3). The linkage of *nal* to *trp* decreased from 69% for the SM31 cross to 51% for the SM19 cross, consistent with the established RV79 map order *met trp nal his* (S. R. Johnson, Ph.D. thesis, University of California, Los Angeles, 1978).

Since the frequency of the Δ*ctx-7922* allele in the trp^+ nal^s double recombinant class was low in both crosses, and the Δ*ctx-7922* allele showed a higher linkage to *nal* than to *trp*. These data suggest that the overall gene order is *met trp nal ctx his*.

To further exclude the possibility that *ctx* is located to the left of *trp*, we constructed a VcA1-facilitated donor, SM204(pSJ15), which donates the *met trp* region originating from a *thy*::VcA1 insertion mutation (Fig. 2). The polarity of transfer from the *thy*::VcA1 origin was determined to be counterclockwise since the *str-79* allele was transferred at a frequency at least 50-fold lower than *trp-1*. The donor strain, SM204(pSJ15), was constructed from strain SM201, a derivative of RV79 which carries the Δ*ctxA23P* Km^r allele, the second *ctx* structural gene alteration we have genetically mapped.

As shown schematically in Fig. 3, the two *ctx* copies of strain RV79 were replaced in SM201 with the in vitro construction carried by plasmid pJM290.14. This construction (Δ*ctxA23P* Km^r) contains both an inserted 1,400-bp fragment encoding resistance to kanamycin and a 450-bp *ctxA* deletion. It was recombined onto the *V. cholerae* RV79 chromosome by an in vivo marker exchange procedure and thus occupies the same chromosomal position as the original *ctx* tandem duplication of RV79. The Km^r marker of SM204(pSJ15) allowed us to directly select for acquisition of the donor *ctx* allele in our three-factor crosses and also simplified the scoring of this *ctx* allele when used as an unselected marker.

Three-factor crosses were performed with the SM204(pSJ15) donor and two recipients, SM102 and SM101, in which Met^+, Trp^+, Nal^r, or Km^r (*ctx*) recombinants were each independently selected and then scored for the other unselected markers. Table 4 presents the compiled data from these crosses. No linkage of Km^r to the met^+ allele was observed in crosses between SM204(pSJ15) and SM101 (Table 4). In contrast, linkage of Km^r to *nal-204* was detected in this cross and was higher (39%) when Km^r was selected marker than when Nal^r was the selected marker (10%). This result indicates that *nal-204* is the proximal marker and Km^r (Δ*ctxA23P* Km^r) is the distal marker donated in this cross. Similarly, crosses between SM204(pSJ15) and SM102 indicated that *nal* was between *trp* and *ctx* and probably closer to *trp* than to *ctx*. Thus, when the most distal marker (Km^r) was selected, linkage of *nal* to *trp* was 107 of 137 (78%), and linkage of *trp* to Km^r was 137 of 405 (34%). When the most proximally transferred marker trp^+ was selected, linkage of *nal* to *trp* was reduced to 179 of 418 (43%), whereas linkage of Km^r to *trp* dropped precipitously to 23 of 418 (5.5%). These data continue to support the gene order *thy met trp nal ctx his*.

In each of the three-factor crosses discussed above, several randomly chosen Km^r recombinants were analyzed by Southern blot hybridization to confirm that their *ctx* structure was identical to that of the Km^r donor used. The donor strain SM204(pSJ15) and two Km^r recombinants

TABLE 3. Linkage of *ctx* to *trp* and *nal* with RV79-derived donors carrying the Δ*ctx-7922* allele and an RV79 recipient

Donor[a]	Recipient	Donor marker selected	Marker class scored[b]	No. with non-selected marker/total (%)	No. with Δ*ctx-7922* allele[c]
SM19(pSJ15)	SM103	trp^+	nal^s	51/100 (51.0)	5
			nal^r	49/100 (49.0)	1
SM31(pSJ15)	SM103	trp^+	nal^s	69/100 (69.0)	5
			nal^r	31/100 (31.0)	1

[a] Donors were counterselected with rifampin.
[b] The nal^s marker represents the original donor allele, and the nal^r marker represents the recipient allele.
[c] The Δ*ctx-7922* allele was scored by colony blot hybridization with the CT-1 probe.

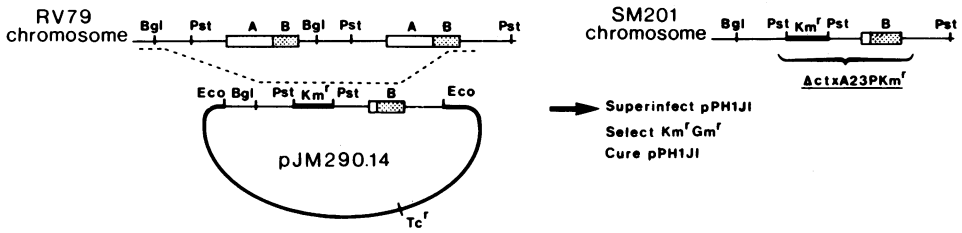

FIG. 3. Marker exchange of the Δ*ctxA23P* Km^r allele. This schematic representation shows the homologous recombinational events (dashed lines) which allowed the replacement of the two *ctxAB* operon copies (open boxes) of RV79 with the Δ*ctxA23P* Km^r allele carried by plasmid pJM290.14. The resultant strain SM201 has the Δ*ctxA23P* Km^r construction in the same chromosomal position as the original tandemly duplicated DNA carrying the two *ctxAB* copies of strains RV79.

displayed the single 7.45-kb *Xba*I fragment characteristic of the Δ*ctxA23P* Km^r allele of strain SM201 (Fig. 4). This fragment fails to hybridize to the LT-A1 probe since it carries a 450-bp deletion in the *ctxA* region (see above).

To support our localization of *ctx* between *nal* and *his*, we converted the Δ*ctx-7922* allele of the *his*::VcA1-facilitated donor SM31(pSJ15) to the Km^r construction of SM201 by transduction of the Km^r gene of SM201 into SM31 with the generalized transducing vibriophage CP-T1 (23). Southern blot analysis indicated that these transductants had the same *ctx* structure as strain SM201 (data not shown). The fact that such transductants can be obtained indicates that the Δ*ctx-7922* deletion must be smaller than the amount of DNA that CP-T1 can package. One of these transductants, SM231(pSJ15), was mated with strain SM103, and Km^r and Trp$^+$ recombinants were separately selected and then scored for their frequency of unselected markers. When Km^r was selected, linkage of *nal* to Km^r was 28% (109 of 389), down from 38% (153 of 405) in the analogous cross with the SM204(pSJ15) donor, where Km^r was transferred as a distal marker (Table 4). However, linkage of *nal* to Km^r increased to 63% (66 of 105) within the class of Km^r recombinants that had also received Trp$^+$ from the SM231(pSJ15) donor. This latter result continues to place *nal-103* between *trp-1* and Δ*ctxA23P* Km^r. Similarly, when Trp$^+$ was the selected marker from the SM231(pSJ15) donor, linkage of *nal* to *trp* increased to 51% (204 of 399), up from the 43% linkage seen when Trp$^+$ was the proximal marker selected in the SM204(pSJ15) × SM102 cross (Table 4). These data support the conclusion that the *his*::VcA1 insertion in donor SM231(pSJ15) directs the transfer of the *trp nal ctx* region in the opposite orientation from that of the *thy*::VcA1-facilitated donor SM204(pSJ15) and therefore provide additional evidence that *ctx* is located between *nal* and *his* on the *V. cholerae* RV79 genetic map.

We performed three-factor crosses to directly demon-

TABLE 4. Linkage of *ctx* to *met*, *trp*, and *nal* in three-factor crosses with RV79-derived donors and recipients

Donor marker selected[a]	Other donor markers in recombinants	Frequency of unselected markers obtained/ total (%)
	SM204(pSJ15)[b] × SM102[c]	
Km^r (*ctx*)	*nal*	46/405 (11.4)
	trp	30/405 (7.4)
	nal trp	107/405 (26.4)
nal^r	*ctx*	11/327 (3.4)
	trp	164/327 (50.2)
	ctx trp	21/327 (6.4)
trp$^+$	*nal*	165/418 (39.5)
	ctx	9/418 (2.2)
	nal ctx	14/418 (3.3)
	SM204(pSJ15)[b] × SM101	
Km^r (*ctx*)	*nal*	38/98 (38.8)
	met	0/98 (0)
	nal met	0/98 (0)
nal^r	*ctx*	10/100 (10.0)
	met	0/100 (0)
	ctx met	0/100 (0)
met$^+$	*ctx*	0/100 (0)
	nal	0/100 (0)
	ctx nal	0/100 (0)

[a] Transfer frequencies were in the range of 5×10^{-3} to 1×10^{-5} for all selected donor markers.

[b] Donors were counterselected with either rifampin or spectinomycin.

[c] The results shown are data from five matings combined.

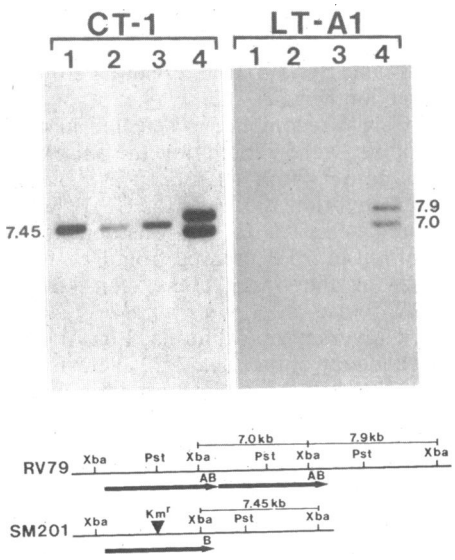

FIG. 4. Southern blot analysis of DNA from El Tor donor, recipient, and recombinant strains. DNA (about 1 μg) from the various strains was digested with *Xba*I, fractionated by electrophoresis in 0.7% agarose gels, transferred to nitrocellulose filters, and hybridized to either radioactive CT-1 or LT-A1 probe. Below the autoradiograms is a schematic diagram that shows the origins of the 7.0-, 7.45-, and 7.9-kb signals obtained. The lanes contain DNA from donor strain SM204(pSJ15) (lane 1), two Trp$^+$ Km^r recombinants from the SM204(pSJ15) × SM102 cross (lanes 2 and 3), and the recipient strain SM102 (lane 4). The donor strain SM204(pSJ15) carries the Δ*ctxA23P* Km^r allele of strain SM201 as shown, and the recipient strain SM102 carries the same tandemly duplicated wild-type *ctxAB* region (arrows) as strain RV79.

strate the close linkage of *ctx* and *his*. Strain SM798 was mated to SM204(pSJ15), and Km^r recombinants were selected and scored for linkage to *his*$^+$ and *nal*^{r}. Of a total of 126 Km^r recombinants scored, 14 received *his*$^+$ only, 4 received *nal*^{r} only, and 27 received both *his*$^+$ and *nal*^{r}. Similar results were obtained with the *his-1* allele when a VcA1 lysogen of strain RV792 was mated with SM204(pSJ15) (data not shown). The segregation pattern of these markers strongly supports the proposed map order *nal ctx his*.

These three-factor cross data are also consistent with the mobilization data for the *ctxA*::VcA1-facilitated donor, DC24(pSJ15). Since DC24(pSJ15) transferred *trp*$^+$ at a higher frequency than *his*$^+$ in RV79 homologous crosses (Table 2), this donor probably initiates chromosomal transfer at *ctx* and proceeds in a clockwise direction on the *V. cholerae* map (Fig. 2).

Three-factor crosses between RV79-derived donor strains and classical recipients. All *V. cholerae* strains of the classical biotype contain a structurally similar duplication of the *ctx* operon (Mekalanos, in press). Southern blot analysis of the classical strain 569B shows that one of its two *ctx* copies is in common with the El Tor strain RV79. When genomic digests are analyzed with CT-1 probe, both RV79 and 569B display two bands, one of which is in common between these two strains (Fig. 5). The other band, which is present in RV79, but not in 569B, remains a constant size of 7 kb with the different enzymes used, indicating that it represents the restriction fragment that spans the novel joint of the 7-kb tandem duplication carried by this strain (Mekalanos, in press). Thus, the size of the other *ctx* copy of RV79 must reflect the position of restriction enzyme sites located in DNA sequences adjacent to the tandem duplication carrying *ctx* in RV79. Since these adjacent DNA sequences are shared by one of the two *ctx* copies of strain 569B, we have

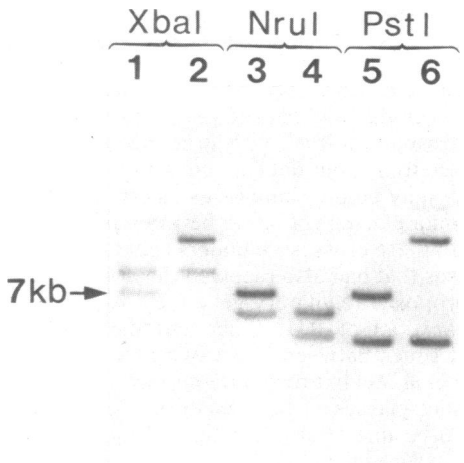

FIG. 5. Comparison of the Southern blot hybridization patterns of DNA from strains RV79 and 569B. Odd-numbered lanes contain RV79 DNA (1 μg), and even-numbered lanes contain 569B DNA (1 μg), that was digested with the indicated restriction enzymes. After agarose gel electrophoresis, the DNA fragments were transferred to a nitrocellulose sheet and hybridized with radioactive CT-1 probe. The position of the 7-kb band containing the novel joint of the 7-kb tandem duplication carrying *ctxAB* in RV79 is indicated and is present all three RV79 digests. The other RV79 band comigrates with one of the two bands seen with strain 569B.

TABLE 5. Linkage of *ctx* to *nal* and *met* obtained in a heterologous cross

Donor marker selected	Transfer frequency[a]	Other donor markers in recombinants	Frequency of unselected markers obtained/total (%)
		SM204(pSJ15)[b] × RV508[c]	
Km^r (*ctx*)	9.7×10^{-4}	*nal*	50/200 (25.0)
		met	22/200 (11.0)
		nal met	23/200 (11.5)
nal^r	8.4×10^{-5}	*ctx*	8/169 (4.7)
		met	2/169 (1.2)
		ctx met	2/169 (1.2)
met$^+$	3.2×10^{-5}	*nal*	27/133 (20.3)
		ctx	9/133 (6.8)
		nal ctx	84/133 (63.2)

[a] Recombination frequencies are reported as recombinants per input donor.
[b] The donor was counterselected with spectinomycin.
[c] The results shown are data from two matings combined.

begun to investigate whether this particular *ctx* copy of 569B maps in the same position as the *ctx* locus of strain RV79. Accordingly, we have analyzed in three-factor crosses between RV79 donors and 569B recipients the linkage of *ctx* to various chromosomal markers in the *thy met trp nal his* cluster. A detailed discussion of these results will follow in another paper, but these data do in general support the hypothesis that the *ctx* copy shared by strains 569B and RV79 does reside in the *nal* region of the *V. cholerae* chromosome. However, these heterologous cross data are complicated by evidence that suggests that a chromosomal rearrangement has occurred in this region between RV79 and 569B. One particular cross that supports the possibility is presented in Table 5. The *thy*::VcA1-facilitated donor SM204(pSJ15) was mated with the 569B-derived recipient RV508 and Nal^r, Km^r (*ctx*), or Met$^+$ recombinants were selected and scored for unselected markers.

Contrary to RV79 homologous crosses with the same donor, linkage between *met* and Km^r could be demonstrated. However, transfer of the distal marker, Km^r, was significantly higher than transfer of the proximal markers *nal*^{r} and *met*$^+$ (Table 5). Moreover, the three-factor cross results, together with the fact that linkage of Km^r to *met-2* was higher when Met$^+$ was selected than when Km^r was selected, suggested that the gene order and polarity of transfer from the SM204(pSJ15) donor was *thy nal ctx met*. Since these latter results contradict the known gene order and donor characteristics of this donor strain in RV79 homologous crosses, we conclude that this heterologous cross must be subject to some genetic artifact that reduces the recovery of Met$^+$ recombinants. One of several possible explanations would be the existence of a genetic inversion of the chromosomal DNA between *met* and *nal* (e.g., containing *trp*; Fig. 2) in strain 569B relative to strain RV79. If one end of such an inversion was located very close to *met*, then it would have the effect of greatly reducing the recovery of recombinants receiving only Met$^+$ from the donor while artificially increasing the apparent linkage of Met$^+$ to downstream markers such as *nal*. When Met$^+$ recombinants were selected, linkage to *nal-204* was 83%; but when Nal^r recombinants were selected, linkage to *met*$^+$ dropped to less than 3% (Table 5). This, once again, is exactly the opposite result from that which one would expect, given the known RV79 gene order and donor characteristics of SM204(pSJ15) in RV79 homologous crosses. The possibility that the *met-2*

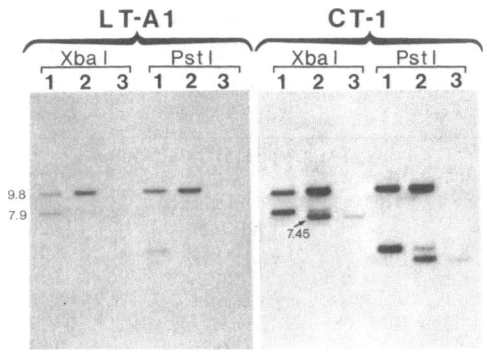

FIG. 6. Southern blot analysis of donor, recombinant and recipient DNA from an El Tor-classical heterologous cross. DNA (about 1 μg) was digested with the indicated restriction enzyme, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with either the LT-A1 or the CT-1 probe. The lanes contain DNA from either the recipient strain RV508 (lane 1), a Km^r Nal^r recombinant from the SM204(pSJ15) × RV508 cross (lane 2), or the donor strain SM204(pSJ15) (lane 3). The sizes of the bands are indicated in kilobase pairs.

allele is not located in the usual map position is unlikely inasmuch as a total of 10 independent *met* alleles in both classical and El Tor strains have been shown to map between *trp* and *thy* (24; Johnson, Ph.D. thesis).

Although these results demonstrate that the potential for ambiguous linkage and transfer properties exists in these heterologous crosses between classical and El Tor strains of *V. cholerae*, the recovery of RV508 Km^r Nal^r recombinants still allowed us to directly test the hypothesis that one of the two *ctx* copies of 569B is located in the *nal* region of the *V. cholerae* chromosome. Figure 6 shows a Southern blot analysis with the enzyme *Xba*I of a Km^r Nal^r recombinant obtained in the mating between SM204(pSJ15) and RV508. The recipient strain RV508 displays two bands, 9.8 and 7.9 kb in size, which both hybridize equally well with the CT-1 probe. The 7.9-kb band is in common with RV79. In contrast, a typical Km^r Nal^r recombinant shows three CT-1-homologous bands of 9.8, 7.9, and 7.45 kb in size. The 7.45-kb band comigrates with the SM204 donor *ctx* gene copy and is smaller than the 7.9-kb band of RV508 by the size of the *ctxA* deletion in the Δ*ctxA23P* Km^r allele incorporated into strain SM204 (Fig. 3 and 4). Consistent with this interpretation is the fact that the 7.45-kb band fails to hybridize with the LT-A1 probe, which does not recognize the DNA remaining in the Δ*ctxA23P* Km^r construction carried by plasmid pJM290.14 (Fig. 6).

The 7.9-kb band in the Km^r recombinant is very weak in intensity, indicating that this signal is present in less than one copy per cell on average. Recent experiments indicate that this weak signal represents low-frequency homogenotization events occurring spontaneously in a heterozygous recombinant strain which replace the Δ*ctxA23P* Km^r allele in one recombined 569B *ctx* copy with the wild-type *ctx* sequences from the other 569B nonrecombined *ctx* copy (unpublished data).

The results indicate that the *ctxAB* copy present on the 7.9-kb *Xba*I fragment of RV508 has been replaced in the Km^r Nal^r recombinants with the Δ*ctxA23P* Km^r allele present in the SM204(pSJ15) donor. Southern blot analysis with the enzyme *Pst*I gave results similar those seen with *Xba*I (Fig. 6). Together these results strongly argue that the 569B *ctx* copy that is in common with RV79 in Southern blot analysis is closely linked to the *nal* locus and is genetically separable from the other *ctx* locus in this strain.

DISCUSSION

In this report, we have described the genetic mapping of the cholera toxin structural genes, *ctxAB*, in the El Tor strain RV79. Three different *ctx* structural gene alterations were used as genetic markers in this analysis. Initially, an insertion mutation of the mutagenic vibriophage VcA1 (13) into the *ctxA* gene was used to construct a VcA1-facilitated donor by the method of Johnson et al. (13). The *ctxA*::VcA1-facilitated donor strain, DC24(pSJ15), was shown to transfer *trp*$^+$ at a high frequency to RV79 recipients, indicating that the chromosomal *ctxA*::VcA1 insertion in this strain was near the *trp* region on the chromosome. Subsequent three-factor crosses using RV79-derived, VcA1-facilitated donors and RV79 recipients showed that the *ctx* locus mapped between the *nal* and *his* genes in the *trp nal his* cluster (Johnson, Ph.D. thesis). In these three-factors crosses, two structural gene alterations of *ctx* were used as genetic markers. The first was a total deletion of the *ctx* region of RV79 (Δ*ctx-7922*), whereas the second was a *ctxA* deletion-Km^r insertion mutation (Δ*ctxA23P* Km^r) recombined in vivo in place of the two resident *ctxAB* copies of RV79. These structural gene alterations allowed the unambiguous scoring of *ctx* mutant alleles since the detection of these was not dependent on toxin production phenotypes in the recombinants. Thus either Km^r or the loss of *V. cholerae* chromosomal DNA homologous to the LT-A1 probe could be used to recognize *ctx* recombinants without the confounding influence of overt or cryptic regulatory mutations in the donor or recipient strains.

Toxin regulatory differences between various strains and mutants of *V. cholerae* have previously complicated the genetic analysis of the toxin structural genes (2, 19, 20, 29, 30). For example, Saunders et al. (29) reported the mapping of a *V. cholerae* gene, *vct*, which is responsible for an antigenic variation in the cholera toxin produced by the two El Tor strains 3083 and RJ1 (the latter is the same as strain RV79). These authors concluded that the *vct-1* allele of RJ1 maps between *met* and *trp* and proposed that the *vct-1* allele might correspond to the toxin structural gene. Inasmuch as *ctx* is linked to *trp*, our data are consistent with the possibility that *vct* may be the same locus as *ctx*. If this is the case, then the data placing *vct* or *ctx* between *met* and *trp* may be in error. In these crosses, Saunders et al. discarded recombinant classes that had also received the donor RJ1 locus, *tox-1000*, a proposed regulatory gene they mapped between the *trp* and *his* loci of RJ1. Since our data place the toxin structural genes between these two loci, it is possible that Saunders et al. did not recognize certain important *vct* or *ctx* recombinant classes in their analysis. The conflicting data might also be due to the fact that Saunders et al. (29, 30) used, in these genetic analyses, heterologous crosses between two different El Tor strains, RJ1 and 3083.

We have observed that heterologous crosses between RV79 and the classical strain 569B gave ambiguous transfer and linkage frequencies for markers in the *met nal ctx* region. One explanation for these results might be that an inversion exists in the *trp* region between RV79 and 569B. This would not constitute the first inversion observed between RV79 and a classical strain. The data of Johnson and Romig (14) and Sublett and Romig (32) support the existence of an inversion of the *ilv lys* region between RV79 and classical strain RV33. Heterologous crosses between differ-

ent El Tor strains may also be subject to error caused by chromosomal inversions or other rearrangements and therefore must be interpreted with caution.

Our genetic analysis of *ctx* has definitively shown that the cholera toxin genes of RV79 are indeed located on the *V. cholerae* chromosome. At least one of the two 569B *ctx* copies also appears to occupy the same chromosomal location as the tandemly duplicated *ctx* region of RV79 (i.e., in the *nal* region). The precise location of the other *ctx* copy of the strain 569B is not yet known.

Other mutations affecting toxin production previously mapped on the *V. cholerae* 569B chromosome all appear to be regulatory in nature. Regulatory mutations in the *htx* or *ltx* locus map in the *str rif* region (19, 20), quite distant from *ctx*. Mutations in another regulatory locus, *tox*, appear to be linked to the *his* locus, but are located on the opposite side of *his* from *ctx* (Fig. 2). Recent cloning and physical characterization of the DNA adjacent to the *ctx* operons of strains 569B, RV79, E7946, and 2125 indicate that about 6 kb of DNA upstream from each of these *ctx* copies is identical in all strains (Mekalanos, in press). If different *ctx* copies from the same or different strains turn out to have variable chromosomal locations, then this shared DNA upstream of *ctx* might be part of a genetic element involved in the transposition or rearrangement of *ctx* sequences on the *V. cholerae* chromosome. Given the possibility that the *ctx* genes may be located on a mobile genetic element (Mekalanos, in press), the lack of close physical clustering of the *ctx* structural and regulatory genes is of interest.

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