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Molecular Epidemiological Studies of United States Gulf Coast *Vibrio cholerae* Strains: Integration Site of Mutator Vibriophage VcA-3

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Environmental and clinical *Vibrio cholerae* O-1 strains isolated from the U.S. Gulf Coast region were found to be lysogenic for a vibriophage which we have designated VcA-3. Comparison of VcA-3 with the previously described vibriophages VcA-1 and VcA-2 has shown that VcA-1 and VcA-3 are homoimmune, have extensive sequence homology, but have markedly different restriction endonuclease digestion patterns. VcA-3 was found to randomly integrate into the *V. cholerae* RV79 chromosome and to introduce stable auxotrophic mutations. We show that all U.S. Gulf Coast environmental and clinical isolates that are lysogenic for VcA-3, including both tox^+ and tox^- isolates, contain the prophage integrated at an identical chromosomal site. Given the known stability of temperate mutator bacteriophages, these results suggest that there is a clonal relationship among the *V. cholerae* O-1 strains examined in this study, including the tox^+ and tox^- isolates.

Since the single-case outbreak in Port Lavaca, Tex. (1973), sporadic outbreaks of cholera have occurred along the Gulf Coast of the United States (3, 19). Epidemiological investigations have revealed that most reported cases have been caused by toxinogenic (Tox^+) strains of *Vibrio cholerae* O-1 of the El Tor biotype and Inaba serotype. In addition, these strains have been shown to possess a rare vibriophage typing pattern (3, 10). Nontoxinogenic (Tox^-) *V. cholerae* with the same characteristics as described above have been repeatedly isolated from the Gulf Coast environment as well as from one clinical case (3, 11). The relationship between these Tox^+ and Tox^- strains of *V. cholerae* has remained unclear. Southern blot analysis using a ^{32}P-labeled cloned *Escherichia coli* heat-labile enterotoxin gene probe has demonstrated that the Tox^- phenotype was due to the absence of cholera toxin structural gene sequences (11). Whereas the bacteriological and epidemiological evidence suggested that a single strain of *V. cholerae* was responsible for clinical outbreaks of disease in the Gulf coast region in 1978 to 1981, the genotypic differences between the Tox^+ and Tox^- *V. cholerae* were unexpected and raised questions with regard to the epidemiological findings (10).

In this paper, we demonstrate that all human *V. cholerae* isolates from the U.S. Gulf Coast region since 1973 are lysogenic for a temperate vibriophage, which we have designated VcA-3. We show that vibriophage VcA-3 is mutagenic and will apparently randomly integrate into the bacterial chromosome. Furthermore, we show that whereas VcA-3 will randomly integrate into the *V. cholerae* RV79 chromosome, all U.S. Gulf Coast clinical isolates of *V. cholerae* carry VcA-3 integrated in the same chromosomal location and are thus most likely to be clonally related. Two nontoxinogenic *V. cholerae* O-1 strains were also found to be lysogenic for vibriophage VcA-3. Interestingly, the site of VcA-3 integration in the chromosome of these strains was identical to that in the tox^+ strains. Given the identical biogram of the many tox^+ and tox^- strains of *V. cholerae* isolated from patients and the Gulf Coast environment and the apparent identical integration site of vibriophage VcA-3 in these strains, we confirmed and extended the observations of Kaper et al. (10) that there is a clonal relationship between *V. cholerae* E507 from Port Lavaca, Texas and the *V. cholerae* subsequently isolated from focal outbreaks of cholera in Louisiana and Florida. Furthermore, our observation that vibriophage VcA-3 is integrated in an identical site in both tox^+ and tox^- *V. cholerae* O-1 strains strongly suggests that the deletion (or addition) of the cholera enterotoxin structural gene(s) has occurred in these tox^- (or tox^+) strains.

MATERIALS AND METHODS

Bacterial strains. The *V. cholerae* strains used in this study are shown in Table 1. *V. cholerae* RV79 has been previously described (8). Environmental and

TABLE 1. *Vibrio cholerae* strains

V. cholerae strain	Source	Tox phenotype	Auxotrophic phenotype	Reference
E507	John P. Craig[a]	Tox^+	Prototrophic	NPD[b]
2164-78	Paul Blake[c]	Tox^+	Prototrophic	NPD
2560-78	Paul Blake	Tox^+	Prototrophic	NPD
1077-79	Paul Blake	Tox^-	Prototrophic	NPD
2740-80	Paul Blake	Tox^-	Prototrophic	NPD
2801-80	Paul Blake	Tox^-	Prototrophic	NPD
2843-80A	Paul Blake	Tox^-	Prototrophic	NPD
SG N 7698	Nell Roberts[d]	Tox^+	Prototrophic	NPD
4371	Nell Roberts	Tox^+	Prototrophic	NPD
RV79	W. Romig[e]	Tox^+	Prototrophic	8
SG100	This study	Tox^+	Trp^-	This study
SG101	This study	Tox^+	Trp^-	This study
SG102	This study	Tox^+	Trp^-	This study
SG103	This study	Tox^+	Met^-	This study
SG104	This study	Tox^+	Met^-	This stduy
SG105	This study	Tox^+	Met^-	This study
SG106	This study	Tox^+	Met^- or Cys^-	This study
SG107	This study	Tox^+	Met^- or Cys^-	This study
SG108	This study	Tox^+	Cys^-	This study
SG109	This study	Tox^+	Cys^-	This study
SG110	This study	Tox^+	Iso^- and Val^-	This study
SG111	This study	Tox^+	His^-	This study
SG112	This study	Tox^+	Leu^-	This study
SG201	This study	Tox^+	Prototrophic	This study
SG202	This study	Tox^+	Prototrophic	This study
SG203	This study	Tox^+	Prototrophic	This stydy
SG204	This study	Tox^+	Prototrophic	This study
SG205	This study	Tox^+	Prototrophic	This study
SG206	This study	Tox^+	Prototrophic	This study
SG207	This study	Tox^+	Prototrophic	This study
SG208	This study	Tox^+	Prototrophic	This study

[a] J.P.C., Downstate Medical Center, Brooklyn, N.Y.
[b] NPD, Not previously described.
[c] P.B., Centers for Disease Control, Atlanta, Ga.
[d] N.R., Louisiana Department of Health and Human Resources, New Orleans.
[e] W.R., University of California at Los Angeles.

clinical isolates of *V. cholerae* were kindly supplied by Paul Blake, Centers for Disease Control, Atlanta, Ga.; Nell Roberts, State of Louisiana Department of Health and Human Resources, New Orleans, La.; and John P. Craig, Downstate Medical Center, Brooklyn, N.Y. All strains are serotype Inaba, El Tor biotype. Vibriophage VcA-3 lysogenic derivatives of *V. cholerae* RV79 were isolated in this laboratory.

Media. Bacterial strains were grown in TYCC medium (10 g of Tryptone [Difco Laboratories, Detroit, Mich.]; 1 g of yeast extract [Difco]; 1 g of glucose; 8 g of NaCl; and 0.5 g of $CaCl_2$ per liter). TYCC agar medium and TYCC soft agar medium contained 1.7% (wt/vol) and 0.5% (wt/vol) of agar (Bacto; Difco), respectively. Minimal M63 glucose agar plates (3 g of KH_2PO_4, 7 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, 2.5 mg of $FeSO_4$, 0.2 g of $MgSO_4$, 15 ml of sterile 20% [wt/vol] glucose solution, and 17 g of agar [Bacto; Difco] per liter) supplemented with 200 μg of nutrient broth (Difco) per ml were used for the isolation of vibriophage VcA-3-induced auxotrophic mutants of *V. cholerae* RV79.

Isolation and propagation of vibriophage VcA-3. *V. cholerae* E507 was grown overnight in TYCC broth at 37°C. The bacterial cells (1 ml) were pelleted by centrifugation at 13,000 × *g* for 1 min in a microcentrifuge (model 235A; Fisher Scientific Co.), and a loopful of the supernatant fluid was streaked onto a TYCC agar plate. Two Pasteur pipette drops of a late-logarithmic-phase culture of strain RV79 were added to 3 ml of molten TYCC top agar at 45°C, vortexed, and poured onto the surface of the inoculated plate. After overnight incubation at 37°C, a single turbid plaque was picked with a sterile Pasteur pipette, transferred to a sterile test tube containing 0.3 ml of buffer C (10 mM Tris-hydrochloride–10 mM $MgCl_2$–10 mM NaCl [pH 7.9]) plus 1 drop of $CHCl_3$, and replated, as above, on a lawn of *V. cholerae* RV79. After overnight incubation, a single plaque was picked, suspended in buffer C, and designated vibriophage VcA-3. High-titer vibriophage stocks were prepared from plate lysates on *V. cholerae* RV79 essentially as described by Adams (1).

Isolation of *V. cholerae* RV79(VcA-3) lysogens. An

overnight culture of *V. cholerae* RV79 was grown in TYCC broth and diluted 1/50 into fresh medium; after a 2-h incubation at 37°C with gentle shaking, the culture was infected with vibriophage VcA-3 at a multiplicity of infection of ca. 1.0. The culture was further incubated, and after 1 h, extensive lysis occurred. After an additional 1-h incubation, a portion of the culture was streaked onto a TYCC plate for the isolation of single bacterial colonies. Individual colonies were tested for lysogeny by toothpick stab inoculation into lawns of *V. cholerae* RV79. After incubation, a zone of clearing in the bacterial lawn around an individual colony indicated phage release and thus lysogeny. In the case of nonlysogenic colonies, the growth of the bacterial lawn was contiguous with the colony. Lysogenic colonies were restreaked for single colonies, which were then retested for phage release. In addition, culture supernatant fluids of the twice-purified colony, after overnight growth in TYCC broth, were checked for the formation of individual plaques on RV79.

Auxotroph isolation. Vibriophage VcA-3-induced auxotrophic strains of *V. cholerae* RV79 were isolated from lysogenic pools which were prepared as described above. After infection with VcA-3 and incubation, bacteria were diluted in buffer C and plated onto M63 glucose agar medium plates supplemented with 200 μg of nutrient broth (Difco) per ml to give ca. 500 colonies per plate. After incubation at 37°C for 2 days, both large and tiny colonies were obtained. Tiny colonies were separately transferred with a sterile toothpick and streaked onto M63 minimal glucose agar medium as well as M63 glucose agar supplemented with nutrient broth. Isolates which failed to grow on unsupplemented minimal medium were characterized for their ability to grow on M63 glucose plates supplemented with various amino acids (9, 17). Auxotrophic strains were then examined for lysogeny as described above.

Determination of lysogenic immunity. (i) Spot test. Lawns of lysogenic strains were prepared on TYCC agar plates. One drop of a high-titer phage stock, containing ca. 10^7 PFU, was spotted on the bacterial lawn. After overnight incubation at 37°C, the plate was examined for vibriophage-induced lysis.

(ii) Plaque assay. To determine if individual plaques could form on various lysogenic strains of *V. cholerae*, vibriophages were streaked onto the surface of a TYCC agar plate and covered with a lawn of the strain to be tested. After overnight incubation at 37°C, plates were examined for the presence of single plaques.

Phage DNA preparation. Early-logarithmic-phase TYCC broth cultures (50 or 500 ml) of nonlysogenic *V. cholerae* RV79 were used as the host to make vibriophage lysates. Bacteria were infected with vibriophage at a multiplicity of infection of ca. 0.01. After bacterial lysis, debris was removed by centrifugation at 8,000 × *g* for 15 min in a Beckman JA-10 rotor, and the phage were precipitated by the addition of polyethylene glycol 6000 (PEG) and NaCl to 7% and 0.5 M, respectively (20). Vibriophage were sedimented by centrifugation in a Beckman Ti50 rotor at 40,000 rpm for 40 min, and the phage pellet was resuspended in 0.5 ml of buffer C. DNA was released and purified from vibriophage by phenol (equilibrated with TE buffer [10 mM Tris-hydrochloride (pH 8.0)–1 mM disodium EDTA]) and chloroform extraction and precipitated with ethanol. After centrifugation, purified phage DNA was suspended in TE buffer and stored at 4°C until used.

Chromosomal DNA extraction. Chromosomal DNA was extracted and purified from *V. cholerae* essentially as described by Brenner et al. (4).

Restriction endonuclease digestion. Phage DNA (2 μg) or *V. cholerae* chromosomal DNA (3 μg) was digested with 4 or 6 U of an individual restriction endonuclease, respectively, under conditions specified by the manufacturer (New England Biolabs, Beverly, Mass.). Digested DNA was electrophoresed on a 0.7% horizontal agarose gel in TBE (89 mM Tris-hydrochloride–89 mM boric acid–2.5 mM disodium EDTA) buffer for 4 h at 90 V or 18 h at 30 V. Both the agarose gel and electrophoresis buffer contained 0.5 μg of ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) per ml. After electrophoresis, the agarose gel was rinsed with distilled water and photographed under UV light through a red filter with Polaroid type 107 film.

Southern blot analysis. Vibriophage VcA-3 DNA was nick translated by the method of Rigby et al. (15). [^{32}P]dCTP (>400 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Southern blot analysis was performed as previously described (16). The ^{32}P-labeled *E. coli* heat-labile enterotoxin (LT) DNA probe (13) was kindly provided by J. Mekalanos, Harvard Medical School, Boston, Mass. Nitrocellulose paper (type B85) was obtained from Schleicher & Schuell Co., Keene, N.H. Hybridization of the [^{32}P]-DNA probe to the DNA on nitrocellulose filters was performed as previously described (16). After being washed extensively, the filters were autoradiographed on Kodak XR-1 film at −70°C with a Dupont Cronex intensifying screen.

RESULTS

Isolation and characterization of vibriophage VcA-3. *V. cholerae* E507 was grown overnight in TYCC medium, and cell-free supernatant fluids were plated on lawns of the El Tor indicator strain *V. cholerae* RV79. A single plaque of typical morphology, a clear center with a turbid outer ring, was picked, and a lysogenic RV79 derivative was purified. The vibriophage released by this strain was designated VcA-3 in accordance with the nomenclature adopted by Gerdes and Romig (8). Vibriophage VcA-3 was compared to the well-studied vibriophages VcA-1 and VcA-2 (8) with respect to lysogenic immunity. VcA-3 was able to form single plaques on lawns of RV79 lysogenic for VcA-2, RV79 (VcA-2), but not on lawns of RV79 (VcA-1) (Table 2). Similarly, VcA-1 was able to form plaques on lawns of RV79 (VcA-2), but not on RV79 (VcA-3). Since VcA-3 and VcA-1 therefore appeared to be homoimmune, it was of further interest to compare restriction endonuclease digests of their respective vegetative genomes. As can be seen in Fig. 1, endonuclease digestion analysis revealed that vibriophages VcA-1 and VcA-3 had similar, but not identical, digestion profiles for some restriction endonu-

TABLE 2. Host range of vibriophages for lysogenic and nonlysogenic *V. cholerae*RV79

Vibriophage	Ability to form plaques on *V. cholerae* RV79 host strain[a]			
	RV79	RV79 (VcA-1)	RV79 (VcA-2)	RV79 (VcA-3)
VcA-1	+	−	+	−
VcA-2	+	+	−	+
VcA-3	+	−	+	−

[a] +, Able to form plaques; −, not able to form plaques.

cleases (e.g., *Bgl*II); however, markedly different digestion patterns were observed with many other restriction endonucleases (e.g., *Hin*dIII, *Cla*I, *Pvu*II). Further analysis of the relationship between VcA-1 and VcA-3 was performed by DNA-DNA hybridization with the Southern blot method using ^{32}P-labeled VcA-3 DNA to probe VcA-1 digests and vice versa. We have found that, regardless of the endonuclease used, there is sufficient homology between all restriction fragments of VcA-1 and VcA-3 to give rise to a positive signal (data not shown).

Vibriophage VcA-3 is a mutator phage. Johnson et al. (9) had previously demonstrated that vibriophage VcA-1 was similar to coliphage Mu (5) in that it was capable of integrating randomly into the RV79 chromosome and thereby introducing a wide variety of auxotrophic mutations. Given the lysogenic immunity and sequence homology between VcA-1 and VcA-3, we examined the ability of VcA-3 to induce auxotrophic mutations in the RV79 strain of *V. cholerae*. In a typical experiment, we found that 15 of 2,493 colonies examined (0.6%) were auxotrophic. Furthermore, the characterization of these auxotrophic strains revealed that the mutations were randomly distributed in the RV79 chromosome (3, *trp*; 3, *met*; 2, *met* + *cys*; 1, *ilv*; 1, *his*; 1, *leu*; strains SG100-SG112 in Table 1) (14). These results suggested that VcA-3, like VcA-1, was able to introduce mutations by apparently integrating randomly into the *V. cholerae* chromosome.

Coliphage Mu has been shown to contain diverse fragments of bacterial DNA on both ends of its vegetative genome (12). Were this also the case for vibriophage VcA-3, one would anticipate that ^{32}P-labeled nick-translated *V. cholerae* RV79 DNA would hybridize to Southern blots of restriction endonuclease-digested vegetative phage DNA. ^{32}P-labeled RV79 DNA specifically hybridized to blots of digested VcA-3 DNA (Fig. 2A). Furthermore, the hybridization pattern was similar to that observed for coliphage Mu. The discrete band may represent a small diverse fragment of bacterial DNA on one end of the VcA-3 genome, whereas the disperse area of hybridization may reflect a larger fragment of diverse bacterial DNA containing randomly located restriction endonuclease sites. In addition, this heterogeneity may reflect varying sizes of bacterial DNA packaged into the VcA-3 virion.

Since coliphage Mu does not circularize before integration into the bacterial chromosome (12), Southern blots of endonuclease-digested lysogen DNA probed with ^{32}P-labeled vegetative phage DNA reveal a change in the electrophoretic mobility of both end fragments. This change in hybridization pattern is also seen in Southern blots of RV79 (VcA-3) lysogen DNA probed with ^{32}P-labeled VcA-3 DNA (strains SG201 to SG208) (Fig. 2B). Both end fragments of VcA-3 vegetative DNA shown in Fig. 2B, lane 1, change in their electrophoretic mobility. Moreover, in the case of *Hpa*I-digested DNAs, the fragments containing the junction between VcA-3 and the bacterial genome have different electrophoretic mobilities in each independently

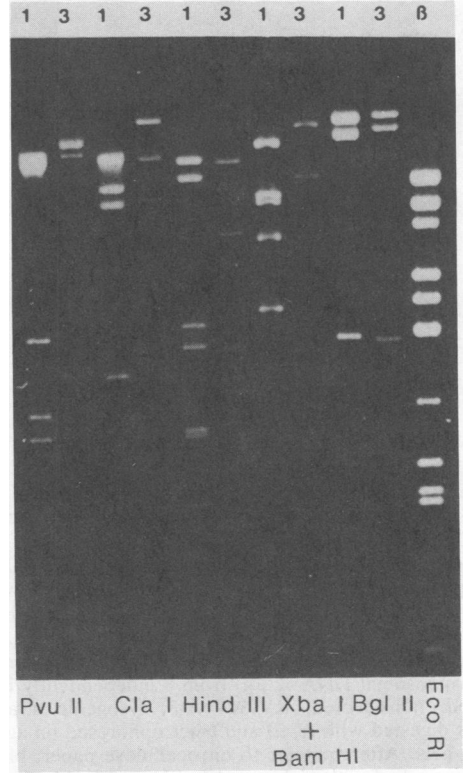

FIG. 1. Comparison of the restriction endonuclease digestion profiles of vibriophages VcA-1 (lanes 1) and VcA-3 (lanes 3). Vibriophage DNA (2 μg) was digested with 4 U of restriction endonuclease and electrophoresed as described in the text. β, Corynephage β^{tox+} DNA digested with *Eco*RI was used as an internal electrophoretic mobility standard (7).

isolated lysogen so far examined. This observation further suggests that VcA-3 is apparently able to integrate randomly into the *V. cholerae* RV79 chromosome.

Common integration site of vibriophage VcA-3 in the chromosome of environmental and clinical *V. cholerae* isolates. Since we demonstrated that vibriophage VcA-3 was able to introduce a wide variety of auxotrophic mutations and apparently randomly integrate into the RV79 chromosome, it was of further interest to examine some of the U.S. Gulf Coast clinical and environmental *V. cholerae* isolates for a temperate vibriophage. Seven of the nine strains examined were found to release a vibriophage identical to VcA-3, as analyzed by both restriction endonuclease digestion profiles and Southern blot hybridization (data not shown).

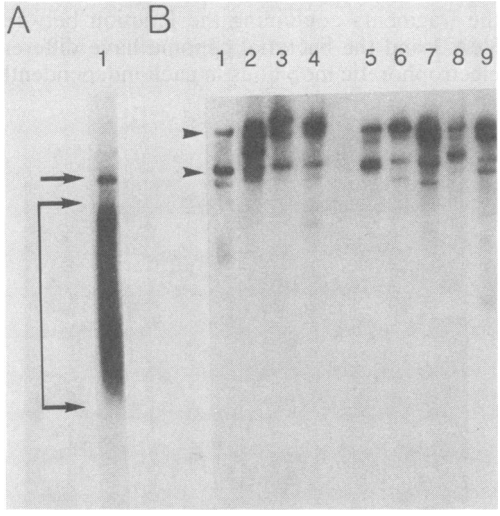

FIG. 2. (A) Southern blot analysis of the presence of RV79 chromosomal DNA in vegetative VcA-3 DNA. DNA (1 μg) from lytically grown VcA3 (see text) was digested with *Hpa*I and electrophoresed on agarose gels. After transfer to nitrocellulose paper, the blot was probed with nick-translated, unrestricted RV79 chromosomal DNA. The arrow indicates the discrete-sized end fragment containing RV79 DNA sequences. Bracket shows the end fragment with variable amounts of RV79 DNA. (B) Southern blot analysis of the relative site of vibriophage VcA-3 integration into the *V. cholerae* RV79 chromosome. Chromosomal DNA (2 μg) from 8 independently isolated, nonselected, RV79(VcA-3) lysogenic strains was digested wtih *Hpa*I and electrophoresed on agarose gels. After transfer to nitrocellulose paper, blots were probed with nick-translated VcA-3 DNA. Autoradiographs were exposed at −70°C for 12 to 48 h. Arrows show common internal *Hpa*I fragments. (A) and (B) are at the same scale with regard to the relative mobilities of restriction fragments. Lanes 1, VcA-3; lane 2, SG201; lane 3, SG202; lane 4, SG203; lane 5, SG204; lane 6, SG205; lane 7, SG206; lane 8, SG207; and lane 9, SG208.

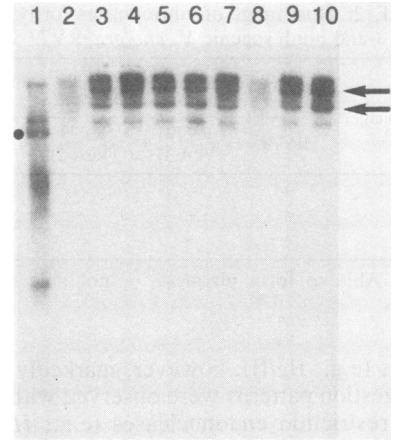

FIG. 3. Southern blot analysis of the relative site of vibriophage VcA-3 integration into the chromosomes of environmental and clinical U.S. Gulf Coast *V. cholerae* isolates after *Hpa*I digestion. Undigested, nick-translated VcA-3 DNA was used as the probe. Arrows indicate new fragments seen in lysogenic strains; dot indicates the fragment from VcA-3 corresponding to the discrete end fragment seen in Fig. 2A. Lane 1, VcA-3; lane 2, 1077-79; lane 3, E507; lane 4, SG N 7698; lane 5, 4371; lane 6, 2560-78; lane 7, 2164-78; lane 8, 2801-80; lane 9, 2843-80A; lane 10, 2740-80.

From the previous results, we assumed that the probability of any two independently isolated VcA-3 lysogens having the same chromosomal prophage integration site was small. With this in mind, chromosomal DNA from nine *V. cholerae* strains was extracted and digested with the endonuclease *Hpa*I. After electrophoresis, Southern blots of these DNAs were probed with ^{32}P-labeled nick-translated VcA-3 DNA, as described. Two of the seven isolates were found to lack the VcA-3-like prophage. The seven strains which released vibriophage VcA-3 were found to have an identical prophage integration site (Fig. 3; Table 3).

To determine if the U.S. Gulf Coast isolates had cholera toxin structural gene sequences, chromosomal digests were analyzed with a ^{32}P-labeled *Escherichia coli* heat-labile enterotoxin gene probe (13). The results indicate that only five of the seven vibriophage VcA-3 lysogenic strains carried cholera toxin structural sequences (data not shown). It is of particular interest that among the seven *V. cholerae* isolates which were found to be lysogenic for vibriophage VcA-3, we found both *tox*$^+$ (e.g., 2164, 4371, 2560) and *tox*$^-$ (e.g., 2740, 2843) strains.

DISCUSSION

We have shown that *V. cholerae* E507, which was originally isolated from the single-case chol-

TABLE 3. Tox phenotype and VcA-3 lysogenicity of *V. cholerae* environmental and clinical strains used in this study

V. cholerae strain	Source and place (yr) of isolation	Tox phenotype	Lysogenic for VcA-3
E507	Patient, Texas (1973)	+	yes
2164-78	Patient, Louisiana (1978)	+	yes
2560-78	Patient, Louisiana (1978)	+	yes
1077-79	Patient, Florida (1979)	−	no
2740-80	Patient, Florida (1980)	−	yes
2801-80	River, Florida (1980)	−	no
2843-80A	Sewer, Florida (1980)	−	yes
SG N 7698	Sewer, Louisiana (1980)	+	yes
4371	Patient, Louisiana (1981)	+	yes
RV79	W.R. Romig,	+	no
RV79 (VcA-3)	This study	+	yes

era outbreak in Port Lavaca, Tex., in 1973, is lysogenic for the mutator vibriophage VcA-3. Electron microscopic analysis has revealed that VcA-3 belongs to the Bradley group A vibriophages and is morphologically similar to the Kappa vibriophage described by Takeya et al. (18). Vibriophage VcA-3 appears to be homoimmune with the closely related vibriophage VcA-1.

The genetic relatedness of vibriophages VcA-1 and VcA-3 was also demonstrated by DNA-DNA hybridization experiments in which Southern blots of restriction endonuclease fragments of each vibriophage were probed with [^{32}P]DNA from the heterologous phage. Despite their similar virion morphologies, homolysogenic immunity determinants, and DNA sequence homologies, VcA-1 and VcA-3 are clearly different vibriophages on the basis of their comparative restriction endonuclease digestion patterns. The extent of these differences strongly suggests a considerable evolutionary divergence between VcA-1 and VcA-3.

Vibriophage VcA-3, like VcA-1 (9, 13) and coliphage Mu (17), is mutagenic and is apparently able to integrate randomly into the *V. cholerae* RV79 chromosome. We have shown that ca. 0.6% of the lysogens found after infection of RV79 with VcA-3 are auxotrophic. Furthermore, these auxotrophic mutations are distributed throughout the chromosome, rather than clustered.

With the exception of coliphage P1, the integration of temperate bacteriophage into the bacterial chromosome has been shown to proceed by two general mechanisms. One class of bacteriophage (e.g., coliphage λ) have linear vegetative genomes with cohesive ends. Upon introduction into the bacterial host, these linear genomes circularize and integrate by site-specific recombination into the bacterial chromosome by using their phage attachment sites (*att*P) (6). The resulting prophage genetic map is a circular permutation of the vegetative map. The second class of bacteriophage (e.g., coliphage Mu) has a linear vegetative genome which does not circularize but, rather, integrates directly into the bacterial genome by insertion of the ends of the vegetative phage DNA genome. We have shown by Southern blot analysis that integration of vibriophage VcA-3 into the *V. cholerae* chromosome is analogous to that of coliphage Mu. The analysis of the site of VcA-3 integration into the chromosome of independently isolated, auxotrophic and prototrophic, lysogenic RV79 (VcA-3) strains has physically shown that VcA-3 is able to integrate into different sites on the chromosome. This observation is consistent with the ability of VcA-3 to introduce random mutations in the *V. cholerae* RV79 chromosome.

We have further shown that many of the environmental and clinical isolates of *Vibrio cholerae* from the U.S. Gulf Coast area since 1978 are lysogenic for a temperate vibriophage. These phages are, on the basis of Southern blot analysis and restriction endonuclease digestion patterns, indistinguishable from vibriophage VcA-3. Southern blot analysis has clearly shown that the Gulf Coast isolates are lysogenic for vibriophage VcA-3, and the site of integration of this phage into the chromosomes of these different isolates is indistinguishable.

Since VcA-3 is capable of apparently random integration into the *V. cholerae* RV79 chromosome but appears to be at the same site in all seven of the lysogenic Gulf Coast isolates examined, it seems likely that all seven lysogens are clonally derived from a single parental lysogen. Furthermore, these results strongly suggest a clonal relationship between the isolates and *V. cholerae* E507, which was isolated from the recent Gulf Coast single-case outbreak of cholera in Port Lavaca, Tex., in 1973. In addition, restriction endonuclease digestion analysis has shown that these strains have an indistinguishable profile (S. Goldberg, unpublished data).

We have confirmed and extended the observations of Kaper et al. (10) who showed that the chromosomal restriction endonuclease digestion patterns of the cholera *tox* gene sequences in the Gulf Coast environmental and clinical *V. cholerae* isolates were markedly similar. We have demonstrated, however, that only by using the

VcA-3 prophage and its relative integration site in the *V. cholerae* chromosome as an epidemiological marker could a clonal relationship be inferred between the tox^+ and tox^- O-1 strains used in this study. In addition, these results demonstrate that the molecular epidemiology of these strains based solely upon hybridization of cloned *E. coli* LT gene or cholera toxin gene probes (10) is not sufficient in determining the relationship among tox^+ and tox^- isolates. Given the well-accepted stability of temperate bacteriophage in their lysogenic hosts (2), we conclude that there is a mechanism(s) for the addition, deletion, or both, of the cholera enterotoxin structural genes in *Vibrio cholerae*.

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