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Isolation of enterotoxin structural gene deletion mutations in *Vibrio cholerae* induced by two mutagenic vibriophages

(cholera toxin mutants/mutagenic bacteriophages/live oral vaccines)

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ABSTRACT **Phenotypically nontoxinogenic mutants of *Vibrio cholerae* were isolated after infection with either of two mutagenic vibriophages, VcA1 and VcA2cts1. DNA isolated from these mutants was analyzed for toxin gene sequences by the Southern blotting method with ^{32}P-labeled probes derived from the cloned A and B subunit genes for the heat-labile enterotoxin of *Escherichia coli*, designated LT. Several of the mutant isolates were shown by this method to have lost all sequences hybridizing to the LT probes, indicating that these clones contain deletion mutations that removed the structural gene(s) for cholera toxin. The mutants were prototrophic and grew normally, *in vitro*, demonstrating that the toxin is not essential for the growth and viability of *V. cholerae*. Moreover, the toxin gene deletion mutants multiplied well *in vivo* in ligated rabbit intestine. Because of these growth properties and the stability of deletion mutations, these strains are promising candidates for testing as live oral vaccine strains for protection against cholera.**

Colonization of the upper bowel of man by toxinogenic *Vibrio cholerae*, the etiologic agent of Asiatic cholera, results in a diarrheal syndrome primarily due to the action of cholera toxin, which stimulates fluid secretion by activating adenylate cyclase in intestinal epithelial cells (1). The toxin is a multimeric protein (M_r, 84,000) composed of one A subunit (M_r, 28,000) and five B subunits (M_r, 11,600) (2). The B subunits mediate binding to ganglioside cell surface receptors and perhaps also facilitate entry of the A subunit into the eukaryotic cell (2, 3). The A subunit promotes the activation of adenylate cyclase in target cells by catalyzing the transfer of the ADP-ribosyl moiety of NAD to a GTPase-regulatory component of the cyclase complex (4, 5).

Recently, the heat-labile enterotoxin of *Escherichia coli*, designated LT, has been shown to be highly similar to cholera toxin in both structure and mode of action (6, 7). Whereas the LT A- and B-subunit genes have been found to exist on a variety of plasmids, the gene(s) encoding cholera toxin has not been definitely located, although there are data to suggest that it resides on the *V. cholerae* chromosome (8–10). Although several laboratories have reported isolation of toxin (Tox) mutations that affect the production of one or both of the toxin subunits, none of these mutants has been shown to produce structurally altered polypeptides (11–17).

The isolation of deletion mutations in the toxin structural gene(s) would facilitate its unambiguous genetic mapping. Furthermore, the high stability of deletion mutations would have practical value in the construction of stably attenuated strains of *V. cholerae* for use as live oral vaccines against cholera. In this study, we report the isolation of deletion mutations in the cholera toxin structural gene. Furthermore, several mutants with complete deletions of the toxin gene(s) exhibit little impairment of growth in adult rabbit intestine. These strains may be useful as stable and safe prototypes for a live oral cholera vaccine.

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MATERIALS AND METHODS

Bacterial Strains and Bacteriophage. Wild-type and mutant strains used in this study are listed in Table 1. Phage VcA1 was grown and partially purified as described (20). Phage VcA2cts1 is a thermoinducible mutant of phage VcA2 (R. H. Hudson and W. R. Romig, personal communication) which displayed better virion stability and plaque morphology than the parental phage (20).

Mutant Isolation and Toxin Assays. Mutagenesis of strain RV79 with VcA1 and VcA2cts1 was performed by preparing a lysogen population from plate lysates (10^4 plaques per plate) grown at 30°C in Tryptone/yeast extract medium (20). Colonies from these lysogen populations were screened for loss of toxin production by the ganglioside filter assay (13) or for auxotrophic mutations on M63 minimal medium (21).

Toxin production in liquid culture was measured with the S49 mouse lymphosarcoma cell assay (15). Toxin antigen was measured with an enzyme-linked immunosorbent assay performed essentially as described by Harris *et al.* (22). Purified cholera toxin (19) served as the standard in the above assays. Toxin production and growth *in vivo* was assayed by using the ligated intestinal-loop model in rabbits as described (13). For each strain, approximately 10^7 cells were injected in a 5-cm intestinal loop; 18 hr later, the loops were scored for fluid accumulation and for viable counts after homogenization of the tissue. Serial passage of bacteria was performed without intermediate culturing of the test strains (e.g., intestine-to-intestine transfer).

DNA Preparation and Analysis. DNA was prepared from bacterial cells by the method of Brenner *et al.* (23). DNA was prepared from partially purified phage by extraction with phenol equilibrated with 50 mM Tris/50 mM EDTA, pH 8.0, followed by ethanol precipitation of the aqueous phase. Digestion of bacterial and phage DNA with restriction endonucleases was carried out under the conditions suggested by the manufacturer.

LT sequence probes from plasmid EWD299 (24) were prepared by the methods of Moseley and Falkow (9). A 1275-base-pair *Hin*cII fragment and a 590-base-pair *Eco*RI–*Hin*dIII fragment were used as the LT A- and LT B-subunit sequence probes, respectively. Labeling of these LT fragments and purified VcA1 DNA with [^{32}P]dCTP (New England Nuclear) to a specific activity of about 10^7 cpm/μg was carried out by nick translation (25). Electrophoresis in 0.7% agarose gels and Southern blot hybridization was performed as described (9, 26).

Table 1. *V. cholerae* strains and bacteriophage

Strain/phage	Genotype/ phenotype	Other information
RV79	Prototroph, Tox$^+$, El tor biotype	Synonymous with RJ1 (18)
RV79 derivatives		
M7922	Prototroph, Tox$^-$	Tox$^-$ mutant induced by VcA1 but nonlysogenic
M7923	Prototroph, Tox$^-$, (VcA1)	VcA1-induced Tox$^-$ mutant
M7928	Prototroph, Tox$^-$, (VcA2cts1)	VcA2cts1-induced Tox$^-$ mutant
M7929	Prototroph, Tox$^-$, (VcA2cts1)	VcA2cts1-induced Tox$^-$ mutant
M7930	Prototroph, Tox$^-$, (VcA2cts1)	VcA2cts1-induced Tox$^-$ mutant
M7941	Prototroph, Tox$^-$	Derivative of strain M7928 cured of VcA2cts1 prophage
M7942	Prototroph, Tox$^-$	Derivative of strain M7930 cured of VcA2cts1 prophage
569B	Prototroph, Tox$^+$, Classical biotype	(8–15, 17, 19)
569B derivatives		
RM3	*his*-1, *tox*-302 str	Previously RV501 1tx-3 (13)
RM6	*his*-1, *tox*-305 str	Previously RV501 1tx-2 (13)
M13	Prototroph, *tox*-2	(12)
Bacteriophage		
VcA1	Wild type	(20)
VcA2cts1	*cts*-1	Thermoinducible derivative of VcA2 (R. Hudson and W. R. Romig, personal communication; ref. 20)

RESULTS

Mutagenic Properties of Bacteriophage VcA1 and VcA2cts1. Early work focusing on the isolation of toxin mutations in *V. cholerae* had largely utilized nitrosoguanidine as a mutagen (11–17), which usually induces point mutations of the transition variety. Because we were interested in isolating deletion mutations in the cholera toxin structural gene, we decided to investigate other means of mutagenesis. UV light and nitrous acid, which generate deletions ("deletionogenic") in *E. coli* (21), were ineffective mutagens in *V. cholerae* primarily because of a high level of cell killing by these agents.

Recent observations in another laboratory suggested that the *V. cholerae* bacteriophage VcA1 had properties consistent with its being capable of transposition and insertion into many different sites on the *V. cholerae* chromosome (18). These properties suggested that VcA1 might have biological properties similar to those of bacteriophage Mu in *E. coli* (27). Consistent with this prediction, Johnson *et al.* (28) have recently observed the induction of auxotrophic mutations by VcA1. Independently, we also have observed that both VcA1 and VcA2, a serologically related and heteroimmune κ-type vibriophage (20), were capable of inducing auxotrophic mutations coincident with lysogenization in *V. cholerae* strain RV79.

Phages VcA1 and VcA2cts1 (a thermoinducible mutant of VcA2) induced auxotrophic mutations at frequencies of about 0.6% and 0.2%, respectively, in lysogen populations of RV79 (Table 2). The frequency of spontaneous auxotrophy in untreated cultures was below detectable levels (less than 0.05%). Furthermore, the frequency of auxotrophy induced by both of the two phages increased to about 1–2% when colonies were picked from rich media to minimal plates, suggesting that cross-feeding on the replica plates resulted in failure to identify a large fraction of auxotrophic mutants. Therefore, the frequency of auxotrophy induced by lysogeny with these vibriophages is comparable to that seen with bacteriophage Mu in *E. coli* (27).

The variety and multiplicity of auxotrophies observed suggests that these phages are capable of random insertion in the cholera chromosome (18, 29). We have obtained physical evidence that at least one of the two phages (VcA1) is indeed integrated at different sites in different lysogens of *V. cholerae* strain RV79. VcA1 [^{32}P]DNA prepared by nick translation was used as the probe in the Southern blot analysis of chromosomal DNA derived from several independent VcA1 lysogens. This analysis (data not shown) demonstrated that in different lysogens the VcA1 prophage was integrated at different positions relative to chromosomal restriction enzyme sites (unpublished results). These results are consistant with recent independent genetic evidence that indicates that the VcA1 prophage is located very close to phage-induced mutations (28). Similar analysis of VcA2cts1 mutations has been precluded by our inability to obtain enough sufficiently pure VcA2cts1 DNA to do these experiments.

The high stability observed for both VcA1- and VcA2cts1-induced mutations is consistent with their being phage Mu-like in nature. No detectable reversion (less than 10^{-10}) of several tested VcA1- or VcA2cts1-induced auxotrophic mutations was observed, suggesting that these vibriophages might also resemble wild-type phage Mu in being essentially incapable of precise excision (27).

Isolation of Phage-Induced Nontoxinogenic Mutants of *V. cholerae*. The high stability of the phage-induced auxotrophic mutations suggested that mutagenesis of *V. cholerae* with VcA1 and VcA2cts1 might be an excellent means of generating stable mutations in the toxin structural gene. Therefore, VcA1 and VcA2cts1 lysogen populations of *V. cholerae* strain RV79 were screened for nontoxinogenic mutants by the ganglioside filter assay (13). Phenotypically nontoxinogenic clones were present in VcA1 and VcA2cts1 lysogen pools at a frequency of about 0.05%.

Five nontoxinogenic mutants were analyzed for toxin production by the S49 lymphosarcoma cell assay (15) and for toxin antigen production by an enzyme-linked immunosorbent assay (22). Table 3 shows that no toxin (<1 ng/ml) or toxin antigen

Table 2. Frequency and type of auxotrophic mutants induced by VcA1 and VcA2cts1*

	Frequency	Type
VcA1	0.6%	*ilv, cys, his, arg* (2), *asn* (2), *met* (2), *thr* (2), *aro* (4), undetermined (6).
VcA2cts1	0.2%	*thr, arg, thy,* undetermined (3).
RV79	<0.05%	—

* Approximately 2–3 × 10^3 colonies of strain RV79 and mixed RV79 lysogens of either VcA1 or VcA2cts1 were screened for auxotrophic mutants by velvet replica plating. Auxotrophs were picked and identified where possible. Marker designations used are those of Parker *et al.* (28), and the numbers in parentheses represent multiple clones having the same phenotype.

(<10 ng/ml) was detectable by either assay in culture supernatant fluids of any of these five mutants. In contrast, the toxin produced by three nitrosoguanidine-induced, hypotoxinogenic mutants (M13, RM3, and RM6) of strain 569B was detected by both assays. Therefore, the phage-induced nontoxinogenic mutants described here are highly defective in production of active or immunologically detectable toxin gene products.

Identification of Deletion Mutations in the Cholera Toxin Structural Gene. Moseley and Falkow (9) recently reported that ^{32}P-labeled probes derived from the cloned genes for the LT A and B subunits of *E. coli* would hybridize to chromosomal fragments of *V. cholerae* DNA under conditions of reduced stringency. The *V. cholerae* restriction enzyme fragments showing homology with these LT probes were predicted to be those containing part or all of the structural gene(s) for cholera toxin. We have used these LT A- and B-subunit gene probes to analyze the structure of the cholera toxin gene in RV79 and in the five nontoxinogenic, phage-induced mutants.

Southern blot analysis of *Hin*dIII-digested DNA prepared from these strains is shown in Fig. 1. Unexpectedly, VcA1-induced mutant M7922 and two VcA2cts1-induced mutants, M7928 and M7930 had lost all detectable sequences hybridizing to the LT A-subunit gene probe. In contrast, VcA1-induced mutant M7923, like the parental strain RV79, displayed a single 20-kilobase fragment that hybridized to the LT A-subunit gene probe. VcA2cts1-induced mutant M7929 also had lost most of the hybridization signal originally present in RV79 but displayed a weak signal of about 23 kilobases in size.

A similar analysis utilizing the LT B-subunit probe produced the same hybridization signals seen with the A-subunit probe for all strains, with the exception that M7929 was completely unreactive with the LT B-subunit probe (data not shown). This result indicates that, as in the case of other strains of *V. cholerae* (9, 10), the DNA sequences coding for cholera toxin's A and B subunits are located within 20 kilobases of each other in strain RV79.

These data indicate that mutants M7922, M7928, and M7930 have deletion mutations that have removed the structural gene(s) coding for cholera toxin. Mutant M7923 may be an insertion or deletion mutant that has inactivated a toxin regulatory element required for expression of the toxin structural gene(s). These results also suggest that none of the toxin mutants appear to have sustained a simple point insertion of either the VcA1 or VcA2cts1 prophages into a toxin structural gene. Such an insertion should have manifested itself as a change in the number or molecular weight, or both, of the bands hybridizing to the

Table 3. Toxin production by wild-type and mutant strains of *V. cholerae**

Strain	Toxin equivalents, μg/ml	
	Toxicity†	Toxin antigen‡
569B	15	15
M13	0.016	0.05
RM3	0.008	0.02
RM6	0.005	0.02
RV79	0.1	0.1
M7922	<0.001	<0.01
M7923	<0.001	<0.01
M7928	<0.001	<0.01
M7929	<0.001	<0.01
M7930	<0.001	<0.01

* Wild-type and mutant strains were grown as described (17).
† Determined in the S49 lymphosarcoma cell culture assay (15).
‡ Determined by enzyme-linked immunosorbant assay as described by Harris *et al.* (22).

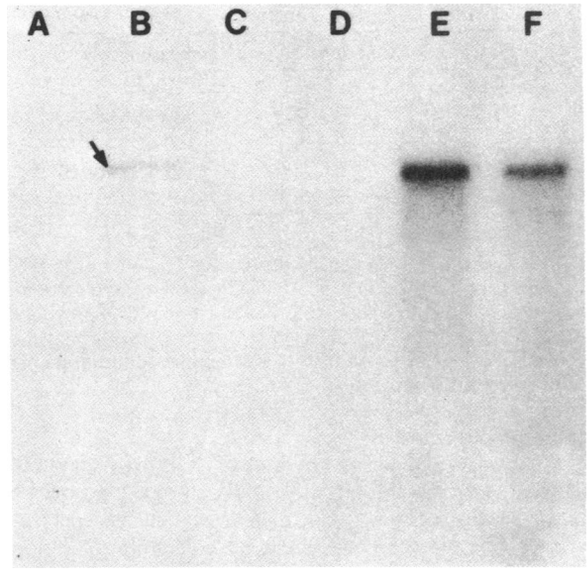

FIG. 1. Autoradiograph of Southern blot analysis of *V. cholerae* DNA from wild-type and nontoxinogenic mutant strains. Approximately 1 μg of DNA from each strain was digested with *Hin*dIII at 37°C for 16 hr. The resultant fragments were separated by electrophoresis in a 0.7% agarose gel and transferred to nitrocellulose as described (26). The nitrocellulose sheet was then incubated with the ^{32}P-labeled LT-A probe, washed, and autoradiographed as described (9). Lanes: A, M7928; B, M7929; C, M7930; D, M7922; E, M7923; F, RV79. Arrow, position of a weak band of 23-kilobase material.

LT probes. Indeed, mutant M7922 appears to have lost its VcA1 prophage (assuming it was originally lysogenized) because this strain was found to be VcA1-sensitive and negative for the VcA1 sequence in Southern blots. Perhaps the toxin gene deletion event in M7922 was associated with loss of the VcA1 prophage from this strain. In contrast, M7928, M7929, and M7930 were all lysogenic for VcA2cts1. The absence of spontaneously occurring nontoxinogenic mutants of strain RV79 (frequency less than 0.02%) argues that the toxin gene deletion events that we have observed are directly related to infection and lysogenization of this strain with VcA1 and VcA2cts1.

Because all of the toxin gene deletion mutants were prototrophic and grew with normal division rates, the deletion events responsible for loss of the toxin gene have been confined to DNA sequences that are nonessential for growth of these strains *in vitro*. The mutants were also motile and sensitive to a variety of *V. cholerae* typing phages, showing that no major alterations in the cell surface occurred concomitantly with the toxin gene deletion mutations.

Intestinal Colonization by Toxin Gene Deletion Mutants. The availability of isogenic wild-type and toxin gene deletion mutants has allowed us to begin evaluation of the role of the toxin in intestinal colonization of *V. cholerae*. The three strains known to have total toxin gene deletion mutations were selected for these studies (M7922, M7928, and M7930). With the latter two it was necessary to cure the VcA2cts1 prophage and to eliminate the temperature-sensitive phenotype from these strains. This was accomplished simply by isolating clones resistant to 42°C that had regained sensitivity to VcA2cts1 phage. Two cured derivatives were selected by this procedure (M7941 and M7942); these remained prototrophic.

Table 4 shows the result of serial intestinal passage of these mutant strains in rabbits. For each strain, approximately 10^7 cells were injected in a 5-cm intestinal loop; 18 hr later, the loops were scored for fluid accumulation and for viable counts

Table 4. Growth of wild-type and mutant strains *in vivo**

Strain	Cell density†	Fluid response‡
RV79	16	0.96
M7922	7.8	<0.05
M7941	3.6	<0.05
M7942	2.6	<0.05
569B	10	1.4

* Measured in the rabbit ileal loop assay as described (13).
† The average bacterial cell density attained in 16–18 hr, expressed in colony-forming units $\times 10^{-9}$ per cm of intestine (three determinations). One centimeter of intestine was equivalent to approximately 0.4 g of tissue.
‡ The average fluid response in 16–18 hr expressed in ml/cm of intestine (three determinations).

after homogenization of the gut tissue. All three toxin deletion mutants multiplied 100- to 1000-fold, attaining average cell densities *in vivo* of 2–8 $\times 10^9$ colony forming units per cm of intestine. The three mutants induced no significant fluid accumulation, and, as expected for total toxin gene(s) deletion mutants, they generated no Tox^+ revertants detectable by the ganglioside filter assay. In contrast, the parental strain RV79 attained only a 2- to 6-fold higher average cell density than the mutant strains, even though it induced a large secretory response. These experiments suggest that the cholera toxin gene does not play an essential role in the process of intestinal growth and survival of *V. cholerae* in this animal model.

DISCUSSION

Mutations altering toxin production in *V. cholerae* have been described (11–17), but their molecular natures have not been determined. Analysis of VcA1- and VcA2cts1-induced nontoxinogenic mutants of *V. cholerae* strain RV79 has led us to conclude that several of these clones possess deletion mutations in the structural gene encoding cholera toxin. This definitive demonstration of a deletion mutation of the cholera toxin structural gene will eventually allow us to map genetically the location of the toxin gene in *V. cholerae* strain RV79.

The demonstration that the toxin deletion mutants show no major defect in their ability to grow in the laboratory or in the gut of susceptible animals argues strongly that the cholera toxin gene is nonessential in *V. cholerae*. Growth defects in other toxin mutant strains, which have been attributed to possible physiological roles for the toxin in *V. cholerae* (16), may more likely represent pleiotropic effects of repeated mutagenesis with nitrosoguanidine. Several of the mutations we characterized proved to be complete deletions of the toxin gene. However, we also found that one mutant (M7929) retained some DNA homologous to the LT A-subunit probe but had lost all sequences hybridizing to the LT B-subunit probe. Thus, the methods described here can also be used to construct partial deletions of the toxin gene. Whether these methods are generally applicable to other stains of *V. cholerae* would depend mainly on the sensitivity of these strains to VcA1 and VcA2cts1.

The induction of toxin deletion mutations by the two vibriophages used in this study is curious in the light of the fact that at least one of them (VcA1) is likely to induce mutations by the insertion of its DNA into random sites on the *V. cholerae* chromosome (26, 28). The event responsible for deletion of the toxin gene(s) concomitant with VcA1 and VcA2cts1 lysogenization is at present unclear. Daniell *et al.* (30) have reported that approximately 15% of *lacZ* mutants induced by phage Mu in *E. coli* repressed for *lac* expression were deletion mutations. In contrast, Mu lysogenization concomitant with active transcription of the *lac* operon, produced either by induction with IPTG or through the use of a *lacI* constitutive mutant, increased the frequency of deletions observed among *lacZ* mutations to 57% and 93%, respectively. These investigators concluded that active transcription of *lac* reduced the ability of phage Mu to insert its DNA into the *lacZ* gene but did not alter its ability to cause complete deletions of this gene. Because RV79 produces toxin in the phage growth medium used here, active transcription of the toxin gene may have similarly interfered with the point insertion of the VcA1 and VcA2cts1 phages into this gene(s). As with phage Mu and *lac*, phage-induced deletion events would then become responsible for a large fraction of the mutations isolated. Experiments are in progress to examine the influence of transcription on the target specificity of VcA1 and VcA2cts1 in *V. cholerae*.

Finally, we have characterized several mutant strains of *V. cholerae* that might have practical value as prototype live oral cholera vaccines. Strains previously developed for this purpose have displayed instability *in vivo*, as evidenced by the appearance of toxinogenic revertant clones in the stools of animals (12, 13) and human volunteers (31). The toxin gene deletion mutants described here would be predicted to be stably attenuated because they have lost the genetic information encoding the toxin. In addition, these deletion mutants can colonize the mucosal surface and multiply within the gut in the rabbit ligated-loop model. It remains to be determined whether the mutants will colonize as well in other animal models in which the gut remains open [e.g., chinchillas (32)]. Colonization of man by such mutants should induce protective, secretory IgA antibody that would inhibit bacterial attachment to the mucosa, motility, chemotaxis, protease and neuraminidase activity, and other undefined activities required by *V. cholerae* for virulence (33–35). Antitoxin, of course, would not be induced by these mutants. However, the antitoxin role in immunity to cholera is currently in dispute, and it actually may be of little protective value (36). Moreover, in the event that an antitoxin response is a significant component of immunity to cholera, the methods described here could be used to construct strains with deletion mutations in the A-subunit gene that continue to produce the highly antigenic B subunit of the toxin (16). Alternatively, the deletion mutants we have characterized could also serve as hosts for the reintroduction of an altered *tox* gene (constructed by recombinant DNA methods) that codes for an immunologically crossreactive nontoxic analogue of the toxin.

Note Added in Proof. We recently have cloned the toxin genes of *V. cholerae* strain 569B on a 5.1-kilobase *Eco*RI–*Pst* I insert in plasmid pBR322 (unpublished data). We also have demonstrated that inactivation of the cloned A-subunit gene *in vitro* results in production of only the nontoxic B subunit of the toxin in *E. coli*. Introduction of the cloned B-subunit gene into the *V. cholerae* deletion mutants described in this report is, therefore, a technically feasible way of constructing prototype vaccine strains.

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1. Collier, R. J. & Mekalanos, J. J. (1980) *Multifunctional Proteins*, eds. Bisswanger, H. & Schmincke-Ott, E. (Wiley, New York), pp. 261–291.
2. Gill, D. M. (1976) *Biochemistry* **15,** 1242–1248.
3. Cuatrecases, P. (1973) *Biochemistry* **12,** 3577–3581.
4. Cassel, D. & Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2669–2673.
5. Gill, D. M. & Meren, R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3050–3054.
6. Clements, J. D., Yancey, R. J. & Finkelstein, R. A. (1980) *Infect. Immun.* **29,** 91–97.
7. Gill, D. M., Evans, D. J. & Evans, D. G. (1976) *J. Infect. Dis.* **133,** S103–S107.
8. Vasil, M. I., Holmes, R. K. & Finkelstein, R. A. (1975) *Science* **187,** 849–850.
9. Moseley, S. L. & Falkow, S. (1980) *J. Bacteriol.* **144,** 444–446.
10. Kaper, J. B., Moseley, S. L. & Falkow, S. (1981) *Infect. Immun.* **32,** 661–667.
11. Howard, B. D. (1971) *Nature (London)* **230,** 97–99.
12. Finkelstein, R. a., Vasil, M. L. & Holmes, R. K. (1974) *J. Infect. Dis.* **129,** 117–123.
13. Mekalanos, J. J., Romig, W. R. & Collier, R. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 941–945.
14. Mekalanos, J. J., Sublett, R. & Romig, W. R. (1979) *J. Bacteriol.* **139,** 859–865.
15. Ruch, F. E., Murphy, J. R., Graf, L. H. & Field, M. (1978) *J. Infect. Dis.* **137,** 747–755.
16. Honda, T. & Finkelstein, R. A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2052–2056.
17. Mekalanos, J. J. & Murphy, J. R. (1980) *J. Bacteriol.* **141,** 570–576.
18. Johnson, S. R. & Romig, W. R. (1981) *J. Bacteriol.* **146,** 632–638.
19. Mekalanos, J. J., Collier, R. J. & Romig, W. R. (1978) *Infect. Immun.* **20,** 552–558.
20. Gerdes, J. C. & Romig, W. R. (1975) *J. Virol.* **15,** 1231–1238.
21. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
22. Harris, C. C., Yolken, R. H., Krokan, H. & Hsu, I. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5336–5339.
23. Brenner, D. J., Fanning, G. R., Johnson, K. E., Citarella, R. V. & Falkow, S. (1969) *J. Bacteriol.* **98,** 637–650.
24. Dallas, W. S., Gill, D. M. & Falkow, S. (1979) *J. Bacteriol.* **139,** 850–858.
25. Maniatis, T., Jeffry, A. & Kleid, A. G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1184–1188.
26. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
27. Bukhari, A. I. (1976) *Annu. Rev. Genet.* **10,** 389–412.
28. Johnson, S. R., Liu, B. C. S. & Romig, W. R. (1981) *FEMS Microbiol. Lett.* **11,** 13–16.
29. Parker, C. D., Gauthier, D., Tate, A., Richardson, K. & Romig, W. R. (1979) *Genetics* **91,** 191–214.
30. Daniell, E., Roberts, R. & Abelson, J. (1972) *J. Mol. Biol.* **69,** 1–8.
31. Woodward, W. E., Gilman, R., Hornick, R., Libonati, J. & Cash, R. (1975) *Proceedings of the 11th Joint Conference on Cholera* (National Institutes of Health, Bethesda, MD), p. 330.
32. Blackman, U., Graboff, S. R., Hagg, G. E., Gottfeld, E. & Pickett, M. J. (1974) *Infect. Immun.* **10,** 1098–1104.
33. Freter, R. (1972) *Infect. Immun.* **6,** 134–141.
34. Guentzel, M. N. & Berry, L. J. (1975) *Infect. Immun.* **11,** 890–897.
35. Baselski, V. S., Upchurch, S. & Parker, C. D. (1978) *Infect. Immun.* **22,** 181–188.
36. Cash, R. A., Music, S. I., Libonati, J. P., Craig, J. P., Pierce, N. F. & Hornick, R. B. (1974) *J. Infect. Dis.* **130,** 325–333.