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Research Article

Spontaneous and chemically induced mutants with reduced ability to produce cholera enterotoxin (choleragen) as an extracellular protein were isolated from Vibrio cholerae strains 569B Inaba, a classical cholera vibrio, and 3083-2 Ogawa, an El Tor vibrio. By qualitative and quantitative immunological assay in vitro such mutants could be separated into different classes characterized either by production of no detectable choleragen (tox minus), or of small quantities of extracellular choleragen, or of large quantities of cell-associated choleragen but little extracellular choleragen. Analysis of proteins in concentrated culture supernates by electrophoresis in polyacrylamide gels showed that cultures from tox minus strains lacked proteins with electrophoretic mobilities corresponding with choleragen or the spontaneously formed toxoid (choleragenoid). Infant rabbits infected with the tox minus strains remained asymptomatic or developed milder symptoms than rabbits infected with the tox+ parental strains. When symptoms of cholera developed after inoculation with tox minus mutants, detectable numbers of tox+ revertants could be isolated from the intestines of the infected animals. Two tox minus strains, designated M13 and M27, caused no sumptoms and showed no evidence of reversion to tox+ during single passage in infant rabbits, and mutant M13 also remained avirulent and stably tox minus during six cycles of serial passage in infant rabbits. Strains M13 and M27 were also noncholeragenic in acult rabbit ileal loops. Quantitative cultures of […]

Studies on Toxinogenesis in *Vibrio cholerae*

III. CHARACTERIZATION OF NONTOXINOGENIC MUTANTS IN VITRO AND IN EXPERIMENTAL ANIMALS

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ABSTRACT Spontaneous and chemically induced mutants with reduced ability to produce cholera enterotoxin (choleragen) as an extracellular protein were isolated from *Vibrio cholerae* strains 569B Inaba, a classical cholera vibrio, and 3083-2 Ogawa, an El Tor vibrio. By qualitative and quantitative immunological assays in vitro such mutants could be separated into different classes characterized either by production of no detectable choleragen (*tox*$^-$), or of small quantities of extracellular choleragen, or of large quantities of cell-associated choleragen but little extracellular choleragen. Analysis of proteins in concentrated culture supernates by electrophoresis in polyacrylamide gels showed that cultures from *tox*$^-$ strains lacked proteins with electrophoretic mobilities corresponding with choleragen or the spontaneously formed toxoid (choleragenoid). Infant rabbits infected with the *tox*$^-$ strains remained asymptomatic or developed milder symptoms than rabbits infected with the *tox*$^+$ parental strains. When symptoms of cholera developed after inoculation with *tox*$^-$ mutants, detectable numbers of *tox*$^+$ revertants could be isolated from the intestines of the infected animals. Two *tox*$^-$ strains, designated M13 and M27, caused no symptoms and showed no evidence of reversion to *tox*$^+$ during single passage in infant rabbits, and mutant M13 also remained avirulent and stably *tox*$^-$ during six cycles of serial passage in infant rabbits. Strains M13 and M27 were also noncholeragenic in adult rabbit ileal loops. Quantitative cultures of the intestines from infected infant rabbits demonstrated that the avirulent mutant M13 can multiply in vivo and can persist in the intestinal tract for at least 48 h.

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INTRODUCTION

Cholera enterotoxin (choleragen) is an extracellular protein of *Vibrio cholerae* that has been purified to homogeneity (1), crystallized (2), and studied extensively in many laboratories (3). When purified choleragen is administered into the lumen of the small intestine of susceptible animals, it elicits a secretory response resulting in profuse diarrhea that mimics the symptoms of cholera in man (4). Choleragen binds to specific receptors, identical or similar to the ganglioside designated G_{M1} or GGnSLC, in the plasma membrane of mammalian cells (5, 6). This is followed by activation of adenylate cyclase, leading to an increase in the intracellular concentration of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) (7–9). The biological activities of choleragen on the intestinal mucosa as well as on other target cells in man and in experimental animals are believed to be mediated by cyclic AMP (3). Although other properties of *V. cholerae* may be important as determinants of virulence, the ability to produce enterotoxin is indispensable for choleragenicity (3, 10).

Little information is available concerning the regulation of enterotoxin synthesis by *V. cholerae*, and the role of such regulatory mechanisms in the pathogenesis of cholera is unknown. Some enteropathogenic strains of *Escherichia coli* produce an enterotoxin that cross-reacts immunologically with choleragen (11) and resembles choleragen in its mode of action (12). In such enterotoxigenic strains of *E. coli* the *ent* gene controlling enterotoxin production can be present on extrachromosomal genetic elements called plasmids (13). Although a

conjugal mating system has been discovered and has been exploited for genetic mapping of the chromosome of *V. cholerae* (14, 15), the genes that determine enterotoxin production in *V. cholerae* have not yet been analyzed.

To begin studies on the genetic regulation of toxinogenesis in *V. cholerae,* methods were developed for the isolation of nontoxinogenic mutants and for the differentiation of *tox*$^+$ from *tox*$^-$ bacterial strains by rapid immunological techniques based on precipitin reactions that can be scored visually (10, 16). A collection of independently derived, nontoxinogenic mutants of *V. cholerae* has been isolated. In the present study these *tox*$^-$ mutants have been compared with the *tox*$^+$ parental strains both in vitro and in two models of cholera in experimental animals, the intraintestinal infection of infant rabbits (17), and the intraintestinal inoculation of ligated ileal loops in adult rabbits (18).

METHODS

Bacterial strains. V. cholerae strain 569B Inaba is a classical cholera vibrio that produces large quantities of enterotoxin in vitro, and *V. cholerae* 3083-2 variant, hereafter designated 3083-2, is a toxinogenic Ogawa strain of the El Tor biotype (10). Ogawa and Inaba serotypes of parental and mutant strains of *V. cholerae* were determined by slide agglutination tests (19).

Media and bacterial cultures. All bacterial strains were stored as lyophilized cultures. Syncase broth, meat extract agar, minimal agar, and antitoxin agar have been described previously (10). Unless otherwise noted, all cultures were incubated at 37°C and broth cultures were aerated by rotary shaking at 240 rpm. After rehydration of lyophilized cultures, samples were inoculated onto meat extract agar either for confluent growth or for single colony isolation. Liquid cultures in syncase broth were inoculated from single colonies when clones of cells were required for genetic experiments. Routine broth cultures were inoculated with specimens from plates with confluent bacterial growth. Broth cultures in sterile Erlenmeyer flasks plugged with gauze contained 10-ml vol in 125-ml flasks or 200-ml vol in 1-liter flasks. For production of enterotoxin, cultures were incubated at 30°C with reciprocal shaking as described previously (10).

Viable bacteria in broth cultures or in homogenates of intestines from infected animals were enumerated by spreading aliquots of appropriately diluted specimens on the surface of meat extract agar and counting colonies that developed after incubation of the plates for 18 h.

Induction of mutations in V. cholerae and selection of nontoxinogenic mutants. The procedure used for mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)[1] (Sigma Chemical Co., St. Louis, Mo.) was modified from the method of Adelberg, Mandel, and Chen (20) and has been described previously (10). Mutagenesis with ethyl methanesulfonate (EMS) (Eastman Kodak Co., Rochester, N. Y.) was based on the method of Loveless and Howarth (21). Exponentially growing cultures in 10 ml of syncase broth containing 1.7×10^8 viable cells/ml were harvested by centrifugation, and the cells were resuspended in 3-ml vol of EMS and incubated at 37°C for 15 min. After incubation, the mutagenized cells were washed by centrifugation three times in syncase broth and then resuspended in 10 ml of syncase broth and incubated overnight at 30°C with rotary shaking. As determined by viable counts performed before and immediately after incubation in EMS, approximately 16% of the *V. cholerae* cells present initially survived treatment with EMS. Mutagenesis with ethidium bromide (EB) (Sigma Chemical Co.) was based on the method of Bouanchaud, Scavizzi, and Chabbert (22). Exponentially growing cultures of *V. cholerae* in syncase broth were diluted in the same medium to a final concentration of 2×10^4 cells/ml, and EB was added at a concentration of 2.5×10^{-5} M, sufficient to inhibit growth slightly, or 1.0×10^{-5} M, a subinhibitory concentration. Cultures were incubated with agitation for 18 h. After each of the above mutagenic treatments, cultures were diluted appropriately and inoculated into pour plates containing antitoxin agar. In this medium colonies of the toxinogenic parental strains are surrounded by halos of toxin-antitoxin precipitate and can be differentiated visually from mutant colonies that lack halos (10). Mutants of *V. cholerae* 569B with altered toxinogenicity were designated arbitrarily by the prefix M and numbered sequentially in order of isolation.

In vitro tests for production of enterotoxin by wild type and mutant strains of V. cholerae. Precipitin tests for cholera enterotoxin were carried out with cultures of cholera vibrios or were performed with antigens prepared either from extracellular products in cultures of *V. cholerae* or from cell-associated products obtained by disruption of washed bacterial cells. Cultivation of *V. cholerae* in pour plates containing antitoxin agar was found to be particularly useful for the recognition of *tox*$^-$ mutants in cultures (10). When large numbers of bacterial strains must be checked for their ability to produce enterotoxin, Elek plates are more convenient than pour plates with antitoxin agar, because many cultures can be tested on a single Elek plate. In an Elek test, cultures are streaked on the surface of the agar at right angles to a strip of filter paper impregnated with antitoxin, and toxin-antitoxin precipitin lines form adjacent to growing bacteria that produce enterotoxin (16). To detect enterotoxin released from bacterial cells into syncase broth, cells were removed from 18-h cultures by centrifugation, the supernates were sterilized by passage through 0.45-μm filters (Millipore Corp., Bedford, Mass.) and the cell-free supernates were used as antigen after they were concentrated 20- or 100-fold by ultrafiltration with membrane filters (PM-10, Amicon Corp., Lexington, Mass.). To detect cell-associated enterotoxin, cells were collected by centrifugation from 200-ml vol of 18-h syncase broth cultures, suspended in 5-ml samples of syncase broth, maintained in an ice bath, and disrupted by sonication for 3–5 min with a Branson sonicator (Branson Instruments Co., Stamford, Conn.). Similar results were obtained in immunodiffusion assays for enterotoxin when disrupted cell preparations were used as antigen either before or after removal of cellular debris by centrifugation. Quantitative assays for choleragen by immunodiffusion in agar gels were based on the methods of Oakley and Fulthorpe (23) and of Mancini, Carbonara, and Heremans (24), and qualitative immunodiffusion tests were modified from the procedure of Ouchterlony (25), as previously described (4, 10). A highly specific equine anticholeragenoid antiserum was used throughout this study (26). Choleragen and choleragenoid used as standards for immunodiffusion, for electrophoresis,

[1] *Abbreviations used in this paper:* EB, ethidium bromide; EMS, ethyl methanesulfonate; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

TABLE I

Induction of Mutations that Alter Toxinogenicity in V. cholerae 569B

Experiment	Mutagen*	Total colonies	Scorable colonies	Colonies without halos‡	
				n	%
Control	None	12,636	7,271	0	0
1	NTG	3,108	1,588	27	1.7
2	NTG	1,668	809	18	2.2
3	NTG	2,325	1,194	24	2.0
4	NTG	1,947	971	13	1.3
5	NTG	1,350	699	9	1.3
6	NTG	1,494	766	12	1.6
7	EMS	5,920	3,161	15	0.25
8	EB, 10^{-5} M	5,729	2,916	2	0.035
9	EB, 2.5×10^{-5} M	7,126	3,713	0	0

* Abbreviations: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; EMS, ethyl methanesulfonate; EB, ethidium bromide.

‡ Suitable dilutions of control or mutagenized cultures of *V. cholerae* 569B were inoculated into pour plates containing antitoxin agar. Colonies that produce normal amounts of choleragen are surrounded by halos of toxin-antitoxin precipitate in this medium.

and for other purposes throughout this study were purified by methods described previously (1, 10).

Biological assays for production of enterotoxin by V. cholerae. Strains of *V. cholerae* to be tested were grown in syncase broth to a cell density between 10^8 and 10^9 cells/ml. Infant rabbits were infected at laparotomy by injection of 0.5 ml of the bacterial culture into the lumen of the terminal ileum (17). The severity of diarrheal disease in individual animals was expressed as the choleragenic score (4). A minimum of three infant rabbits was tested with each strain, and mean choleragenic scores were used as a measure of the relative virulence for infant rabbits of parental and mutant strains of *V. cholerae.* Autopsies on infant rabbits were performed at the times indicated in the text. The intestinal tract from pylorus to rectum was excised and rinsed. The intestine and its contents were then minced in 25 ml of syncase broth and homogenized in an Ultra-Turrax tissue homogenizer (Tekmar Co., Cincinnati, Ohio). Appropriate dilutions of each homogenate were inoculated on meat extract agar for determination of viable counts of *V. cholerae* and of other aerobic bacteria from the intestinal tracts of the infected rabbits. For tests with adult rabbits, ligated ileal loops were established at laparotomy (18), and 1-ml samples of the cultures to be tested were inoculated into the lumen of the ligated loops. The animals were sacrificed and examined 12–18 h after inoculation, and the secretory responses were expressed as milliliters of accumulated fluid per centimeter of ligated intestinal segment.

Analysis of proteins by electrophoresis in polyacrylamide gels. Formulations for sample gels and spacer gels employing a high pH discontinuous buffer system were as described by Maizel (27). Electrophoresis was performed in a model 1,200 bath assembly (Canalco, Inc., Rockville, Md.) and the length of the sample gels was 6 cm. 100-μl samples contained either 80–90 μg of extracellular proteins from concentrated syncase broth cultures of *V. cholerae* or 10-μg samples of purified choleragen or choleragenoid. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall using bovine serum albumin as the standard (28). Electrophoresis was carried out at 4 mA/gel until the tracking dye had migrated to a position near the distal ends of the gels (approximately 1.5 h). Gels were fixed in 20% trichloroacetic acid (TCA), stained with 0.2% Coomassie brilliant blue in 20% TCA, decolorized with 40% ethanol, and stored in 7% acetic acid.

RESULTS

V. cholerae 569B Inaba and 3083-2 Ogawa are virulent strains capable of producing cholera in man and experimental animals. Both strains produce choleragen in vitro in syncase broth cultures. They are both prototrophic and grow on the unsupplemented minimal medium used in these studies. Strains 569B and 3083-2 were selected as parental types for our studies on the genetic control of toxinogenesis in *V. cholerae.*

When strain 569B or strain 3083-2 is grown in pour

TABLE II

Phenotypes of V. cholerae Mutants with Altered Toxinogenicity

Parental strain	Representative mutant	*Tox* phenotype	Associated phenotypic changes
569B Inaba	M1	Less toxin	None
	M5	*Tox*$^-$, reverting	None
	M13	*Tox*$^-$, nonreverting	None
	M14	Cell-associated toxin	None
3083-2 El Tor Ogawa	Var-1	*Tox*$^-$, reverting	Colonial morphology

TABLE III

In Vitro Studies of V. cholerae Mutants with Altered Toxinogenicity

Strain*	Serotype	Tests for enterotoxin‡		
		Extracellular antigen	Cell-associated antigen	Electrophoretically similar protein
		μg/ml	μg/ml	
569B	Inaba	20	0.3	Yes
M1	Inaba	3	—	Yes
M5	Inaba	—	—	—
M13	Inaba	—	—	—
M14	Inaba	0.5	5	Yes
M18	Inaba	—	—	—
M23	Inaba	—	—	—
M27	Inaba	—	—	±
M32	Inaba	—	—	—
M37	Inaba	—	—	±
M40 (EMS)	Inaba	—	NT	NT
M44 (EB)	Inaba	—	NT	NT
3083-2	Ogawa	13	—	Yes
3083-2 var-1	Ogawa	—	—	—

* Strains 569B and 3083-2 are the parental *tox*$^+$ strains. Strains designated by the prefix M are independently derived mutants of 569B induced by NTG unless otherwise noted (EMS or EB). 3083-2 var-1 is a spontaneous mutant of 3083-2. All strains listed in this table are photorophic.

‡ The concentration of cell-associated antigen is expressed as micrograms per milliliter of crude culture. (−) indicates not detectable. (±) indicates that a very faintly stained band was present with the mobility of choleragen or choleragenoid. (NT) indicates not tested. Samples tested by electrophoresis in polyacrylamide gels were concentrated supernates of syncase broth cultures.

plates containing syncase agar with specific antitoxin, the lenticulate subsurface colonies produce choleragen in amounts detectable by a precipitin reaction. In this medium *tox*$^+$ colonies are surrounded by halos of toxin-antitoxin precipitate and can therefore be distinguished visually from *tox*$^-$ colonies that lack halos. For technical reasons, only colonies that can be viewed on edge are scorable.

The proportion of bacteria that form colonies without halos has been measured in cloned cultures of *V. cholerae* 569B before and after treatment of the bacteria with the mutagenic agents NTG, EMS, and EB (Table I). Among 7,271 scorable colonies of the parental strain 569B, none lacked halos. The frequency of spontaneous mutants of 569B with the haloless phenotype is therefore low, less than 0.014% in our experiments. After treatment of six cloned cultures of 569B with NTG (experiments 1–6, Table I), haloless mutants were consistently detected at frequencies between 1.3 and 2.2% of scorable colonies. The frequency of haloless mutants induced by NTG is therefore at least 100 times as great as the frequency of spontaneous mutants. Because NTG is such a potent mutagen, it is possible that undetected secondary mutations may be present in some of the independently isolated haloless strains. EMS was 5- to 10-fold less effective than NTG for inducing haloless mutants, but such mutants could be isolated with ease after EMS mutagenesis (experiment 7, Table I). After treatment with EB only two haloless colonies were found among 6,629 scorable colonies (experiments 8 and 9, Table I). Because of this low frequency, it is not certain whether they were induced by EB or had occurred spontaneously. All haloless mutants of strain 569B formed surface colonies on meat extract agar indistinguishable from those of the parental strain 569B. In contrast, cloned cultures of strain 3083-2 contained variants at a frequency of 1% or greater that formed colonies that were more opaque in oblique transmitted light than those of the parental strain 3083-2. These morphological variants of strain 3083-2 also formed haloless colonies in antitoxin agar. Because of the high frequency of spontaneously occurring haloless mutants, chemical mutagenesis was not used with strain 3083-2.

Haloless mutants derived from strains 569B and 3083-2 were subjected to further tests in vitro to establish how the haloloess phenotype correlates with specific alterations in toxinogenesis. Several different classes of mutants were identified, and the phenotypic properties of representative mutants from each class are summarized in Table II. Specific data concerning some of the qualitative and quantitative tests used for the

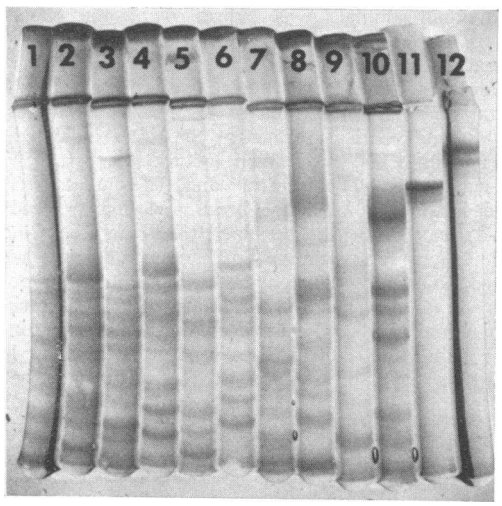

FIGURE 1 Polyacrylamide gel electrophoresis of extracellular proteins from *tox*$^+$ and *tox*$^-$ strains of *V. cholerae*. Samples contained either 80–90 μg of extracellular protein from *V. cholerae* or 10 μg of purified choleragen or choleragenoid. Samples containing extracellular proteins were prepared from the following strains of *V. cholerae:* (1) M18, (2) M23, (3) M27, (4) M32, (5) M37, (6) M5, (7) M13, (8) 3082-2 wild type, (9) 3083-2 var-1, and (10) 569B wild type. Controls contained: (11) choleragen and (12) choleragenoid.

characterization of these mutants in vitro are presented in Table III and in Figs. 1 and 2. All strains were serotyped by slide agglutination tests and were examined for colonial morphology and for growth on minimal medium. These tests were performed to identify the putative mutants as *V. cholerae* and to verify that secondary mutations altering either the serotypes or the nutritional requirements of these strains had not occurred during mutagenesis. All of the mutants discussed below are prototrophic and have the same serotypes as their parental strains. Oakley-Fulthorpe, radial immunodiffusion, and Ouchterlony tests for choleragen were performed with supernates of syncase broth cultures before and after 20-fold concentration. Similar tests were also carried out with antigens prepared from disrupted cells of the mutant strains. Polyacrylamide gel electrophoresis was performed with culture supernates concentrated at least 100-fold.

Strain M1 is representative of mutants that synthesize small amounts of enterotoxin insufficient to produce visible halos in antitoxin agar but detectable by more sensitive precipitin tests in vitro (Table II). The amount of choleragen produced by mutants of this type did not exceed 15% of the amount synthesized by the parental strain 569B in syncase broth cultures (Table III and data not presented).

The *tox*$^-$ mutants belonging to the classes represented by strains M5 and M13 (Table II) make no extracellular choleragen detected by precipitin tests. The tests used could have detected 0.5% of the choleragen produced by the parental strain 569B (Table III). Concentrated culture supernates from eight of these *tox*$^-$ mutants were examined by electrophoresis in polyacrylamide gels (Table III and Fig. 1). In the parental strains 569B and 3083-2, choleragen is the most abundant extracellular protein, although many other proteins are also present in smaller quantities. The extracellular proteins of these *tox*$^-$ mutants had mobilities similar to proteins that were also present in cultures of the parental strains. None of the mutants had a strongly stained protein band corresponding in mobility to choleragen or to choleragenoid. In addition, none of the mutants produced any single protein that might be a nonantigenic, electrophoretic variant of choleragen in quantities that were comparable to the amount of choleragen in cultures of the parental strains. The 10 *tox*$^-$ strains investigated were also separated into two groups differing in their ability to revert from *tox*$^-$ to *tox*$^+$ (Table II). The phenomenon of reversion to *tox*$^+$ in these strains will be discussed later.

One haloless strain, M14, elaborates small amounts of extracellular enterotoxin but differs from both the parental strain 569B and from other mutants that give low yields of choleragen. The unique property of strain M14 is that it produces large amounts of cell-associated enterotoxin, as demonstrated by immunodiffusion tests with sonicated cell extracts as antigen (Fig. 2 and Table III).

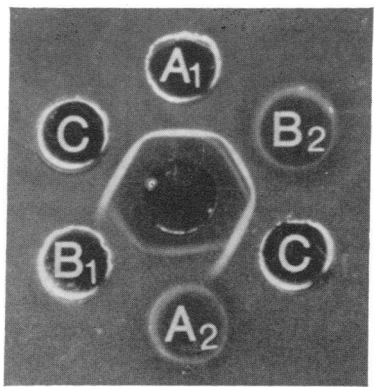

FIGURE 2 Demonstration of extracellular and cell-associated choleragen produced by *V. cholerae* strains 569B and M14. Preparation of antigens is described in Methods. Wells in Ouchterlony gel-diffusion plates contained the following specimens: A_1, 569B culture supernate; A_2, 569B sonicated cells; B_1, M14 culture supernate; B_2, M14 sonicated cells; C purified choleragen, 200 μg/ml; center well, equine anticholeragenoid serum. Antigen from 569B-supernate and from M14-sonicated cells forms a line of identity with purified choleragen. A second antigen unrelated to choleragen is detected by this antiserum (10) and is responsible for the weak precipitin lines formed between the antiserum and samples A_2, B_1, and B_2.

Strain 3083-2 var-1 is representative of the spontaneous *tox*$^-$ colonial variants of strain 3083-2 that occur with high frequency (Tables II and III). *Tox*$^+$ revertants derived from 3083-2 var-1 have the colonial morphology of the parental strain 3083-2. In several successive cycles of forward and reverse mutation with derivatives of strain 3083-2, colonial morphology and toxinogenicity always changed at the same time. These properties suggest that alterations of toxinogenicity and colonial morphology in strain 3083-2 may be pleiotropic effects of a single mutation.

Eight of the *tox*$^-$ mutants in Table III that produced no detectable choleragen with the most sensitive immunological tests used in vitro were examined for choleragenicity in experimental animals. Data derived from intraintestinal infection of infant rabbits by the *tox*$^+$ parental and *tox*$^-$ mutant strains of *V. cholerae* 569B and 3083-2 are summarized in Table IV. In these experiments large inocula of living vibrios, between 10^8 and 10^9 per infant rabbit, were used to provide a sensitive test for virulence. Animals that died were autopsied at 18–24 h postinfection, and survivors were sacrificed and autopsied at 48 h postinfection. The wild type *tox*$^+$ strains 569B and 3083-2 produced fatal infections in all animals within 24 h, and the mean cho-

leragenic scores with these strains were above 8. In contrast, all of the *tox*⁻ mutants were less virulent than the parental strains. None of the rabbits succumbed to infection with any of the *tox*⁻ mutants in 48 h. Strains M13 and M27 were totally avirulent and produced no signs of diarrheal illness in any of the infected animals. In contrast, other strains like M5 and M37 produced mild diarrheal disease and had mean cholerangenic scores less than 4.

Quantitative bacterial counts were performed after excision and homogenization of the intestines and their contents obtained at autopsy from the infected infant rabbits. Cholera vibrios were present and easily cultured from all specimens. In most cases they constituted the major component of the cultivable aerobic intestinal microflora. The total numbers of vibrios recovered per intestine were usually greater than 10^9 with the *tox*⁺ parental strains, but with the *tox*⁻ avirulent mutant M13 the total counts varied between 10^6 and 10^9. The *tox*⁻ mutants that produced mild disease were usually recovered from infant rabbits in larger numbers than found with strain M13. Selected colonies of *V. cholerae* isolated from each infected rabbit were examined by slide agglutination to verify their serotypes and were tested to determine their ability to produce choleragen. All colonies tested from animals infected with the avirulent strains M13 and M27 were *tox*⁻. In contrast, a significant proportion of the vibrios recovered from rabbits infected with strains that produced mild diarrheal illness were found to be *tox*⁺. When control cultures of the *tox*⁻ strains were tested for the presence of *tox*⁺ revertants without passage in infant rabbits, none were detected. Passage of *tox*⁻ mutants of *V. cholerae* in infant rabbits therefore provides a selective environment for growth of *tox*⁺ revertants. Strain M13 has been serially passaged six times in rabbits with no evidence of virulence or of reversion to *tox*⁺. Strain M13 has now been tested in a total of 113 infant rabbits over a period of 2 yr, and none has developed symptoms of diarrheal disease.

The *tox*⁻ mutants M13 and M27 were also tested in ileal loops in adult rabbits. No secretory response was elicited with either mutant strain, although controls with the parental strain 569B were strongly positive (2.2–2.4 ml/cm). After culture from the contents of the infected intestinal loops, 200 colonies of M13 and 100 colonies of M27 were tested for enterotoxin production on Elek plates, and none was positive. Among 100 colonies of *V. cholerae* recovered from loops infected with the parental strain 569B, all gave positive tests for enterotoxin. Based on all of our experiments to date, including 26 ileal loop tests with M13 and 4 tests with M27 in addition to the studies with infant rabbits described above, these mutants appear to be stable and nonreverting *tox*⁻ strains of *V. cholerae*.

Several experiments were performed to compare colonization of infant rabbits by the virulent parental strain 569B and by the stably *tox*⁻ avirulent mutant M13 (Table V). Inocula of 4.8×10^2 viable cells of 569B multiplied to yield an average of 6.1×10^9 progeny and produced severe diarrheal disease in all infant rabbits. In contrast, inocula of 3.8×10^2–3.8×10^8 viable cells of M13 produced no signs of illness in any rabbit, although strain M13 multiplied and persisted in the intestinal tract for at least 48 h after inoculation. The numbers of vibrios recovered from the intestines of animals infected with M13 were smaller than from animals infected with the parental strain 569B. To determine whether or not the development of a secretory diarrhea facilitates colonization of the intestinal

TABLE IV

Choleragenicity of Selected Tox⁻ Mutants of V. cholerae in the Infant Rabbit Model

Infecting strain	Number of animals		Mean choleragenic score	Reisolation of *V. cholerae*	
	Total	Dead		*V. cholerae* present	*Tox*⁺ colonies/ Colonies tested
569B	4	4	9.5	Yes	NT*
M5	7	0	4.0	Yes	7/21
M13	62	0	0	Yes	0/100
M18	3	0	0.7	Yes	21/60
M23	3	0	3.7	Yes	51/60
M27	3	0	0	Yes	0/60
M32	3	0	0	Yes	3/60
M37	3	0	4.0	Yes	51/60
3083-2	7	7	8.4	Yes	NT
3083-2 var-1	4	0	3.25	Yes	w.t. colonies 10/10 Var-1 colonies 0/20

* NT = not tested; w.t. = wild type.

TABLE V

Colonization of Infant Rabbits after Intraintestinal Infection with V. cholerae 569B tox⁺ or M13 tox⁻

Strain	Inoculum	Number of animals	Time of autopsy	Pretreatment*	Mean choleragenic score	Average counts of *V. cholerae* per intestine‡
			h			
569B	4.8×10^2	3	48	—	7.3	6.1×10^9
M13	3.8×10^8	4	18	—	0	2.6×10^9
	3.8×10^6	4	18	—	0	2.5×10^8
	3.8×10^4	4	18	—	0	6.7×10^6
	3.8×10^2	4	18	—	0	1.0×10^6
M13	3.8×10^8	4	48	—	0	2.8×10^7
	3.8×10^6	4	48	—	0	6.6×10^8
	3.8×10^4	4	48	—	0	7.6×10^6
	3.8×10^2	4	48	—	0	$<5.4 \times 10^3$
M13	5×10^5	4	9	Buffer	0	3.9×10^8
	5×10^5	4	9	Choleragen, 5 μg	4.3	2.9×10^7
	1.5×10^5	4	18	Buffer	0	1.5×10^8
	1.5×10^5	3	18	Choleragen, 2 μg	5.3	5.4×10^8
	1.5×10^4	3	18	Buffer	0	1.4×10^6
	1.5×10^4	3	18	Choleragen, 2 μg	8.3	4.2×10^6
	1.5×10^3	3	18	Buffer	0	3.4×10^6
	1.5×10^3	3	18	Choleragen, 2 μg	2.0	5.0×10^5

* Animals were pretreated by intragastric instillation of 5 ml of 0.1 M Tris-Cl buffer at pH 8 alone or containing purified choleragen in the doses indicated. (—) indicates that pretreatment was omitted.
‡ Expressed as geometric means.

tract by nontoxinogenic strains of *V. cholerae,* 2–5 μg of purified choleragen was administered to infant rabbits intragastrically in buffer 1 h before the rabbits were infected intraintestinally with various inocula of mutant M13 (Table V). All animals became colonized, and there were no striking or consistent differences in the numbers of M13 recovered at autopsy from animals pretreated with choleragen and from animals pretreated with buffer alone. The differences in mean choleragenic scores between the two groups of animals indicate that diarrheal illness was produced by the administered choleragen. It is clear that strain M13 can multiply in vivo and can colonize the intestinal tract of infant rabbits for periods up to 48 h. However, the size of the inoculum required for successful colonization may be somewhat larger and the population of vibrios obtained in the intestine may be somewhat smaller for mutant M13 than for the parental strain 569B.

DISCUSSION

In the present study mutants that are altered in their ability to synthesize or release choleragen have been isolated from *V. cholerae* strains 569B Inaba and 3083-2 Ogawa biotype El Tor. Isolation and characterization of such mutants is the initial step in studying the genetics of toxinogenesis in *V. cholerae.* Genetic analysis can help to define the mechanisms that regulate production of choleragen in *V. cholerae* and to clarify the role of such regulatory mechanisms in the pathogenesis of cholera.

Although the regulation of toxinogenesis in *V. cholerae* has not been studied in detail, several observations are important as background for a discussion of this problem. Choleragen has been highly purified and is an oligomeric protein of molecular weight 84,000 without detectable quantities of carbohydrate or lipid (3). Under various conditions it can be dissociated into subunits that are not identical (29, 30). Unless the subunits are formed by cleavage of a single polypeptide chain, separate structural genes must be required for the synthesis of each district polypeptide subunit of the choleragen molecule. In addition, detectable yields of choleragen are formed in some but not in all media that support the growth of *V. cholerae* 569B or 3083-2, and other strains of *V. cholerae* may differ in their requirements for optimal production of enterotoxin in vitro (1, 31). Because choleragen is not formed constitutively by *tox⁺* strains of *V. cholerae,* it is likely that specific regulatory systems control the synthesis and secretion of choleragen in a manner that is distinct from the regulation of bulk protein synthesis.

Recent evidence suggests that production of choleragen by *V. cholerae* may be regulated by mechanisms that require cyclic AMP (32). If the above observations are interpreted by analogy with other well-studied bacterial systems (33), the control of toxinogenesis in *V. cholerae* should involve coordinated interactions of two or more structural genes with specific regulatory genes and sites (repressors, operators, promotors, etc.) that determine the production and expression of positive or negative regulatory products.

Our independently isolated mutants of *V. cholerae* with altered toxinogenicity could be separated into groups with different phenotypic properties (Tables II and III, Figs. 1 and 2). The mutants that we have designated *tox*$^-$ make no choleragen detectable in vitro by precipitin test or by electrophoresis in polyacrylamide gels. Supernates of mutant M13 concentrated 100-fold also gave no reactions in Ouchterlony tests (unpublished observations) with antisera specific for the immunologically noncross-reacting subunits A and B of choleragen described recently (31). Taken together, these data suggest that none of the structural genes required for synthesis of choleragen is expressed in our *tox*$^-$ mutants and indicate that such structural genes may be coordinately regulated. It is possible, therefore, that the *tox*$^-$ phenotypes of our mutant strains could reflect either mutations in regulatory genes or mutations in structural genes that have strong polar effects.

We have previously described an antigenic difference, detected by an immunological cross-reaction in precipitin tests, between the enterotoxins of strains 569B and 3083-2 (10). It may be possible, therefore, to use this antigenic difference as a marker for a structural gene for choleragen in genetic studies with strains 569B and 3083-2 of *V. cholerae*. The observation that toxinogenesis and colonial morphology are altered simultaneously by single mutations in *V. cholerae* 3083-2 could be explained either by coordinate regulation of *tox* with other genes controlling colonial morphology or by some biochemical activity of choleragen required for normal colonial morphology in strain 3083-2.

The other mutants described in the present studies have phenotypes that suggest the alteration of specific regulatory functions that control toxinogenesis in *V. cholerae*. Some mutants like M1 (Tables II and III) make detectable yields of antigenically normal and biologically active choleragen, indicating that the structural genes for choleragen are intact. Strains like these that make reduced yields of choleragen under optimal conditions of cultivation might harbor mutations in promotors or mutations that produce polar effects. Strains like M14 (Tables II and III), that synthesize intracellular choleragen but produce small yields of extracellular toxin, may be altered in specific functions associated with the secretion of choleragen. Although the precise localization of the cell-associated choleragen in strain M14 is not established, the fact that it is released by sonication to a form that is freely detectable in immunodiffusion experiments suggests that it is located within the cell or its periplasmic space rather than absorbed nonspecifically to the external surface of the bacterial cell. Physiological studies of strains like M14 might therefore elucidate processes of general biological significance for the secretion of specific extracellular enzymes, proteins, or toxins in bacteria.

Although the hypotheses described above provide plausible explanations for the properties of the mutants we have observed, there are no direct data at present to confirm these or to exclude alternative formulations. Nevertheless, such hypotheses can be subjected to experimental tests by formal genetic studies, since the various genetic elements have specific properties that have been defined in other systems. A conjugal mating system exists in *V. cholerae* and is dependent on a sex factor designated P that is analogous in many respects to the classical fertility factor F of *E. coli* (15, 34). Mapping of chromosomal genes that determine nutritional requirements, antibiotic resistance, and surface antigens in *V. cholerae* were begun by Bhaskaran and his colleagues (14, 15) and extended by Parker, Gauthier, and Romig (35) and Parker, Tate, Richardson, Gauthier, and Romig (36). It should therefore be feasible to apply the techniques of formal genetics to the analysis of our mutants of *V. cholerae* with altered toxinogenicity. In addition, studies in our laboratories with *tox*$^-$ strains of *V. cholerae* unrelated to the mutants described here have demonstrated that genes controlling toxinogenesis can be transferred by conjugation and have established that one such gene is located on the chromosome of *V. cholerae*.[2] Thus, regulation of toxinogenesis in *V. cholerae* may differ from the plasmid-mediated system for control of enterotoxin synthesis reported in *E. coli* (13).

Our observations on the virulence of mutants of *V. cholerae* in experimental animals (Tables IV and V) may be relevant in considering the pathogenesis and the natural history of cholera. The observation that nonreverting *tox*$^-$ mutants are totally avirulent even though they can colonize the intestinal tract provides genetic evidence confirming the conclusion that choleragen plays an indispensable role in producing the secretory diarrhea of cholera. Our data show that mutants that produce quantitatively less extracellular choleragen

[2] Vasil, M. L., R. K. Holmes, and R. A. Finkelstein. Conjugal transfer of a chromosomal gene determining production of enterotoxin in *Vibrio cholerae*. *Science* (*Wash. D. C.*). In press.

produce milder diarrheal disease than the parental strains. In addition, in the infected animal *tox*$^+$ strains have a selective growth advantage relative to *tox*$^-$ strains. When virulent strains of *V. cholerae* are examined in vitro by Elek tests, classical strains give positive tests but most El Tor strains appear *tox*$^-$ although they are choleragenic in man and animals (16). Since El Tor vibrios are now recognized as capable of causing pandemic cholera (3), it seems likely that production of high maximal yields of choleragen in vitro is not a major determinant of survival value among *tox*$^+$ strains of *V. cholerae* in nature. However, these observations do correlate with the increased ratio of asymptomatic carriers to cases in El Tor infections as compared with classical *V. cholerae* infections (3).

The immunological responses of man and experimental animals to infection with cholera vibrios and the protective effects of antitoxic and antibacterial immunity against cholera have been recently reviewed (3). Immunological responses of animals to infection with mutants of *V. cholerae* altered in toxinogenicity are of considerable interest but were not included in the studies reported here because the animal systems used were short-term, terminal models. The idea that attenuated strains of *V. cholerae* isolated from nature (37) or developed in the laboratory (38–40) might be useful as live vaccines is not new. For several reasons, however, the strains we have isolated offer advantages over strains used by previous investigators for such experimental studies of immunization against cholera. Our *tox*$^-$ mutants are prototrophic and are antigenically similar to the parental strains. They appear to differ from the parental strains only in toxinogenicity. They can also multiply in the intestines of infected animals and are capable of colonizing the intestinal tract. *Tox*$^-$ strains like M13 are totally avirulent and have not been observed to revert to *tox*$^+$. Such *tox*$^-$ strains may therefore be able to elicit antibacterial immunity to cholera in appropriately infected experimental animals. Attempts to isolate stably *tox*$^-$ strains that produce immunologically cross-reacting but biologically inactive choleragen have not yet been successful, but such mutants would also be of even greater interest as possible vaccine strains.

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