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Regulation of Cholera Toxin Production in *Vibrio cholerae*: Genetic Analysis of Phenotypic Instability in Hypertoxinogenic Mutants

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Hypertoxinogenic mutants of *Vibrio cholerae* frequently possess mutations in a chromosomal locus called *htx*. Spontaneously occurring phenotypic revertants were shown to fall into three classes. One class retained the *htx* mutation and therefore represented a second-site mutation(s) capable of suppressing the Htx phenotype, whereas the other two classes represented strains that had lost the *htx* mutation. One of the latter two classes appeared to be composed of true genetic revertants, whereas the third class consisted of clones that had replaced *htx* with a new mutation conferring a hypotoxinogenic phenotype. Several rare *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced hypotoxinogenic mutants selected by *rif* comutation also map in the same region as *htx*. This new locus, which mediates the hypotoxinogenic phenotype in these strains and potentially in some hypertoxinogenic phenotypic revertants, has been designated *ltx*. *htx* and *ltx* appear to be regulatory loci, since mutations in both sites alter the level of cholera toxin A and B subunit production coordinately. The genetic data also support a model in which *htx*$^-$ and *ltx*$^-$ are allelic states of the same Tox regulatory locus.

Little is known of the location and structural organization of the genetic information coding for the production of cholera toxin in *Vibrio cholerae*. Mutants altered in the production of cholera toxin (Tox mutants) have been isolated in several laboratories (4-6, 10, 14, 16, 18, 19). Tox mutants have, in general, fallen into several phenotypic classes: nontoxinogenic (Ntx); hypotoxinogenic (Ltx); and hypertoxinogenic (Htx). Recently, the first of these three classes has been shown to be a subset of Ltx, since all nontoxinogenic mutants that have been carefully examined appear to produce small amounts of holotoxin. Until recently all the *tox* mutations appeared to be in regulatory loci, since only the level of holotoxin produced was affected. That is, the level of expression of subunits A and B was affected coordinately.

The mutation conferring one of the hypotoxinogenic phenotypes, *tox-1* (and several potentially allelic mutations) has been mapped on the vibrio chromosome and shows weak linkage to the *his-1* locus (2, 19). Mutations in a locus called *htx* cause hyperproduction of toxin and have been shown to map between the streptomycin resistance (*str*) and the rifampin resistance (*rif*) loci of *V. cholerae*, a site that is distant from the *his-1* locus on the chromosome (14, 17). It is important to note that the mutations defining the *htx* and *tox-1* loci do not appear to be in the structural genes for cholera toxin.

Recently, mutant strains of *V. cholerae* that appear to release only subunit B of toxin into the culture medium (5), as well as strains that produce high levels of subunit A relative to subunit B (16), have been isolated. The mutations that allow for the uncoordinated production of cholera toxin subunits in these mutant strains have not, as yet, been mapped. Mutants of both types have been shown to be nonvirulent in vivo and may serve as prototypes for the development of live oral vaccines for cholera and related enterotoxic enteropathies (3).

In this communication we describe the isolation of several classes of Tox regulatory mutants recognized by their ability to suppress the effect of an *htx* mutation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *V. cholerae* and plasmids used in this study are listed in Table 1. In the text the plasmid content of a strain is given in parentheses following the strain number. Strains were stored as frozen stocks at −70°C in brain heart infusion broth (Difco) containing 15% (vol/vol) glycerol. Casamino Acids-yeast extract (CYE) (11) agar was used as the solid nutrient medium. Antibiotics were used at the following concentrations (μg/ml): streptomycin (Sm), 100; spectinomycin (Sp), 100; rifampin (Rf), 30; and ampicillin (Ap), 700.

Mutant isolation. Mutagenesis was performed by

TABLE 1. *V. cholerae strains and plasmids*

Strain/plasmid	Genotype/phenotype[a]	Other information
569B	Prototroph, Tox+	Classical biotype; serotype, Inaba (3)
569B derivatives		
RV503	*arg-1 spc*	(14)
RV506	*arg-1 his-2 tox-306 spc*	From RV503; hypotoxinogenic phenotype (*tox-306*) was coinduced with *his-2* mutation after NTG mutagenesis.
RM71	*his-1 thy-1*::Tn*1 htx-3 str* Apr	Hypertoxinogenic mutant carrying Tn*1* insertion in *thy* (14)
RM72 to RM81	*his-1 thy-1*::Tn*1 str rif* Apr	From RM71 spontaneously induced phenotypic revertants of *htx-3*; also spontaneously induced Rifr
RM100	*his-1 thy-2*::Tn*1 str* Apr	(14)
RM101	*his-1 thy-2*::Tn*1 htx-4 str rif* Apr	(14)
RM102	*his-1 thy-2*::Tn*1 ltx-5 str rif* Apr	From RM100 by NTG-induced *rif* comutation
RM103	*his-1 thy-2*::Tn*1 ltx-6 str rif* Apr	From RM100 by NTG-induced *rif* comutation
MN1	Prototrophic, *tox-TI101*	From 569B; hypotoxinogenic mutant induced by growth at 42°C; produces subunit A and low levels of subunit B (16)
M13	Prototrophic, *tox-2*	From 569B by NTG mutagenesis; hypotoxinogenic mutant (2, 4)
Other strains		
RV79	Prototrophic, El tor biotype	Synonymous with RJ1 (7)
NIH41	Prototrophic, classical biotype	(14)
0395	Prototrophic, classical biotype, serotype Ogawa	(14)
Plasmids		
pSJ13	P::Tn*1* hybrid, Apr	Tn*1* in (−) orientation (7); in strains RM71, RM100, and their derivatives, this plasmid mediates polarized transfer of the *str* . . . *rif* region as described (14).

[a] Designations correspond to those of Johnson and Romig (7) for *V. cholerae.*

treatment of bacteria with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) by the method of Adelberg et al. (1). NTG-induced *rif* comutation (7) was performed as previously described (14). Other antibiotic resistance mutants were obtained by spontaneous, one-step mutations.

Mapping by transposon-facilitated recombination. Transposon-facilitated recombination (7) was used in genetic mapping studies as previously described (14). In these crosses a single donor allele (Smr) was selected, and Sp was used to counterselect the donor. Recombinants were transferred to the same selective medium and scored for unselected markers by replica plating.

Determination of cholera toxin production on solid medium. The ganglioside filter assay (10) was routinely used to score the level of toxin production in recombinants grown on CYE agar medium. Recombinants were inoculated from gridded master plates to the surface of ganglioside filters by velvet replica plating. After growth at 30°C for 16 h the filters were washed, processed with ^{125}I-labeled anti-subunit B immunoglobulin G, and autoradiographed as previously described (10). Since all strains used in this investigation (with the exception of MN1) coordinately express the A and the B subunits of the toxin, the level of response in the ganglioside filter assay closely correlated with the level of toxin produced by a given strain.

Determination of toxin production in liquid culture. *V. cholerae* strains were grown in CYE broth for 18 to 20 h at 30°C with vigorous aeration. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant fluid was assayed for cholera toxin and its subunits.

(i) Subunit B determination. Immunoradial diffusion (10) was used to measure the concentration of subunit B, using antiserum against purified choleragenoid (9). Purified cholera toxin (11) was diluted in sterile CYE medium and served as the standard.

(ii) Subunit A determination. The [^{125}I]guanyltyramine ([^{125}I]GT) assay (12) was used to measure the enzymic activity of subunit A in culture supernatant fluids. The reaction was initiated by the addition of 20 μl of culture supernatant fluid to 40 μl of a solution containing 10 mM NAD, 50 mM dithiothreitol, 0.2 μCi of [^{125}I]GT, 0.01% (wt/vol) Triton X-100, and 0.2 M sodium phosphate buffer (pH 7.0). Reaction mixtures were incubated at 30°C for 3 h and stopped by the addition of 7.5 ml of 20 mM sodium borate buffer (pH 8.5). The fluid was then filtered through

two stacked DE81 filter disks (Whatman) and washed with 100 ml of borate buffer. The washed filters were dried and counted in Econofluor fluid (New England Nuclear Corp., Boston, Mass.) by liquid scintillation. Purified cholera toxin diluted in sterile CYE broth was the standard. Under the conditions described above, cholera toxin concentrations ranging from 0.5 to 50 μg/ml gave levels of activity corresponding to initial rates of ADP ribosylation of [^{125}I]GT. Less than 10% of the available substrates were consumed after 3 h of incubation.

(iii) Cholera toxin determination. The S49 mouse lymphosarcoma cell assay (18) was used to measure the level of cholera toxin in culture supernatant fluids. Purified cholera toxin served as the standard.

Cell-associated toxin subunits. Strains were grown in CYE broth at 30°C with vigorous aeration until early stationary phase. Cells were collected by centrifugation, washed in CYE, and suspended in 0.1 volume of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. After 30 min at 4°C, the lysis debris was removed by centrifugation in a Brinkman microcentrifuge at 15,000 rpm for 5 min. The supernatant fluid (Triton extract) was then assayed for ADP-ribosylation activity by using the [^{125}I]GT assay and for toxin antigen by immunoradial diffusion. Alternatively, suspended cells were disrupted in a 0.1 volume of 1 M potassium phosphate buffer (pH 7.0) by sonication at 4°C for 2 min. Debris was removed by centrifugation, and the supernatant fluid (KP extract) was assayed for ADP-ribosylation activity by using the [^{125}I]GT assay and for toxin antigen by immunoradial diffusion.

RESULTS

Genetic analysis of phenotypic instability in Htx mutants. All confirmed Htx mutants of strain RV503 that were isolated after NTG mutagenesis and Rf selection were found to have a small-colony phenotype on CYE agar medium relative to the parental strain. In broth culture, many of these mutant strains have a filamentous morphology and rapidly settle out of stationary culture. Spread plates prepared from broth cultures of some Htx mutants revealed a high frequency of reversion to normal colony morphology. Subsequent analysis showed that these colony variants also reverted to either wild type or a hypotoxinogenic phenotype as well.

It was of interest to determine whether spontaneous revertants of Htx mutants were true genetic revertants or pseudorevertants due to the effect of a second-site mutation. It has been previously reported that the *htx-3* allele was linked to the *str*...*rif* region of the *V. cholerae* chromosome by 95 to 98% in transposon-facilitated recombination crosses (14). Accordingly, we scored for the loss of the *htx-3* allele in phenotypic revertants of RM71 by transferring the *str*...*rif* region of these strains to a suitable Tox$^+$ recipient strain (RV503).

Ten independent phenotypic revertants of RM71 were obtained after extended growth on CYE agar at 39°C. Spontaneously induced rifampin resistance was introduced into RM71 and each of these revertant clones (RM72 to RM81) as a marker for subsequent mating experiments, and all clones were made transposon-facilitated recombination donors by introduction of the plasmid pSJ13. These donor strains were then mated to RV503, and Sm^r Sp^r transconjugants were selected and scored for their resistance to Rf and for Tox phenotype.

Three classes of phenotypic revertants were recognized by this genetic analysis. The first class (represented by RM73 and RM75 to RM80) consisted of donor strains that retained the ability to transfer the Htx phenotype to the recipient strain. For example, among the RV503 transconjugates receiving both the RM73(pSJ13) donor's *str* and *rif* alleles, 96% also received the Htx phenotype (Table 2, Fig. 1). Therefore, these revertant strains are likely to carry a second-site mutation(s) that is capable of suppressing the Htx phenotype. Furthermore, transconjugates that received the *htx*$^-$ allele from RM73(pSJ13) also displayed the small-colony phenotype, suggesting that the *htx*$^-$ allele causes growth anomalies only when expressed in the form of hyperproduction of cholera toxin and not when suppressed by second-site mutation(s).

The second class of phenotypic revertants [represented by RM74(pSJ13)] was not found to transfer the Htx phenotype in conjugal crosses. RV503 transconjugates receiving the RM74(pSJ13) donor's *str* and *rif* loci displayed the same Tox phenotype as RM74, which was essentially identical to the RV503 recipient (Fig.

TABLE 2. *Mapping of htx and ltx loci by segregational analysis*[a]

Cross	Recombinant class	Relative frequency	Percent
RM73(pSJ13)	*rif*	2/166	1.2
× RV503	*htx*	2/166	1.2
	htx rif	48/166	28.9
RM101(pSJ13)	*rif*	3/176	1.7
× RV503	*htx*	2/176	1.1
	htx rif	49/176	27.8
RM102(pSJ13)	*rif*	6/336	1.8
× RV503	*ltx*	3/336	0.9
	ltx rif	92/336	27.4
RM103(pSJ13)	*rif*	1/144	0.7
× RV503	*ltx*	2/144	1.4
	ltx rif	41/144	28.5

[a] Each donor was mated separately to the recipient strain RV503. Sm^r was the selected donor marker, and the donor was counter-selected with spectinomycin. Recombinant clones were then scored for the other indicated donor markers.

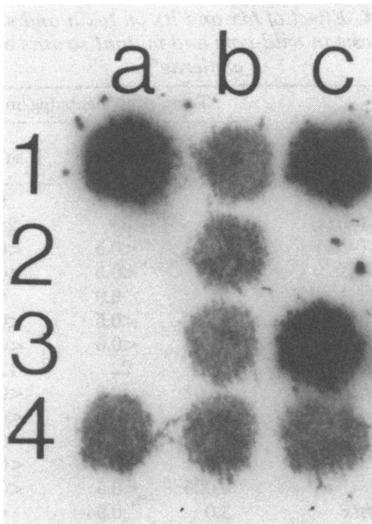

FIG. 1. Ganglioside filter assay autoradiograph. Donor strains RM71(pSJ13) and three phenotypic revertants, RM72(pSJ13), RM73(pSJ13), and RM74(pSJ13), were mated separately to tox⁺ recipient strain RV503. An Sm^r Sp^r transconjugate which had also received the donor rif^r allele was selected for each cross and scored for their tox phenotypes by the ganglioside filter assay. Position 1a, RM71(pSJ13); 2a, RM72(pSJ13); 3a, RM73(pSJ13); 4a, RM74(pSJ13); 1b–4b, RV503; 1c–4c, Sm^r Sp^r Rf^r transconjugants from crosses RM71(pSJ13) × RV503, RM72(pSJ13) × RV503, RM73(pSJ13) × RV503, and RM74(pSJ13) × RV503, respectively.

1). The transconjugates from this cross received the donor's *rif* allele linked to the selected *str* locus by about 30%, indicating that the *str*... *rif* region was transferred in this cross. A mutation (unlinked to the *str*... *rif* region) conferring pigment production, present in RM74 and all RM71 derivatives (14), was also scored in these crosses but was not present in the recombinant population, indicating that no donor clones had survived the counterselection with spectinomycin. Thus, the Sm^r Sp^r Rf^r clones represent true transconjugates, and we conclude that their Tox phenotype must indicate the loss of the *htx* mutation in RM74. Since the Tox phenotype displayed by RM74 was very close to the parental strain Tox phenotype, these data suggest that RM74 is probably a true genetic revertant of RM71.

The third class of phenotypic revertants (represented by RM72 and RM81) were found to transfer a hypotoxinogenic phenotype in place of Htx in these crosses (Fig. 1). The genetic linkage of these transferred hypotoxinogenic phenotypes to *rif* was identical to that of *htx*: ca. 97%. This third class of phenotypic revertant, therefore, may contain clones which harbor a mutation(s) whose characteristics are those of an intragenic suppressor (e.g., a new mutant allele of the *htx* locus).

Isolation of hypotoxinogenic mutants by NTG-induced comutation. We tested the possibility that hypotoxinogenic phenotypes could be mediated by mutations in a locus which was either closely linked or identical to the *htx* locus by employing NTG-induced *rif* comutation (14). *V. cholerae* RM100 was mutagenized, survivors were plated on Rf-CYE agar plates, and antibiotic-resistant clones were scored for Tox phenotype by the ganglioside filter assay. As was the case with *V. cholerae* RV503, approximately 10% of the RM100 resistant clones were found to be both Htx and of small-colony morphology. In contrast, clones producing very low levels of toxin by the ganglioside filter assay were rare, occurring at a frequency of less than 0.1%. Two independently isolated hypotoxinogenic (Ltx) clones (RM102 and RM103) were selected for genetic mapping studies and were converted to transposon-facilitated recombination donor strains by the introduction of the plasmid pSJ13. These two donor strains, and a hypertoxinogenic derivative [RM101(pSJ13)], were separately mated to RV503. Sm^r Sp^r transconjugants were selected and scored for Rf^r and Tox phenotype.

The Ltx donor strains and Htx donor strain transferred their respective Tox phenotypes with linkage frequencies to *str* and *rif* that were virtually identical (Table 2). These data suggest that a genetic site which we have called *ltx* maps in a position very close to the *htx* locus. The phenotypes of these two mutations are opposite: strains that carry mutations in *htx* are hyperproducers of toxin, whereas strains that carry mutations in *ltx* are hypoproducers of toxin. However, we cannot rule out the possibility that both *htx* and *ltx* mutations are different classes of mutations in the same genetic site.

Regulatory nature of mutations in the *htx* and *ltx* loci. Mekalanos et al. (10, 14) previously demonstrated the toxin-specific effects of mutations in the *htx* locus. Similarly, the effect of the *ltx* mutation appears to be specific for cholera toxin, since only two bands corresponding to the A and B subunits of the toxin were lost when concentrated culture supernatant fluids of RM102 and RM103 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Consistent with this observation, the level of protease (active against gelatin) produced by these *ltx* mutants was also not significantly different from the parental strain RM100.

The possibility that mutations in the *ltx* and *htx* loci affect secretion or release of the toxin from cells was also investigated. Table 3 shows

TABLE 3. *Determination of cell-associated A subunit*

Strain	ADP-ribosylation activity[a]	
	Triton extracts	KP extracts
RM100	3,162 (0.6)	ND (<0.5)
RM101	6,580 (1.2)	12,251 (2.9)
RM102	ND (<0.5)	ND (<0.5)

[a] Prepared as described in the text. Expressed as counts per minute of ADP-ribosyl-[^{125}I]GT formed, corrected for background incorporation of buffers. The number in parentheses gives the corresponding amount of toxin equivalents (micrograms) per milliliter in the extract. ND, Not detectable.

the level of A subunit present in cell extracts of the parental strain RM100 and its *htx* and *ltx* derivatives, RM101 and RM102, respectively. Significant levels of the A subunit were present in extracts of the hypertoxinogenic mutant strain RM101 and, to a lesser extent, the parental strain RM100. No detectable A subunit was present in extracts of RM102. In addition, no B subunit (less than 0.1 μg/ml) was detected in any of the extracts by immunoradial diffusion. Since RM100 and RM101 produced about 10 and 30 μg of toxin per ml, respectively, in their culture supernatants (Table 4), the level of A subunit that is cell associated is on the order of only 1% of the total produced.

Clearly, the effect of the *ltx-5* mutation is not related to a block of toxin release, whereas the *htx-4* mutation does not simply facilitate the release of toxin from cells. However, strains RM102 and RM103 were shown to produce small amounts of toxic activity in the S49 lymphosarcoma assay (18) corresponding to about 50 and 200 pg of toxin per ml in their culture supernatant fluids, respectively. Therefore, mutations in the *htx* and *ltx* loci appear to be regulatory in nature, affecting the level of production of both extracellular and cell-associated toxin subunits.

Effects of *htx* and *ltx* mutations on wild-type and mutant strains of *V. cholerae*. It was of interest to examine the effects of mutations in these two loci in strains of *V. cholerae* other than the exceptionally toxinogenic 569B strain. Table 4 shows that the introduction of *htx-4* and *ltx-5* mutations into *V. cholerae* 0395, NIH41, and RV79 by conjugation results in stimulation of toxin production and depression of toxin production, respectively. In the case of the *htx-4* mutation, a clear coordinate effect can be seen on A and B subunit production in these recombinants.

The introduction of the *htx-4* and *ltx-5* mutations into previously reported hypotoxinogenic mutants of *V. cholerae* 569B gave somewhat different results. As can be seen in Table 4, the hypotoxinogenic mutant RV506 showed nearly complete suppression of its hypotoxinogenic phenotype after acquisition of the *htx-4* locus, whereas toxin production of *V. cholerae* M13 was not stimulated by the *htx-4* locus. Baine et al. (2) have reported that the mutation causing the hypotoxinogenic phenotype of M13 is linked to the *his-1* locus on the *V. cholerae* chromosome. We have shown (unpublished data) that the hypotoxinogenic phenotype of RV506 also appears linked to the *his* locus. The differential results following the introduction of the *htx-4* mutation into M13 and RV506 suggest that the hypotoxinogenic locus located in the *his* region of the vibrio chromosome might have multiple allelic forms. Alternatively, there may be multiple loci in the *his* region which interact differently with *htx* gene product.

Nichols et al. (16) have reported the isolation

TABLE 4. *Effect of htx and ltx on toxin and subunit synthesis in wild-type and mutant strains of V. cholerae*[a]

Strain	Toxin equivalents (μg/ml)		
	Toxicity[b]	A subunit[c]	B subunit[d]
RM100	10	12	9.5
RM101 (-*htx*)	30	39	28
RM102 (-*ltx*)	<0.001	<0.5	<0.1
O395	0.04	<0.5	<0.1
O395-*htx*	3.3	6.9	6.6
O395-*ltx*	0.009	<0.5	<0.1
NIH41	0.014	<0.5	<0.1
NIH41-*htx*	10	22	12.5
NIH41-*ltx*	<0.001	<0.5	<0.1
RV79	0.12	<0.5	<0.1
RV79-*htx*	1.1	1.1	0.5
RV79-*ltx*	<0.001	<0.5	<0.1
RV506	0.025	<0.5	<0.1
RV506-*htx*	3.0	0.94	4.0
RV506-*ltx*	<0.001	<0.5	<0.1
M13	0.005	<0.5	<0.1
M13-*htx*	0.005	<0.5	<0.1
M13-*ltx*	<0.001	<0.5	<0.1
MN1	<0.001	<0.5	<0.1
MN1-*htx*	<0.001	0.55	<0.1
MN1-*ltx*	<0.001	<0.5	<0.1

[a] Sp^r derivatives of the listed strains were mated to either RM101 or RM102. Sp^r Sm^r transconjugates were selected and scored for the donor's *rif*^{r} allele. A transconjugate clone receiving both the donor's *str* and *rif* loci was selected for each of these crosses and scored for the effect of the indicated locus on production in liquid culture of toxin and the A and B subunits of the toxin.

[b] Determined in the S49 lymphosarcoma cell culture assay (18).

[c] Determined by the [^{125}I]GT assay (12).

[d] Determined by immunoradial diffusion (10).

of hypotoxinogenic variants of strain 569B after growth at elevated temperatures. One of these variants, MN1, was reported to produce greatly reduced levels of subunit B while producing only a fivefold lower level of subunit A. Table 4 shows the effect of the *htx-4* and *ltx-5* mutations on MN1. Although we detected no A subunit activity in MN1, we did detect significant activity in the *htx-4* derivative of this strain. (Our failure to detect enzymatically active A subunit in MN1 may be related to differences in the amount of "nicked," proteolytically processed A subunit produced by MN1 and the relative activity of this precursor form in the pigeon erythrocyte lysate assay [16] versus the [^{125}I]GT assay [12, 13; J. Mekalanos, J. Nichols, and J. Murphy, unpublished data]). No increase in either S49 cell toxicity or B subunit production was noted in this MN1 *htx-4* derivative. These data suggest that the noncoordinated expression of the A subunit in MN1 may remain sensitive to *htx*-mediated elevation.

DISCUSSION

The mechanism by which hypertoxinogenicity induces growth abnormalities in *V. cholerae* Htx mutants is not known but may be related to similar pleiotropic effects of amylase and protease hyperproduction mutations in *Bacillus subtilis* (20). The growth effects of *htx* mutations resulted in a strong selection pressure for phenotypic suppressor mutations (most Htx mutants display growth rates of about one-third those of parental and revertant strains).

The genetic analysis of spontaneously occurring revertants of *V. cholerae* Htx mutants has resulted in their separation into three different classes. The first class is composed of strains that produce reduced levels of cholera toxin, but have retained the ability to donate the hypertoxinogenic phenotype to recipient strains. The mutation(s) responsible for suppression of the Htx phenotype in these strains does not map in the *str...rif* region of the vibrio chromosome and has not as yet been mapped. The second class of phenotypic revertants contains strains which display parental Tox phenotypes and have the properties of true genetic revertants of the *htx-3* mutation. Together these first two classes probably account for about 80 to 90% of the spontaneous phenotypic reversion events observed in Htx mutants of *V. cholerae*.

The last class of Htx phenotypic revertants was composed of strains that had apparently replaced the *htx-3* mutation of RM71 with a new mutation conferring a hypotoxinogenic phenotype. We have been unable to rescue the *htx-3* mutation from this last class of revertants in genetic crosses in which we have scored several hundred *str*$^+$ recombinants. This result suggests that the latter class of Htx revertants represents strains with intragenic suppressor mutations in the *htx* locus. However, we cannot rule out the possibility that an extragenic suppressor mutation, very closely linked to *htx*, is responsible for these results.

Comutation with NTG allowed us to isolate a new class of hypotoxinogenic mutants possibly related to the class of intragenic suppressor mutations postulated above. The *ltx* locus was shown to have linkage properties which were virtually identical to those of the *htx* locus. We observed the mutation frequency of *htx* to be approximately 100-fold higher than *ltx* in an NTG-induced *rif*-comutagenized population. This compares to a similar low mutation frequency in the non-comutagenized population of 0.1% for mutants which display either the Htx or Ltx phenotype (11, 14). However, approximately 90% (seven out of eight tested) of the Ltx mutants isolated from *rif* comutagenized populations showed strong linkage of their Ltx phenotype to *str* and *rif*, whereas none (out of five tested) of the Ltx mutants obtained from non-comutagenized populations exhibited this property. Consistent with this latter result is the observation that most hypotoxinogenic mutants obtained from random NTG-mutagenized populations show linkage of their Tox phenotypes to the *his* region (2; J. Mekalanos, unpublished data), which is quite distant from the *str...rif* region on the *V. cholerae* map (17). Thus, *ltx* mutations are apparently rare mutations detectable only when enrichment techniques such as NTG comutation are employed.

Although the data do allow room for other interpretations, the assumption that *htx* and *ltx* mutations are different allelic states of the same locus enables us to propose certain characteristics of the *htx/ltx* site. Since the mutation frequency to *htx* is two orders of magnitude greater than to *ltx*, one can predict that the *htx*$^-$ allele represents a loss of function, whereas the *ltx*$^-$ allele represents an alteration in function of the gene product. Since the *htx* mutation results in elevation of toxin production, the *htx/ltx* gene product has the characteristics of a negative control element, or repressor. Accordingly, *ltx* mutations may be analogous to i^s mutations in the *lac* system (15) and result in an altered repressor molecule which is not inactivated by normal Tox inducers. Different growth conditions (8) as well as mutations in other Tox regulatory or structural genes may, of course, lead to alterations in *htx/ltx*-mediated regulation of cholera toxin production. However, the

observation that *htx* and *ltx* mutations were active in regulating toxin production in several strains of *V. cholerae* of diverse origin suggests that the *htx/ltx* gene product(s) does play a significant role in the regulation of cholera toxin production.

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