JOURNAL OF BACTERIOLOGY, Sept. 1979, p. 859-865
0021-9193/79/09-0859/07$02.00/0

Vol. 139, No. 3

# Genetic Mapping of Toxin Regulatory Mutations in *Vibrio cholerae*

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Received for publication 26 April 1979

We have mapped a regulatory site mediating the hyperproduction of cholera toxin in mutants of *Vibrio cholerae* strain 569B. Mutations in this locus, called *htx*, result in the hypertoxinogenic phenotype, as measured by the ganglioside filter assay and immunoradial diffusion. Transposon-facilitated recombination was used to construct improved genetic donors in 569B parental and hypertoxinogenic mutant strains. Subsequent mapping by conjugation indicated that the *htx* locus was closely linked to the *rif*, *str*, and *ilv* loci of *V. cholerae*. Analysis of recombinants from these crosses suggested the following gene order: *thy str htx rif ilv arg*. The close genetic linkage of *htx* to *rif* (as high as 98%) resulted in a high comutation frequency of these two loci by nitrosoguanidine mutagenesis. Transfer of the *htx* mutant locus from a hypertoxinogenic donor to several unrelated $Tox^+$ strains of *V. cholerae* caused a detectable elevation of toxin production in the recipients. These results suggest that toxin production in diverse strains of *V. cholerae* is controlled by a common regulatory mechanism in which the *htx* gene product plays a significant role.

Although impressive advances have been made in understanding the molecular structure and action of *Vibrio cholerae* enterotoxin in the past two decades (5; R. J. Collier and J. J. Mekalanos, *in* H. Biswanger and E. M. Shimke-Ott, ed., *Multifunctional Proteins*, in press), relatively little is known concerning the genetic factors involved in the production of this toxic protein. In contrast to diphtheria toxin, whose structural gene was shown to be carried by the temperate bacteriophage $\beta$ (18), the location of the gene(s) coding for the A and B subunits of cholera toxin is not known. The primary reason for this uncertainty is that well-characterized mutations in the structural gene for cholera toxin have not been available. It seems likely that these genes are located on the *V. cholerae* chromosome, because neither bacteriophages (7) nor transmissible plasmids (7, 11) appear to be required for toxin production by these bacteria. Clearly, further studies are necessary before the genetic site(s) of the toxin genes can be reliably determined.

Toxin production in vitro is affected by a number of genetic and biochemical factors including the composition of the media and growth conditions employed (e.g., pH, degree of aeration, and temperature; 4, 21). Under standardized conditions, widely varying amounts of toxin are produced by various toxinogenic *V. cholerae* strains, although the toxin they produce appears to be identical (5).

Mutants altered in the production of toxin (*tox* mutants) have been isolated in several laboratories, but most of these appear to be regulatory mutants that produce greatly reduced levels of toxin (6, 10, 16, 22). A *tox* regulatory site that mediates the hypoproduction of cholera toxin was reported by Vasil and co-workers (3, 23) to be weakly linked to the *his-1* locus of *V. cholerae*. Several presumably allelic mutations were also mapped in the same region of the chromosome, but the nature of these mutations was not determined. More recently, Mekalanos et al. (15, 16) isolated mutants that produced elevated levels of cholera toxin compared to the wild-type parent strain. These hypertoxinogenic (Htx) mutants appeared to produce equivalently elevated amounts of both the A and B chains of the toxin. This behavior was consistent with the apparent coordinate control of A and B subunit synthesis previously observed for various hypotoxinogenic mutants (6, 22). These observations suggested that hypertoxinogenic mutations, as well as all *tox* mutations (6, 10, 16, 22) thus far described, belong to the broad class of "regulatory" mutations which alter the levels of toxin produced rather than the toxin molecule per se.

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However, the mutant strain that was reported by Honda and Finkelstein (9) to produce B subunits without detectable A subunits may be an exception.

In the present investigation, procedures were developed for genetically analyzing properties of *V. cholerae* mutants that produced altered levels of toxin. For this purpose, we used the recently developed transposon-facilitated recombination (Tfr) system of Johnson and Romig (11) to construct similar Hfr-like donors for mapping the regulatory site that appears to mediate the hyperproduction of toxin in *V. cholerae* 569B. This locus, termed *htx*, maps close to the *rif* and *str* loci of *V. cholerae* and is therefore distinct from the other possible regulatory sites previously mapped near the *his* region of the map (3). The proximity of the *htx* locus to *rif* allowed us to employ *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced comutation (8) to greatly enrich for hypertoxinogenic mutants in a number of strains of *V. cholerae*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Strains of *V. cholerae* and plasmids are listed in Table 1. Cultures were stored in the lyophilized state or as frozen stocks at −70°C in brain heart infusion broth (Difco) containing 15% (vol/vol) glycerol.

**Media.** Meat extract agar (19) and Casamino Acids-yeast extract agar (16) were used as nutrient solid media. Brain heart infusion broth supplemented with 0.2% of yeast extract was used in mating experiments. Minimal medium A (17) supplemented with required amino acids at 100 μg/ml was used as the solid synthetic medium. For strain RJ1 and its derivatives, medium A was also supplemented with 50 μg of adenine per ml to satisfy an undefined purine requirement. Antibiotics were used at the following concentrations: 100 μg of streptomycin (Sm), 100 μg of spectinomycin (Sp), 30 μg of rifampin (Rif), 700 μg of ampicillin or penicillin G (Ap), and 15 μg of chloramphenicol (Cm) per ml.

**Mutant isolation.** Mutagenesis was performed by treating cells with NTG by the procedures of Adelberg et al. (1). NTG-induced *rif* comutation (8) was used as follows to isolate several auxototrophic strains and Htx mutants. Frozen stocks of outgrown mutagenized cells were plated on rifampin-containing Casamino Acids-yeast extract agar and incubated at 30°C for 20 h. Rif$^r$ colonies were scored for auxotrophic mutations by replica plating to minimal media or for *tox* mutations by the ganglioside filter assay (15). Other antibiotic-resistant mutants were obtained as spontaneous, one-step mutations.

**Construction of Tfr genetic donors.** The methods of Johnson and Romig (11) were used to construct strains containing chromosomally inserted copies of the ampicillin transposon Tn*1* in the *thy* locus of *V. cholerae*. The wild-type *tox* parental strain RV501 and its Htx mutant, RM7, were mated to RJ1(pSJ26), and transconjugants carrying pSJ26 were selected on ampicillin–meat extract agar plates. In this and subsequent matings, plasmid donors were counterselected with appropriate alternative antibiotics (see Table 1). Strains that contained extensively deleted, transfer-deficient derivatives of pSJ26 were isolated after in-

TABLE 1. *V. cholerae strains and plasmids*

| Strain | Genotype/phenotype | Other information |
|---|---|---|
| 569B | Prototroph, Tox$^+$ | Classical biotype; serotype, Inaba (5) |
| 569B derivatives | | |
| RV501 | *his-1 str* | (15) |
| RV502 | Prototroph, *spc* | (15) |
| RV503 | *arg-1 spc* | From RV502 by NTG[a] |
| RV504 | *arg-1 ilv-1 rif spc* | From RV503 by NTG-induced *rif* comutation |
| RV505 | *arg-1 trp-1 rif spc* | From RV503 by NTG-induced *rif* comutation |
| RM5 | *htx-2 spc* | Formerly Htx-2 (15,16) |
| RM7 | *his-1 htx-3 str* | Formerly Htx-3 (15, 16); RM7 and derivatives also produce a brown pigment |
| RM71 | *his-1 thy-1*::Tn*1 htx-3 str* Ap$^r$ | From RM7 by Tn*1* insertion into *thy* |
| RM100 | *his-1 thy-2*::Tn*1 str* Ap$^r$ | From RV501 by Tn*1* insertion into *thy* |
| RM101 | *his-1 thy-2*::Tn*1 htx-4 str rif* Ap$^r$ | From RM100 by NTG-induced *rif* comutation |
| Other strains | | |
| RV177 | *his-1 arg-1 ilv-1 met-2 trp-1 spc* | Derivative of classical strain 162 (19) |
| RJ1 | Prototroph, El Tor biotype | Synonymous with RV79 (11), nonlysogenic (7) |
| NIH41 | Prototroph, classical biotype | Lysogenic for VCA-1 and VCA-2 (7) |
| O395 | Prototroph, classical biotype, serotype Ogawa | (20) |
| Plasmids | | |
| P | *V. cholerae* conjugative plasmid | (12) |
| pSJ5-' | P::Tn*1* hybrid, Ap$^r$ | Tn*1* in (+) orientation (11) |
| pSJ13- | P::Tn*1* hybrid, Ap$^r$ | Tn*1* in (−) orientation (11) |
| pSJ25 | Thermosensitive P::Tn*9* hybrid, Cm$^r$ | (11) |
| pSJ26 | pSJ25::Tn*1*::Tn*9*, Cm$^r$, Ap$^r$, thermosensitive | (11) |
| pJM1 and JM2 | Transfer-deficient, deletion mutant of pSJ26, Cm$^s$, Ap$^r$ | Analogous to pSJ7 (11) |

[a] Designations correspond to those of Parker et al. (19) and Johnson and Romig (11) for *V. cholerae*.

cubation at 42°C as previously described (11). The plasmid deletion mutants pJM1 and pJM2 still carried the Tn*1* transposon and expressed P incompatibility functions. The thermosensitive plasmid pSJ25 was transferred from RJ1(pSJ25) into the resulting strains, RV501(pJM1) and RM7(pJM2), to eliminate the incompatible Tn*1* plasmid vector, and transconjugants were selected with both ampicillin and chloramphenicol at 30°C. The RV501(pSJ25) and RM7(pSJ25) derivatives thus obtained contained Tn*1* insertions in their chromosomes. These Tn*1*-containing strains were selected with trimethoprim (11, 17) to isolate *thy*::Tn*1* insertion mutants. The thermosensitive pSJ25 plasmids were eliminated from the *thy*::Tn*1* mutants by incubating them at 42°C. The resulting strains, RM101 and RM71, were converted into Tfr donors by separately transferring the pSJ5 and pSJ13 conjugative plasmids into them. The donor properties of these Tfr donors are described in the Results section.

**Quantitative mating procedures.** Conjugal transfer mediated by P::Tn*1* sex factors in Tn*1* chromosomal insertion strains (Tfr conjugation) was performed as previously described (11). Logarithmically growing donor and recipient cultures were mixed at a ratio of 1:10 in brain heart infusion broth and shaken at 100 rpm at 37°C for 150 min. Dilutions of the mating mixture were spread on appropriate synthetic or other selective media, and the donor strain was counterselected with an antibiotic (usually spectinomycin). Transfer frequencies are reported as recombinants per input donor. For linkage analyses, single donor alleles were selected on media supplemented with the other nutritional requirements of the donor and recipient. Recombinants were transferred to gridded areas on the same kind of selective medium and scored for unselected markers by replica plating.

**Toxin assays.** The ganglioside filter assay was routinely used to score the level of toxin produced by donor and recipient strains (15). Recombinants were inoculated onto ganglioside filters from gridded master plates by velvet-replica plating and processed with $^{125}$I-labeled anti-B subunit antibody as described. The use of Quanta-3 enhancing screens (Dupont, Houston, Tex.) and XR-5 film (Eastman, Rochester, N.Y.) reduced the necessary X-ray film exposure time to 2 to 3 h at −70°C. Toxin production in liquid cultures was quantified by immunoradial diffusion (15) using antiserum against the purified B subunits (14) of the toxin, with purified toxin (16) as the standard. The ganglioside filter assay and immunoradial diffusion specifically measure the B subunit of cholera toxin. The A subunit was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and enzymological methods (16).

## RESULTS

**NTG-induced comutation of Htx and Rif$^r$.** It was previously found that NTG-induced mutations to rifampin resistance were particularly useful for generating coinduced auxotrophic mutations (8, 11). Using the conditions described, approximately 5% of the Rif$^r$ mutants induced with NTG in *V. cholerae* RV502 also acquired new auxotrophic mutations (Mekalanos, unpublished data). The success of this procedure prompted us to investigate *rif* comutation as a means for enriching mutations affecting cholera toxin production. The ganglioside filter assay was used to screen NTG-induced Rif$^r$ mutants for their Tox phenotype (15).

A typical ganglioside filter assay of a population of NTG-induced Rif$^r$ colonies of RV502 is presented in Fig. 1. Approximately 10% of the Rif$^r$ clones displayed a markedly higher level of film exposure than the parental Rif$^s$ strain. Immunoradial diffusion assays were used to confirm that the Htx phenotype of these high-level mutations resulted from the increased production of B-subunit antigen. The Htx mutants tested by this procedure produced three- to fivefold more B-subunit antigen than the approximately 7 μg/ml produced by the RV502 parental culture. Analyses of cell-free culture fluids or of cell-associated A and B subunits released by sonic treatment from the Rif$^r$ hypertoxinogenic mutants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that they produced equivalently elevated amounts of both the A and B toxin chains. Similar results were found for the previously reported randomly isolated hypertoxinogenic mutants of 569B (15, 16). In addition, by our previously described enzymological and immunological criteria (16), the toxin purified from several representatives of both kinds of mutants was indistinguishable from the

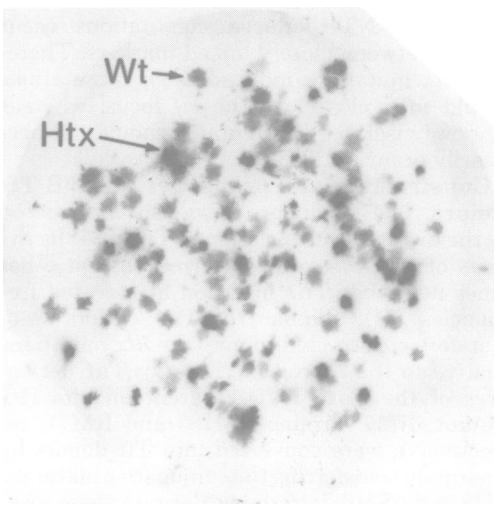


FIG. 1. *NTG-induced comutation of the hypertoxinogenic phenotype with rifampin resistance. Strain RV502 was mutagenized with NTG and selected for resistance to rifampin. Rif$^r$ clones were screened for coinduced toxin mutations by the ganglioside filter assay (15). Colonies displaying the hypertoxinogenic (Htx) and wild-type (Wt) phenotypes are indicated.*

wild-type toxin. These results suggested that the *htx* mutations coinduced with Rif$^r$ were regulatory and that they affected the expression of both chains of the toxin in a coordinate manner.

The frequency of hypertoxinogenic mutants (about 10%) obtained as NTG-induced Rif$^r$ comutations was about 100-fold higher than the frequency previously observed in unselected, NTG-induced populations (15). In contrast, the frequency of hypotoxinogenic mutants that produced at least 100-fold less toxin than the parental strain was about 0.05%, or slightly decreased among the Rif$^r$ population. Thus, Rif$^r$ comutation did not randomly increase the frequency of both classes of *tox* mutations.

Two previously isolated 569B Htx mutants, RM5 (15) and RM7 (16), were taken through the same NTG mutagenesis-rifampin selection protocol described above. However, no enrichment of mutants further increased in toxin production over the starting level was observed. Two possibilities for the lack of enrichment of these kinds of mutants were considered: (i) that additional mutations at another site (specifically, the *rif* comutable locus) could not further increase toxin production of existing Htx mutants; or (ii) that the mutations responsible for the phenotype of existing Htx mutants originally occurred in the *rif* comutable locus, and therefore these strains were not susceptible to further enrichment by this procedure. These possibilities were tested by mapping the *htx* mutations in both kinds of isolates. According to Guerola et al. (8), NTG-induced comutations occur mainly between closely linked markers. Therefore, *htx* mutations induced as *rif* comutations should map closely to the *rif* locus, whereas randomly isolated Htx mutants should not necessarily occur in this area.

**Construction and properties of 569B Tfr donors.** The Tfr donors previously constructed by the directed transposition of Tn*1* into the *thy* locus of *V. cholerae* transferred *rif* and other genes near that Tn*1* insertion at elevated frequencies (11). Similar *thy*::Tn*1* donors were therefore constructed to map the *htx* mutations relative to the *rif* region. The *thy*::Tn*1* derivatives of the Tox$^+$ RV501 parent and its Htx mutant, RM7 (strains RM100 and RM71, respectively), were converted into Tfr donors by separately transferring the conjugative plasmids, pSJ5 and pSJ13, into them. Because these plasmids contain Tn*1* inserted in opposite orientations, the ability of the resulting donors to transfer chromosomal markers in opposite directions was determined. For testing polarity, transfer frequencies were measured for the *ilv* and *trp* markers, which were previously mapped on opposite sides of the *thy* locus (11). To minimize potential strain differences that might affect toxin production, the recipients and donors were derived from strain 569B. Spectinomycin was used to counterselect donors because *spc* mutations mapped outside the genetic region considered (19).

Consistent with the polarized transfer found for previously characterized *thy*::Tn*1* donors, Tfr donor RM71(pSJ13) transferred *ilv* at elevated frequency, whereas the oppositely located *trp* marker was transferred at elevated frequency by RM71(pSJ5) (Table 2). Similar transfer frequencies for both markers were obtained with RM100(pSJ13) and RM100(pSJ5) (data not shown).

**Linkage of *htx* to *rif*, *str*, and *ilv-1*.** The hypertoxinogenic Tfr donor RM71(pSJ13) was mated to the Tox$^+$ recipient, strain RV504, and recombinants were selected for the donor *ilv*, *arg*, and *str* alleles. The use of the ganglioside filter assay for scoring the Htx phenotype is illustrated in Fig. 2. Only two levels of $^{125}$I binding were found among these recombinants, one corresponding to the Tox$^+$ recipient and the other to the Htx donor phenotype. Thus, the scoring of the *htx* allele in these crosses was usually unambiguous.

As shown in Table 3, cotransfer frequencies of the unselected *str*, *rif*, and *htx-3* markers with the donor *ilv* marker ranged from 14 to 20%. Linkage of *ilv-1* to *arg-1*, although detectable, was low. In contrast, when Arg$^+$ recombinants were selected (Table 3), the linkage of *ilv-1* to *arg-1* increased to 10%. These results indicated that *ilv-1* was the proximal of the two markers relative to the *thy*::Tn*1* origin and that *arg-1* was the distal gene. The *htx-3*, *str*, and *rif* markers were all linked at about 1 to 2% to *arg-1*, indicating that these loci were more closely linked to *ilv-1* than to *arg-1*. Among selected Arg$^+$ recombinants, *arg-1* was more closely linked to *ilv-1* than to *htx-3*, *str*, or *rif*, indicating that the latter genes were located between *ilv-1* and the *thy-1*::Tn*1* origin. The possible cotrans-

TABLE 2. *Transfer properties of thy-1::Tn1 donor strain RM71 containing oppositely oriented conjugative plasmids, pSJ5 and pSJ13*

| Recipient | Selected markers[a] | Recombination frequency[b] | |
|---|---|---|---|
| | | RM71(pSJ5) | RM71(pSJ13) |
| RV504 | *ilv*$^+$ | $4.0 \times 10^{-5}$ | $6.0 \times 10^{-5}$ |
| RV505 | *trp*$^+$ | $6.3 \times 10^{-5}$ | $6.5 \times 10^{-5}$ |

[a] Donors were counterselected with spectinomycin.
[b] Frequencies are presented as recombinants per input donor.

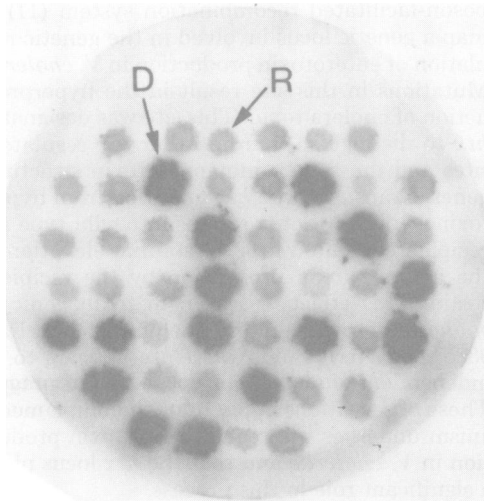


FIG. 2. *Scoring recombinants for the htx-3 locus by the ganglioside filter assay. Strain RM71(pSJ13) was mated to RV504, and randomly chosen Str$^r$ recombinants were scored for acquisition of the Htx phenotype. Representative clones displaying recipient (R) and donor (D) toxin phenotypes are indicated on the resultant autoradiographic image.*

TABLE 3. *Cotransfer of selected and unselected donor markers in the htx-rif region*[a]

| Selected donor marker | Marker tested[b] | | | | |
|---|---|---|---|---|---|
| | *ilv*$^+$ | *arg*$^+$ | *str*$^r$ | *rif*$^r$ | *htx-3* |
| *ilv*$^+$ | (336) | 5 | 63 | 48 | 64 |
| *arg*$^+$ | 30 | (288) | 3 | 4 | 7 |
| *str*$^r$ | 5 | 0 | (144) | 52 | 52 |

[a] Donor: RM71(pSJ13) *his-1 thy-1*::Tn*1 htx-3 str*. Recipient: RV504 *arg-1 ilv-1 rif spc*.

[b] Number of recombinants that received unselected donor markers; total numbers of recombinants that inherited the selected donor marker are in parentheses. Donors were counterselected with spectinomycin.

fer of a pigment mutation that originated in RM7 and persisted in RM71 was also scored in these crosses. The brown-pigmented phenotype was not transferred with any genes in the *htx* region of the chromosome, but this mutation was linked near the *trp-1* marker in strains RV505 and RV177 (data not shown).

The linkage of *str* to *htx-3* and *rif* decreased from the values of 63% and 52%, respectively, found in selected Ilv$^+$ recombinants to 36% in recombinants that were selected for Str$^r$ (compare Tables 3 and 4). This decreased linkage suggested that transfer of *htx-3* and *rif* to recombinants selected for Str$^r$ was limited by prezygotic exclusion and that *str* was the proximal gene in this cross. Segregational analyses of recombinants selected for Ilv$^+$ and Str$^r$ also showed that *htx-3* most often appeared in recombinants that received the *rif* and *str* donor alleles (Table 4). Conversely, recombinants that received the latter two donor markers without *htx-3* represented the least frequent recombinant class in either cross. Taken together, these results favor the gene order presented in Fig. 3B, which is analogous to the map order proposed for the El Tor strain, RJ1 (11; Fig. 3A).

The close genetic linkage of the randomly isolated *htx-3* mutation to the *rif* marker made it likely that *htx* mutations coinduced with Rif$^r$ by NTG mutagenesis also occurred in this genetic locus (8). This assumption was directly tested by mapping an *htx* mutation obtained by *rif* comutation. The Tox$^+$ *thy-2*::Tn*1* strain, RM100, was mutagenized with NTG, and Rif$^r$ mutants were scored for coinduced *htx* mutations. The resulting Htx mutant, strain RM101 (*htx-4*), was converted into a donor with the

TABLE 4. *Mapping the hypertoxinogenic locus htx-3 by segregational analysis*[a]

| Donor marker selected | Donor markers in recombinants | No. observed |
|---|---|---|
| *ilv*$^+$ | *ilv* | 244 |
| | *ilv str* | 21 |
| | *ilv rif* | 5 |
| | *ilv htx* | 12 |
| | *ilv rif str* | 2 |
| | *ilv rif htx* | 10 |
| | *ilv str htx* | 9 |
| | *ilv rif str htx* | 28 |
| *str*$^r$ | *str* | 91 |
| | *str rif* | 1 |
| | *str htx* | 1 |
| | *str htx rif*[b] | 51 |

[a] Individual recombinants from the cross described in Table 3 were analyzed.

[b] The five *ilv* recombinants detected were in this class.

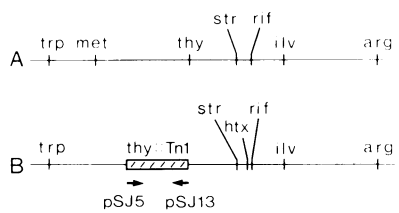


FIG. 3. *(A) Positions of genes in the rif ... str region of the V. cholerae RJ1 genetic map, as determined by Tfr conjugation (11). Scale is approximate. (B) Position of the htx-3 locus in strain RM71 as determined by Tfr conjugation. Arrows indicate the direction of chromosomal transfer from the thy::Tn1 insertion using the conjugative plasmids pSJ5 or pSJ13.*

conjugative plasmid pSJ13. Selected Str$^r$ recombinants, obtained by mating RM101(pSJ13) to RV503, were scored for the unselected *htx-4* and *rif* markers. Linkage of *htx-4* to *rif* was 97% (59/61), and linkage to *str* was 19% (61/324). These linkage values were closely similar to those obtained for the *htx-3* mutation (Table 3) and indicated that both mutations probably occurred in the same locus. The Tox$^+$ parental strain, RM100(pSJ13), was also mated to RV503 by the above procedures. None of the selected Str$^r$ recombinants exhibited the Htx phenotype, indicating that "cryptic" *htx* mutations were probably not present in RM100 before the *htx-4* mutation was induced. Thus, the class of *htx* mutations coinduced with rifampin resistance were similar in both Tox phenotype and genetic location to *htx* mutations obtained without this selection.

**Effect of *htx* on other *V. cholerae* strains.** The *htx* locus was transferred to other strains as an unselected marker with donor *str* and *rif* alleles. In these experiments, Tfr donor strain RM101(pSJ13) was separately mated to Sp$^r$ derivatives of the unrelated Tox$^+$ strains RV502, RJ1, 0385, and NIH41. Str$^r$ recombinants were selected, and those that also acquired the donor *rif* allele were subsequently scored for their Tox phenotype.

Increased toxin production by recombinants from all four strains of *V. cholerae* was detected by the ganglioside filter assay. In strain 0395, acquisition of the *htx-4* locus by one of the three recombinants tested resulted in a 32-fold increase in toxin production, as measured by immunoradial diffusion. However, more variation in the effect of the *htx-4* locus on the level of toxin production was observed in strains RJ1, 0395, and NIH41 than in strain RV502. This variability may indicate that other genetic parameters are involved in expression of the Htx phenotype in these less toxinogenic strains. Linkage aberrations between these unrelated strains and the RM101 Tfr donor might also explain the variation in Tox phenotypes of these recombinants. Quantitative effects of the acquisition of the *htx* mutation by various recombinants are currently being investigated (Mekalanos and R. J. Murphy, personal communication). We recently observed that NTG-induced *rif* comutation induces Htx mutations at high frequency in strains RJ1 and 0395. Thus, a locus having linkage and comutation properties similar to the *htx* locus appears effective in regulating toxin production in several strains of *V. cholerae*.

## DISCUSSION

We have used the recently developed transposon-facilitated recombination system (11) to map a genetic locus involved in the genetic regulation of enterotoxin production in *V. cholerae*. Mutations in this site result in the hyperproduction of cholera toxin. This site was designated *htx* to distinguish it from other *tox* regulatory sites and the as yet unmapped toxin structural genes. Transfer of the *htx* mutation from hypertoxinogenic Tfr donor strains into wild-type *tox* recipients resulted in a substantial elevation in the average toxin production by the recipient strain. This effect was seen in toxinogenic *V. cholerae* of diverse origin, including 569B, RJ1, 0395, and NIH41, and therefore appears to be independent of serotype, biotype, or phage type. These observations imply that a common mechanism underlies the regulation of toxin production in *V. cholerae* and that the *htx* locus plays a significant role in this process.

The nature of the *htx* gene product is still speculative. The possibility that the *htx* mutation causes an increased ability in the transport and release of toxin from inside the cell to the outside seems unlikely because almost threefold more cell-bound toxin was released by sonic treatment from *htx* mutants than from wild-type cells. The *htx* locus may code for a negative control element or repressor, whose inactivation by an *htx* mutation results in constitutive toxin production. This model also predicts another class of mutations in the *htx* locus analogous to the $i^-$ mutations in the *lac* system of *E. coli* (17). These mutations should result in hypo- or nontoxinogenic phenotypes due to improperly controlled repression of toxin synthesis. Several hypotoxinogenic mutants isolated by NTG induced *rif* comutation appear to map in the *str* . . . *rif* region, but their interactions with wild-type and hypertoxinogenic mutant alleles have not been established.

The high comutation frequency between the *rif* and *htx* loci after NTG mutagenesis is probably a consequence of the close genetic proximity of these two markers. Guerola et al. (8) observed comutation frequencies of about 3% between *azi* and *leu* in *E. coli* after NTG mutagenesis. Since these two loci map within 0.5 min of each other (2, 8), the 10% comutation frequency observed for *rif* and *htx* is consistent with their close linkage in *V. cholerae*.

It may be significant that the chromosomal site of the *htx* locus in *V. cholerae* is in a region that, in *E. coli*, is enriched for genes that code for protein synthetic machinery (2). Thus, *htx* may be a mutation that affects ribosomal functions related to toxin expression. Subinhibitory concentrations of the antibiotic lincomycin have been shown to hyperinduce toxin and periplasmic protein synthesis in *V. cholerae* (13;

Mekalanos, unpublished data). Accordingly, mutations in the *htx* locus and the lincomycin effect may be related.

Since all *htx* mutations thus far mapped appear to be tightly linked to both *rif* and *str*, the *htx* locus appears to be the major site of hypertoxinogenic mutations in *V. cholerae* 569B. Vasil and colleagues (3, 23) reported that the hypotoxinogenic *tox-1* locus in *V. cholerae* strain RV31 was weakly linked to the *his* locus of strain 569B. Because the *his* locus of *V. cholerae* is quite distant from the *str* . . . *rif* region (19), the *tox-1* locus mapped in their study is probably distinct from the *htx* locus we have described.

The ultimate practical goal of these studies is the development of a stably nontoxinogenic, live oral vaccine for cholera. The *htx* locus described here may be useful for characterizing the stability of potential nontoxinogenic vaccine candidates. Several hypotoxinogenic mutants isolated by ourselves (15) and others (6) have been shown to be unstable in vivo. The response of *tox* mutants to acquisition of an *htx* locus may facilitate classification of these mutants into various regulatory groups. It has been suggested (5) that the most effective live oral vaccine would be one producing immunologically cross-reacting material to the toxin, which was virtually free of toxic activity. Should such a mutant strain be developed, the *htx* locus might well be used to amplify the production of cross-reacting material and thereby improve the immunogenicity of such a vaccine strain.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-07877 and AI-08024 from the National Institute of Allergy and Infectious Diseases. J.J.M. was a genetics trainee under Public Health Service grant GM-07104 from the National Institute of General Medical Sciences, and was subsequently supported by funds from Public Health Service grant AI-07877 to R. John Collier from the National Institute of Allergy and Infectious Diseases.

We appreciate the continued interest and support of John Collier during the course of this work, and we thank him and Alison Weiss for helpful suggestions on the manuscript.

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