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Mapping of a Gene That Regulates Hemolysin Production in *Vibrio cholerae*

SUSAN VON MECHOW, AKHIL B. VAIDYA, AND MICHAEL G. BRAMUCCI*

Department of Microbiology and Immunology, Hahnemann University School of Medicine, Philadelphia, Pennsylvania 19102

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A gene that regulates the hemolysin structural gene (*hly*) was found to be tightly linked to the *tox-1000* locus of *Vibrio cholerae* RJ1 and separated from *hly* by a large section of the *V. cholerae* genetic map. This hemolysin regulatory gene was designated *hlyR*.

The amount of cholera toxin (CT) produced under a certain set of culture conditions can vary greatly from one wild-type strain of *Vibrio cholerae* to another (5, 11, 15). One of the loci that regulates the amount of CT synthesized by *V. cholerae* is *tox*. The *tox-1* locus was originally described in the classical strain 569B (1, 19). The genes *tox-1000* and *tox-2000* are probably alleles of the *tox-1* locus (15, 16). Wild-type El Tor strain RJ1 and recombinants with *tox-1000* are designated Tox$^-$ because these strains do not produce quantities of CT that can be detected in the Elek test or the radial passive immune hemolysis assay for CT, whereas wild-type El Tor strain 3083-2 and recombinants with *tox-2000* are toxinogenic in these tests. Genetic mapping experiments indicated that *tox-1000* is separated from the CT structural genes of strain RJ1 by at least one gene that is not related to CT synthesis (*trp*), implying that *tox-1000* transregulates the CT structural genes (14–16). Miller and Mekalanos (12) and Sporecke et al. (17) subsequently provided evidence supporting a similar conclusion for the CT regulatory and structural genes of strain 569B. Their data indicated that the 569B *toxR* gene (which is most likely the same as *tox-1*) is a positive regulator of CT synthesis and acts at the transcriptional level (12).

If *tox* transregulates CT synthesis, then *tox*-mediated regulation may not be limited to CT. Accordingly, we investigated the possibility that *tox* controlled the level of synthesis of other extracellular proteins. Although we have not found *tox*-mediated regulation of a protein other than CT, we provide evidence in the present report that a gene tightly linked to *tox-1000* transregulates expression of hemolysin in *V. cholerae*.

In assays for extracellular protease, DNase, and hemagglutinin (4, 7, 8), strains RJ1 and 3083-2 had similar phenotypes (data not shown). However, these strains were found to have significantly different hemolysin (Hly) phenotypes. The Hly and Tox phenotypes of parental strains, recombinants, and mutants are demonstrated on the modified radial passive immune hemolysis assay plate (2, 3) shown in Fig. 1. Strain MB1602, an auxotrophic mutant of strain 3083-2, was Hly$^+$, with a large hemolytic region surrounding the area of bacterial growth (Fig. 1A). Strain RJ1 was Hly$^-$, with little or no hemolysis surrounding the area of bacterial growth (Fig. 1A). When goat anti-CT and complement were added to the assay plate, a large region of specific immune hemolysis developed around the MB1602 stab, whereas no immune hemolysis developed around the RJ1 stab, indicating that in this assay these strains are Tox$^+$ and Tox$^-$, respectively (Fig. 1B).

Since the Hly phenotypes of strains RJ1 and 3083-2 were similar to the Tox phenotypes of these strains, we tested the possibility that *tox-1000* controlled both phenotypes in strain RJ1 by conjugally transferring the *tox-1000* allele into the 3083-2 recipient strain MB1602. The data in Table 1 indicate that a gene causing severe depression of hemolysin production by MB1602 cotransferred with *his* from the donor RJ1(pSJ13) into the recipient MB1602 at a frequency of ca. 70%. Although the MB1602 recombinants were sometimes slightly more hemolytic than strain RJ1, the phenotype of these recombinants was designated Hly$^-$ because the recombinants were significantly less hemolytic than strain MB1602. The Hly phenotype of a typical recombinant, MB4001, is demonstrated in Fig. 1.

All of the Hly$^-$ recombinants were also Tox$^-$ (Table 1). However, it is not likely that *tox-1000* was responsible for the Hly$^-$ phenotype of RJ1 and the Hly$^-$ recombinants. One recombinant, MB4003, was Hly$^+$ but Tox$^-$ in the radial passive immune hemolysis assay (Fig. 1). MB4003 was also negative in the Elek test for CT (data not shown). Although we previously reported data concerning the restriction nuclease digestion patterns of the DNA of Tox$^+$ recombinants derived from strains RJ1 and 3083-2 (14), we did not demonstrate the restriction nuclease pattern of any Tox$^-$ recombinant. Since RJ1 and 3083-2 are heterologous strains, it was possible that the Tox$^-$ recombinants were the result of a deletion or other genetic rearrangement caused by recombination between the two genomes. Indeed, we described a Tox$^+$ recombinant derived from strains RJ1 and 3083-2 in which some CT structural gene sequences were evidently deleted (14). Accordingly, we examined the restriction nuclease digestion patterns of several Tox$^-$ recombinants.

Plasmid pCVD002 (10), which contains cloned CT A-subunit and B-subunit genes, was ^{32}P nick translated and used in Southern hybridization analysis of *Pst*I-cleaved whole cellular DNA from strain RJ1, strain MB1602, and several Tox$^-$ recombinants as previously described (14), except that the hybridization was performed under more stringent conditions as follows. The prehybridization (4 h at 42°C) and hybridization (overnight at 42°C) incubations were performed in 50% formamide–0.05 M Tris–5× Denhardt solution–100 μg of calf thymus DNA per ml–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After the hybridization incubation, the blot was washed for three 10-min periods in 2× SSC with 1% sodium dodecyl sulfate at room temperature, for three 10-min periods in 0.1× SSC

* Corresponding author.

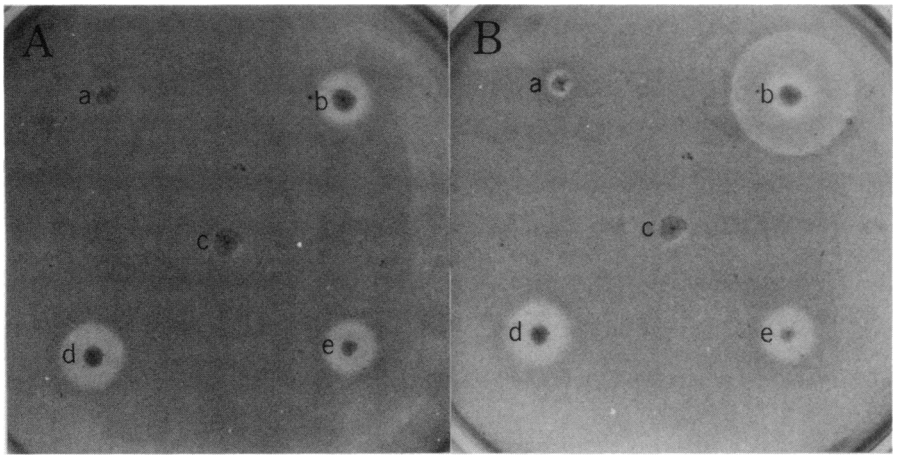

FIG. 1. Radial passive immune hemolysis assay blood agar plate demonstrating the Hly and Tox phenotypes of *V. cholerae* wild-type, recombinant, and mutant strains. The strains were: RJ1, an Hly$^-$ Tox$^-$ wild-type strain (a); MB1602, an Hly$^+$ Tox$^+$ auxotrophic mutant of wild-type strain 3083-2 (b); MB4001, an Hly$^-$ Tox$^-$ recombinant of RJ1(pSJ13) and MB1602 (c); MB4003, an Hly$^+$ Tox$^-$ recombinant of RJ1(pSJ13) and MB1602 (d); and MB4014, an Hly$^+$ Tox$^-$ mutant of the Hly$^-$ Tox$^-$ recombinant MB4004 (e). Each strain was stabbed into the blood agar, incubated overnight at 37°C, and examined for hemolysis (A) before the addition of anti-CT and complement (B) for determination of the Tox phenotype.

with 1% sodium dodecyl sulfate at 50°C, and one time for 5 min in 2× SSC at room temperature.

Strains MB1602, MB4001 (Tox$^-$ Hly$^-$), and MB4003 (Tox$^-$ Hly$^+$) all had a major nuclease restriction fragment that was ca. 8.0 kilobase pairs (kb) in size (Fig. 2), as previously reported for other derivatives of strain 3083-2 (14). In addition, the CT probe used in this study weakly hybridized with a nuclease restriction fragment ca. 11 kb in size that was not detected in the previous study. Two nuclease restriction fragments that were ca. 12 and 2.4 kb in size were detected in the recombinants but not in strains RJ1 or MB1602. Two observations suggest that it is most likely that the 12- and 2.4-kb nuclease restriction fragments were derived from pSJ13, the plasmid that was used to mobilize the donor chromosome. First, we previously reported that ^{32}P-nick-translated pBR322 DNA hybridizes with a 2.4-kb *Pst*I nuclease restriction fragment in RJ1(pSJ13) whole cellular DNA but not in RJ1 whole cellular DNA (14). We suggested that the β-lactamase gene of pBR322 hybridized to the β-lactamase gene of pSJ13. Second, the 12- and 2.4-kb nuclease restriction fragments were detected by the pCV002 CT probe (Fig. 2) and the pSG1012 hemolysin structural gene (*hly*) probe (Fig. 3) that is described below. Plasmids pCVD002 and pSG1012 are derived from closely related plasmids, i.e., pBR325 and pBR322, respectively (6, 10). The appearance of the 12- and 2.4-kb nuclease restriction fragments in the recombinants does not alter the fundamental conclusion derived from the data in Fig. 1, i.e., the Tox$^-$ phenotypes of the typical Hly$^-$ recombinant MB4001 and the Hly$^+$ recombinant MB4003 were not due to a detectable deletion of CT structural genes.

Plasmid pSG1012, which contains the cloned RJ1 *hly* gene (6), was ^{32}P nick translated and used in Southern hybridiza-

TABLE 1. Hly and Tox phenotypes of His$^+$ recombinants[a]

Phenotype of recombinants	No. of recombinants	% Of total
Hly$^-$ Tox$^-$	110	67.5
Hly$^-$ Tox$^+$	0	0.0
Hly$^+$ Tox$^+$	52	31.9
Hly$^+$ Tox$^-$	1	0.6

[a] The Hly$^-$ Tox$^-$ donor strain RJ1(pSJ13) was mated with the Hly$^+$ Tox$^+$ 3083-2 recipient strain MB1602 (*tox-2000 str-5000 his-5001 ilv-5001*). The bacterial strains, bacteriological media, routine culture conditions, and mating conditions have been previously described (15). His$^+$ recombinants were selected by plating the mating cultures on minimal medium supplemented with isoleucine and valine. Streptomycin was used to counterselect the donor strain. The Hly and Tox phenotypes of all of the resulting His$^+$ recombinants were determined as described in the legend of Fig. 1. The results of three independent experiments were combined for presentation.

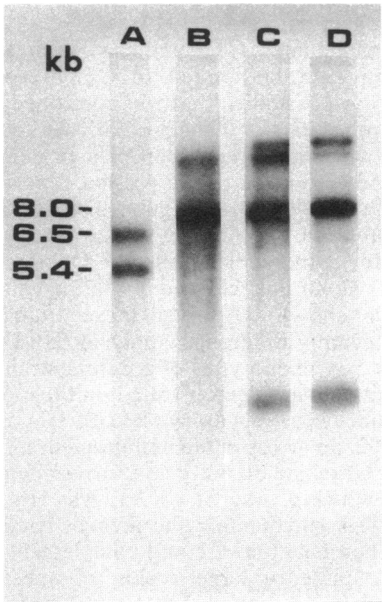

FIG. 2. Autoradiography showing hybridization of the *V. cholerae* CT probe from plasmid pCVD002 to *Pst*I restriction fragments of the whole cellular DNA of *V. cholerae* strains. Lanes A, RJ1; B, MB1602; C, MB4001; D, MB4003.

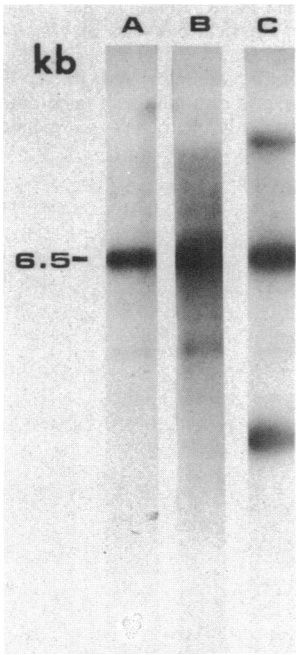

FIG. 3. Autoradiography showing hybridization of the *V. cholerae hly* probe from plasmid pSG1012 to *Pst*I restriction fragments of the whole cellular DNA of *V. cholerae* strains. Lanes: A, RJ1; B, MB1602; C, MB4001.

tion analysis of *Pst*I-cleaved whole cellular DNA from strain RJ1, strain MB1602, and the recombinant MB4001 under the conditions described for the CT probe. The *hly* probe hybridized with a single nuclease restriction fragment of ca. 6.5 kb in each of the tested strains (Fig. 3). In addition, the *hly* probe hybridized with nuclease restriction fragments of 12 and 2.4 kb in the whole cellular DNA of the recombinant MB4001. As already suggested, the 12- and 2.4-kb nuclease restriction fragments were most likely derived from pSJ13. The data obtained with the *hly* probe suggest that the Hly^- phenotype of the recombinants was not the result of a detectable deletion of the hemolysin structural gene.

The recombinant MB4004 had a Hly^- Tox^- phenotype (data not shown). We attempted to mutate the recombinant phenotype of MB4004 to the wild-type phenotype of MB1602 by mutagenesis with ethyl methane sulfonate (9). A log-phase brain heart infusion broth (Difco Laboratories) culture of MB4004 was treated with ethyl methane sulfonate by washing the cells one time with 1× minimal salts solution (3) and incubating the cells for 15 min with 0.4 M ethyl methane sulfonate in 1× minimal salts solution at 37°C. Survivors of the ethyl methane sulfonate treatment were washed with brain heart infusion, grown overnight in brain heart infusion, and plated on blood agar. Several Hly^+ mutants were independently isolated. However, only one mutant, MB4014, had a stable Hly^+ phenotype. MB4014 was found to be Tox^- in the radial passive immune hemolysis assay (Fig. 1) and the Elek test for CT (data not shown). Hybridization of *Pst*I-cut MB4014 whole cellular DNA with the CT gene probe and the *hly* gene probe indicated that the *Pst*I restriction fragments that carried the CT structural genes and the hemolysin structural gene had not been detectably altered by the mutagenic treatment (data not shown).

The data presented in this paper are consistent with several conclusions. First, a gene that controls the Hly phenotype is located in the *his-tox* region of the RJ1 chromosome. Second, since the Hly^- and Tox^- phenotypes are apparently separable by recombination and mutatgenesis, it is not likely that the gene controlling the Hly phenotype is *tox-1000*. We have designated the gene that is linked to *tox-1000* and determines the Hly phenotype of RJ1 as *hlyR1*, and the 3083-2 allele of *hlyR1* as *hlyR2*. Finally, since *hlyR1* is tightly linked to *tox-1000* (Table 1) and the hemolysin structural gene *hlyA* is located between *ilv* and *arg* (6), the *arg-his* region of the *V. cholerae* genetic map (13, 18) most likely separates *hlyR1* from *hlyA*. This observation suggests that expression of *hlyA* is transregulated by *hlyR1*.

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