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# Cloning and Characterization of the Hemolysin Determinants from *Vibrio cholerae* RV79(Hly$^+$), RV79(Hly$^-$), and 569B

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**The Hly region from the chromosome of *Vibrio cholerae* El Tor strain RV79(Hly$^-$) and the nonhemolytic classical strain 569B were cloned into plasmid vector pBR322. *Escherichia coli* K-12 transformants possessing these recombinant plasmids were nonhemolytic and were detected with a $^{32}$P-labeled *hly*-specific DNA probe. Restriction endonuclease *Sau*3AI digestions of the cloned *hly* loci of two independently obtained RV79(Hly$^+$) convertants, when compared with the digests of cloned RV79(Hly$^-$) loci, revealed that an apparent alteration (10 to 15 base pairs) had occurred. In contrast, an apparent 20-base-pair deletion was present in the cloned *hly* locus of the classical biotype *V. cholerae* strain 569B. Maxicell analysis and immunoprecipitation of labeled proteins of *E. coli* which are encoded by the cloned *hly* loci of RV79(Hly$^+$) and from nuclease BAL 31-deleted plasmids, as well as immunoprecipitation of [$^{35}$S]methionine-labeled *V. cholerae* proteins, suggest that the hemolysin is an 84,000-dalton polypeptide.**

*Vibrio cholerae* strains of the El Tor biotype are capable of synthesizing and secreting a hemolysin into the culture medium, whereas classical biotype isolates are invariably nonhemolytic. However, El Tor isolates which secrete little or none of this factor have been repeatedly isolated (6, 8). In addition, individual El Tor isolates are capable of hemolytic phenotype variation. In our laboratory, strain RV79(Hly$^-$) was found to convert to the hemolytic phenotype at moderate frequencies under certain conditions (9).

To study the genetics and regulation of the *V. cholerae* hemolysin gene(s) and to examine the variability of the Hly phenotype, we recently cloned the *hly* structural gene from an Hly$^+$ convertant of RV79 (9). The hemolysin was expressed but was not secreted from *Escherichia coli* transformants that carry recombinant plasmids with *hly* sequences. Southern blot hybridizations of Hly$^+$ and Hly$^-$ El Tor and Hly$^-$ classical *V. cholerae* isolates revealed a common hybridizing fragment in all of the strains examined. These results failed to correlate the variability of the Hly phenotype with major differences in either the DNA content or structure of the *hly* region of these strains (9).

In this study we have extended our analysis of the Hly phenotype of *V. cholerae* by examining the DNA sequences of cloned *hly* loci from RV79(Hly$^+$), RV79(Hly$^-$), and classical strain 569B(Hly$^-$). *E. coli* K-12 transformants that carried the *hly* chromosomal region from these strains were detected by colony blot hybridization with a $^{32}$P-labeled *hly*-specific DNA probe (9). We performed maxicell analysis and immunoprecipitation studies with [$^{35}$S]methionine-labeled proteins expressed by *E. coli* transformants which carry the *hly* locus, as well as by transformants which contain nuclease BAL 31-deleted *hly* genes. We also immunoprecipitated $^{35}$S-labeled proteins expressed by an Hly$^+$ *V. cholerae* strain and an isogenic Hly$^-$ mutant (*hly*::VcA-3) isolate. The results of these experiments demonstrate that hemolytic activity is associated with the expression of an 84,000-dalton protein in both *E. coli* and *V. cholerae*. In addition, the results suggest that specific DNA alterations observed in the cloned RV79(Hly$^-$) and 569B determinants afftect the synthesis of this hemolysin-related polypeptide and may be responsible for the Hly$^-$ phenotypes of these strains.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *V. cholerae* strains were supplied by John P. Craig, Downstate Medical Center, Brooklyn, N.Y. and W. R. Romig, University of California, Los Angeles. *E. coli* strain SY327 was supplied by John Mekalanos, Harvard Medical School, Boston.

**Media and antibiotics.** LB medium contained 10 g of Tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 5 g of NaCl per liter. TYCC medium consisted of 10 g of Tryptone, 1 g of yeast extract, 8 g of NaCl, and 0.5 g of $CaCl_2$ per liter. For LB and TYCC agar plates, 1.7% (wt/vol) Bacto-Agar (Difco) was included. Brain heart infusion medium was purchased from Difco. For blood agar plates, 5% (vol/vol) preparation of defibrinated, washed, and packed sheep erythrocytes (Northeast Laboratories, Waterville, Maine) was added to tryptic soy agar medium (Difco). Minimal M63 medium contained 3 g of $KH_2PO_4$, 7 g of $K_2PO_4$, 2 g of $(NH_4)_2SO_4$, 25 mg of $FeSO_4$, 0.2 g of $MgSO_4$, and 20 ml of a sterile 20% (wt/vol) glucose solution per liter. M63 medium supplemented with 1% Casamino Acids (Difco) and thiamine (0.1 mg/ml) was used for maxicell experiments. Starvation medium contained M63 medium with all amino acids except methionine. AG medium (5) was made in 0.05 M potassium phosphate buffer (pH 7.5) and contained 0.25% NaCl, 0.25% asparagine, 0.25% glucose, and 0.1% (vol/vol) trace metal solution (5% $MgSO_4$, 0.5% $MnSO_4$, and 0.5% $FeCl_3$ in 0.4% nitrilotriacetic acid).

Antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. and were used at the following concentrations: tetracycline 15 μg/ml; kanamycin 50 μg/ml; ampicillin 50 μg/ml; and chloramphenicol, 30 μg/ml.

**Preparation of DNA.** Chromosomal DNA was prepared according to the procedure of Brenner et al. (4). Plasmid DNAs were prepared by the alkaline lysis method and purified on cesium chloride–ethidium bromide gradients as

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previously described (14). DNA restriction fragments were purified from low-gelling-temperature agarose (type VII; Sigma) as previously described (14).

**Restriction enzymes and agarose gel electrophoresis.** DNA was digested with restriction endonucleases as recommended by the manufacturer (New England Biolabs, Inc., Beverly, Mass.). Restriction fragments were electrophoresed in 0.8% horizontal agarose gels for 18 h at 30 V in TBE buffer (89 mM boric acid, 89 mM Trizma base, 2.5 mM disodium EDTA [pH 7.0]). Agarose gels and running buffer contained 0.5 μg of ethidium bromide (Sigma) per ml. Gels were photographed on a UV light transilluminator (Ultraviolet Products, Inc., San Gabriel, Calif.), with Polaroid type 667 film with red and yellow filters.

**Molecular cloning.** Recombinant plasmids were constructed by using pBR322 as the vector. Competent *E. coli* K-12 SY327 cells were transformed according to the method of Mandel and Higa (13). *E. coli* transformants containing putative hemolysin-expressing plasmids were scored on blood agar plates containing an appropriate selective antibiotic after 48 h of incubation at 37°C.

**Colony blot hybridization.** The procedure of Grunstein and Hogness (10) was used to identify *E. coli* colonies possessing plasmids containing DNA sequences homologous to the *hly* region. The *hly*-specific *Eco*RI-*Nru*I fragment of pSG1012 (9) was isolated from low-gelling-temperature agarose and then nick translated as described previously (14). [$^{32}$P]dCTP (>3,000 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Nitrocellulose paper (type BA85) was purchased from Schleicher and Schuell, Keene, N.H. Hybridizations with $^{32}$P-labeled DNA were performed as described by Moseley and Falkow (16). Filters were autoradiographed on Kodak XR-1 film with a DuPont Cronex intensifying screen at −70°C for 18 h.

**Hemolysin assays.** To detect cell-associated hemolysin in *E. coli*, bacteria were first grown overnight in maxicell medium at 37°C. The cells were pelleted by centrifugation for 5 min at 8,000 rpm in a Beckman JA-20 rotor. The supernatant fluid was saved, and the cells were washed twice with equal volumes of 10 mM phosphate-buffered saline (pH 7.0). After resuspension in 1/10 volume of phosphate-buffered saline, the bacteria were disrupted with a Branson model 200 Sonifier (Branson Sonic Power Co., Danbury, Conn.). Debris was removed by centrifugation in a microcentrifuge (model 254A; Fisher Scientific Co., Medford, Mass.) for 5 min at 4°C. Samples of the supernatant fluid and sonicate (30 μl) were placed into 2-mm-diameter circular wells which had been punched into a blood agar plate containing chloramphenicol (to prevent bacterial contamination). The plates were incubated for 18 h at 37°C before being examined for the presence of hemolytic activity.

**Polyacrylamide gel electrophoresis.** DNA restriction fragments were analyzed on vertical 8% polyacrylamide gels made in TBE buffer. Gels were electrophoresed in TBE buffer at 200 V for 2.5 h, stained with ethidium bromide (1 μg/ml) for 45 min, rinsed in water, and photographed under UV light.

Protein samples were electrophoresed in 1-mm 12.5% polyacrylamide–0.1% sodium dodecyl sulfate (SDS) gels as described by Laemmli (12) for 12 h at 40 V. Gels were fixed in 30% trichloroacetic acid–10% methanol–10% acetic acid for 1 h followed by two changes of 10% acetic acid for an additional 1 h. After being rinsed in water for 15 min, gels were soaked in Autofluor (National Diagnostics, Somerville, N.J.) for 1 h. Gels were then placed onto Autofluor-saturated Whatman 3MM filter paper and dried for 1.5 h under vacuum at 79°C on a Hoeffer model SE540 slab gel drier (Hoeffer Instruments, Palo Alto, Calif.). Autoradiographs were exposed as described above.

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant biotype, phenotype, or genotype | Reference or source |
|---|---|---|
| *V. cholerae*[a] | | |
| RV79(Hly$^-$) | Hly$^-$ | 17 |
| RV79(Hly$^+$) | Hly$^+$ | This study |
| HK-1 | Hly$^+$ | J. Craig |
| SG23 | HK-1, *hly*::VcA-3 | This study |
| 569B | Classical, Hly$^-$ | 17 |
| *E. coli* | | |
| SY327 | F$^-$ *araD* Δ(*lac-pro*) *argE*(Am)Rif$^r$ *nalA recA56* | J. Mekalanos |
| Plasmid | | |
| pBR322 | Ap$^r$ Tc$^r$ | 3 |
| pSG1012 | Hly$^+$ Ap$^r$ | 9 |
| pSG1015 | Hly$^+$ Tc$^r$ | This study |
| pSG1015-2 | Hly$^+$ Tc$^r$ | This study |
| pSG201 | Hly$^-$ Tc$^r$ (derived from *V. cholerae* RV79 [Hly$^-$]) | This study |
| pSG202 | Hly$^-$ Tc$^r$ (derived from *V. cholerae* 569B) | This study |
| pSG401 | BAL 31 deletion; Hly$^+$ Km$^r$ | This study |
| pSG402 | BAL 31 deletion; Hly$^-$ Km$^r$ | 9 |
| pSG403 | BAL 31 deletion; Hly$^+$ Km$^r$ | This study |
| pSG404 | BAL 31 deletion; Hly$^-$ Km$^r$ | 9 |

[a] All *V. cholerae* strains are El Tor biotype except where indicated.

**Maxicell analysis.** Maxicell analysis was based on the procedure of Sancar et al. (18). The final cell pellet was suspended in 50 μl of SDS loading buffer (0.0625 M Tris-HCl [pH 8.0], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromphenol blue) and incubated in a boiling water bath for 5 min. Cell debris was removed by centrifugation for 2.5 min. Up to 20 μl of the samples was applied to an SDS-polyacrylamide gel for analysis.

**Immunoprecipitation. (i) Labeling.** *E. coli* cells were grown in maxicell medium to an optical density at 600 nm of 0.7 ± 0.1. A 0.5-ml sample was washed two times with M63 salts, suspended in starvation medium, and incubated at 37°C for 1 h. [$^{35}$S]methionine was added (40 μCi/ml), and the culture was grown for 1 h at 37°C. The cells were pelleted in a microfuge, washed twice with 10 mM Tris-HCl (pH 8.0), and suspended in 50 μl of 1% SDS–1 mM EDTA–50 mM Tris-HCl (pH 8.0). The samples were heated in a boiling water bath for 2 min. *Vibrio cholerae* proteins were labeled by using a modification of a procedure by Fairweather et al. (7). Bacteria were grown in 100 ml of brain heart infusion medium to an optical density at 590 nm of 1.4. A 20-ml sample was removed and centrifuged in a Beckman JA-20 rotor at 8,000 rpm for 5 min at 20°C. The pellet was washed once with AG medium and resuspended in 5 ml of the same medium. [$^{35}$S]methionine was added (150 μCi), and the culture was incubated at 37°C for 1 h. The bacteria were again pelleted, and a 1-ml portion of the supernatant was removed for immunoprecipitation. The pellet was washed twice with phosphate-buffered saline, suspended in 1 ml of lysozyme buffer (25% [wt/vol] sucrose; 30 mM Tris-HCl [pH 8.0]; 50 mM EDTA; 50 mM NaCl; lysozyme [Sigma], 1 mg/ml) and incubated at 37°C for 1 h. The cells were

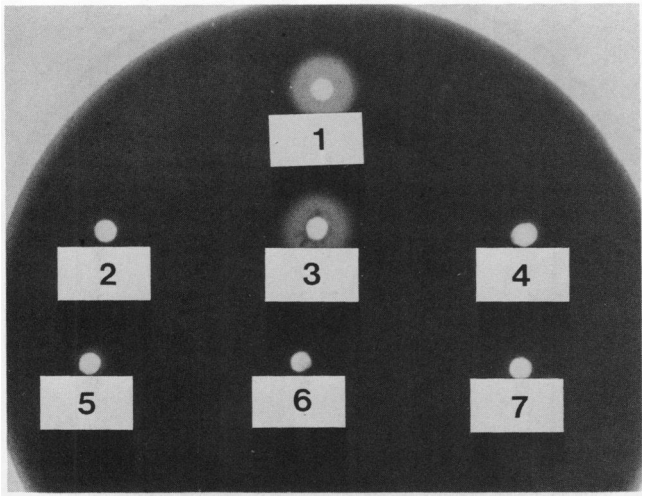



FIG. 1. Test for hemolytic activity in sonicated extracts of *E. coli* transformants carrying Hly$^+$ and Hly$^-$ recombinant plasmids. Wells: 1, supernatant from *V. cholerae* HK-1(Hly$^+$); 2, supernatant from *V. cholerae* SG23(*hly*::VcA-3); 3, sonicate from SY327(pSG1015); 4, sonicate fro SY327(pSG201); 5, sonicate from SY327(pSG202); 6, sonicate from SY327; 7, phosphate-buffered saline solution.

disrupted by sonication, and the debris was removed by centrifugation in a microfuge for 5 min at 4°C. The supernatant fluid was retained for immunoprecipitation.

**(ii) Antibody precipitation.** All steps of the antibody precipitation procedure were performed at 0 to 4°C. A 20- or 100-μl sample of labeled *E. coli* or *V. cholerae* protein, respectively, was added to 0.65 ml of 50 mM Tris-HCl (pH 8.0)–1 M NaCl–0.1 mM EDTA–2% Triton X-100 (the precipitation solution). The mixture was vortexed gently, and the cell debris was removed by centrifugation for 5 min in a microfuge. The supernatant was added to a 5-μl sample of *V. cholerae* antihemolysin serum (a gift from Richard Finkelstein, University of Missouri School of Medicine, Columbia) which had been preadsorbed with 50 μl of a sonicated extract of *E. coli* SY327(pBR322) or *V. cholerae* SG23 for 24 h at 4°C. After overnight incubation, 50 μl of IgGsorb (The Enzyme Center, Boston, Mass.) that had been washed three times in the precipitation solution was added and mixed occasionally for 1 h. The cells were precipitated and washed once with the precipitation solution, once with altered precipitation solution (0.5 M LiCl replaced the 1 M NaCl), and twice with 10 mM Tris-HCl (pH 8.0). The final cell pellet was treated as described above for the maxicell analysis.

## RESULTS

**Molecular cloning of Hly$^-$ determinants from *V. cholerae* strains RV79(Hly$^-$) and 569B.** To examine in more detail the DNA relationships between RV79(Hly$^+$), RV79(Hly$^-$), and 569B(Hly$^-$), chromosomal regions from the two Hly$^-$ strains homologous to the *hly* locus of RV79(Hly$^+$) were cloned in plasmid vector pBR322. Chromosomal DNAs from RV79(Hly$^-$) and 569B(Hly$^-$) were digested with restriction endonucleases *Eco*RI plus *Pst*I and electrophoresed on an 0.8% low-gelling-temperature agarose gel. The electrophoretic mobilities of chromosomal DNA restriction fragments containing the *hly* locus from these strains were previously shown to be indistinguishable by Southern blot hybridization analysis (9). We therefore isolated DNA from the region corresponding to the size of the RV79(Hly$^+$) *Eco*RI-*Pst*I fragment containing the *hly* locus (3.4 kilobases [kb]). This DNA was purified and ligated into the large *Eco*RI-*Pst*I fragment of pBR322 which had also been isolated from a low-gelling-temperature agarose gel. As a result of the vectorial cloning strategy, Tc$^r$ colonies which appeared after transformation possessed *V. cholerae* DNA in pBR322. Single colonies were processed by colony blot hybridization and probed with the $^{32}$P-labeled, *hly*-specific *Eco*RI-*Nru*I fragment of pSG1012 (9).

Of ca. 800 colonies examined in each cloning experiment, two transformants that carried DNA inserts from RV79(Hly$^-$) and one containing an insert from 569B were found to possess DNA homologous to the *hly*-specific probe. DNA restriction endonuclease digestion patterns of plasmids from these transformants were compared to that of the cloned Hly determinant of RV79(Hly$^+$). Since digestion patterns resulting from restriction endonuclease cleavage with *Hin*cII and *Bst*NI were identical, we concluded that the desired chromosomal region from RV79(Hly$^-$) and 569B had been cloned. Transformants which were grown for 3 days at 37°C on blood agar plates containing tetracycline were nonhemolytic. As a positive control, the *Eco*RI-*Pst*I fragment of pSG1012 was recloned into the *amp* locus of pBR322. *E. coli* containing this plasmid (pSG1015) were clearly hemolytic after 3 days of growth on blood-tetracycline agar medium. We also tested sonicated extracts of the transformants for hemolytic activity; only extracts from bacteria containing pSG1015 were found to be hemolytic (Fig. 1).

By using the 4-bp (base pairs) recognition site restriction enzymes *Hpa*II, *Sau*3AI, *Rsa*I, *Hae*III, *Taq*I, and *Fnu*DII, a further digestion analysis of the cloned DNA sequences was performed. The *Sau*3AI and *Taq*I restriction profiles of the cloned *hly* regions from RV79(Hly$^+$) (pSG1015), RV79(Hly$^-$) (pSG201), and 569B (pSG202) are shown in Fig. 2. In both digests of pSG202, a DNA fragment which is common to both pSG1015 and pSG201 was reduced in size by ca. 20 bp (lanes 2, 3, 5, and 6). In contrast, there was an increase of ca. 10 to 15 bp in the electrophoretic mobility of a single *Sau*3AI fragment from pSG1015 compared with digests of pSG201 (lanes 4 and 5). Interestingly, we have confirmed that an identical change in molecular weight had occurred in cloned DNA obtained from a second, independently isolated RV79(Hly$^+$) convertant (pSG1015-2; data not shown). By isolating specific restriction fragments within the Hly determinant and repeating the *Sau*3AI digestions, we found that the apparent deletion in pSG202 was located on the right side of the *hly* gene (in pSG1012), whereas the small increase in pSG1015 was within the left 1.0-kb of the 2.3-kb *hly* locus (data not shown).

**Maxicell analysis.** Proteins encoded by recombinant plasmids with *V. cholerae* inserts were radioactively labeled with [$^{35}$S]methionine by the maxicell procedure of Sancar et al. (18). The samples were electrophoresed on SDS-polyacrylamide gels and autoradiographed. Proteins expressed from the vector, pBR322, are shown in Fig. 3 (lane 1). The two prominent protein bands with electrophoretic mobilities corresponding to 38,000 and 28,000 daltons are the tetracycline-resistant protein and β-lactamase, respectively (18). Since plasmids pSG1015, pSG201, and pSG202 contain cloned DNA within the *amp* locus, β-lactamase was absent and a unique protein (ca. 84,000 daltons) appeared among the proteins expressed from the Hly$^+$ plasmid, pSG1015 (lane 2). A band of identical electrophoretic mobility appeared among proteins encoded by the cloned *hly* locus carried by pSG1015-2 (lane 3). A novel polypeptide (ca.

64,000 daltons) was encoded by pSG201, whereas no new proteins were produced by cells containing pSG202 (lanes 4 and 5, respectively).

$^{35}$S-labeled proteins from strains carrying nuclease BAL 31-deleted plasmids pSG402 or pSG404 were also analyzed by the maxicell method. Transformants that carry these plasmids were phenotypically nonhemolytic. pSG402 and pSG404 had deletions extending ca. 300 and 100 bp into the left and right ends of the pSG1012 *hly* gene, respectively. As shown in Fig. 3 (lane 6), a novel protein of 72,000 daltons was expressed from pSG402. In marked contrast, a hemolysin-related protein could not be detected in extracts from SY327(pSG404) (lane 7). Thus, those plasmids with deletions at the right end of the *hly* gene (pSG202 and pSG404) apparently fail to express a hemolysin-related protein; deletions on the left end of the locus result in a proportional loss in the size of the hemolysin protein.

**Immunoprecipitation of *E. coli* extracts.** Antihemolysin serum was used to immunoprecipitate labeled proteins from the same strains used in the maxicell analysis. Figure 4 shows that an 84,000-dalton polypeptide is expressed by transformants which carry either pSG1015 or pSG1015-2 (lanes 2 and 3). These results support the hypothesis that the

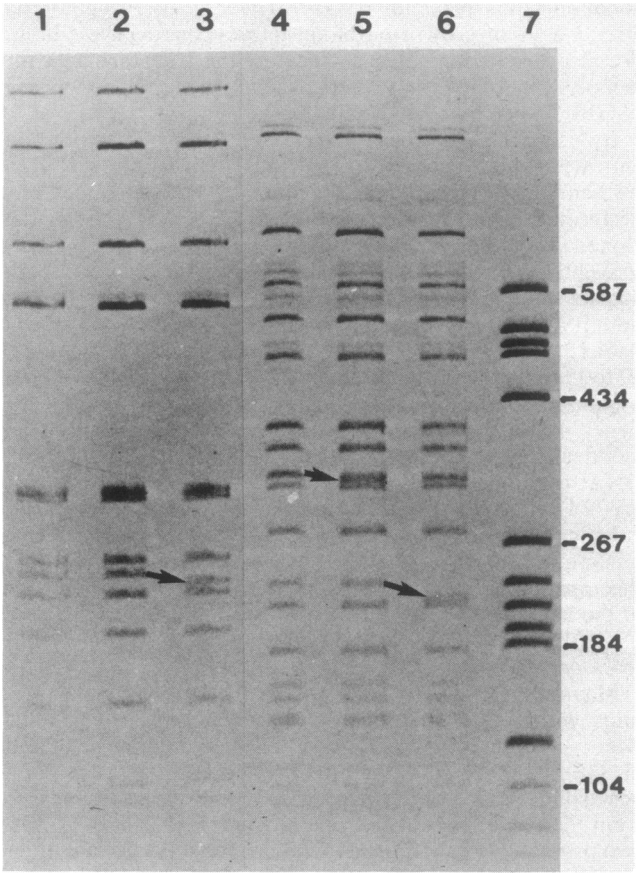



FIG. 2. Restriction enzyme profiles of pSG1015, pSG201, and pSG202. Plasmid DNA was digested with restriction endonuclease *Taq*I (lanes 1 through 3) or *Sau*3AI (lanes 4 through 6). Lanes: 1 and 4, pSG1015; 2 and 5, pSG201; 3 and 6, pSG202. Arrows between lanes 2 and 3 and between lanes 5 and 6 show the apparent 20-bp deletion in pSG202. Arrow between lanes 4 and 5 indicates the increase (10 to 15 bp) seen in pSG1015. *Hae*III digest of pBR322 DNA (lane 7) was used as a molecular weight standard. Sizes of restriction fragments are given in base pairs.

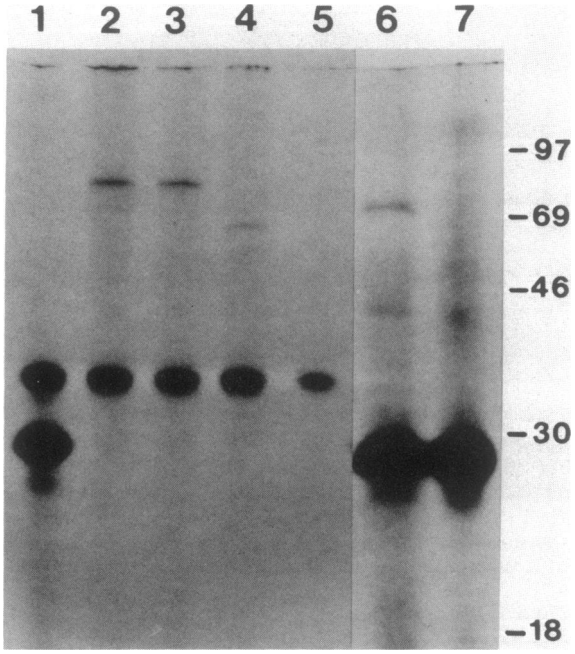



FIG. 3. Maxicell analysis of labeled proteins from *E. coli* transformants possessing recombinant plasmids. Lanes: 1, SY327(pBR322) Amp$^r$ Tet$^r$; 2, SY327(pSG1015) Tet$^r$; 3, SY327 (pSG1015-2) Tet$^r$; 4, SY327(pSG201) Tet$^r$; 5, SY327(pSG202) Tet$^r$; 6, SY327(pSG402) Amp$^r$; 7, SY327(pSG404) Amp$^r$. The mobilities of labeled protein size standards are given in kilodaltons. Lanes 6 and 7 are taken from an autoradiograph of the same gel which had been exposed for a longer period.

84,000-dalton polypeptide is the *V. cholerae* hemolysin. In addition, the immunoprecipitation of a 72,000-dalton protein from extracts of SY327(pSG402) (lane 6) establishes an antigenic relationship between this polypeptide and the 84,000-dalton hemolysin-related protein. However, the 64,000-dalton protein which is expressed by transformants that carry pSG201 is not immunoprecipitated (Fig. 4, lane 5). Plasmids with BAL 31 deletions which do not extend into the 2.3-kb *hly* locus are, as expected, hemolytic and express an immunoprecipitable 84,000-dalton protein. A summary of the above results is given in Fig. 5 along with our proposed model of the orientation of the *hly* gene.

**Immunoprecipitation of *V. cholerae* extracts.** Since the size of the purified *V. cholerae* hemolysin was reported to be ca. 20,000 daltons (11), it was possible that the 84,000-dalton hemolysin-related protein observed was a larger, precursor form of the hemolysin which was not processed by *E. coli*. To test this hypothesis, we performed immunoprecipitation experiments with [$^{35}$S]methionine-labeled proteins from the supernatant fluids and the sonicated extracts of *V. cholerae* strains HK-1 and SG23(HK-1, *hly*::VcA-3) and then analyzed the samples as described above. As shown in Fig. 6, an 84,000-dalton protein present in the culture supernatant fluid of HK-1(Hly$^+$) was immunoprecipitated by antihemolysin serum. In marked contrast, a hemolysin-related polypeptide could not be detected in the culture supernatant fluid of SG23.

## DISCUSSION

We have previously suggested that a large change in DNA structure (>200 bp) is not responsible for the variation in the Hly phenotype of *V. cholerae* strains (9). We therefore analyzed the cloned DNA of *hly* loci from hemolytic and

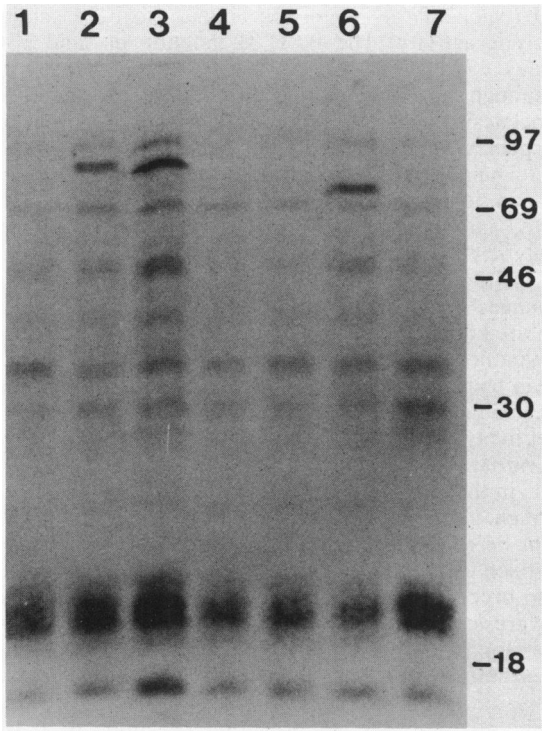



FIG. 4. Immunoprecipitation of $^{35}$S-labeled proteins from *E. coli* transformants carrying recombinant plasmids. Lanes: 1, SY327(pBR322); 2, SY327(pSG1015); 3, SY327(pSG1015-2); 4, SY327(pSG201); 5, SY327(pSG202); 6, SY327(pSG402); 7, SY327(pSG404). The mobilities of labeled protein standards are given in kilodaltons.

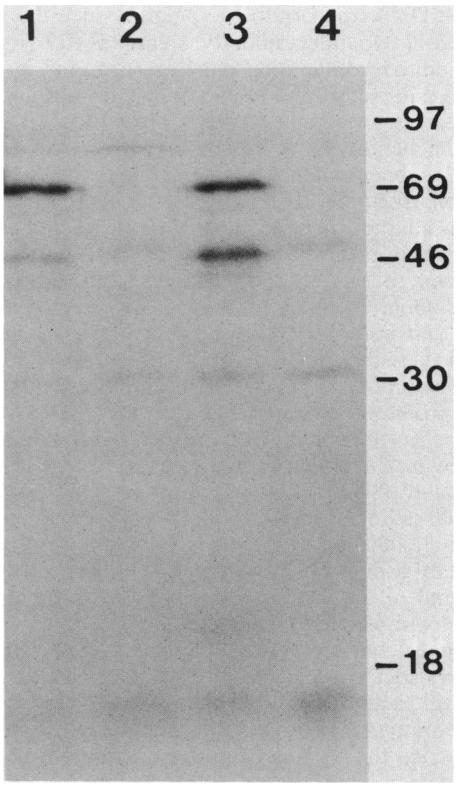
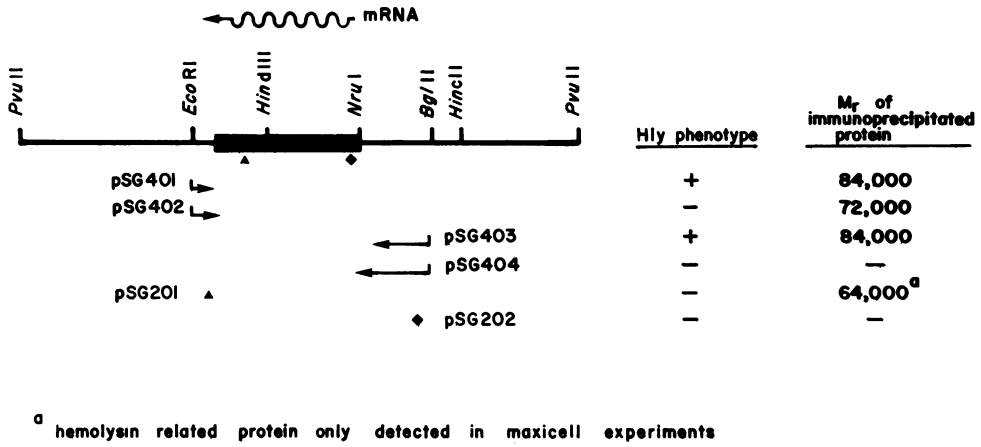



FIG. 6. Immunoprecipitation of $^{35}$S-labeled proteins from *V. cholerae* using antihemolysin serum. Lanes: 1, sonicate from HK-1; 2, supernatant fluids from HK-1; 3, sonicate from SG23(HK-1, *hly*::VcA-3); 4, supernatant fluids from SG23.

nonhemolytic *V. cholerae* isolates using a series of 4-bp recognition site restriction enzymes. Small alterations in the electrophoretic mobility of certain restriction fragments were observed by using the 4-bp-recognizing enzymes *Sau*3AI and *Taq*I. An apparent 20-bp decrease in the size of a single pSG202 fragment (*V. cholerae* 569B Hly$^-$) compared with that of pSG1015 (*V. cholerae* RV79 Hly$^+$) was observed with both of these enzymes. This alteration was subsequently localized to the right 1.3-kb region of the *hly* locus. We also demonstrated that the conversion RV79(Hly$^-$) to RV79(Hly$^+$) is correlated with an increase of ca. 10 to 15 bp in a specific *Sau*3AI restriction fragment. This increase is located between the *Eco*RI and *Nru*I sites of pSG1012, which includes the left 1.0 kb of the Hly determinant. This




[a] hemolysin related protein only detected in maxicell experiments

FIG. 5. Summary of maxicell and immunoprecipitation data showing proposed extent and orientation of the *hly* locus in the *V. cholerae* chromosome. Symbols: ▲, approximate position of the alteration in the cloned *hly* region from *V. cholerae* RV79(Hly$^-$); ◆, approximate position of the alteration in the cloned *hly* region from *V. cholerae* 569B(Hly$^-$). Plasmids pSG401 through pSG404 have been treated with BAL 31 nuclease, and the arrows indicate the approximate size of the deletions. The Hly phenotype and electrophoretic mobility of the immunoprecipitated hemolysin-related proteins expressed from each plasmid are indicated.

change in DNA appears to be specific, since the cloned *hly* regions from two independently isolated RV79(Hly$^+$) strains were found to have identical changes. Although we observed an increase in electrophoretic mobility that is suggestive of a DNA insertion, we cannot rule out an inversion of a region within the hemolysin gene. In this case, we would have expected a concomitant decrease in the mobility of a second restriction fragment. If this decrease were small and occurred in a large fragment, it would not be detected by the methods used in this study.

Assuming there are 110 daltons per amino acid, the 2.3-kb *hly* locus could encode a protein of 84,333 daltons. Maxicell analysis and immunoprecipitation of labeled proteins from *E. coli* possessing the cloned Hly$^+$ determinants carried by pSG1015 and pSG1015-2 demonstrated that a polypeptide of ca. 84,000 daltons was correlated with the hemolytic phenotype. As shown through nuclease BAL 31 deletion analysis, the removal of DNA sequences at the right end of the *hly* region (as in pSG404) eliminated all detectable synthesis of the 84,000-dalton protein, while a polypeptide of reduced size (ca. 72,000 daltons) was visible among the proteins encoded by a plasmid (pSG402) with a deletion in DNA at the left end of the *hly* gene. *E. coli* transformants carrying either deleted plasmid are nonhemolytic.

Analogous changes were seen in the proteins expressed by cloned *hly* loci from RV79(Hly$^-$) and 569B. In the latter case, novel proteins were not observed in either the maxicell or the immunoprecipitation experiments with transformants which carried the *hly* loci from these strains. It is likely that the products of transcription or translation in 569B are either greatly truncated or reduced in quantity by the apparent 20-bp deletion involving the right side of the *hly* gene. As observed in the analysis of pSG402, the alteration in DNA structure of the cloned *hly* region of RV79(Hly$^-$) compared with that of RV79(Hly$^+$) involved the left side of the Hly determinant and resulted in the synthesis of a truncated protein of ca. 64,000 daltons, which was expressed in maxicells at levels comparable to the 84,000-dalton polypeptide.

The previously reported size of the purified El Tor hemolysin is 20,000 daltons (11). We have demonstrated that hemolytic activity is associated with an 84,000-dalton protein by maxicell analysis and after immunoprecipitation of proteins from *E. coli* containing cloned Hly$^+$ sequences. In addition, a protein of the same size was immunoprecipitated from the supernatant fluid of *V. cholerae* strain HK-1 but not from the supernatant fluid of HK-1 containing a VcA-3 insertion in the *hly* locus (strain SG23). At present there is no evidence of a unique 20,000-dalton polypeptide representing a processed hemolysin. Since many hemolysins, including the immunologically related hemolysin from non-O1 *V. cholerae* strains, interact with gel matrices (19, 23), the molecular mass of the El Tor hemolysin may have been underestimated.

The data presented here suggest that transcription of *hly* begins near the *Nru*I site of pSG1012 and proceeds towards the *Eco*RI site. If the apparent 20-bp deletion of the *hly* region of classical strain 569B was located in the *hly* regulatory sequences, the invariable nonreverting Hly$^-$ phenotype of this classical biotype isolate would be anticipated. The 64,000-dalton polypeptide expressed by the cloned *hly* locus of RV79(Hly$^-$) may represent a truncated version of the complete active hemolysin. If so, the failure to immunoprecipitate this protein would suggest that the major antigenic determinants of the hemolysin protein are located within its C-terminal 20,000 daltons. Furthermore, the C-terminal region of the hemolysin appears to be essential for hemolytic activity. The BAL 31 deletion plasmid pSG402 encodes a 72,000-dalton protein which is inactive.

Although the contribution of the *V. cholerae* hemolysin to pathogenesis is not yet known, the virulence of other bacterial species is significantly enhanced by their ability to secrete hemolysin (1, 2, 21). In addition, the purified *V. cholerae* hemolysin is cytotoxic and rapidly lethal when injected into mice (11). Therefore it is possible that the hemolytic phenotype plays a role in the infectious process of *V. cholerae*.

Changes which involve rearrangements of chromosomal DNA are known to play an important role in the virulence of many pathogenic organisms. In *Neisseria gonorrhoeae* and African trypanosomes, DNA rearrangements determine the expression of the pilus protein (15) and surface antigens (22), respectively. An inversion in a DNA segment of *Salmonella typhimurium* containing a promoter for the flagella gene results in the expression of either the H1 or H2 protein (24). Although an inversion within the *V. cholerae hly* locus cannot be ruled out, any substantial rearrangement would have been detected by our DNA restriction analysis. There is also precedent for smaller changes in DNA which affect the expression of a virulence factor. The spontaneous loss of expression of the M protein of *Streptococcus pyogenes*, a surface antigen which permits these bacteria to resist phagocytosis, is apparently due to a deletion (ca. 50 bp) in a specific restriction fragment within or adjacent to the M protein coding sequence (20).

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