Identity of Hemolysins Produced by *Vibrio cholerae* Non-O1 and *V. cholerae* O1, Biotype El Tor

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Hemolysins purified from non-O1 *Vibrio cholerae* (non-O1 hemolysin) and a *Vibrio cholerae* O1, biotype El Tor (El Tor hemolysin) were investigated for their homology. The hemolysins were isolated from the culture supernatant fluids by ammonium sulfate precipitation and gel filtration on Sephadex G-100 columns. The purified hemolysins gave single bands with an identical mobility on conventional polyacrylamide gel disc electrophoresis. The molecular weights of the non-O1 and El Tor hemolysins were estimated to be about 60,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the amino acid compositions of the hemolysins were very similar. The specific activities of the hemolysins were identical, and both hemolysins were neutralized to the same extent with antisera against the homologous and heterologous hemolysins. Ouchterlony double immunodiffusion tests with both hemolysins and antihemolysin serum gave a common (fused) precipitin line. These data indicate that the non-O1 hemolysin is biologically, physicochemically, and immunologically indistinguishable from the El Tor hemolysin.

Vibrio cholerae other than serovar O1 (non-O1 *V. cholerae*) is known to cause gastroenteritis, although it rarely causes epidemic disease or person-to-person infection. Some non-O1 *V. cholerae* isolates produce an enterotoxin which is similar to cholera enterotoxin (24, 25). However, many clinical isolates of non-O1 *V. cholerae* do not produce choleralike enterotoxin. This suggests that an unknown diarrheagic factor(s) is produced by these organisms. It is known that most non-O1 *V. cholerae* strains synthesize a large amount of hemolysin (non-O1 hemolysin) which resembles hemolysin produced by *V. cholerae* O1 biotype El Tor (El Tor hemolysin) (15, 23). Non-O1 hemolysin was purified and was reported to have a molecular weight of 60,000 and to exhibit partial immunological relatedness to the El Tor hemolysin (23). Our earlier observations (23) were made with purified non-O1 and crude El Tor hemolysins. However, more recently we found complete fusion of immunoprecipitin lines of non-O1 and El Tor hemolysins in Ouchterlony immunodiffusion tests when the crude El Tor hemolysin was freshly prepared (unpublished observation). Thus, in this study we compared the properties of purified preparations of the two hemolysins to rigorously evaluate our inconsistent observations. We did not observe any biological, physicochemical, or immunological differences between the two purified hemolysins.

MATERIALS AND METHODS

Bacterial strains. Non-O1 *V. cholerae* strain S7, obtained from Yutaka Zinnaka, Defense Medical College, Tokorozawa, Saitama, Japan, was used to purify the non-O1 hemolysin. This strain was isolated from a diarrheal patient in Sudan in 1969. It produces choleralike enterotoxin which is biologically similar but physicochemically not identical to cholera enterotoxin (25). *V. cholerae* O1, biotype El Tor, serotype Inaba, strain N86 was used for purification of El Tor hemolysin. This strain and the other eight El Tor strains were

all obtained from Toshio Miwatani, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan.

Preparation of crude hemolysin. Crude preparations of the non-O1 and El Tor hemolysins were prepared as described previously (23). Unless otherwise noted, all steps were done at ca. 4°C. In brief, a portion (1.5 ml) of an overnight heart infusion broth culture grown at 37°C was inoculated into Roux bottles containing 150 ml of syncase medium supplemented with 3% glycerol. Cultivation was carried out for 48 h at 30°C with a surface/volume ratio of 2 cm^2/ml. Ammonium sulfate (390 g/liter) was dissolved in the pooled culture supernatant fluids; the precipitate was recovered by centrifugation, dissolved in 10 ml of 50 mM Tris hydrochloride–1 mM EDTA–3 mM NaN_3, pH 8.0 (TEA buffer), and dialyzed overnight against TEA buffer. The dialyzed sample was centrifuged for 20 min at 20,000 × *g*, and the supernatant fluids were used as crude hemolysin.

Determination of hemolytic activity. Hemolytic activity was assayed as previously described (23). Briefly, 1 ml of appropriately diluted sample in 10 mM phosphate-buffered 0.9% NaCl, pH 7.0 (PBS), containing 0.1% gelatin was incubated for 30 min at 37°C with 1 ml of a 2% washed sheep erythrocyte suspension in PBS-gelatin. The mixture was centrifuged for 2 min at 1,000 × *g*, and the released hemoglobin in the supernatant fluids was measured spectrophotometrically at 540 nm. One hemolytic unit is defined as the amount of hemolysin which lyses 50% of the erythrocytes in the standardized erythrocyte suspension.

Preparation of antiserum. Hemolysin (100 μg) in 1 ml of PBS emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) was injected into the footpads of 2-kg rabbits. At 2 and 4 weeks after the first injection, the rabbits were injected intravenously with 100 μg of the purified hemolysin in PBS. Antiserum was obtained 5 weeks after the first injection. Rabbit immunoglobulin specific for the purified non-O1 hemolysin was obtained from the antiserum by affinity column chromatography on CNBr-activated Sepharose 4B (Pharmacia Fine

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Chemicals Inc., Uppsala, Sweden) coupled with the purified non-O1 hemolysin as described previously (21).

Ouchterlony immunodiffusion test. Double immunodiffusion in agar was carried out by the method of Ouchterlony (16). Wells (5-mm diameter) placed 10 mm apart were cut in a gel composed of 5 ml of 1.2% Noble agar gel (Difco) in 10 mM phosphate buffer (pH 7.0) on a glass plate (2.6 by 2.6 cm). Each well received 40 μl of antiserum or antigen, and the agar plate was incubated for 18 h at 37°C in a petri dish to retain moisture. After incubation, the gel was dialyzed against a solution containing 0.4% NaCl and 0.4% $Na_2B_4O_7$, dried at 37°C, stained with 0.1% Coomasie brilliant blue, and destained with 50% (vol/vol) methanol–10% (vol/vol) acetic acid (vol/vol).

Electrophoresis. Conventional polyacrylamide gel disc electrophoresis and sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis were carried out essentially by the methods of Davis (4) and Laemmli (13), respectively. Equine cytochrome c and cross-linked cytochrome c (Oriental Yeast Co., Tokyo, Japan) were used as molecular weight marker proteins.

Western blotting. Immunochemical detection of antigen on nitrocellulose was carried out by a modification of the method of Towbin et al. (20). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis the separated proteins in the gel were transferred to nitrocellulose by electrophoresis at a constant voltage of 40 V for 12 h at 20°C. The electrode buffer contained 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (pH 8.3). The nitrocellulose membrane was incubated for 30 min with the affinity-purified rabbit antibody raised against the non-O1 hemolysin. After washing three times, the membrane was incubated for 30 min with affinity-purified goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Cappel Laboratories, Inc., Cochranville, Pa.) and washed three times. All of the incubation and washing procedures were done by gently agitating the membrane at room temperature with 20 ml of PBS containing 0.05% Tween 20. After washing with PBS, the nitrocellulose membrane was immersed for 10 to 30 min in PBS containing 0.02% H_2O_2 and 0.06% 1-chloro-4-naphthol, and color development was stopped by washing with distilled water.

Amino acid analysis. Approximately 10 μg of purified hemolysin was hydrolyzed by the method of Simpson et al. (18) with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indol. The amount of cystine and cysteine (half-cystine) was estimated by determining the cysteic acid content of the performic acid-oxidized protein. Amino acid composition was determined with a high-pressure liquid chromatograph (Simadzu LC-4A; Shimadzu Rika Instruments Co., Tokyo, Japan).

Protein determination. Protein content was assayed by the method of Bradford (2) with bovine serum albumin as the standard.

RESULTS

Western blotting of crude hemolysin preparations. Crude hemolysin preparations from nine O1 (El Tor) strains and from one non-O1 strain were examined by Western blotting analysis with anti-non-O1 hemolysin antibody to detect antigens cross-reacting with the non-O1 hemolysin (Fig. 1). All of the preparations gave several reactive bands, and a common 60,000-molecular-weight protein band was found. This molecular weight coincides with that of the non-O1 hemolysin (23). Non-O1 hemolysin has been suggested to be immunologically cross-reactive but only partially identical to
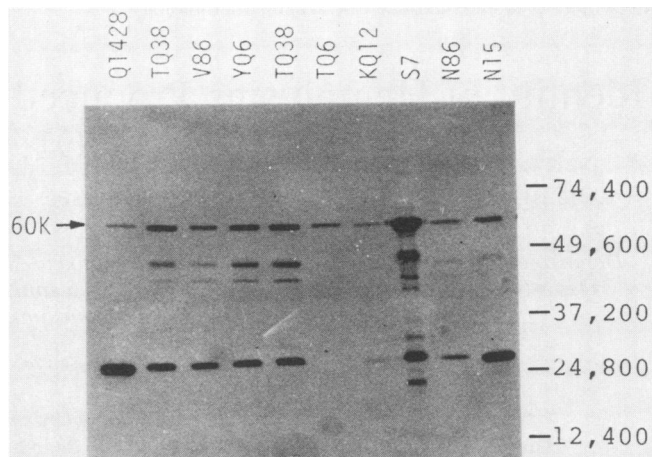

FIG. 1. Detection of immunoreactive proteins in crude El Tor and non-O1 hemolysins by Western blotting with anti-non-O1 hemolysin antibody. Strain names are noted above the lanes. All the strains except S7 (non-O1 *V. cholerae*) are *V. cholerae* O1, biotype El Tor. The numbers on the right show the molecular weights of standard protein markers. 60K indicates the molecular weight (60,000) of non-O1 hemolysin (23).

the El Tor hemolysin (23). However, the Western blotting data in Fig. 1 suggest that the El Tor hemolysin may have the same molecular weight (60,000) as the non-O1 hemolysin. Several bands having a molecular weight smaller than 60,000 were also observed (Fig. 1), and they may be proteolytically digested epitopes of the hemolysin.

Purification of hemolysins. A crude El Tor hemolysin preparation from strain N86 and a crude non-O1 hemolysin preparation from strain S7 were chromatographed on a Sephadex G-100 gel column (Fig. 2A). Both hemolysins showed similar elution patterns, i.e., most of the activity was eluted after the bed volume (fraction no. 60) of the gel. In the El Tor hemolysin minor hemolytic activity (less than 3% of the total activity) was found at the void volume of the column (fraction no. 21). This minor activity is likely to be the altered El Tor hemolysin (maybe aggregates). Because this activity was neutralized by anti-non-O1 hemolysin and because a small amount of activity was also found when the major active fractions were pooled, concentrated, and rechromatographed on the same column equilibrated with same TEA buffer (data not shown). The major active fractions were concentrated and further purified by a column of Sephadex G-100 superfine gel equilibrated with TEA buffer containing 0.25 M glucose to interfere with the interaction between the hemolysins and the gel. Hemolytic activity was found at the same eluting position (fraction no. 33) before the bed volume (fraction no. 65) in both hemolysins (Fig. 2B). The active fractions were pooled and used for the following comparison of properties.

Electrophoresis. The purities of the non-O1 and El Tor hemolysins were examined by conventional polyacrylamide gel disc electrophoresis (Fig. 3). The hemolysins gave a single band and had an identical mobility (columns 1 and 2). Their hemolytic activities (columns 3 and 4) coincided with the protein bands. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the hemolysins showed an identical molecular weight, 60,000 (Fig. 4). The mobility was not altered in the presence or absence of 2-mercaptoethanol or dithiothreitol.

Specific activities. The dose-response curves for the purified non-O1 and El Tor hemolysins were almost the same
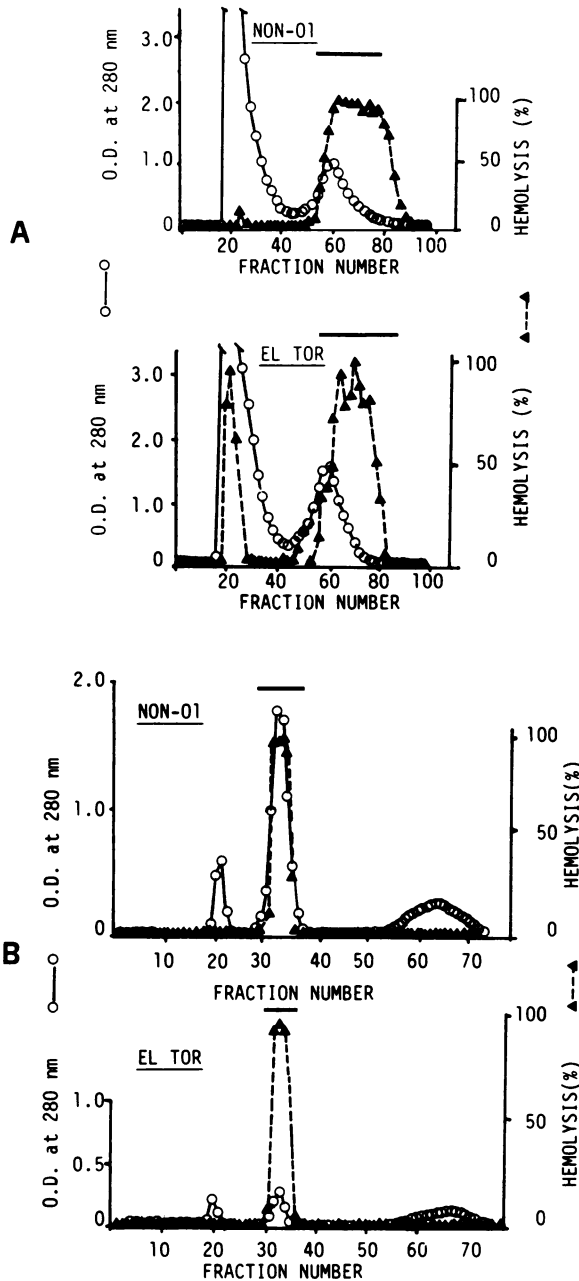
FIG. 2. Gel filtrations on Sephadex G-100 columns of non-O1 and El Tor hemolysins. Protein content (absorbance at 280 nm) (○) and hemolysis (absorbance at 540 nm) (▲) were determined. Horizontal bars indicate pooled active fractions. (A) Crude hemolysin preparations from a 4-liter culture were applied to a Sephadex G-100 regular column (4 by 72 cm) with 15 ml per fraction; samples diluted 500-fold (non-O1) or 100-fold (El Tor) were assayed as described in Materials and Methods. (B) Concentrated active fractions in Fig. 1A were chromatographed in a Sephadex G-100 superfine column (2.5 by 67 cm) with 5 ml per fraction; samples diluted 5,000-fold (non-O1) or 1,000-fold (El Tor) were assayed for hemolysis.

(Fig. 5). One hemolytic unit of the El Tor and non-O1 hemolysins was 12.6 and 10.2 ng, respectively.

Amino acid composition. The amino acid compositions of both purified hemolysin preparations were in good agreement (Table 1). Particularly, there were a few moles of half-cystine and methionine residues in the hemolysins.
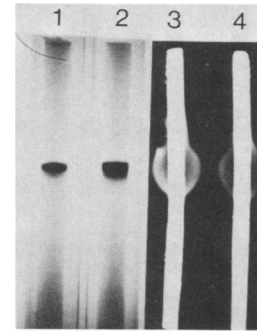

FIG. 3. Polyacrylamide gel disc electrophoresis of El Tor and non-O1 hemolysins. Columns 1 and 2 contain Coomasie brilliant blue-stained hemolysins (50 μg). For columns 3 and 4, the gel was immersed into melted blood agar, solidified, and incubated for 1 h at 37°C; 5-μg samples of hemolysins were applied. Columns 1 and 3 contain El Tor hemolysin; columns 2 and 4 contain non-O1 hemolysin.

Immunological identity. The ability of the homologous and heterologous antisera to inhibit both purified hemolysin was almost identical, although the maximal dilutions of the antisera to neutralize hemolysis were different (Fig. 6.)

Ouchterlony immunodiffusion of the purified non-O1 and El Tor hemolysins with the anti-non-O1 and anti-El Tor hemolysin sera revealed that each antisera gave a single fused immunoprecipitin line (no spur formation) with both hemolysins (Fig. 7). These results indicate that non-O1 and El Tor hemolysins are immunologically identical.

DISCUSSION

In this communication, we showed that non-O1 hemolysin was indistinguishable from El Tor hemolysin in specific activity, physicochemical and immunological properties, and amino acid composition. Previously, partial nonidentity of non-O1 hemolysin from El Tor hemolysin was suggested, although the two hemolysins were immunologically related (23). That is, in Ouchterlony immunodiffusion test, spur formation was found between precipitin lines of purified non-O1 and crude El Tor hemolysins. In this study, how-
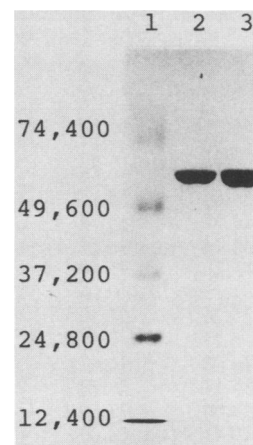

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of El Tor and non-O1 hemolysins. Samples of about 20 μg of El Tor (lane 2) and non-O1 (lane 3) hemolysins were applied. The numbers on the left show molecular weights of standard marker proteins in lane 1.
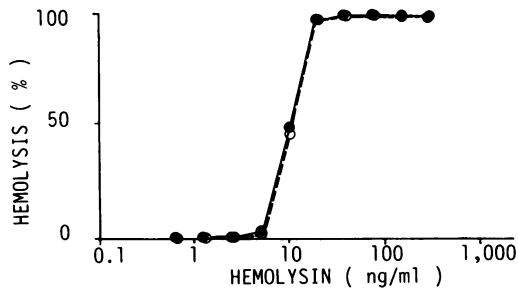
FIG. 5. Dose-response curves for non-O1 and El Tor hemolysins. Non-O1 (●) and El Tor (○) hemolysins were assayed as described in Materials and Methods. The indicated amounts denote final concentrations of hemolysin protein.
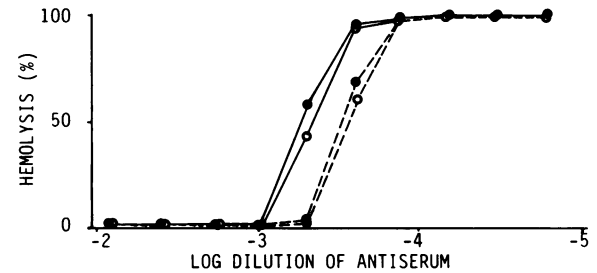

FIG. 6. Inhibition of hemolysis of El Tor and non-O1 hemolysin by antisera. Samples of 0.5 ml of anti-El Tor (– – –) or anti-non-O1 (———) serum diluted as indicated were mixed with 0.5 ml (100 ng/ml) of El Tor (○) and non-O1 (●) hemolysins. After incubation for 30 min at 37°C, 1 ml of 2% sheep erythorcytes was added to the mixture, and the hemolysis was determined at 540 nm as described in Materials and Methods.

ever, the purified hemolysins did not give any spur in the Ouchterlony test (Fig. 7). Rapid loss of hemolytic activity is widely observed in culture supernatant fluids of El Tor biotype *V. cholerae* O1, which suggests the coexistence of a hemolysin-destructive factor in the crude hemolysin preparation (10, 22). Thus, the spur formation previously found (23) might be due to alteration of antigenic determinants in the El Tor hemolysin by contaminating proteases.

El Tor biotype *V. cholerae* O1, the causative agent of the seventh cholera pandemic, was first separated from the classical biotype because of its hemolytic properties, but later hemolysis has not been as frequently observed in most of the isolates obtained during 1960s and 1970s (5). Sakazaki et al. (17), however, showed that more than 99% of non-O1 *V. cholerae* isolates were hemolytic. Kudoh et al. (12), on the other hand, noted the closer characteristics of non-O1 *V. cholerae* to El Tor biotype *V. cholerae* O1 than to the classical ones, namely, hemaggulutination of chicken erythrocytes, positive Voges-Proskauer reaction, and resistance to polymyxin B, cholera phage IV, and hemolysis. They reported that 92% of non-O1 *V. cholerae* isolates were hemolytic, whereas only 42% of *V. cholerae* El Tor isolates

were hemolytic. Our results suggest that hemolysis by non-O1 *V. cholerae* is caused by the same hemolysin of *V. cholerae* O1 El Tor.

Craig (3) compared the influence of various culture conditions on the production of enterotoxin by O1 and non-O1 *V. cholerae*. He described that enterotoxin production by non-O1 *V. cholerae* is similar to El Tor vibrios but distinct from classical ones. The enterotoxin of non-O1 *V. cholerae* was purified and demonstrated to be basically the same as cholera enterotoxin (24, 25). These observations and our results strongly suggest a close relationship between non-O1 *V. cholerae* to O1 *Vibrio cholerae*, especially to biotype El Tor, in the homology of enterotoxin and hemolysin.

Honda and Finkelstein (9) estimated the molecular weight of the El Tor hemolysin to be about 20,000 by gel filtration. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4) showed that the molecular weight of the El Tor hemolysin and the non-O1 hemolysin is about 60,000. The retarded migration of the El Tor hemolysin during Sephadex gel filtration (Fig. 2) suggests that the molecular weight of the hemolysin was previously underestimated because of interaction of the hemolysin with the Sephadex gel. Similar interaction between gel matrices and other cytolytic proteins has been reported (1, 8, 11, 19).

Structural information on the El Tor hemolysin from molecular cloning of the gene (6, 7, 14) does not agree with our results. Goldberg and Murphy (7) and Manning et al. (14) estimated the molecular weight of the El Tor hemolysin to be 84,000 and 80,000, respectively, when the hemolysin gene was expressed in *Escherichia coli* by minicell or maxicell analysis. The smaller molecular weight (60,000) we estimate for the El Tor hemolysin (Fig. 4) suggests that the gene product studied by Goldberg and Murphy and Manning et al.

TABLE 1. Amino acid compositions of El Tor and non-O1 hemolysins[a]

Amino acid	No. of residues			
	Obtained by hydrolysis		To nearest interger	
	El Tor	Non-O1	El Tor	non-O1
Aspartic acid	66.01	67.03	66	67
Threonine	34.79	35.21	35	35
Serine	46.63	46.86	47	47
Glutamic acid	54.04	55.74	54	56
Proline	13.57	14.79	14	15
Glycine	42.73	45.58	43	46
Alanine	45.28	45.58	45	46
Half-cystine	1.43	0.96	1	1
Valine	25.24	25.30	25	25
Methionine	1.25	1.59	1	2
Isoleucine	16.02	16.07	16	16
Leucine	35.14	36.37	35	36
Tyrosine	23.00	23.66	23	24
Phenylalanine	18.41	18.03	18	18
Lysine	16.68	17.07	17	17
Histidine	7.44	8.27	7	8
Arginine	22.92	23.49	23	23

[a] Data are expressed as the number of residues per molecule of the hemolysin (molecular weight, 60,000).
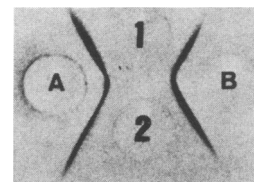

FIG. 7. Ouchterlony agar gel double immunodiffusion test of El Tor and non-O1 hemolysin with anti-El Tor and anti-non-O1 hemolysins. Wells: 1, non-O1 hemolysin, 50 μg; 2, El Tor hemolysin, 50 μg; A, anti-non-O1 hemolysin serum, 8-fold diluted; B, anti-El Tor hemolysin serum, 16-fold diluted.

may be the precursor form of El Tor hemolysin, which is not processed in *E. coli*. We did not find 84,000- or 80,000-dalton proteins in the supernatant of *V. cholerae* by the Western blotting method (Fig. 1). This is not unreasonable, however, if *V. cholerae* does not secrete the precursor protein extracellularly. Recently, Yoh et al. reported that, besides the common El Tor hemolysin, some strains of *V. cholerae* produces a hemolysin which is immunologically related and biologically similar to the thermostable direct hemolysin of *Vibrio parahaemolyticus* (M. Yoh, T. Honda, and T. Miwatani, Abstr. Annu. Meet. Jpn. Soc. Bacteriol. **40**:143, 1985). This suggests the possibility that some *V. cholerae* isolates may produce more than one hemolysin. Thus, it is possible that the cloned hemolysin gene may code for a different hemolysin than the hemolysin we studied.

ACKNOWLEDGMENTS

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