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Cytolytic Activity and Virulence of *Vibrio damsela*

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A correlation was observed between the ability of 19 isolates of *Vibrio damsela*, a halophilic bacterium recently recognized as a human pathogen, to cause disease in mice and to produce large amounts of a cytolytic toxin in vitro. The yield of toxin in the culture supernatant fluids was optimal during the mid- and late-logarithmic phases of growth in medium containing 0.5% Na^+ ion, was stable during the stationary growth phase, and was significantly reduced in culture medium containing ≥0.8% Na^+ ion, even though Na^+ ion concentrations ranging from 0.8% to 2% significantly enhanced growth of the bacterium. The activity in toxin preparations partially purified by ammonium sulfate precipitation was deleteriously affected by heat, low and high pH, proteases, dithiothreitol, and chelating agents, but was unaffected by cholesterol, trypan blue, and mixed gangliosides. The toxin had a molecular weight (estimated by gel filtration) of ca. 57,000 and an isoelectric point of ca. 5.7 and was antigenically distinct from previously described cytolysins produced by *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and the El Tor biotype of *Vibrio cholerae*. Bacteriologically sterile, partially purified toxin preparations were lethal for mice after intraperitoneal, intravenous, and subcutaneous administration, and subcutaneous injection elicited grossly observable changes similar to those observed during the lethal experimental infection caused by subcutaneous injection of *V. damsela*.

Seven new *Vibrio* species have been recognized since 1976 as possible causes of human disease (2, 6, 8, 14). One of these, *Vibrio damsela* (originally known as group EF-5 *Vibrio* species), is a halophilic bacterium that has been reported to cause skin ulcers and fatal disease in temperate-water damselfish and wound infections in humans (11, 19, 23). Some of the clinical and epidemiological characteristics of human disease caused by *V. damsela* have been described previously (11, 23); however, previous studies have not dealt with the possible production of toxins or other potential virulence factors by the bacterium. This paper reports a correlation between the virulence of *V. damsela* for mice and in vitro production of large amounts of an extracellular cytolysin by the bacterium. In addition, some of the biological and physicochemical properties of the toxin are described.

MATERIALS AND METHODS

Bacteria. Nineteen strains of *V. damsela* were kindly supplied by F. Hickman-Brenner of the Centers for Disease Control (Atlanta, Ga.). Frozen specimens of the bacteria were prepared by growing the strains with shaking at 30°C to the mid-logarithmic phase of growth in heart infusion (HI) broth (Difco Laboratories, Detroit, Mich.) and freezing portions (1 ml) of the cultures with sterile calf serum (0.5 ml) at −70°C. Seed cultures were prepared before each experiment by inoculating Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants with 1 loopful of rapidly thawed specimens of the bacteria, incubating the cultures for ca. 24 h at 30°C, and inoculating Columbia agar (BBL; contained in 100- by 15-mm petri dishes) for confluent growth with 2 loopsful of growth from the Trypticase soy agar slant cultures. After incubation for ca. 24 h at 30°C, the surface growth on the Columbia agar was collected with sterile 0.85% NaCl (5 ml per petri dish culture), and the suspensions were adjusted to an optical density at 650 nm of ca. 10 (ca. 10^{10} CFU/ml).

The markedly hemolytic *Vibrio cholerae* strain 26-3, serotype Ogawa, biotype El Tor (15), was kindly supplied by R. A. Finkelstein (University of Missouri, Columbia). HI broth supplemented with 3% glycerol (200 ml in a 2-liter flask) was inoculated with ca. 10^9 CFU of the bacterium and was incubated statically for 20 h at 37°C. The culture supernatant fluids obtained by centrifugation (16,000 × *g*, 15 min) contained ca. 360 hemolytic units per ml (assayed against washed mouse erythrocytes) and were used as the source of *V. cholerae* biotype El Tor cytolysin during the cytolysin-antiserum neutralization studies.

Preparation of HID broth. The deionized water and dialysis tubing used to prepare HI diffusate (HID) were sterilized by autoclaving and ethylene oxide treatment, respectively. Approximately 1,440 ml of 10-times-concentrated (250 g/liter) HI broth was prepared by dissolving the powdered medium in boiling deionized water. The solution was divided equally among 16 dialysis bags made of regenerated cellulose tubing having a flat width of 75 mm (American Scientific Products, Charlotte, N.C.) and was dialyzed at 4°C against 7.2 liters of deionized water in a model C Pope multiple dialyzer (A. H. Thomas Co., Philadelphia, Pa.). The bottom baffle of the dialyzer was removed to facilitate movement of the dialysis bags. After 3 days, the contents of the bags were discarded, and the diffusate was autoclaved in 2-liter Erlenmeyer flasks (200 to 210 ml per flask).

Preparation of partially purified cytolysin concentrate. Unless otherwise noted, all steps were done at approximately 4°C. Twelve 2-liter flasks containing 200 ml of HID broth were inoculated with portions (1 ml) of a seed culture suspension (ca. 10^{10} CFU/ml) of *V. damsela* strain 1421-81, and the culture supernatant fluids were obtained by centrifugation (16,000 × *g*, 20 min) after incubation of the cultures for 14 to 15 h at 30°C on a Gyrotory shaker (model G-25; New Brunswick Scientific Co., New Brunswick, N.J.) operating at 220 cycles per min. Ammonium sulfate (enzyme grade; Schwarz/Mann, Spring Valley, N.Y.) was dissolved in the culture supernatant fluids to a final concentration of ca. 60% saturation (420 g/liter); after 4 to 5 h, the precipitate

was recovered by centrifugation and dissolved in 60 ml of 0.05 M ammonium bicarbonate (pH 7.8). The solution was dialyzed for 16 to 18 h against 12 liters of 0.05 M ammonium bicarbonate and was lyophilized after centrifugation (20,000 × *g*, 20 min) to remove a small amount of insoluble residue. The lyophilized preparation was stored at −10°C.

Assays. Cytolytic activity against erythrocytes was determined by the tube assay of Bernheimer and Schwartz (5). One hemolytic unit (HU) is defined as the amount of preparation which causes the release of 50% of the hemoglobin in the standardized (ca. 0.7%, vol/vol) washed erythrocyte suspension.

The median lethal doses (LD_{50} values) of the *V. damsela* strains and for the partially purified cytolysin concentrate were determined with female, 6- to 8-week-old (26- to 30-g), Dub:(ICR) strain, randomly bred albino mice (Dominion Laboratories, Dublin, Va.). The values were calculated by the method of Reed and Muench (24) after observing the animals for 3 days postinjection. The bacteria were grown for the bacterial infection studies by inoculating HI broth (200 ml per 2-liter flask) with 1 ml of seed culture suspension (ca. 10^{10} CFU) and incubating the medium at 30°C for 14 to 15 h on a gyrotory shaker (220 cycles per min). The bacteria in portions (5 ml) of the cultures were collected by centrifugation (5,100 × *g*, 10 min) and washed twice with 20 ml of phosphate-buffered saline (0.02 M Na_2HPO_4–0.15 M NaCl, pH 7.4) before being suspended in and diluted with PBS (pH 7.4). The washed, undiluted bacterial suspensions contained <10 HU of cytolytic activity (against mouse erythrocytes) per ml. Groups of mice (10 mice per group) were injected subcutaneously with portions (0.1 ml) of twofold serial dilutions of the bacterial suspensions, and the number of CFU injected into the mice was determined by plate counts on Columbia agar. The toxin lethality studies were performed by injecting groups of mice (10 mice per group) intraperitoneally (i.p.), intravenously (i.v.), or subcutaneously (s.c.) with portions (0.2 ml for i.p. and i.v. injections, 0.1 ml for s.c. injections) of membrane filter-sterilized solutions of the partially purified cytolysin concentrate in PBS (0.067 M Na_2HPO_4–0.077 M NaCl, pH 7) containing 1 mg of crystalline bovine albumin (Miles Laboratories, Elkhart, Ind.) per ml.

Culture supernatant fluids and samples (500 μg) of partially purified cytolysin preparations in PBS (pH 7.4) were assayed for protease activity against azocasein as previously described (17), except the assay mixtures were incubated for 20 min instead of 10 min at 37°C.

Partially purified cytolysin preparations were assayed for protein by the Bradford method (7), with bovine gamma globulin as the standard. The standard and the assay reagent were obtained from Bio-Rad Laboratories, Inc. (Richmond, Calif.).

Antisera. Rabbit antiserum against a crude preparation of an extracellular cytolysin produced by *Vibrio vulnificus* strain E4125 was obtained as previously described (18). Antiserum against the partially purified cytolysin concentrate of *V. damsela* strain 1421-81 was raised in the same manner, except the initial vaccination dose and the booster vaccination dose (4 weeks later) were 0.2 mg of toxin concentrate. Rabbit antiserum against the thermostable direct (Kanagawa phenomenon-associated) hemolysin of *Vibrio parahaemolyticus* strain WP-1 was kindly supplied by D. J. Grimes and R. R. Colwell (University of Maryland, College Park). Rabbit antiserum raised against the cytolysin produced by *V. cholerae* biotype El Tor was kindly supplied by R. A. Finkelstein (University of Missouri, Columbia).

Inactivation studies. Samples (10,000 HU, assayed against washed mouse erythrocytes) of the partially purified cytolysin concentrate in PBS (pH 7) (1 ml, final volume) were incubated for 15 or 30 min at 37°C with various amounts of soluble Pronase-CB (Calbiochem-Behring, La Jolla, Calif.), bovine pancreatic trypsin (3× crystallized; Worthington Diagnostics, Freehold, N.J.), dithiothreitol (electrophoresis grade; Bio-Rad Laboratories), mixed bovine brain gangliosides (type II grade; Sigma Chemical Co., St. Louis, Mo.), cholesterol (Sigma), trypan blue (Eastman Kodak Co., Rochester, N.Y.), disodium EDTA, and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), and the mixtures were assayed for residual cytolytic activity against washed mouse erythrocytes. Samples of toxin also were tested for heat sensitivity (37, 56, and 100°C for 30 min) at pH 7 and for sensitivity to exposure to low pH (0.1 M acetate buffer, pH 4) and high pH (0.1 M glycine buffer, pH 10) for 24 h at 4°C.

The ability of sera raised against preparations of *V. damsela* cytolysin, *V. vulnificus* cytolysin (18), the thermostable direct (Kanagawa phenomenon-associated) hemolysin of *V. parahaemolyticus* (kindly supplied by D. J. Grimes and R. R. Colwell, University of Maryland), and the cytolysin produced by *V. cholerae* biotype El Tor (15) to neutralize the homologous and heterologous toxins also was examined. Mixtures (1 ml, final volume) containing 10 HU of toxin in PBS (pH 7)–bovine albumin and various amounts of heat-inactivated (56°C, 30 min) antiserum or normal rabbit serum were incubated for 30 min at 37°C. The toxin-serum mixtures then were incubated for 30 min at 37°C with portions (1 ml) of a washed mouse erythrocyte suspension standardized as described by Bernheimer and Schwartz (5), and the amount of serum that reduced the 10 HU of activity to less than 1 HU of activity was determined.

Gel filtration. Gel filtration was performed with a water-cooled (5°C) column (2.6 by 95 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with PBS (pH 7.4). A 30-mg amount of the partially purified cytolysin concentrate dissolved in 3 ml of equilibrating buffer was applied to the column and eluted in the downward flow mode at a flow rate of ca. 20 ml/h (3.8 ml/cm^2 per h) with the equilibrating buffer. Fractions (ca. 5 ml) were collected at 5°C and assayed for cytolytic activity against washed mouse erythrocytes and for absorbance at 280 nm. The peak fractions of cytolytic activity and of a noncytolytic, 280 nm-absorbing peak also were examined for lethal activity in mice. Blue dextran was used to determine the column void volume and the homogeneity of column packing. The apparent molecular weight of the cytolysin was estimated by the Andrews plot method (1).

Isoelectric focusing. The partially purified cytolysin concentrate was fractionated by high-speed electrofocusing (20, 28) in pH 3.5 to 10 and pH 5 to 7 sucrose density gradients formed at 15 W for 18 h with a LKB 8100-1 column (LKB Instruments, Gaithersburg, Md.). Two milligrams of cytolysin concentrate was fractionated in the pH 3.5 to 10 gradient, and 3 mg was used in the pH 5 to 7 gradient. The pH of each fraction (4 ml) was determined at 4°C and the fractions were assayed for cytolytic activity against washed mouse erythrocytes. The peak fractions of cytolytic activity also were examined for lethal activity in mice.

RESULTS AND DISCUSSION

***V. damsela* cytolysin production and virulence for mice.** Of the 19 *V. damsela* strains examined, 17 produced detectable amounts of extracellular cytolysin active against mouse erythrocytes (Table 1). The activity in the culture superna-

TABLE 1. Virulence of and extracellular cytolysin production by *V. damsela* strains

Strain	Isolated from:	LD_{50}[a]	HU/ml[b]
9045-81	Animal (Hawaii)	$>9 \times 10^8$	<2
1471-82	Human (Louisiana)	$>9 \times 10^8$	<2
1424-81	Fish (Senegal)	$>9 \times 10^8$	8
9051-81	Human (Louisiana)	$>9 \times 10^8$	20
1423-81	Fish (Senegal)	$>9 \times 10^8$	25
9046-81	Human (Louisiana)	$>9 \times 10^8$	30
1958-80	Human (Florida)	$>9 \times 10^8$	60
2588-80	Fish (California)	$>9 \times 10^8$	180
9048-81	Human (Florida)	9×10^8	2
0183-79	Human (Hawaii)	9×10^8	30
9049-81	Human (Hawaii)	9×10^8	30
9047-81	Unknown (Puerto Rico)	9×10^8	50
0023-81	Sewage (Florida)	6×10^8	20
9050-81	Human (Michigan)	3×10^8	60
1420-81	Fish (Senegal)	3×10^7	70,000
2227-81	Human (Massachusetts)	3×10^7	28,000
0727-82	Human (Florida)	3×10^7	26,000
1421-81	Fish (Senegal)	2×10^7	75,000
1422-81	Fish (Senegal)	2×10^7	26,000

[a] The bacteria were grown in HI broth, and the LD_{50} values were determined by s.c. injection of mice with washed bacteria as described in the text. The largest challenge dose tested was ca. 9×10^8 CFU.

[b] The bacteria were grown in HI broth, and the culture supernatant fluids were obtained by centrifugation (5,100 × *g*, 10 min) and assayed for cytolytic activity against washed mouse erythrocytes and for protease activity against azocasein as described in the text. Protease activity was not detected in the culture supernatant fluids.

tant fluids from heart infusion broth cultures ranged from 2 to 75,000 HU/ml, and the five strains that produced the largest amounts of toxin (26,000 to 75,000 HU/ml) had LD_{50} values, for subcutaneously infected mice, that were ca. 10-fold to >30-fold lower than those of the 12 strains that produced relatively small amounts of cytolysin (2 to 180 HU/ml). Fatally infected mice usually died 12 to 48 h postinfection, and *V. damsela* (ca. 10^6 CFU/ml) was isolated from the

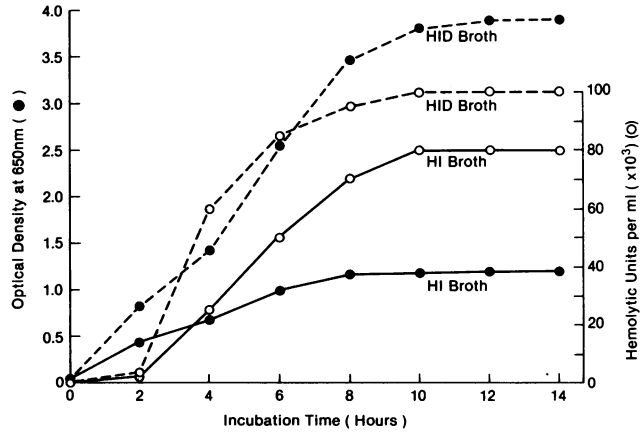

FIG. 1. Growth and cytolysin production by *V. damsela* strain 1421-81 in HI and HID broths. Two-liter flasks containing 200 ml of broth were inoculated with a seed culture suspension prepared as described in the text and were incubated at 30°C on a gyrotory shaker operating at 220 cycles per min. Growth was followed turbidimetrically at 650 nm (1-cm light path) in a model DB-GT spectrophotometer (Beckman Instruments), and culture supernatant fluids were obtained by centrifugation (5,100 × *g*, 10 min) and assayed for cytolytic activity against washed mouse erythrocytes and for protease activity against azocasein. Cytolysin activity was stable for at least 24 h of incubation, and protease activity was not detected in the culture supernatant fluids (data not shown).

TABLE 2. Effect of Na^+ ion concentration on growth and extracellular cytolysin yield by *V. damsela* strain 1421-81 in HID and HI broths

Broth	Na^+ (%)[a]	OD_{650} of culture[b]	% of maximal activity
Basal HID	0.5	4.3	100[c]
HID	0.8	5.1	14
HID	1.2	5.2	3
HID	1.6	5.5	0.7
HID	2.0	5.6	0.3
Basal HI	0.3	1.2	67
HI	0.5	2.6	100

[a] The Na^+ ion concentrations in the autoclaved basal HI and HID broths were determined with an Astra-8 analyzer equipped with a Na^+ ion-specific electrode (Beckman Instruments, Inc., Fullerton, Calif.). The HID broths and the HI broth containing more than 0.5 and 0.3% Na^+, respectively, were prepared by supplementing the basal media with NaCl.

[b] The optical density at 650 nm (OD_{650}) was determined after incubation with agitation at 30°C for ca. 14 h.

[c] Approximately 90,000 HU/ml of culture supernatant fluid, assayed against washed mouse erythrocytes as described in the text.

TABLE 3. Sensitivity of erythrocytes from various animal species to *V. damsela* strain 1421-81 cytolysin partially purified by ammonium sulfate precipitation

Species[a]	Sp act (HU/mg of dry wt)[b]	Relative sensitivity (%)[c]
Mouse	90,000, 100,000, 100,000, 110,000, 110,000	100
Rat	70,000, 70,000, 70,000	70
Rabbit	150, 400, 800, 1,000, 3,000	0.15 to 3
Cat	400	0.4
Goat	250	0.25
Dog	200	0.2
Horse	175	0.18
Pig	150	0.15
Monkey	75, 75, 75, 90	0.08 to 0.09
Burro	60	0.06
Guinea pig	50	0.05
Pigeon	40, 45, 50	0.04 to 0.05
Chicken	20	0.02
Human	15[d]	0.015
Sheep	15	0.015
Cow	1.5	0.0015

[a] All erythrocytes were obtained from young adults. The sources of the bloods used to prepare the erythrocyte suspensions were as follows: mouse, Dub: (ICR) strain (Dominion Laboratories, Dublin, Va.); rat, Sprague-Dawley strain, kindly supplied by R. Joines of the Surgery Research Laboratories of the Bowman Gray School of Medicine; rabbit, New Zealand white (Franklin Rabbitry, Wake Forest, N.C.); cat, dog, burro, and guinea pig, Colorado Serum Co. (Denver, Colo.); goat, horse, pig, chicken, sheep, and cow, Granite Diagnostics, Inc. (Burlington, N.C.); pigeon, Department of Comparative Medicine (Bowman Gray School of Medicine); cynomolgus monkey (*Macaca fasicularis*) and human, kindly supplied by C. Keever and A. Beard, respectively, of the Tissue Typing Laboratory of the Bowman Gray School of Medicine.

[b] Multiple values were obtained with erythrocyte suspensions prepared from individual mice, rats, rabbits, monkeys, and pigeons.

[c] Compared with sensitivity of mouse erythrocytes.

[d] No difference noted among O (Rh-positive), O (Rh-negative), A (Rh-positive), and B (Rh-positive) erythrocytes.

TABLE 4. Stability of *V. damsela* strain 1421-81 cytolysin partially purified by ammonium sulfate precipitation

Treatment[a]	Residual activity (%)
4°C, pH 7, 24 h	100[b]
4°C, pH 4, 24 h	27
4°C, pH 10, 24 h	82
37°C	100
56°C	0.5
100°C	0.5
Pronase, 25 μg	3
Pronase, 10 μg	18
Trypsin, 25 μg	41
Trypsin, 10 μg	64
Dithiothreitol, 5×10^{-3} M, 15 min[c]	50
Dithiothreitol, 1×10^{-3} M, 15 min	91
EDTA, 1×10^{-3} M, 15 min	50
EGTA, 1×10^{-3} M, 15 min	60
Cholesterol, 100 μg[d]	100
Trypan blue, 50 μg[e]	100
Mixed gangliosides, 100 μg	100

[a] Unless otherwise indicated, all treatments were at 37°C and pH 7 for 30 min.

[b] 10,000 HU/ml; assayed against washed mouse erythrocytes as described in the text.

[c] Conditions commonly used to activate thiol-activated (oxygen-labile) cytolysins (4, 9, 26).

[d] Commonly used to inactivate thiol-activated cytolysins (4, 10, 26).

[e] Common used to inactivate streptolysin S (10, 13).

heart blood of dead and dying mice. The infected mice were lethargic and had ruffled fur, encrustations around the eyelids, and severe local edema around the injection site.

Some of the factors influencing the yield of toxin produced by one of the virulent strains (1421-81) were examined. Toxin yield was optimal during the mid- and late-logarithmic phases of growth in medium containing 0.5% Na^+ ion (equivalent to ca. 1.3% NaCl), was stable during the stationary growth phase, and was significantly reduced in culture medium containing ≥0.8% Na^+ ion, even though Na^+ ion concentrations ranging from 0.8 to 2% (equivalent to ca. 2 to 5% NaCl) significantly enhanced growth of the bacterium (Fig. 1 and Table 2). At the present time, it is not known whether the decreased yield of toxin in culture medium containing ≥0.8% Na^+ ion is caused by Na^+ ion-mediated inhibition of toxin production or inhibition of toxin release.

The stability of toxin activity during the stationary growth phase may be explained by the absence of detectable protease production by *V. damsela* and, therefore, the absence of proteolytic degradation of the toxin. Incubation of a virulent strain culture of *V. vulnificus* past the mid-logarithmic phase of growth has been reported to cause a progressive loss of extracellular cytolytic activity, which correlated with the appearance of proteolytic activity in the culture supernatant fluids (18).

The partially purified cytolysin concentrate prepared by ammonium sulfate precipitation of the toxin in culture supernatant fluids of strain 1421-81 contained ca. 1×10^5 HU/mg of dry weight (assayed against mouse erythrocytes) or ca. 6×10^5 HU/mg of protein, and did not contain detectable amounts of protease activity. The recovery of activity was ca. 75%.

Hemolytic spectrum and lethal activity of the partially purified cytolysin preparation. The hemolytic spectrum of the *V. damsela* cytolysin differed from that of cytolysins produced by *V. vulnificus* (18) and *V. cholerae* biotype El Tor (15) and from the thermostable direct (Kanagawa phenomenon-associated) hemolysin of *V. parahaemolyticus* (29). The cytolysin preparation was active against erythrocytes from 16 animal species; however, mouse and rat erythrocytes were significantly more sensitive than were erythrocytes from the other 14 species (Table 3). The narrow hemolytic spectrum of the cytolysin suggests that it may prove useful, as have other membrane-damaging toxins, as a probe in studies of membrane structure (3, 12, 21, 25). At the present time, the reason(s) for the marked sensitivity of mouse and rat erythrocytes to the *V. damsela* cytolysin is not known. However, the observation suggests the possibility, which is open to experimental evaluation, that mouse and rat erythrocytes possess larger numbers of exposed, specific receptors for the toxin than do erythrocytes of other animal species.

The lethal activity of the cytolysin preparation was heat labile (56°C, 30 min) and was observed after i.p., i.v., and s.c. injection in mice. The LD_{50}s by the i.p., i.v., and s.c. routes were ca. 0.83 μg (83 HU) per mouse (30 μg/kg), 1.2 μg (120 HU) per mouse (43 μg/kg), and 10 μg (1,000 HU) per mouse (357 μg/kg), respectively. Mice injected s.c. with 1 LD_{50} (1,000 HU) were lethargic and had ruffled fur, encrustations around the eyelids, and severe local edema similar to that observed in mice lethally infected with *V. damsela* via the s.c. route. The survival time of mice injected with the toxin preparation varied inversely with the quantity of preparation administered. Mice injected i.p. with 2 LD_{50}s usually survived for 3 to 16 h, whereas mice injected with 20 LD_{50}s commonly survived for only 20 to 30 min. The effect of the route of administration on the lethal activity of the *V. damsela* cytolysin preparation differed from that previously observed with *V. vulnificus* cytolysin preparations, which were significantly less lethal to mice when injected by the i.p. route than when administered by the i.v. route (18).

Stability and physicochemical properties of the *V. damsela*

TABLE 5. Sensitivity of *Vibrio* spp. cytolysins to neutralization with homologous and heterologous antisera

Cytolysin source	Amt (μl) of antiserum against cytolysin from the following species that is required to inhibit >90% of the activity of 10 HU[a]:			
	V. damsela	*V. vulnificus*	*V. parahaemolyticus*	*V. cholerae*
V. damsela strain 1421-81	20	>200	>200	>200
V. vulnificus strain E4125	>200	1	>200	>200
V. parahaemolyticus strain WP-1	>200	>200	40	>200
V. cholerae strain 26-3, biotype El Tor	>200	>200	>200	10

[a] The toxin-serum mixtures were incubated for 30 min at 37°C before testing for residual activity. The largest amount of serum tested was 200 μl. Serum from nonvaccinated rabbits did not neutralize the cytolysins.

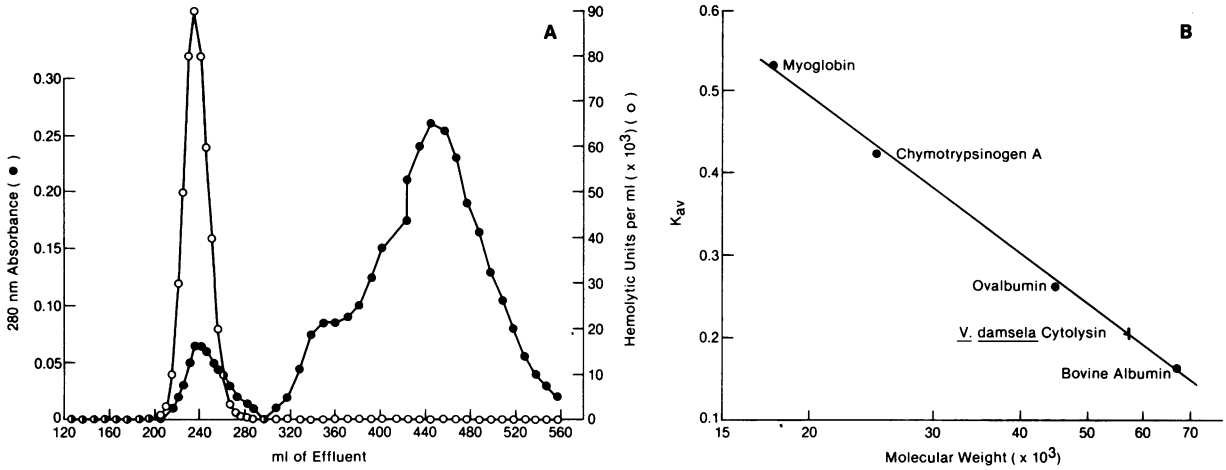

FIG. 2. Fractionation of the partially purified cytolysin concentrate of *V. damsela* 1421-81 by gel filtration with Sephadex G-100. (A) Elution pattern of the cytolysin concentrate. Fractions (ca. 5 ml) were assayed for cytolytic activity against washed mouse erythrocytes and for absorbance at 280 nm. The column void volume and bed volume were ca. 166 and 504 ml, respectively. (B) Estimation of apparent molecular weight of *V. damsela* cytolysin by the Andrews plot method (1).

cytolysin. The cytolysin was heat labile, was unstable at pH 4 and 10, and was inactivated by proteases, chelating agents, and dithiothreitol (Table 4). Activity was unaffected by cholesterol, trypan blue, and a mixed ganglioside preparation. The data indicate that the cytolysin is a protein that requires metal ions for optimal activity and that it may be differentiated from (i) the thermostable direct (Kanagawa phenomenon-associated) hemolysin of *V. parahaemolyticus* (22); (ii) thiol-activated (oxygen-labile) cytolysins, which are activated by dithiothreitol and inactivated by cholesterol (4, 9, 10, 26); (iii) streptolysin S, which is inactivated by trypan blue (10, 13); and (iv) the cytolysin produced by *V. cholerae* biotype El Tor and the Kanagawa phenomenon-associated hemolysin of *V. parahaemolyticus*, which are inactivated by mixed ganglioside preparations (15, 27). In addition, the results of antiserum neutralization studies indicated that the *V. damsela* cytolysin is antigenically distinct from previously described cytolysins produced by other *Vibrio* species (Table 5).

Gel filtration of the partially purified cytolysin preparation with Sephadex G-100 revealed one peak of cytolytic activity that was well resolved from a large, noncytolytic, 280 nm-absorbing peak and had an elution volume consistent with an apparent molecular weight of ca. 57,000 (Fig. 2). The cytolytic peak fractions, but not the noncytolytic peak fractions, also were lethal for mice. The molecular weight estimated for the *V. damsela* cytolysin differs from the molecular weights reported for cytolysins produced by *V. vulnificus* and *V. cholerae* biotype El Tor and for the Kanagawa phenomenon-associated hemolysin of *V. parahaemolyticus*, which have molecular weights (estimated by gel filtration) of ca. 38,500 (18), 20,000 (15), and 42,000 or 44,000 (16, 22), respectively.

Isoelectric focusing of the partially purified cytolysin preparation in pH 3.5 to 10 and pH 5 to 7 sucrose density gradients revealed only one peak of cytolytic activity having an isoelectric point of ca. 5.7 (Fig. 3). The cytolytic peak fractions also were lethal for mice. The isoelectric point of the *V. damsela* cytolysin differs from the published values of 4.2 (16) and 4.9 (22) for the Kanagawa phenomenon-associated hemolysin of *V. parahaemolyticus*.

In conclusion, this paper contains data on the production

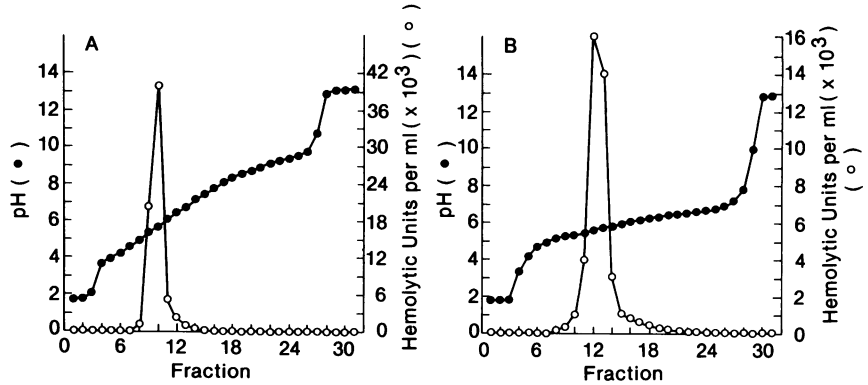

FIG. 3. Isoelectric focusing of the partially purified cytolysin concentrate of *V. damsela* strain 1421-81. The pH of each fraction (ca. 4 ml) was determined at 4°C, and the fractions were assayed for cytolytic activity against mouse erythrocytes. (A) Results obtained with a pH 3.5 to 10 gradient. (B) Results obtained with a pH 5 to 7 gradient.

and some of the properties of a previously undescribed cytolytic toxin produced by *V. damsela*. In addition, examination of 19 strains of the bacterium for mouse virulence and toxin production indicates a correlation between the ability of the bacterium to cause disease in mice and to produce large amounts of the toxin in vitro. Furthermore, s.c. injection of mice with bacteriologically sterile preparations of the partially purified toxin elicits grossly observable changes similar to those observed during the lethal experimental infection caused by s.c. injection of *V. damsela*. At the present time, it is not known whether the toxin plays an important role in the pathogenesis of disease caused by *V. damsela* in its usual hosts (i.e., damselfish and humans). However, the new knowledge concerning the existence of the toxin and the correlation of mouse virulence with toxin production suggests that further studies designed to rigorously examine this question are justified.

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