INFECTION AND IMMUNITY, Oct. 1981, p. 115-119
0019-9567/81/100115-05$02.00/0

Vol. 34, No. 1

Effect of pH on the Production of the Kanagawa Hemolysin by *Vibrio parahaemolyticus*

JOHN W. CHERWONOGRODZKY* AND A. GAVIN CLARK

Department of Microbiology and Parasitology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

Received 13 March 1981/Accepted 25 June 1981

Production of the Kanagawa hemolysin by patient strains of *Vibrio parahaemolyticus* was found to respond to the pH rather than to the type of carbohydrate present in the growth medium. Regardless of the carbohydrate present, hemolysin production in peptone broth cultures occurred only when the pH was between 6.5 and 5.5. Mannitol, the sugar used in the Wagatsuma agar, lowered the pH to within this range, thus providing optimal conditions for hemolysin production. Glucose and mannose, although readily metabolized, lowered the pH below this range, inhibiting growth and hemolysin production. Alkaline cultures either without carbohydrates or containing non-metabolizable sugars showed little hemolytic activity because the pH always remained alkaline. In pH-stat cultures maintained at pH 6.2, higher hemolysin yields were produced irrespective of the presence or absence of mannitol. We conclude that the production of the Kanagawa hemolysin is under pH control. Marine strains of *V. parahaemolyticus*, which are Kanagawa negative, did not express detectable amounts of hemolysin under those conditions shown to stimulate hemolysin production by Kanagawa-positive strains.

The enteropathogenicity of *Vibrio parahaemolyticus* is closely associated with the production of a thermostable hemolysin (6, 7, 14, 18). For over a decade a special mannitol-blood agar, the Wagatsuma agar (16), has proved a reliable test medium for discriminating putatively pathogenic strains of *V. parahaemolyticus* from those saprophytic strains usually found in the marine environment (11). As both types produce other hemolysins (10), whereas only the former produces a thermostable hemolysin (8), it is poorly understood how the Wagatsuma medium is so effective in this differentiation.

Chun et al. (3) observed that although fermentable carbohydrates enhanced production of the general hemolysins, only mannitol in the test agar caused expression of the Kanagawa thermostable hemolysin by the pathogenic strains. This paper explores the effect of pH on hemolysin production by *V. parahaemolyticus* and gives insight into why mannitol, the carbohydrate in the Wagatsuma agar, selectively distinguishes the nonhemolytic marine isolates from the hemolytic, putatively pathogenic strains of *V. parahaemolyticus*.

MATERIALS AND METHODS

Cultures examined. Test strains of *V. parahaemolyticus* (Table 1) were kindly supplied by G. I. Barrow, Public Health Laboratory Service, Truro, U.K.; R. R. Colwell, Department of Microbiology, University of Maryland, College Park, Md.; the late M. Fishbein, Food and Drug Administration, Washington, D.C.; and R. Sakazaki, National Institute of Health, Tokyo, Japan. Where indicated, strains were obtained from the National Collection of Marine Bacteria (NCMB), Torry Research Station, Aberdeen, Scotland; or the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, London.

Culture media. Kanagawa hemolysis was tested on Wagatsuma blood agar (17). Unless otherwise stated, peptone growth media contained 2.0% peptone (Difco Laboratories) and 3.0% NaCl in 0.03 M KH_2PO_4-K_2HPO_4 buffer at the described pH. Carbohydrates were sterilized separately by filtration through 0.45-μm membranes (Millipore Corp.) before being added to sterilized media to a final concentration of 0.5%. All chemicals were of analytical grade.

Culture conditions. All cultures were incubated at 37°C. Strains were subcultured at least three times in 50 ml of peptone broths (pH 8.0) with shaking at 250 rpm. For each experiment, the third or greater subcultured inoculum was harvested at 8 h, washed in sterile 3.0% NaCl, and diluted in test media to an optical density at 600 nm (OD_{600}) of 0.003 nm. When prolonged hemolysin sampling was necessary, the volumes were increased to 600 ml in 2,800-ml Fernbach flasks, incubated at 37°C, and shaken at 225 rpm to achieve aeration rates equivalent to those of the smaller-volume cultures. For pH-stat studies, a Fernbach flask was modified by adding two sidearms near the base. One opening held a combination pH electrode (Analytical Instruments, Mississauga, Ontario), and the other held the pH control lines. Sterile 1.0 M NaOH and 1.0 M HCl were delivered to the flask by

TABLE 1. *Origin and designation of V. parahaemolyticus strains*

Strain designation	Kanagawa hemolysis[a]	Isolation source	Donor or depositor
NCTC 10885	−	Oysters, United Kingdom	Barrow
FC-1011	−	Crab, Chesapeake Bay	Colwell
162-71	−	Fish, Japan	Colwell (Sakazaki)
5A52B	−	Bluefish, Atlantic	Fishbein
7A82K	−	Sea trout, Atlantic	Fishbein
NCTC 10886	+	Stool, Far East	Barrow
33C10	+	Stool, Japan	Fishbein
8700	+	Stool, Maryland	Fishbein
NCMB 1175	+	Stool, Japan	Sakazaki
NCMB 1190	+	Stool, Japan	Sakazaki

[a] Tested on Wagatsuma agar (17).

Watson-Marlow delta-B pumps connected to a Chemtrix type 45 E pH controller. This latter instrument was standardized against a Fisher Accumet pH meter model 600. The medium volume was 600 ml as described above.

Determination of growth. Growth in the fluid medium was determined turbidimetrically at 600 nm, using a Unicam SP 1800 spectrophotometer (Pye Instruments, Cambridge).

Determination of hemolytic activity. Human erythrocytes, obtained from the Toronto Red Cross, were washed three times in 0.9% NaCl containing 0.01 M KH_2PO_4-K_2HPO_4 (pH 7.0). The packed erythrocytes (1,000 × *g* for 10 min) were resuspended to 1% (vol/vol at 1.4 × 10^6 cells/ml) in the same buffered saline. A standard of 100% lysis was established with 0.1% Na_2CO_3 (15). To assay the hemolytic activity of the cell-free supernatants, partially purified samples (see below) were first heat shocked at 90°C for 10 min to inactivate thermolabile hemolysins (10) before being twofold serially diluted in 1.0 ml of buffered saline. One-milliliter portions of the 1% erythrocyte suspension were added and, after incubation at 37°C in a water bath for 2 h, the cells were allowed to settle for 12 h at 4°C. One hemolytic unit (HU) is that amount of Kanagawa hemolysin which causes 50% lysis of the erythrocytes under the above conditions. As an internal standard against erythrocyte heterogeneity between batches, a partially purified preparation from the culture supernatant of strain NCTC 10886 (Kanagawa positive) which had been diluted to 2 HU/ml, frozen, and stored at −20°C was used in each assay.

Partial purification of the hemolysin. Hemolysin was concentrated and partially purified from the culture supernatant by the method of Honda et al. (7). A 10.5-g amount of ammonium sulfate was dissolved into 30 ml of culture supernatant to give 55% saturation (5). The mixture was chilled at 4°C for 8 h and centrifuged at 12,000 × *g* for 10 min. The pellet was dissolved in 2 ml of buffered saline before being dialyzed for 8 h at 4°C against at least 100 volumes of buffered saline. The hemolytic activity of the culture, measured as described above, was then expressed as HU per milliliter of original supernatant.

Cell lysis solutions. Thirty milliliters of culture was centrifuged at 12,000 × *g* for 10 min, the supernatant was removed, and the cells were suspended in 1 ml of 0.73 M sucrose in 50 mM tris(hydroxymethyl)aminomethane and 1 mM disodium ethylenediaminetetraacetic acid at pH 8.0. To this suspension was added 0.4 ml of a 10-mg/ml concentration of lysozyme in 0.25 M tris(hydroxymethyl)aminomethane at pH 8.0. After incubation at room temperature for 20 min, 0.8 ml of 0.5 M disodium ethylenediaminetetraacetic acid at pH 8.0 was added, and the mixture was further incubated for 20 min at room temperature before being diluted with 27.8 ml of distilled water. Lysis was complete as judged by microscopic examination.

Hemolysin release at different pH values. Strain NCTC 10886 was inoculated into two 600-ml Fernbach cultures, one at pH 6.2 and the other at pH 8.0. When midlogarithmic growth reached 0.4 (OD_{600}), the cultures were centrifuged and the cells from each were suspended separately in 10 ml of sterile 3.0% NaCl. Each suspension was then split between two 300-ml portions of 3.0% NaCl in 0.03 M KH_2PO_4-K_2HPO_4 containing 25 μg of chloramphenicol per ml, one at pH 6.2 and the other at pH 8.0. Over the course of 4 h at 37°C, 30-ml samples were removed from each of the four test solutions and centrifuged at 12,000 × *g* for 10 min, and the supernatant was assayed for hemolytic activity as previously described.

Mannitol determination. Mannitol was assayed by the method of Burton (1).

RESULTS AND DISCUSSION

Effect of pH. When peptone broths with mannitol (pH 8.0) were inoculated with strains of *V. parahaemolyticus*, the hemolytic, Kanagawa-positive isolates produced a thermostable hemolysin, whereas the nonhemolytic, Kanagawa-negative isolates did not (Table 2). However, the sensitivity of the quantitative assay was sufficient to show that even in the absence of mannitol, the Kanagawa-positive cultures produced detectable amounts of hemolysin but the Kanagawa-negative strains did not.

These same strains, when inoculated into

TABLE 2. *Effect of mannitol on hemolysin production by V. parahaemolyticus in peptone media at an initial pH 8.0*

Peptone medium	Strain[a]	Final pH	HU/ml
With mannitol	NCMB 1190 (K+)	5.7	0.12
	NCTC 10886 (K+)	7.7	0.54
	NCTC 10885 (K−)	7.9	0
	5A52B (K−)	7.9	0
Without mannitol	NCMB 1190 (K+)	8.1	0.04
	NCTC 10886 (K+)	8.5	0.24
	NCTC 10885 (K−)	8.1	0
	5A52B (K−)	8.3	0

[a] K+, Kanagawa positive; K−, Kanagawa negative.

either mannitol-free peptone broths (Fig. 1) or peptone broths containing mannitol (data not shown) over a range of initial pH values, gave higher total hemolysin yields with decreasing pH at inoculation.

Despite this evidence that pH affects the production of the thermostable hemolysin, the results did not differentiate between the effect of initial pH alone and the effect of pH changes throughout growth. Further studies with strain NCTC 10886 in a carbohydrate-free peptone medium at pH 8.0 showed that the culture experienced little pH variation over 24 h of growth and that hemolytic activity was below 0.2 HU/ml throughout this time. If the same medium was inoculated at an initial pH of 6.0, the culture became alkaline over the course of 24 h, with marked hemolysin production occurring in the exponential phase when the pH was below 6.4 (Fig. 2). Thereafter the hemolysin production decreased despite a continued growth.

Effect of carbohydrates. If nonfermentable carbohydrates were added (e.g., lactose) to a peptone medium of pH 8.0 (Fig. 3), then growth, pH, and hemolysin production differed little from that obtained with peptone alone at pH 8 as described above. Galactose was poorly fermented by strain NCTC 10886, but once the pH fell below neutrality, hemolytic production started and continued to increase throughout the early phase of the culture (Fig. 3).

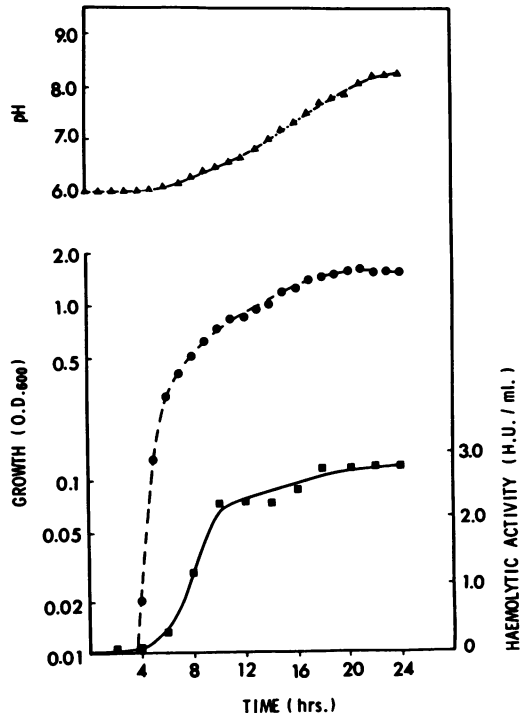

FIG. 2. *Hemolysin production (■), growth (●), and pH changes (▲) of hemolytic strain NCTC 10886 in mannitol-free peptone broth with an initial pH value of 6.0.*

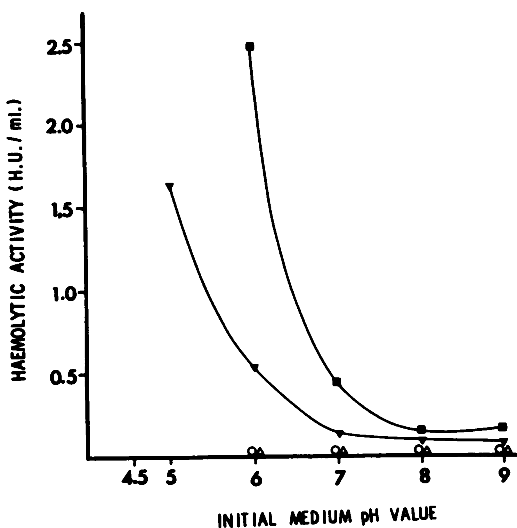

FIG. 1. *Effect of initial medium pH on the final hemolysin yields for hemolytic and nonhemolytic strains of V. parahaemolyticus. Hemolytic (NCTC 10886, ■, NCMB 1190, ▼) and nonhemolytic (NCTC 10885, ○; 5A52B, △) strains were grown for 24 h in mannitol-free peptone broth at the indicated initial pH values. Only strain NCMB 1190 grew at pH 5.0; no strain grew at pH 4.5.*

For the rapidly metabolized carbohydrates, such as glucose and mannose, their utilization had a marked influence on pH during growth (Fig. 4). All caused a rapid decline in the pH of the medium to a value that remained acidic for the remainder of the growth cycle. This minimum pH was reached at the time of an abrupt onset of stationary phase. A short period of hemolysin production was evident in such cases only during the late exponential or early stationary phase of growth. Mannitol was unique. In media of pH 8 initially, the culture experienced a decrease in pH that was both at a slower rate and to a final value less acidic than that obtained with glucose (Fig. 4). The production of the thermostable hemolysin in the mannitol culture began when the pH dropped below 6.5 but did not continue as the pH continued to rise back to pH 8. When the strains listed in Table 1 were also grown in a peptone-mannitol medium at the initial pH of 8.0, all gave similar ranges of growth and pH changes. However, only the Kanagawa-positive isolates showed hemolysin production under these conditions, and all but one correlated with the critical pH range found for NCTC 10886. Strain NCMB 1190, a weak Kanagawa-

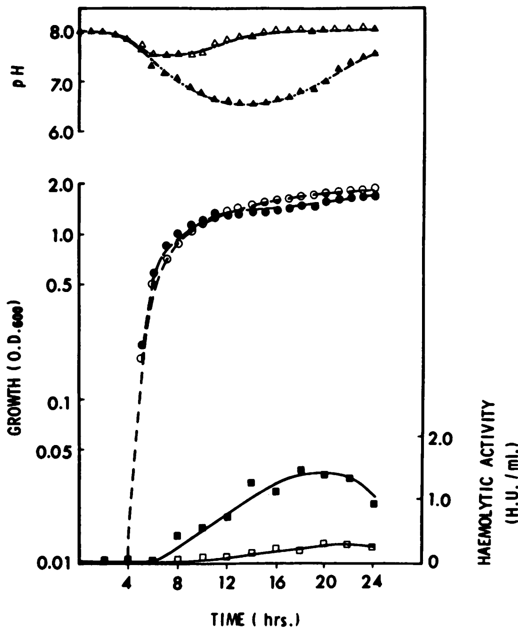

FIG. 3. *Response of Kanagawa-positive V. parahaemolyticus NCTC 10886 to peptone media with galactose (solid symbols) or lactose (open symbols). Growth (●, ○); hemolytic activity (■, □), and medium pH (▲, △).*

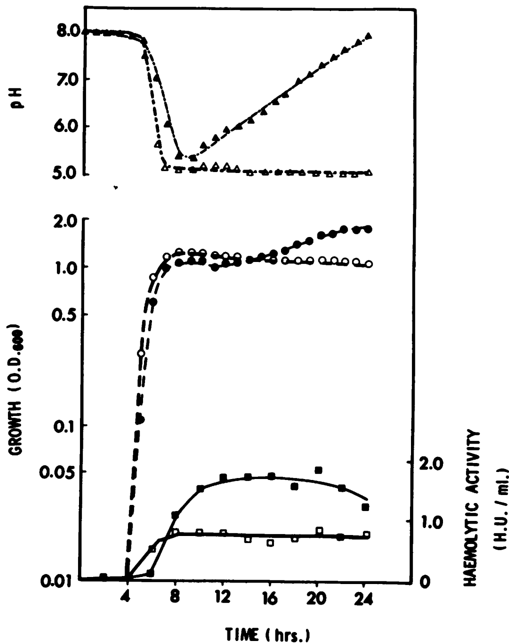

FIG. 4. *Response of Kanagawa-positive V. parahaemolyticus NCTC 10886 to peptone media with mannitol (solid symbols) or glucose (open symbols). Growth (●, ○), hemolytic activity (■, □), and medium pH (▲, △).*

positive strain in the Wagatsuma test, differed from the other patient isolates in that the minimal pH attained in the peptone-mannitol medium was above the usual value; i.e., less acid was produced. As this culture also became alkaline after the mannitol was metabolized, the time spent in the critical pH range needed for hemolysin production was less than that for the other Kanagawa-positive strains, and we subsequently found that the hemolysin yield for NCMB 1190 was also decreased.

For the above findings, other possible explanations for increased hemolytic activity, other than hemolysin production within a critical pH range, were considered and discarded. The possibility that the hemolysin was stable only in acidic conditions was discounted by incubating purified hemolysin in buffers ranging from 5.5 to 9.5 for 24 h at 37°C (J. Cherwonogrodzky and A. G. Clark, unpublished data). The hemolysin did not lose its activity in alkaline pH media. Because several bacterial toxins are released only upon cell lysis (4, 9, 13, 11), NCTC 10886 was grown in a peptone medium with a pH-stat of 8.0 ± 0.1. The cells were lyzed by the lysozyme-ethylenediaminetetraacetic acid-distilled water treatment, but no hemolysin was released irrespective of growth phase. Finally, following the method of Callahan and Richardson (2), the possibility of pH-controlled release of hemolysin was examined. When logarithmic-phase cells, grown in peptone media of pH 6.2 or 8.0, were shifted to the alternate pH in buffered saline with chloramphenicol, the shift to either pH did not cause a release of hemolysin. Therefore, it appeared that the pH was truly controlling hemolysin production rather than hemolysin release in these cultures at pH 5.5 to 6.5.

pH-stat studies. The role of an acidic pH on hemolysin production rather than carbohydrate source was further supported by growing strain NCTC 10886 in a peptone medium without carbohydrates and under pH-stat conditions. The pH was maintained at 6.2 ± 0.1 throughout the growth cycle (Fig. 5). The hemolysin was produced continuously throughout the growth. The yield under these conditions was an order of magnitude greater than that obtained in pH 6.0 batch culture without pH control (Fig. 2) and two orders of magnitude greater than an uncontrolled pH 8.0 culture. When the pH-stat studies were repeated with mannitol in the medium, although the carbohydrate was fully metabolized, there were no significant differences in the hemolysin yields. No Kanagawa-negative strain produced detectable amounts of hemolysin in these same pH-stat conditions.

We conclude, therefore, that it is the pH resulting from growth, rather than the carbohy-

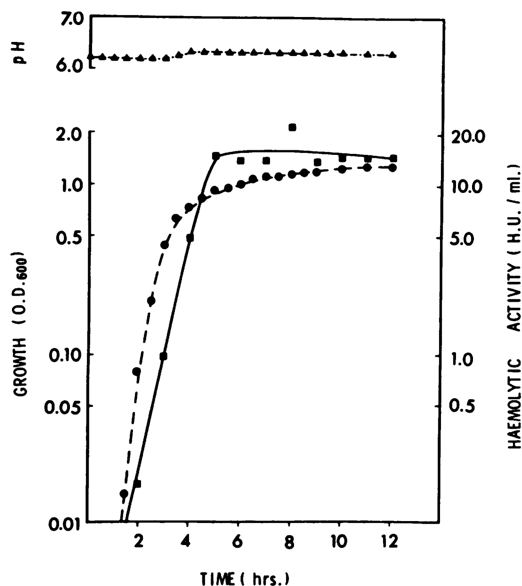

FIG. 5. *V. parahaemolyticus NCTC 10886 (Kanagawa positive) grown under a pH-stat of 6.2 ± 0.1. Growth (●), hemolytic activity (■), and medium pH (▲).*

drates per se, which affects hemolysin production by Kanagawa-positive strains of *V. parahaemolyticus*. The fact that mannitol, the carbohydrate used in the Wagatsuma test medium, is so effective lies in our observations that it lowers the pH to within a range of 5.5 to 6.5, which, in peptone media, is essential for hemolysin production and still suitable for cell growth. We also conclude that the phase of growth appears immaterial. The stationary phase of galactose, the late-logarithmic phase for mannitol, and the entire logarithmic phase for the pH-stat studies all showed hemolysin production provided the critical pH values for hemolysin production were achieved. Unlike *V. cholerae* (2), hemolysin release does not appear to be initiated by or dependent on an alkaline pH.

ACKNOWLEDGMENTS

We acknowledge the assistance of P. Prince and M. Mauldoon of the Toronto Red Cross and the persons mentioned in Table 1 who generously donated strains of *V. parahaemolyticus*. J.W.C. was supported by a Mary H. Beatty fellowship.

LITERATURE CITED

1. **Burton, R. M.** 1957. The determination of glycerol and dihydroxyacetone. Methods Enzymol. **3**:246–249.
2. **Callahan, L. T., III, and S. H. Richardson.** 1973. Biochemistry of *Vibrio cholerae* virulence. III. Nutritional requirements for toxin production and the effects of pH on toxin elaboration in chemically defined media. Infect. Immun. **7**:567–572.
3. **Chun, D., J. K. Chung, R. Tak, and S. Y. Seol.** 1975. Nature of the Kanagawa phenomenon of *Vibrio parahaemolyticus*. Infect. Immun. **12**:81–87.
4. **Granum, P. E., and J. R. Whitaker.** 1980. Improved method for purification of enterotoxin from *Clostridium perfringens* type A. Appl. Environ. Microbiol. **39**:1120–1122.
5. **Green, A. A., and W. L. Hughes.** 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods Enzymol. **1**:67–90.
6. **Honda, T., K. Goshima, Y. Takeda, Y. Sugino, and T. Miwatani.** 1976. Demonstration of cardiotoxic activity of the thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. Infect. Immun. **13**:163–171.
7. **Honda, T., S. Taga, T. Takeda, M. A. Hasibuan, Y. Takeda, and T. Miwatani.** 1976. Identification of lethal toxin with thermostable direct hemolysin produced by *Vibrio parahaemolyticus*, and some physiochemical properties of the purified toxin. Infect. Immun. **13**:133–139.
8. **Kato, T., Y. Obara, H. Ichinoe, K. Nagashima, S. Akiyama, K. Takizawa, A. Matsushima, S. Yamai, and Y. Miyamoto.** 1965. Grouping of *Vibrio parahaemolyticus* (biotype 1) by hemolytic reaction. Shokuhin Eisei Kenkyu **15**:83–86.
9. **Koupal, L. R., and R. H. Deibel.** 1975. Assay, characterization, and localization of an enterotoxin produced by *Salmonella*. Infect. Immun. **11**:14–22.
10. **Miwatani, Z., Y. Takeda, J. Sakurai, A. Yoshihara, and S. Taga.** 1972. Effect of heat (Arrhenius effect) on crude hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **6**:1031–1033.
11. **Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai.** 1969. In vitro hemolytic characteristics of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J. Bacteriol. **100**:1147–1149.
12. **Raichvarg, D., M. Guenounou, C. Brossard, J. Agneray, and J. E. Alouf.** 1980. Comparative study of biological activities and mitogenic effect of extracts from *Haemophilus influenzae* Type a. Med. Microbiol. Immunol. **168**:201–210.
13. **Saito, H., T. Watanabe, and H. Tomioka.** 1979. Purification, properties, and cytotoxic effect of a bacteriocin from *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **15**:504–509.
14. **Sakazaki, R., K. Tamura, A. Nakamura, T. Kurata, A. Ghoda, and Y. Kazuno.** 1974. Enteropathogenic activity of *Vibrio parahaemolyticus*, p. 231–235. *In* T. Fujino, G. Sakajuchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
15. **Sakurai, J., M. A. Bahavar, Y. Jinguji, and T. Miwatani.** 1975. Interaction of thermostable direct hemolysin of *Vibrio parahaemolyticus* with human erythrocytes. Biken J. **18**:187–192.
16. **Wagatsuma, S.** 1968. On a medium for hemolytic reaction. Media Circle **13**:159–162.
17. **Wagatsuma, S.** 1974. Ecological studies on Kanagawa phenomenon positive strains of *Vibrio parahaemolyticus*, p. 91–96. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
18. **Zen-Yoji, H., Y. Kudoh, H. Igarashi, K. Ohta, and K. Fukai.** 1974. Purification and identification on enteropathogenic toxins "a" and "a′" produced by *Vibrio parahaemolyticus* and their biological and pathological activities, p. 237-243. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.