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# Comparison of *Vibrio parahaemolyticus* Grown in Estuarine Water and Rich Medium†

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**Cell envelope composition and selected physiological traits of *Vibrio parahaemolyticus* were studied in regard to the Kanagawa phenomenon and growth conditions. Cell envelopes were prepared from cells cultured in Proteose Peptone-beef extract (Difco Laboratories, Detroit, Mich.) medium or filtered estuarine water. Protein, phospholipid, and lipopolysaccharide contents varied with culture conditions. The phospholipids present in the cell envelopes were identified as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Phosphatidylethanolamine decreased and phosphatidylglycerol increased in cells grown in estuarine water. Profiles of proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated numerous protein species, with four to six predominant proteins ranging from 26,000 to 120,000 in molecular weight. The profile of *V. parahaemolyticus* cell envelope proteins was unique and might be useful in the identification of the organism. Alkaline phosphatase activity was slightly higher in Kanagawa-negative strains and was higher in cells grown in estuarine water than in cells grown in rich laboratory medium. The DNA levels in estuarine water-grown cells increased, while RNA levels and cell volume decreased. Bacteriophage sensitivity typing demonstrated a close intraspecies relationship. Results indicated that Kanagawa-positive and -negative strains were closely related, but they could be grouped separately and may have undergone starvation-related physiological changes when cultured in estuarine water.**

Since it was first isolated during a food-poisoning outbreak in Japan, *Vibrio parahaemolyticus* has been shown to be widely distributed (6, 16). It has been commonly found in estuarine and coastal waters and is closely associated with zooplankton (29, 34, 51). The bacterium has been isolated from marine animals, and outbreaks of foodborne illness attributable to *V. parahaemolyticus* have occurred mainly as the result of the consumption of fish, crustaceans, and mollusks (9, 14, 18, 50, 59).

Strains of *V. parahaemolyticus* isolated from the stools of patients are almost always Kanagawa positive ($K^+$), producing hemolysis on a high-salt blood agar, while those from seafoods are Kanagawa negative ($K^-$) (40). The reason for the differences in the Kanagawa reaction between strains isolated from different sources has not been determined. Studies have suggested that culture methods might enhance the isolation of $K^-$ strains over $K^+$ strains or that a different physiological or nonculturable state might exist for these microorganisms in the estuarine environment (14, 15, 20, 54, 57).

The cell envelopes of gram-negative bacteria play an important role in the physiology and pathogenicity of the organism (26, 44). Proteins of the cell envelope may participate in the production of disease, and lipopolysaccharide may be toxic (24, 26, 44, 45). Application of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) can determine the cell envelope protein profiles, which are consistent under defined growth conditions, and with bacteriophage sensitivity typing and phospholipid determination, the taxonomic relationships of closely related organisms can be determined (5, 13, 36).

This study was undertaken to determine the cell envelope composition of *V. parahaemolyticus* and the relationship of the cell envelope to the Kanagawa phenomenon and growth conditions. Major cell envelope proteins and protein profiles, differences in cell envelope phospholipids, lipopolysaccharide content, and the relationship between $K^+$ and $K^-$ strains were examined by bacteriophage sensitivity typing.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Seven strains each of confirmed $K^+$ and $K^-$ *V. parahaemolyticus* strains were used in this study (Table 1). They were donated by R. Colwell and A. Delisle (University of Maryland), D. Johnson (Veterans Administration), and R. Twedt (Food and Drug Administration). Overnight cultures were inoculated 1/1,000-fold into Proteose Peptone-beef extract (PPBE; Difco Laboratories, Detroit, Mich.) broth with 0.5% NaCl added or into filtered (pore size, 0.22 μm) estuarine water. The cultures were incubated at 35°C in a rotary shaker at 250 rpm for 15 h. PPBE broth contained 1% Proteose Peptone no. 3 (Difco), 0.2% beef extract (Difco), and 0.5% NaCl (12). Estuarine water used for culture was taken in the morning high tide in the spring from downstream Choptank River northwest of Cambridge, Md. The estuarine water used had an average composition (per liter) of 34 μg-atoms of total nitrogen, 16 μg-atoms of $NO_3$, 0.9 μg-atoms of total phosphorus, and 58 μg-atoms of particulate carbon. It had a salinity of 10.1‰ and a pH of 7.3 (60).

Estuarine water-chitin was prepared by adding 0.5% preground chitin (ICN Biomedicals, Cleveland, Ohio) into filtered estuarine water and then was autoclaved. The chitin was ground with a micromill (CRC, Cleveland, Ohio) at 50,000 rpm to a particle size of 100 μm. Cells were grown in estuarine water-chitin for 15 h at 35°C to determine the effect of chitin on the growth of the organism. After growth the chitin was eliminated by centrifugation (1,500 × *g*, 10 min), and unattached cells from the supernatant were quantified.

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TABLE 1. Strains of *V. parahaemolyticus* used in this study[a]

| Strain | Kanagawa phenomenon | Serotype | Isolation source | Donor |
|---|---|---|---|---|
| P1 | + | O4:K11 | Stool | A. Delisle |
| P7 | + | O4:K4 | Stool | A. Delisle |
| P26 | + | O2:K3 | Stool | A. Delisle |
| 38C1 | + | O4:K11 | Blue crab hemolymph | R. Colwell |
| NAK | + | N | Hemolymph | R. Colwell |
| V10136 | + | O4:K8 | Stool | R. Twedt |
| V6074 | + | O3:K57 | Seawater | R. Twedt |
| P6 | − | O2:K30 | Blue crab | A. Delisle |
| P14 | − | N | Stool | D. Johnson |
| P15 | − | O5:K17 | Crab basket | A. Delisle |
| P68 | − | O4:K34 | Fish | A. Delisle |
| FC1011 | − | O1:K38 | Blue crab hemolymph | R. Colwell |
| 38C6 | − | O3:K30 | Hemolymph | R. Colwell |
| V10145 | − | O2:K28 | Stool | R. Twedt |

[a] Abbreviations: +, positive; −, negative; N, not known.

The pellet was rinsed with 3% NaCl, and a second count was performed to determine the number of attached cells. Cell numbers were determined by using appropriate dilutions in PPBE broth and by spread plating the cells onto PPBE agar plates.

**Flow cytometric analyses.** Forward-angle light scatter and cellular DNA and RNA contents of cells grown in PPBE broth or estuarine water were determined. Cells were permeabilized with 0.08 N HCl–0.15 M NaCl–0.1% Triton X-100 and stained with 13 μM acridine orange in 1 mM disodium EDTA (10, 56, 58). Assays were performed by using an Epics profile instrument (Coulter Electronics, Hialeah, Fla.), with excitation measured at 488 nm and fluorescence measured at 530 and 650 nm.

**Cell envelope preparation.** Cells in the late log phase were harvested by centrifugation and washed once in 0.85% NaCl. The cell pellets were suspended in 50 mM Tris hydrochloride (pH 7.8). The cell suspension was disrupted by passing it through a French pressure cell at 18,000 lb/in$^2$ (13). The clear mixture of broken cells was centrifuged at 2,500 × *g* for 20 min to remove any intact cells or debris. The supernatant was centrifuged at 270,000 × *g* for 1 h at 2°C. The pellets, which primarily contained cell envelope materials, were suspended and washed once (12). The final pellets were suspended in a small amount of 50 mM Tris hydrochloride.

**Chemical analysis.** Total protein of the cell envelopes was determined by the method of Lowry et al. (35), using bovine serum albumin as a standard.

Total phospholipid content was determined by measuring the total phosphorous content as described by Ames and Dubin (2) and by using an average molecular weight of 700 for phospholipids. The $A_{820}$ was measured, and a standard curve prepared from various concentrations of an $Na_2HPO_4$ solution was used for comparison.

Total lipopolysaccharide of the cell envelopes was determined by the method described by Watson et al. (61) by using the *Limulus* amebocyte lysate (Associates of Cape Cod, Woods Hole, Mass.). The $A_{369}$ was measured, and a standard curve was prepared from different quantities of purified endotoxin from *Escherichia coli* 0113 (Associates of Cape Cod).

**Phospholipid extraction and thin-layer chromatography.** Phospholipids were extracted from cell envelopes with chloroform-methanol by the method of Bligh and Dyer (8). The extraction was repeated three times. The aqueous and organic layers were separated and were assayed for phospholipids by thin-layer chromatography (TLC).

TLC was carried out on silica gel plates (20 by 20 cm) with a coating thickness of 0.5 mm (J. T. Baker Chemical Co., Phillipsburg, N.J.). The solvent system used was $CHCl_3$-$CH_3OH$-$CH_3COOH$ (65:25:8) (3). Phospholipids separated by TLC were visualized with iodine vapor and a modified molybdate spray reagent (48). Phospholipids were identified by comparing their relative mobilities with those of standard phospholipids. The standard phospholipids used were cardiolipin (diphosphatidylglycerol), phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine (Sigma Chemical Co., St. Louis, Mo.). Phospholipids separated by TLC were scraped from the plates after evaporation of the iodine vapor, and they were extracted with chloroform-methanol-water-acetic acid (48.5:48.5:2:1) and quantitated by determination of the phosphorus content (2).

Two-dimensional TLC was carried out to determine the purity of the phospholipid species detected by one-dimensional TLC. The chromatogram was developed in the first dimension with $CHCl_3$-$CH_3OH$-$CH_3COOH$-$H_2O$ (250:74:19:3), and after air-drying the plate was developed in the second dimension with $CHCl_3$-$CH_3OH$-$NH_4OH$ (230:90:15) (M. Blecher, unpublished data). The phospholipids were visualized with iodine vapor.

**SDS-PAGE.** The cell envelope protein profile was determined by SDS-PAGE with a dual slab gel apparatus (height, 32 cm) in a discontinuous buffer system by a method modified from that of Laemmli (12, 36). The separation gel contained 11.5% acrylamide, 0.1% SDS, and 3.0% urea.

The cell envelopes were solubilized in a sample buffer composed of 12.5% glycerol, 2.5% SDS, and 1.25% 2-mercaptoethanol in 0.125 M Tris hydrochloride (pH 6.8) at 100°C for 5 min. A molecular weight standard (Bio-Rad Laboratories, Richmond, Calif.) was run for comparison. The molecular weight standards included phosphorylase *b* (92,500), bovine serum albumin (66,300), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,100). The gels were stained in Coomassie brilliant blue G and destained in 10% acetic acid.

**Bacteriophage study.** Bacteriophages for *V. parahaemolyticus* were isolated from freshly shucked oyster liquor from oysters harvested in the Chesapeake Bay. Isolation was carried out by the soft agar overlayer technique of Adams (1). Single plaques were purified three times by the overlay method to ensure purity.

Bacteriophage sensitivity typing was carried out with a tissue culture microplate and a 48-tip replicator. Host cells were grown on PPBE agar and spread plated onto PPBE agar for phage sensitivity typing. The phage typing range was determined as described by Blair and Williams (7). Phage typing was carried out by using a phage stock determined for each phage.

**Alkaline phosphatase and chitinase activities.** Assay of alkaline phosphatase activity was carried out on a French pressure cell-disrupted cell suspension. For the assay 0.2 M Tris hydrochloride buffer (pH 8.5) and *p*-nitrophenyl phosphate (5.0 mg/ml; Sigma) were used, and the solution was incubated at 37°C for 15 min. The optical density was determined at 420 nm, and units of activity were defined as the optical density at 420 nm per milliliter minute (49, 52). Chitinase activity was determined similarly. For the chitinase activity assay 0.2 M $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH

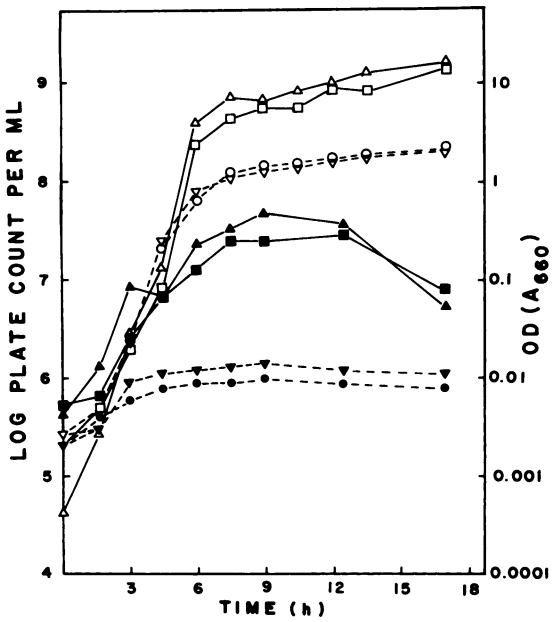


FIG. 1. Growth curves of *V. parahaemolyticus* $K^+$ strains grown in different media. Symbols: △ and □, PPBE cell numbers; ▽ and ○, PPBE culture absorbance; ▲ and ■, estuarine water cell numbers; ▼ and ●, estuarine water culture absorbance; △, ▲, ▽, and ▼, strain P7; □, ■, ○, and ●, strain 38C1. OD, Optical density.

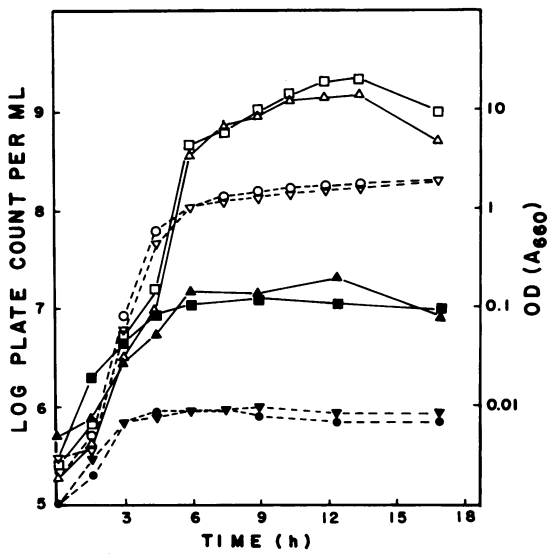


FIG. 2. Growth curves of *V. parahaemolyticus* $K^-$ strains grown in different media. Symbols: △ and □, PPBE cell numbers; ▽ and ○, PPBE culture absorbance; ▲ and ■, estuarine water cell numbers; ▼ and ●, estuarine water culture absorbance; △, ▲, ▽, and ▼, strain P 15; □, ■, ○, and ●, strain 38C6. OD, Optical density.

7.5) and *p*-nitrophenyl-*N*-acetyl-β-D-glucosinamide (6.0 mg/ml, Sigma) were used as the substrate (41, 47, 62).

## RESULTS

Determination of the growth characteristics of *V. parahaemolyticus* indicated that growth was much faster and to a higher level in rich laboratory medium than in estuarine water (Fig. 1 and 2). Generation times in estuarine water were more than twice as long as those in PPBE broth during the logarithmic growth phases in each medium. Cells grown in PPBE broth reached levels greater than $1 \times 10^9$/ml, while only about $5 \times 10^7$/ml were produced in estuarine water. The time for $K^+$ strains to reach the stationary growth phase was similar in both media, but negative strains required longer periods of growth in estuarine water to reach the stationary growth phase. Viable cells of $K^+$ strains continued to increase in PPBE broth until 18 h, but decreased after 12 h when they were grown in estuarine water. On the contrary, $K^-$ viable cells decreased at 14 h when grown in PPBE broth and remained at a relatively constant level in estuarine water at 14 h.

The addition of chitin to estuarine water enhanced the growth of both $K^+$ and $K^-$ strains (Table 2). The cell numbers of microorganisms were 2.3 to 2.8 log units higher in estuarine water with chitin than in the estuarine water control. The numbers of cells attached to the chitin particles were 2.1 to 2.7 times more than the numbers of unattached cells in the estuarine water.

Flow cytometric analyses showed that the cell volume for five of the six strains assayed decreased during growth in estuarine water (Table 3 and Fig. 3 and 4). During the 6 to 8 h of growth in estuarine water, the cell volume decreased as much as 24% compared with that of cells grown in PPBE broth. Plots of forward-angle light scatter versus 90° light scatter indicated that $K^-$ cells grown in estuarine water had a rougher surface and were more diverse in regard to this trait. In $K^+$ strains, very little difference was noted between estuarine water- and PPBE broth-grown cells. Cells grown in estuarine water had an increased DNA content in each cell but a decreased RNA content compared with that of PPBE broth-grown cells. The DNA/RNA ratios were lower in $K^+$ cells than in $K^-$ cells grown in estuarine water. This ratio in $K^+$ cells increased by an average of about 51%, however, while the ratio in $K^-$ cells increased by an average of more than 114% over that in cells grown in PPBE broth (Table 3). RNA levels were higher in $K^+$ than in $K^-$ cells.

Cell envelopes of the $K^+$ strains grown in PPBE broth had a mean total protein content of 50.1 ± 6.2%, while negative strains had a mean total protein content of 53.6 ± 5.9% (Table 4). $K^+$ strains grown in estuarine water had a mean protein content of 45.2 ± 3.2%, and $K^-$ strains had a mean protein content of 61.5 ± 3.1 %.

PPBE broth-grown cells had a relatively constant total phospholipid content, regardless of the presence or absence of the Kanagawa phenomenon, and contained an average of

TABLE 2. Effect of chitin on *V. parahaemolyticus* $K^+$ and $K^-$ strains grown in estuarine water

| Strain | CFU/ml in the following cells: | | | | |
|---|---|---|---|---|---|
| | Initial | Attached | Unattached | Attached: unattached | Estuarine water control |
| $K^+$ | | | | | |
| P7 | $4.0 \times 10^5$ | $2.0 \times 10^9$ | $8.8 \times 10^8$ | 2.27 | $5.2 \times 10^6$ |
| 38C1 | $4.5 \times 10^5$ | $2.6 \times 10^9$ | $9.8 \times 10^8$ | 2.65 | $8.0 \times 10^6$ |
| V6074 | $4.3 \times 10^5$ | $2.5 \times 10^9$ | $1.0 \times 10^9$ | 2.50 | $7.4 \times 10^6$ |
| $K^-$ | | | | | |
| P6 | $3.4 \times 10^5$ | $3.4 \times 10^9$ | $9.1 \times 10^8$ | 3.74 | $7.5 \times 10^6$ |
| P15 | $3.7 \times 10^5$ | $2.1 \times 10^9$ | $8.2 \times 10^8$ | 2.56 | $8.3 \times 10^6$ |
| 38C6 | $2.1 \times 10^5$ | $1.4 \times 10^9$ | $6.8 \times 10^8$ | 2.06 | $9.8 \times 10^6$ |

TABLE 3. Flow cytometric analyses of cellular DNA, RNA, and volume of *V. parahaemolyticus*

| Strain | Flow cytometric results[a] under the following growth conditions: | | | | | |
|---|---|---|---|---|---|---|
| | PPBE broth | | | Estuarine water | | |
| | LFS[b] | DNA[c] | RNA[d] | LFS | DNA | RNA |
| $K^+$ | | | | | | |
| P7 | 41.9 ± 23.0 | 91.6 ± 23.5 | 571.6 ± 100.4 | 46.3 ± 25.4 | 111.0 ± 27.1 | 435.3 ± 113.2 |
| 38C1 | 52.7 ± 27.2 | 63.8 ± 30.3 | 512.5 ± 123.0 | 40.1 ± 22.7 | 108.8 ± 25.3 | 488.1 ± 104.4 |
| V6074 | 43.8 ± 24.2 | 89.8 ± 29.5 | 489.4 ± 117.6 | 39.1 ± 21.4 | 101.2 ± 24.3 | 491.2 ± 100.9 |
| K | | | | | | |
| P6 | 45.7 ± 24.7 | 74.1 ± 28.8 | 566.3 ± 120.8 | 43.0 ± 26.2 | 105.6 ± 31.7 | 361.6 ± 115.0 |
| P15 | 49.2 ± 25.9 | 78.0 ± 29.6 | 534.9 ± 125.5 | 42.9 ± 26.5 | 79.2 ± 23.8 | 294.8 ± 76.5 |
| 38C6 | 45.8 ± 25.4 | 62.7 ± 29.6 | 543.4 ± 125.3 | 40.3 ± 24.4 | 93.5 ± 25.6 | 329.1 ± 100.9 |

[a] Arbitrary units.
[b] LFS, Forward-angle light scatter is proportional to cell volume.
[c] DNA, Green fluorescence (530 nm) reported as the mean channel ± standard deviation.
[d] RNA, Red fluorescence (650 nm) reported as the mean channel ± standard deviation.

17 or 18% phospholipid in the total envelope (Table 5). On the contrary, $K^+$ strains grown in estuarine water had a mean content of 46.6 ± 2.3% cell envelope phospholipids and $K^-$ strains had a mean content of 19.2 ± 6.4%.

Total lipopolysaccharide contents of cell envelopes from PPBE broth-grown $K^+$ strains were similar to those of cell envelopes from $K^-$ strains, with both containing about one-third of the cell envelopes (Table 6). Growth in estuarine water caused a decrease in the lipopolysaccharide level in the cell envelope in $K^+$ and $K^-$ strains. $K^+$ strains grown in estuarine water had a mean of 8.2 ± 2.7%, and $K^-$ strains had a mean of 19.3 ± 4.9%.

Alkaline phosphatase is located in the cell envelope and has been used for the detection of cell subfractions (21). We performed an assay for this enzyme to determine whether there is any relationship between its activity and the Kanagawa phenomenon or growth medium. The results indicated that $K^+$ strains have an average lower alkaline phosphatase activity than $K^-$ strains do (Table 7). When grown in PPBE broth the $K^+$ strains had a mean specific activity for alkaline phosphatase of 0.51 ± 0.34 U/ml min mg, while $K^-$ strains had a mean specific activity of 0.75 ± 0.24 U/ml min mg. The activities of cells grown in estuarine water were higher than those of cells grown in rich laboratory medium. $K^+$ cells grown in estuarine water had a mean activity that was slightly higher than that of $K^-$ cells.

Chitinase activities were similar for both PPBE broth- and estuarine water-grown cells, regardless of the presence or absence of the Kanagawa phenomenon (Table 8). Activities ranged from 0.15 to 0.47 units/ml min mg.

Phospholipids were identified by comparing the $R_f$ values of standard phospholipids and extracted phospholipids that were separated by TLC. The standard phospholipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin had $R_f$ values in chloroform-methanol-acetic acid (65:25:8) of 0.63, 0.86, and 0.98, respectively, while extracted phospholipids had $R_f$ values of 0.65, 0.86, and 0.97, respectively (data not shown). Phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin were isolated from all strains that were grown on PPBE and accounted for a mean of 83.0, 12.8, and 4.7%, respectively, of the total phospholipids extracted (Table 9). Two-dimensional TLC of the phospho-

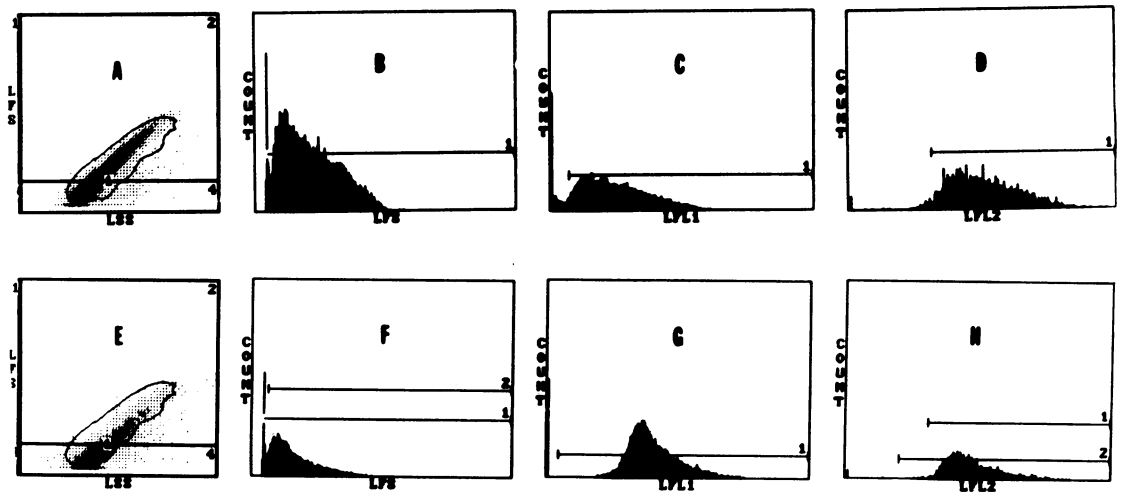


FIG. 3. Flow cytometric analyses of $K^+$ *V. parahaemolyticus* 38C1 cells grown in different media. Panels: A and E, forward-angle light scatter (LFS) versus 90° light scatter (LSS); B and F, cell count versus forward-angle light scatter; C and G, cell count versus forward-angle light scatter (LFL1; green fluorescence, DNA); D and H, cell count versus forward-angle light scatter (LFL2; red fluorescence, RNA); A, to D, PPBE-grown cells; E to H, estuarine water-grown cells.

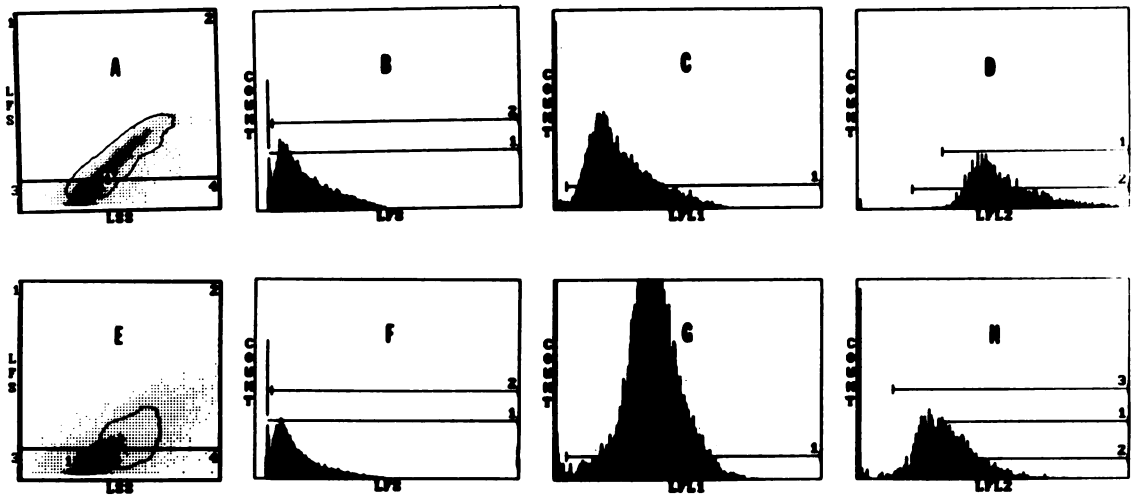


FIG. 4. Flow cytometric analyses of K⁻ *V. parahaemolyticus* 38C6 cells grown in different media. Panels: A and E, forward-angle light scatter versus 90° light scatter (LSS); B and F, cell count versus forward-angle light scatter; C and G, cell count versus forward-angle light scatter (LFL1; green fluorescence, DNA); D and H, cell count versus forward-angle light scatter (LFL2; red fluorescence, RNA); A to D, PPBE-grown cells; E to H, estuarine water-grown cells.

lipids that were extracted indicated that no other species were present than those that were identified by one-dimensional TLC. No difference in phospholipid composition was found between $K^+$ and $K^-$ strains. However, it is not known whether the fatty acid composition had any correlation to the presence or absence of the Kanagawa phenomenon. Cells grown in estuarine water had reduced phosphatidylethanolamine and increased phosphatidylglycerol contents (Table 10). In estuarine water-grown cells phosphatidylethanolamine was decreased by an average of 31%, with a concomitant increase in phosphatidylglycerol, compared the contents of PPBE broth-grown cells. Changes in cardiolipin content were strain related.

Most aquatic sources, including Chesapeake Bay water, estuarine water, river water, and sewage discharge, rarely contained bacteriophages for *V. parahaemolyticus*. The liquor released from oysters during the shucking process was found to be a good source of bacteriophages for this organism. A total of 46% (6 of 13) of bacteriophages isolated for $K^+$ strains attacked only positive strains, and 59% (13 of 22) of bacteriophages isolated for $K^-$ strains attacked only $K^-$ strains grown on the same medium (data not shown). The remainder of the bacteriophages isolated attacked bacterial strains regardless of the Kanagawa phenomenon reaction. None of the bacteriophages isolated from *V. parahaemolyticus* attacked *E. coli* strains, *Proteus vulgaris*, *Pseudomonas*

TABLE 4. Total protein content of the *V. parahaemolyticus* cell envelope

| Strain | Protein content (%) under the following growth conditions: | |
|---|---|---|
| | PPBE broth | Estuarine water |
| $K^+$ | | |
| P1 | 53.6 | 44.0 |
| P7 | 44.5 | 42.8 |
| P26 | 56.5 | 52.0 |
| 38C1 | 49.6 | 44.0 |
| NAK | 38.0 | 42.7 |
| V10136 | 53.6 | 43.3 |
| V6074 | 54.7 | 47.4 |
| Mean ± SD | 50.1 ± 6.2 | 45.2 ± 3.2 |
| $K^-$ | | |
| P6 | 55.5 | 61.0 |
| P14 | 52.8 | 67.0 |
| P15 | 49.3 | 63.0 |
| P68 | 57.1 | 63.1 |
| FC1011 | 56.7 | 60.9 |
| 38C6 | 61.8 | 56.7 |
| V10145 | 42.2 | 59.0 |
| Mean ± SD | 53.6 ± 5.9 | 61.5 ± 3.1 |

TABLE 5. Total phospholipid content of the cell envelope

| Strain | Phospholipid content (%) under the following growth conditions: | |
|---|---|---|
| | PPBE broth | Estuarine water |
| $K^+$ | | |
| P1 | 18.7 | 49.1 |
| P7 | 17.3 | 47.6 |
| P26 | 21.4 | 43.0 |
| 38C1 | 12.5 | 43.0 |
| NAK | 23.9 | 47.8 |
| V10136 | 11.6 | 48.0 |
| V6074 | 13.6 | 47.6 |
| Mean ± SD | 17.0 ± 4.3 | 46.6 ± 2.3 |
| $K^-$ | | |
| P6 | 12.8 | 16.0 |
| P14 | 10.8 | 15.0 |
| P15 | 17.0 | 16.0 |
| P68 | 12.3 | 16.4 |
| FC1011 | 17.5 | 21.9 |
| 38C6 | 27.4 | 33.9 |
| V10145 | 28.7 | 15.0 |
| Mean ± SD | 18.1 ± 6.7 | 19.2 ± 6.4 |

TABLE 6. Total lipopolysaccharide content of the cell envelope

| Strain | Lipopolysaccharide content (%) under the following growth conditions: | |
|---|---|---|
| | PPBE broth | Estuarine water |
| $K^+$ | | |
| P1 | 27.7 | 6.9 |
| P7 | 38.2 | 9.6 |
| P26 | 22.1 | 5.0 |
| 38C1 | 37.9 | 13.0 |
| NAK | 38.0 | 9.5 |
| V10136 | 34.9 | 8.7 |
| V6074 | 31.7 | 5.0 |
| Mean ± SD | 32.9 ± 5.7 | 8.2 ± 2.7 |
| $K^-$ | | |
| P6 | 31.7 | 23.0 |
| P14 | 36.4 | 18.0 |
| P15 | 33.7 | 21.0 |
| P68 | 30.8 | 20.5 |
| FC1011 | 25.8 | 17.2 |
| 38C6 | 14.1 | 9.4 |
| V10145 | 29.0 | 26.0 |
| Mean ± SD | 28.8 ± 6.8 | 19.4 ± 4.9 |

*aeruginosa*, *Salmonella newport*, or several strains of *Vibrio cholerae*.

The rich laboratory medium PPBE broth had been used in our laboratory in the past for the study of cell envelopes of gram-negative bacteria. This medium supports good growth of all gram-negative bacteria, and because it is used extensively, there are good sources of data with which we can compare our results. Results of SDS-PAGE of cell envelope proteins prepared from cells grown in this medium indicated numerous proteins that were found in the cell envelope of *V. parahaemolyticus* with molecular weights ranging from 26,900 to above 100,000 (Fig. 5 and 6). A number of strains, regardless of the presence or absence of the Kanagawa phenomenon reaction, had seven proteins in common; these proteins had molecular weights of 26,900, 27,500, 40,300, 52,400, 53,600, 56,400, and 75,700. $K^+$ strains had a much more uniform protein profile and more proteins in common than the more diverse $K^-$ strains.

Since *V. parahaemolyticus* is an estuarine organism, the cell envelope composition and the physiology of an organism grown in estuarine water would be expected to be different from that of an organism grown in laboratory medium. *V.*

TABLE 7. Specific activity of alkaline phosphatase from disrupted cell suspension

| Strain | Sp act (total units/total protein) under the following growth conditions: | |
|---|---|---|
| | PPBE broth | Estuarine water |
| $K^+$ | | |
| P1 | 0.30 | 3.46 |
| P7 | 0.31 | 2.07 |
| P26 | 1.15 | ND[a] |
| 38C1 | 0.25 | ND |
| NAK | 0.90 | ND |
| V10136 | 0.18 | ND |
| V6074 | 0.51 | 1.93 |
| Mean ± SD | 0.51 ± 0.34 | 2.49 ± 0.69 |
| $K^-$ | | |
| P6 | 0.85 | 1.36 |
| P14 | 0.42 | 1.29 |
| P15 | 0.67 | ND |
| P68 | 1.19 | ND |
| FC1011 | 0.47 | ND |
| 38C6 | 0.84 | 3.60 |
| V10145 | 0.84 | ND |
| Mean ± SD | 0.75 ± 0.24 | 2.08 ± 1.07 |

[a] ND, Not determined.

TABLE 8. Specific activity of chitinase from disrupted cell suspension

| Strain | Sp act (total units/total protein [$OD_{420}$/mg]) under the following growth conditions: | |
|---|---|---|
| | PPBE broth | Estuarine water |
| $K^+$ | | |
| P7 | 0.15 | 0.29 |
| 38C1 | 0.45 | 0.34 |
| V6074 | 0.47 | 0.38 |
| Mean ± SD | 0.36 ± 0.15 | 0.3 ± 0.04 |
| $K^-$ | | |
| P6 | 0.24 | 0.23 |
| P15 | 0.44 | 0.31 |
| 38C6 | 0.43 | 0.38 |
| Mean ± SD | 0.37 ± 0.09 | 0.31 ± 0.06 |

TABLE 9. Composition of phospholipids extracted from cell envelopes of PPBE-grown cells

| Strain | Phospholipid composition (%) | | |
|---|---|---|---|
| | Phosphatidyl-ethanolamine | Phosphatidyl-glycerol | Cardiolipin |
| $K^+$ | | | |
| P1 | 81.4 | 18.5 | 6.1 |
| P7 | 91.7 | 7.6 | 0.69 |
| P26 | 98.6 | 0.99 | 0.41 |
| 38C1 | 81.9 | 12.9 | 5.2 |
| NAK | 93.6 | 4.9 | 1.7 |
| V10136 | 77.7 | 16.9 | 5.4 |
| V6074 | 76.8 | 13.4 | 9.8 |
| Mean ± SD | 86.0 ± 7.9 | 10.7 ± 6.0 | 4.2 ± 3.2 |
| $K^-$ | | | |
| P6 | 82.0 | 12.4 | 5.6 |
| P14 | 82.3 | 12.1 | 5.6 |
| P15 | 87.6 | 12.3 | 0.1 |
| P68 | 86.5 | 13.1 | 0.4 |
| FC1011 | 73.3 | 18.8 | 7.9 |
| 38C6 | 72.8 | 18.3 | 8.8 |
| V10145 | 75.8 | 16.3 | 7.8 |
| Mean ± SD | 80.0 ± 5.7 | 14.8 ± 2.7 | 5.2 ± 3.3 |

TABLE 10. Composition of phospholipids extracted from cell envelopes of estuarine water-grown cells

| Strain | Phospholipid composition (%) | | |
|---|---|---|---|
| | Phosphatidyl-ethanolamine | Phosphatidyl-glycerol | Cardiolipin |
| $K^+$ | | | |
| P7 | 68.8 | 25.1 | 6.1 |
| 38C1 | 58.1 | 27.7 | 14.3 |
| V6074 | 71.7 | 24.2 | 4.1 |
| Mean ± SD | 66.2 ± 5.8 | 25.7 ± 1.5 | 8.2 ± 4.4 |
| $K^-$ | | | |
| P6 | 58.8 | 25.8 | 15.4 |
| P15 | 60.1 | 26.7 | 13.2 |
| 38C6 | 68.2 | 27.2 | 4.6 |
| Mean ± SD | 62.4 ± 4.2 | 26.6 ± 0.6 | 11.1 ± 4.7 |

*parahaemolyticus* strains were grown in filtered estuarine water (salinity, 10 ‰). We examined the cell envelope protein patterns of cells grown in estuarine water and compared them with those of cells grown in laboratory medium. Protein profiles of cells grown in estuarine water showed striking differences; there were 6 to 12 distinct, predominant proteins in $K^-$ strains, while there were only 1 to 4 major proteins in $K^+$ strains. The estuarine water-grown cells had diminished levels of proteins in the 26,900- to 36,100-molecular-weight range and proteins in the 53,400- to 56,400-molecular-weight range (Fig. 7 and 8). New major proteins with molecular weights of approximately 120,000 were observed in estuarine water-grown cells.

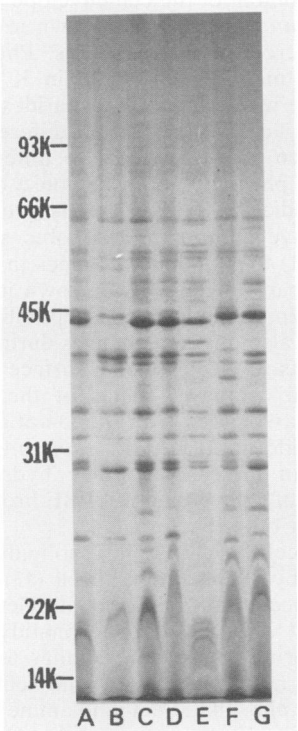


FIG. 5. Stained SDS-polyacrylamide slab gels of cell envelopes prepared from *V. parahaemolyticus* strains grown on PPBE broth containing 0.5% NaCl (see legend to Fig. 6). Numbers on the left of the gels are molecular weights (in thousands [K]); lanes A to G, $K^+$ strains P1, P7, P26, 38C1, NAK, V10136, and V6074, respectively.

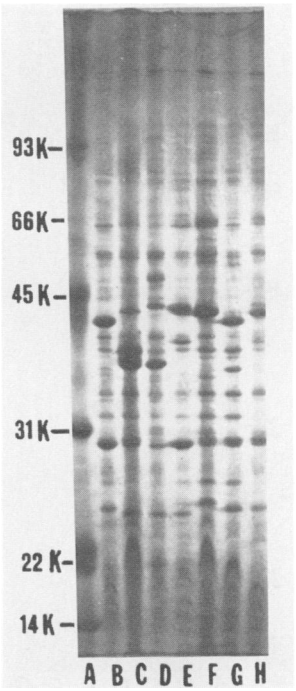


FIG. 6. Stained SDS-polyacrylamide slab gels of cell envelopes prepared from *V. parahaemolyticus* strains grown on PPBE broth containing 0.5% NaCl. The molecular weight standards were lysozyme (14,000), soybean trypsin inhibitor (22,000), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,000), and phosphorylase *b* (93,000). Lane A, Molecular weight standards (in thousands [K]); lanes B to H, $K^-$ strains P6, P14, P15, P68, FC1011, 38C6, and V10145, respectively.

## DISCUSSION

It is commonly expected that the growth rate and maximum cell number for cells grown in rich laboratory medium are much higher than those for cells grown in estuarine water. Supplementation of the estuarine water with chitin resulted in enhanced growth of *V. parahaemolyticus*. This was probably because of the ability of the bacteria to utilize the chitin as a nutrient source. Chitinase activity was demonstrated, and adsorption to the chitin was shown; this might have enhanced the ability of the vibrios to grow in estuarine water. Other investigators (41, 47, 62) have found that chitinases are abundant in bacteria, particularly in members of the genus *Vibrio*. Alkaline phosphatase activity was increased in cells that were grown in estuarine water. This indicates that phosphate levels in estuarine water might be a limiting growth factor. Siuda and Chrost (55) have shown that bacteria produce alkaline phosphatases that may be constitutive or inducible and that can aid the growth of bacteria at low phosphate levels. The availability of phosphatases in these organisms grown in estuarine water is important for the hydrolysis of phosphate esters and the transformation of organic phosphates to a usable form. These enzymes are compartmentalized in the periplasmic space, which is sandwiched between the phospholipid-con-

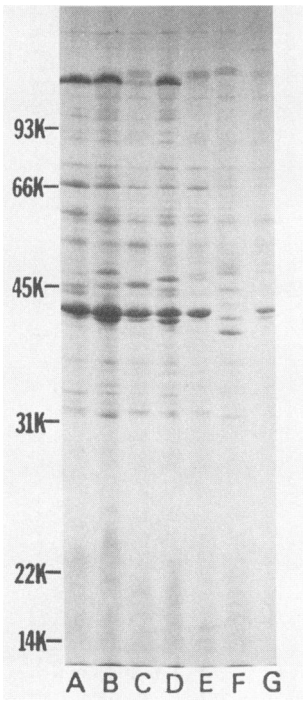


FIG. 7. Stained SDS-polyacrylamide slab gels of cell envelopes prepared from *V. parahaemolyticus* strains grown in estuarine water. See the legend to Fig. 6 for molecular weight standards. Numbers on the left of the gels are molecular weights (in thousands [K]); lanes A to G, $K^+$ strains P1, P7, P26, 38C1, NAK, V10136, and V6074, respectively.

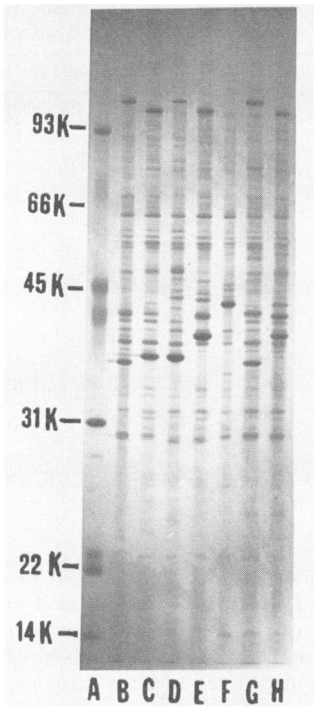


FIG. 8. Stained SDS-polyacrylamide slab gels of cell envelopes prepared from *V. parahaemolyticus* strains grown in estuarine water. See the legend to Fig. 6 for molecular weight standards. Lane A, Molecular weight standards (in thousands [K]); lanes B to H, $K^-$ strains P6, P14, P15, P68, FC1011, 38C6, and V10145, respectively.

taining inner membrane and the outer membrane of the bacterial cell envelope. It is not known whether alkaline phosphatases are involved in energy utilization during starvation of the marine vibrios, during which a large decrease in total phospholipid phosphate and ATP has been detected (43).

$K^+$ strains reached the late log phase of growth at a similar time, regardless of the growth medium. $K^-$ strains required longer growth periods to reach the late log phase in estuarine water. Growth curves for cells grown in estuarine water indicated that $K^+$ cell numbers decreased rapidly after they reached the stationary growth phase, while the optical density decreased only slightly. This indicates that the cells probably had not lysed, but they were nonculturable in estuarine water. DNA levels were higher in all estuarine water-grown cells, and RNA levels were higher in $K^+$ strains. $K^-$ strains had higher DNA/RNA ratios. These results are indicative of DNA synthesis without growth in cells grown in estuarine water. A decrease in cell volume was indicated by forward-angle light scatter. Other investigators have observed a similar phenomenon in bacteria under starvation conditions, including the incorporation of [$^3$H]thymidine into cellular macromolecules (37). Cell fragmentation and continuous size reduction, such as occur during starvation and before the viable nonculturable stage, is reached, require DNA synthesis to proceed (31, 37). It is plausible that the $K^+$ cells might enter the viable nonculturable stage more readily when confronted with nutrient-deficient conditions, such as those found in estuarine water. This suggests that pathogenic strains might be more common in the environment than has been observed previously and that the isolation procedure in use might miss them.

The protein content of the cell envelope was decreased in $K^+$ *V. parahaemolyticus* strains grown in estuarine water, while it was increased in $K^-$ strains. Phospholipid levels were increased more than twofold in $K^+$ cells grown in estuarine water, while lipopolysaccharides were decreased fourfold, but $K^-$ cells displayed no difference in phospholipid levels when grown in the two different media. The distinct level of phospholipid in response to the Kanagawa phenomenon indicates that $K^+$ cells grown in estuarine water might have a more hydrophobic surface than $K^-$ strains. Chai (11) found similar changes in *E. coli* grown in bay water compared with *E. coli* grown in laboratory medium, resulting in an increased susceptibility to detergents. Marine bacteria also undergo changes during starvation that result in an increased hydrophobic surface (31). Hood et al. (25) have found decreased levels of the sugars that are expected to be associated with the O-antigen side chain of lipopolysaccharide in starved *V. cholerae*; this also might have resulted in increased surface hydrophobicity. The protein content of cells grown in PPBE broth was similar to that reported for other bacteria (44).

In our study cell envelope phospholipids were similar to those reported by Oliver and Colwell (42) in other marine bacteria. The decreases in phosphatidylethanolamine and the concomitant increases in phosphatidylglycerol during growth in estuarine water were similar to the results obtained by Oliver and Stringer (43), who demonstrated a 25% decrease in the phosphatidylethanolamine level during the nutrient starvation of a marine vibrio. During starvation, *Vibrio vulnificus* was demonstrated to have a decreased level of the predominant fatty acid species (to less than 12% of the total), and a loss of virulence was observed in the nonrecoverable state (K. Linder and J. Oliver, Abstr. Annu. Meet.

Am. Soc. Microbiol. 1988, N14, p. 32). Variability in the proportion of cardiolipin was probably strain related and was caused by individual responses to changes in the cation content of the growth medium. Miller (38, 39) has shown that the cardiolipin content of a *Planococcus* sp. is affected by the cation content of the medium, and combinations of cations with different valences may result in attenuation and a varied cardiolipin content, depending on the combination of cations (38). Bacterial lipid was degraded during starvation. A 90% decrease in phospholipid fatty acids per gram (dry weight) and a 99% decrease in fatty acids per cell were reported for *V. cholerae* in cells starved for 7 days (25). Guckert et al. (19) further analyzed phospholipid ester-linked fatty acids and found that the amount of the *cis*-monoenoic acids declined, while the amounts of saturated fatty acids, the cyclopropyl derivatives, and *trans*-monoenoic acid increased during starvation.

The lipopolysaccharides of *Vibrio* species have been shown to be similar to those of members of the family *Enterobacteriaceae* and have exhibited endotoxic properties, including lethality, pyrogenicity, and *Limulus* amebocyte lysate gelation (17, 22, 23, 28, 46). Changes in the cell envelope, such as the decrease in the level of lipopolysaccharides of cells grown in estuarine water that we observed, might affect the pathogenicity of the organism. The increased protein content and relatively higher lipopolysaccharide content of $K^-$ strains grown in estuarine water compared with those of $K^+$ cells could be related to the higher surface roughness, as predicted by 90° light scatter, since the lipopolysaccharide side chains extend out from the cell surface.

Koga and Kawata (32) have separated five major proteins from the outer membrane of *V. parahaemolyticus*, some of which corresponded to proteins species that we demonstrated in the present study. We found (unpublished data) four proteins with molecular weights of 47,000, 44,000, 40,000, and 33,000 to be outer membrane proteins; and because of their insolubility in SDS we believe that they are associated with peptidoglycan. Deneke and Colwell (17) have previously demonstrated major cell envelope proteins from *V. parahaemolyticus* with molecular weights of 30,000, 45,000, and 90,000. Discrepancies between the different results might be due to the use of different extraction methods and gel electrophoresis systems or different growth conditions and strain variability. *V. cholerae* has similar outer membrane proteins ranging in molecular weight from 27,000 to 94,000 (27, 30). *V. parahaemolyticus* grown in estuarine water had decreased levels of proteins with molecular weights ranging from 26,900 to 36,100 and 53,400 to 56,400. The level of the 40,000-molecular-weight outer membrane protein was increased, while the level of the 44,000-molecular-weight protein appeared to be decreased. Decreased levels of OmpF in *E. coli* grown in estuarine water have been found by T. Chai (unpublished data). A major 120,000-molecular-weight protein appeared in the cell envelope of *V. parahaemolyticus* grown in estuarine water, and similar high-molecular-weight proteins have been demonstrated in *V. cholerae* cultured under nutrient-limiting conditions (53). Considering the low level of iron in estuarine water (0.06 μg-atom of Fe/liter; T. Fisher, personal communication) and the frequency of siderophore production by other vibrios, the new major protein might be a receptor for chelation-mediated iron uptake. It is interesting that the varied $M_r$s of the 120,000-molecular-weight protein might be related to the K serotype of each strain. However, because of the few strains that were assayed, no definite conclusion could be made.

Koga et al. (33) have isolated 18 bacteriophages for *V. parahaemolyticus* from seawater; these were grouped into four groups by morphology. They reported no correlation between serotype and the host ranges of the bacteriophages, and two bacterial strains tested were not lysed by any bacteriophage. Bacteriophages for *V. parahaemolyticus* were isolated from shellfish, and the quantities of bacteriophages increased with water temperature and mesophilic vibrio levels (4). Baross et al. (5) have found that bacteriophages for *V. parahaemolyticus* can be isolated when only psychrophilic vibrios are present, indicating that these vibrios that are marginally related to *V. parahaemolyticus* can share bacteriophages and can exchange genetic information. Twenty bacteriophages were isolated that produced interspecies lysis of *V. parahaemolyticus*, and those agar-digesting vibrios had up to 70 to 80% differences in DNA sequence homology. Of 28 *V. parahaemolyticus* strains, 7 were not lysed by any bacteriophages (5). In this study, we isolated 35 bacteriophages for *V. parahaemolyticus*, and the results indicate that oyster liquor contains high levels of bacteriophages, indicating that the bivalves concentrate vibrio bacteriophages during their pumping feeding process. The relationship was very close between the sensitivities of the $K^+$ and $K^-$ strains, and 57% (20 of 35) of the bacteriophages showed intergroup lysis. Sixty-three percent (22 of 35) of the bacteriophages for $K^-$ strains were isolated, which reflects the findings of higher levels of *V. parahaemolyticus* $K^-$ strains in Chesapeake Bay water.

Pathogenicity is the capacity of an organism to cause disease in a host. The pathogenicity of *V. parahaemolyticus* has easily been demonstrated by injecting the organism into a rabbit ileal loop, which caused an outpouring of fluid, but the exact mechanism has been difficult to discern (20). In spite of the correlation between pathogenicity and the Kanagawa phenomenon and the extensive work concerning the Kanagawa phenomenon-associated hemolysin, no exact mechanism has been proved (24). It may be that a number of entities are involved in the pathogenicity, and the mechanism may vary from strain to strain, as is found with the mechanisms of *V. cholerae* and non-O1 *V. cholerae*. In this study we presented results that $K^+$ and $K^-$ strains differ somewhat in their cell envelope protein profiles, lipopolysaccharide levels under certain growth conditions, and alkaline phosphatase activities. These results indicate that pathogenicity may be related to the cell envelope composition, and variation in the cell envelope composition may be related to strain variation and cultural conditions. *V. parahaemolyticus* may be able to enter the viable nonculturable state, which might be responsible for the difficulty in isolating $K^+$ strains from the environment. Further work is needed to ascertain the significance of cell envelope changes in regard to growth conditions and pathogenicity.

## ACKNOWLEDGMENTS

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