JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1985, p. 97–101
0095-1137/85/010097-05$02.00/0

Vol. 21, No. 1

Demonstration of a Common Antigen in Sonicated Cells for Identification of *Vibrio vulnificus* Serotypes†

MITSUAKI NISHIBUCHI‡ AND RAMON J. SEIDLER*

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804

Received 4 June 1984/Accepted 4 October 1984

Ouchterlony immunodiffusion of sonicated *Vibrio vulnificus* cells illustrated a single major precipitation line with antiserum prepared from whole cells of the same species. Antigenic analysis by two-dimensional immunoelectrophoresis verified the presence of a single dominant precipitation line. Tandem two-dimensional immunoelectrophoretic analyses of *V. vulnificus* antigens from various strains revealed one fused precipitation line of identity. No fused precipitation lines were seen with other *Vibrio* species tested. This dominant antigen, designated VVA, was not dialyzable, lost antigenicity by heating at 100°C but not at 70°C, and was precipitated by 70%, but not by 50%, saturated ammonium sulfate. VVA was not present in concentrated (20×) spent culture medium. VVA is possibly an intracellular protein specific to *V. vulnificus* species and may be useful in the serological identification of this important human pathogen.

Vibrio vulnificus is a recognized human pathogen which causes wound infections and primary septicemia characterized by unusually rapid disease progress (3–5). In early reports, isolates from patients were mistakenly identified as *V. parahaemolyticus* (13, 19, 20). Originally, *V. vulnificus* was designated as lactose fermenting or lactose positive due to the ability of isolates to ferment lactose. These isolates were often labeled halophilic vibrios because of their tolerance to NaCl (9, 14). The "halophilic lactose-fermenting" *Vibrio* species was shown to be distinct from other *Vibrio* species by DNA hybridization (6). Reichelt et al. (12) named the organism *Beneckea vulnifica*. It was subsequently transferred to the genus *Vibrio* and established as *V. vulnificus* (7, 8). Recently, organisms with biochemical reactions very similar to those of clinical isolates of *V. vulnificus* were isolated from seawater (10), and *Vibrio* isolates from the estuarine environment were identified as *V. vulnificus* by DNA hybridization (17). *Vibrio* isolates from diseased eels cultured in estuarine ponds in Japan were also found to be taxonomically very similar to *V. vulnificus* and were included as a biotype within this species (16).

As reflected in the stormy history of its taxonomy and its diverse ecological habitats, identification of *V. vulnificus* based on biochemical tests is not always correct. For example, it is now known that vibrios other than *V. vulnificus* ferment lactose (2, 3, 15). Identification of this species by molecular techniques based on DNA hybridization is exacting, but such elaborate and time-consuming efforts are unrealistic in the clinical laboratory.

Bang et al. (1) used the moderately conserved enzyme superoxide dismutase to show that serological relationships among *Vibrio* species are generally in agreement with other taxonomic critieria, including DNA homology. In view of such a report, we have initiated a study to establish an identification scheme for *V. vulnificus* serotypes. Since strains of *V. vulnificus* have rather diversified surface antigens when examined by the rapid slide agglutination test (16), sonicated whole-cell antigens were examined by precipitation tests in this study. Ouchterlony immunodiffusion analysis followed by two-dimensional immunoelectrophoresis analyses have demonstrated the presence of an antigen apparently common to *V. vulnificus* strains. A companion report (11) illustrates antigen purification and the specificity of this potentially useful diagnostic tool.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study and their experimental designation and origins are listed in Table 1. Most of the strains were previously used in DNA hybridization studies (15–18). All organisms were stored at −80°C in tryptic soy broth without glucose (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl and glycerol (5% final concentration).

Antigens. Sonicated bacterial cell antigens used for Ouchterlony immunodiffusion analysis and two-dimensional immunoelectrophoresis were prepared as follows. Organisms were grown on tryptic soy broth without glucose supplemented with 0.5% NaCl plus 1.5% agar at 25°C for 24 h and then on this broth prepared in a 32-oz (ca. 1.06-liter) prescription bottle and incubated at 25°C for 20 to 24 h. The cells were harvested with formalinized phosphate-buffered saline (0.01 M sodium phosphate [pH 7.2], 0.74% NaCl, 0.3% Formalin) and washed with formalinized phosphate-buffered saline three times. The washed cells were suspended in formalinized phosphate-buffered saline (0.2 g of cells [wet weight] per ml), sonicated with a Branson Sonifier cell disruptor 200 equipped with a 0.5-in. (ca. 1.27-cm) horn (50 W for four 30-s cycles), and stored at 4°C until used. The sonicated whole-cell antigens prepared in this manner were designated by the lab strain number, e.g., antigen V1.

Antisera. Whole, washed bacterial cells were prepared as described above for antigen preparation. The cells were suspended in formalinized phosphate-buffered saline to an optical density of 1.4 to 1.5 at 600 nm. Two milliliters of the cell suspension was mixed with an equal volume of Freund complete adjuvant (Difco) by using a homogenizer (VirTis 23; The VirTis Co., Inc., Gardiner, N.Y.) equipped with a small blade at 10,000 rpm and was inoculated into the interscapular muscles and footpads of a rabbit. A booster was given with Freund incomplete adjuvant (Difco) into

* Corresponding author.

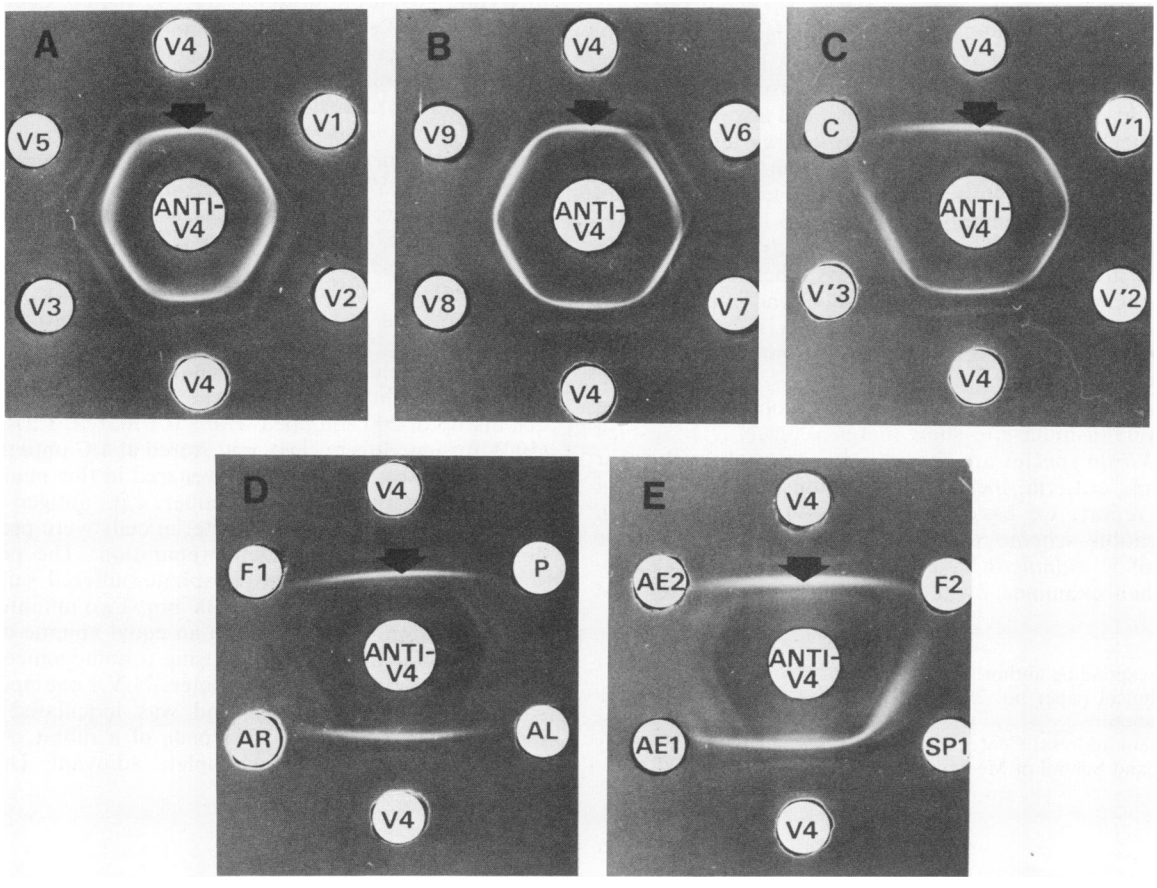
† Technical paper no. 7317 of the Oregon Agricultural Experiment Station.

‡ Present address: Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD 21201.

TABLE 1. Bacterial strains studied

Laboratory designation	Species	Strain no.	Origin
V1	*V. vulnificus*	E9315	Clinical, Centers for Disease Control
V2		CDC B51	Blood, Hawaii
V3		A8867	Finger wounds, Louisiana
V4		80-02-125	Blood, Texas
V5		79-08-9	Seawater, Texas
V6		A38	Crab, Oregon
V7		ATCC 27562	Blood, Florida
V8		LSU 11-3M-30	Crab, Louisiana
V9		79-11-114	Seawater, Texas
V'1	*V. vulnificus* biogroup 2[a]	ET-7617 (= ATCC 33148)	Eel, Japan
V'2		ES-7601 (= ATCC 33147)	Eel, Japan
V'3		KV-1 (= ATCC 33149)	Eel, Japan
C	*V. cholerae* non 0-1	N-3	Crab, Louisiana
P	*V. parahaemolyticus*	CDC A8659	Gastroenteritis, Maryland
AL	*V. alginolyticus*	ATCC 17749	Seawater, Japan
AR	*V. anguillarum*	LS1-74	Salmon, Oregon
F1	*V. fluvialis*	2386	River water, United Kingdon
F2	*V. fluvialis*	DJVP 7225	Gastroenteritis, Indonesia
SP1	*Vibrio* sp.	HOG-3	Oyster, Louisiana
SP2		OY-0-002 (= ATCC 35048)[b]	Oyster, Oregon
SP3		CL-0-001	Clam, Oregon
SP4		F43	Water, Louisiana
AE1	*Aeromonas* sp.	NMRI 1	River water, Maryland
AE2		NMRI 3	River water, Maryland

[a] Tison et al. (16).
[b] This strain was recently designated as the type strain of a newly proposed species, *V. aestuarianus* (18).



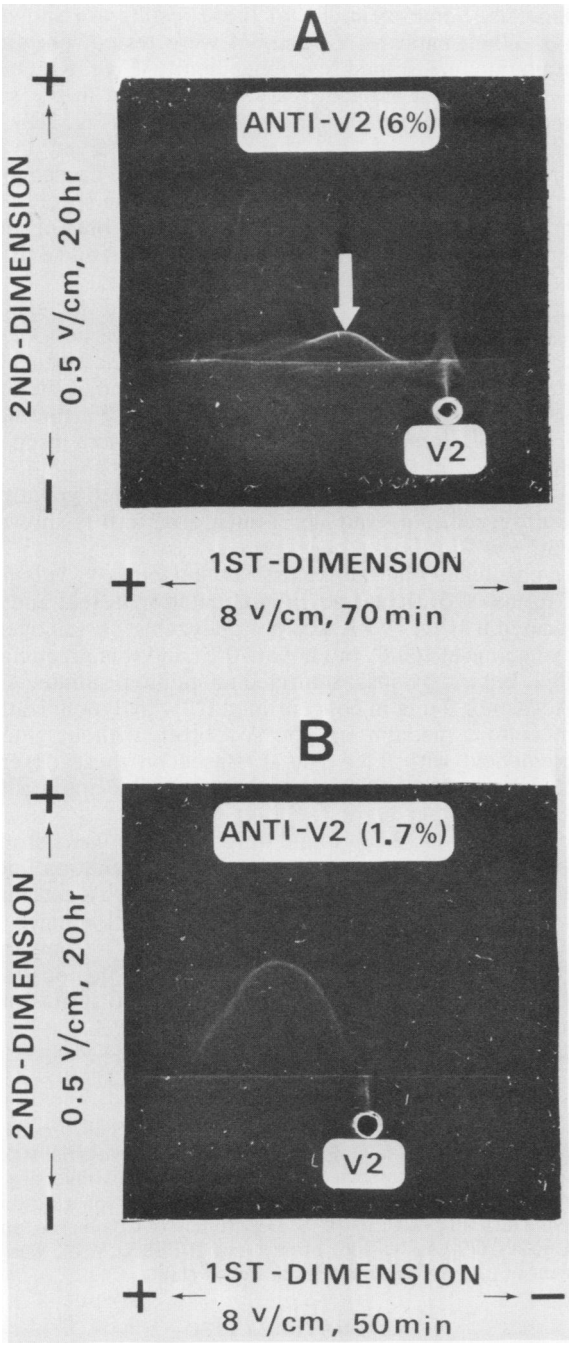


FIG. 2. Antigenic analysis of *V. vulnificus* V2 by two-dimensional immunoelectrophoresis. Sonicated V2 antigen was placed in the well and electrophoresed in 0.8% agarose. Antiserum against whole cells of *V. vulnificus* V2 (anti-V2 serum) was incorporated into the top portion of agarose at the concentration indicated. (A) First-dimension of electrophoresis for 70 min at 8 V/cm; antiserum concentration, 6% (vol/vol). The arrow indicates the group antigen of *V. vulnificus*. (B) First-dimension electrophoresis for 50 min; antiserum concentration, 1.7% (vol/vol).

interscapular muscles 32 days after the initial injection. Serum was prepared from the blood obtained 2, 3, and 4 weeks after the booster. Pooled serum was filter sterilized and stored at 4°C with added 0.01% thimerosal (Sigma Chemical Co.). These antisera prepared against whole bacterial cells were given experimental strain notations, e.g., anti-V1 serum, which is antiserum prepared against whole cells of bacterial strain V1.

Ouchterlony immunodiffusion analysis. Ouchterlony double-diffusion plates were prepared by solidifying buffered agarose, consisting of 0.8% agarose (type II; Sigma) and 0.01% thimerosal in Veronal buffer (pH 8.6; ionic strength, 0.05), in a plastic petri dish (100 by 15 mm). Antisera and antigens placed in the wells were allowed to react in a moist chamber at room temperature for up to 3 days. The results were photographed against dark-field illumination.

Two-dimensional immunoelectrophoresis. About 4.6 ml of melted 0.8% agarose prepared in Veronal buffer was solidified over a glass plate (50 by 50 by 2 mm). Antigen(s) put in the well(s) made at the bottom-right position was electrophoresed at 8 V/cm in the first dimension for 50 or 70 min at room temperature. The upper two-thirds of the gel was then removed. Antiserum was incorporated into 3.5 ml of melted 0.8% agarose gel in Veronal buffer (50°C) at the concentration indicated below, and the gel was casted to cover the top portion of the glass plate. Electrophoresis in the second dimension was conducted at 0.5 V/cm for 20 h at room temperature. The gel plate was incubated at 4°C overnight before the results were photographed against dark-field illumination.

RESULTS AND DISCUSSION

The initial survey for a possible *V. vulnificus* group antigen was conducted by Ouchterlony immunodiffusion analysis with sonicated whole-cell antigens and antisera prepared against whole cells. Sample reactions with anti-V4 serum are illustrated in Fig. 1. The most distinct precipitation line (examples shown by arrows) appeared common to all strains of *V. vulnificus* (V1 to V9 and V'1 to V'3 in Fig. 1A, B, and C) but was not detected in other organisms, with the possible exception of strain SP1 (Fig. 1C, D, and E). A very similar precipitation pattern was also observed when anti-V2 and anti-V'3 sera were used in the place of anti-V4 serum.

Two-dimensional immunoelectrophoresis analysis, which gives a much higher resolution than Ouchterlony immunodiffusion, was performed to determine whether this most distinct precipitation line was common to *V. vulnificus*. One strain of *V. vulnificus* (V2) and its homologous antiserum (anti-V2 serum) were used to optimize the experimental conditions for this antigenic analysis. Preliminary experiments with different concentrations of antiserum and antigens indeed revealed one dominant precipitation line (arrow, Fig. 2A). The concentration of antiserum in the gel was subsequently reduced to the extent that only the most distinct precipitation line could be seen (Fig. 2B).

To determine whether other strains of *V. vulnificus* have the same dominant precipitation line as seen in Fig. 2B, test antigens were placed into a second well and analyzed in tandem with the V2 antigen and antiserum by immunoelec-

FIG. 1. Ouchterlony immunodiffusion analysis of antigens of various *Vibrio* and *Aeromonas* species. Center wells contain rabbit antiserum prepared against whole cells of *V. vulnificus* V4. Outer wells contain sonicated whole-cell antigens of *Vibrio* and *Aeromonas* species. Arrows indicate precipitation lines formed by a presumed group antigen of *V. vulnificus*. See Table 1 for strain designations.

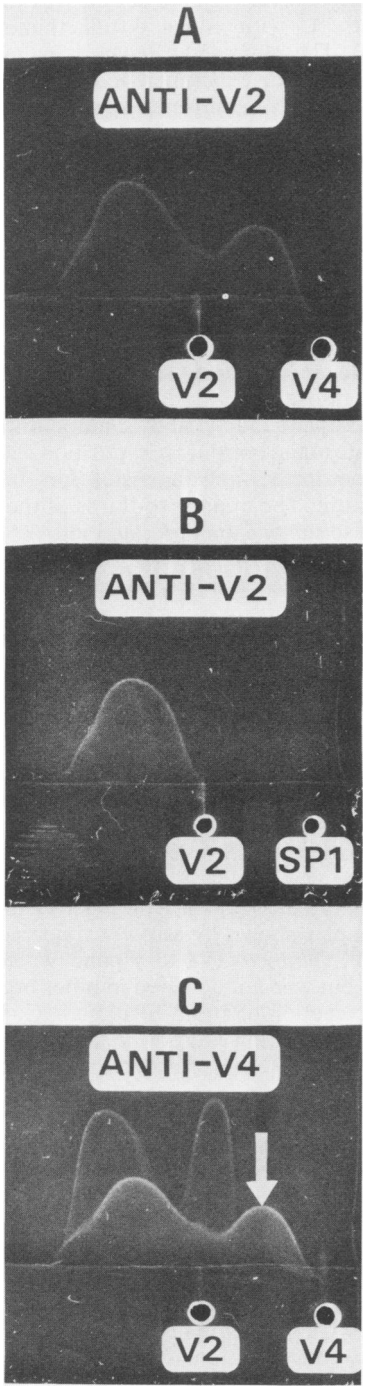


FIG. 3. Reaction of the group antigen of *V. vulnificus* by tandem two-dimensional immunoelectrophoresis. Sonicated whole-cell antigens were electrophoresed in 0.8% agarose gel at 8 V/cm for 50 min in the first dimension. Electrophoresis in the second dimension was performed in 0.8% agarose containing 1.7% (vol/vol) antiserum at 0.5 V/cm for 20 h. (A) Reaction of antigens V2 and V4 with anti-V2 serum. (B) Reaction of antigens V2 and SP1 with anti-V2 serum. (C) Reaction of antigens V2 and V4 with anti-V4 serum. The fused precipitation line in (A) and (C) demonstrate identity of the reciprocal antigen and antisera reactions.

trophoresis. Some examples of these results are shown in Fig. 3. When antigens V2 and V4 were tested, one fused precipitation line formed revealing identity (Fig. 3A). On the other hand, when antigen SP1 was examined in the same manner, there was no fused precipitation line (Fig. 3B). The latter results suggest that the reaction observed in the Ouchterlony immunodiffusion (Fig. 1E) was caused by a different antigen superimposed over that of the species-specific antigen or by an antigen that shares one or more antigenic determinants with that of *V. vulnificus* and exists at significantly lower concentration.

All strains listed in Table 1 were tested by tandem two-dimensional immunoelectrophoresis with anti-V2 serum. Results demonstrate that all *V. vulnificus* strains displayed the same fused line of identity, but none of the other test organisms showed any precipitation line. Furthermore, when anti-V2 serum was replaced by antisera prepared against other strains of *V. vulnificus* (anti-V1, -V3, -V4, -V5, and -V6 sera) for reciprocal tests, identical reaction patterns were observed. An example of such a pattern is shown in Fig. 3C.

We concluded that there exists an antigen in *V. vulnificus* that forms a distinct line in precipitation tests, and we designated it VVA. VVA was not dialyzable, lost antigenicity by heating at 100°C, but not at 70°C, and was precipitated by 70%, but not by 50%, saturated ammonium sulfate. Also, VVA was not found in concentrated (20× by lyophilization) spent culture medium (tryptic soy broth without glucose supplemented with 0.5% NaCl). Based on these observations, VVA was considered to be a possible intracellular protein.

So far, VVA has been found only in *V. vulnificus* strains. In addition, VVA forms a very distinct precipitation line in the simple Ouchterlony immunodiffusion test. Accordingly, it is believed that VVA could be a useful antigen for identification of *V. vulnificus* serotypes. In a companion study (11), the specificity of purified VVA is demonstrated by testing other bacterial species plus some 60 strains of *V. vulnificus* with antiserum prepared against VVA.

ACKNOWLEDGMENTS

The authors thank M. T. Kelly, J. Baross, R. J. Siebeling, N. C. Roberts, J. L. Fryer, and O. P. Daily for providing bacterial strains.

This research was supported by the Oregon State University Sea Grant College Program, National Oceanic and Atmospheric Administration Office of Sea Grant, U.S. Department of Commerce, under grant no. NA79AA-D-00106, project no. R/FSD8. M.N. was the recipient of a J. A. Tartar Research Fellowship.

LITERATURE CITED

1. **Bang, S. S., L. Baumann, M. J. Woolkalis, and P. Baumann.** 1981. Evolutionary relationships in *Vibrio* and *Photobacterium* as determined by immunological studies of superoxide dismutase. Arch. Microbiol. **130**:111–120.
2. **Baumann, P., L. Baumann, and B. G. Hall.** 1981. Lactose utilization by *Vibrio vulnificus*. Curr. Microbiol. **6**:131–135.
3. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine vibrio: clinical characteristics and epidemiology. N. Engl. J. Med. **300**:1–5.
4. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34**:341–367.
5. **Bonner, J. R., A. S. Coker, C. R. Berryman, and H. M. Pollock.** 1983. Spectrum of vibrio infections in a gulf coast community. Ann. Intern. Med. **99**:464–469.
6. **Clark, W. A., and A. G. Steigerwalt.** 1977. Deoxyribonucleic acid reassociation experiments with a halophilic, lactose-fer-

menting vibrio isolated from blood cultures. Int. J. Syst. Bacteriol. **27**:194–199.
7. **Farmer, J. J., III.** 1979. *Vibrio* ("*Beneckea*") *vulnificus*, the bacterium associated with sepsis, septicaemia and the sea. Lancet **ii**:903.
8. **Farmer, J. J., III.** 1980. Revival of the name *Vibrio vulnificus*. Int. J. Syst. Bacteriol. **30**:656.
9. **Hollis, D. G., R. E. Weaver, C. N. Baker, and C. Thornsberry.** 1976. Halophilic *Vibrio* species isolated from blood cultures. J. Clin. Microbiol. **3**:425–431.
10. **Kelly, M. T., and D. M. Avery.** 1980. Lactose-positive *Vibrio* in seawater: a cause of pneumonia and septicemia in a drowning victim. J. Clin. Microbiol. **11**:278–280.
11. **Nishibuchi, M., and R. J. Seidler.** 1984. Rapid microimmunodiffusion method with species-specific antiserum raised to purified antigen for identification of *Vibrio vulnificus* serotypes. J. Clin. Microbiol. **21**:102–107.
12. **Reichelt, J. L., P. Baumann, and L. Baumann.** 1976. Study of genetic relationships among marine species of the genera *Beneckea* and *Photobacterium* by means of in vitro DNA/DNA hybridization. Arch. Microbiol. **110**:101–120.
13. **Roland, F. P.** 1970. Leg gangrene and endotoxin shock due to *Vibrio parahaemolyticus*—an infection acquired in New England coastal waters. N. Engl. J. Med. **282**:1306.
14. **Thorsteinsson, S. B.** 1974. Clinical manifestations of halophilic noncholera vibrio infections. Lancet **ii**:1283–1284.
15. **Tison, D. L., J. Greenwood, M. Nishibuchi, and R. J. Seidler.** 1984. Molecular taxonomy of lactose-fermenting vibrios, p. 217–237. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.
16. **Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler.** 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. **44**:640–646.
17. **Tison, D. L., and R. J. Seidler.** 1981. Genetic relatedness of clinical and environmental isolates of lactose-positive *Vibrio vulnificus*. Curr. Microbiol. **6**:181–184.
18. **Tison, D. L., and R. J. Seidler.** 1983. *Vibrio aestuarianus*: a new species from estuarine waters and shellfish. Int. J. Syst. Bacteriol. **33**:699–702.
19. **Weaver, R. E., and N. J. Ehrenkranz.** 1975. *Vibrio parahaemolyticus* septicemia. Arch. Intern. Med. **13**:197.
20. **Zide, N., J. Davis, and N. J. Ehrenkranz.** 1974. Fulminating *Vibrio parahaemolyticus* septicemia. Arch. Intern. Med. **133**:479–481.