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Characterization and Relatedness of Marine Vibrios Pathogenic to Fish: Physiology, Serology, and Epidemiology

R. E. PACHA[1] AND E. D. KIEHN[2]

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, and Department of Microbiology, University of Washington, Seattle, Washington 98105

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Cultural characteristics and serological relationships of pathogenic marine vibrios isolated from fish in the Pacific Northwest were studied. These organisms were compared with cultures of *Vibrio anguillarum*, a known fish pathogen. On the basis of morphological and cultural characteristics, the Pacific Northwest strains of *Vibrio* were found to be closely related to *V. anguillarum*. Serological analyses of thermostable antigens served to distinguish three serotypes among the vibrios. Serotype 1 was composed of organisms isolated from Northwest salmonids; serotype 2 of strains of *V. anguillarum* from European waters; and serotype 3 of organisms isolated from Pacific herring. The epidemiology of vibrio disease among populations of fish in the Pacific Northwest is discussed.

Marine vibrios were first reported to be pathogenic to fish in 1909 when Bergman (3) described an outbreak of disease in eels from the Baltic Sea. This disease was characterized by the appearance of bloody lesions in the musculature of the infected fish. A marine vibrio, described as *Vibrio anguillarum* was shown to be the etiological agent of this disease. Since Bergman's original description, numerous outbreaks of disease due to marine vibrios have been recognized among a variety of fish hosts in European waters (1-7, 12, 14-16).

In the Pacific Northwest, outbreaks of disease caused by marine vibrios were first reported in young salmonids by Rucker, Earp, and Ordal (13). These outbreaks occurred among young salmon held at the Deception Pass Marine Station of the Washington State Department of Fisheries and among rainbow and steelhead trout at the Vancouver Hatchery of the Washington State Department of Game. The disease which occurred in these fish was characterized by the presence of extensive hemorrhages in the musculature and internal organs, and appeared to be similar to that described by Bergman (3). Severe losses due to this disease occurred among the populations of fish at both of these hatcheries. Additional outbreaks of disease in the Pacific Northwest due to marine vibrios have been noted among herring. At times, mass mortalities have occurred among populations of these fish being held for live bait (13).

Descriptions of *V. anguillarum* in the literature are somewhat variable. Nybelin (12), in 1935, investigated the cultural reactions of a number of strains of *V. anguillarum*. As a result of his studies, two biochemical types were recognized. More recently, a third biochemical type obtained from finnock in Scotland was described by Smith (16). These latter studies also served to extend the description of *V. anguillarum*.

To date, very little attention has been given to the characteristics of the pathogenic vibrios isolated from fish in the Pacific Northwest. The purpose of the present study was to examine the characteristics of these organisms and to compare them with known cultures of *V. anguillarum*. Several approaches have been used to examine the relationships among these bacteria in an effort to provide an accurate classification of the organisms. This paper deals with the cultural characteristics and serology of the organisms. Deoxyribonucleic acid (DNA) base analyses and DNA hybridization studies are reported in a subsequent communication (10).

MATERIALS AND METHODS

Source of strains. Thirteen strains of *Vibrio* and vibrio-like organisms isolated from fish in the Pacific Northwest were studied. Three strains of *Vibrio*

[1] Present address: Department of Biological Sciences, Central Washington State College, Ellensburg, Wash.

[2] Present address: Salk Institute for Biological Studies, La Jolla, Calif.

identified as *V. anguillarum* were examined for comparison. The code numbers and sources of the cultures are shown in Table 1.

Marine vibrios were maintained by inoculating the surface of an agar medium of the following composition: tryptone, 0.05%; yeast extract, 0.05%; beef extract, 0.02%; sodium acetate, 0.02%; Difco agar, 0.4%; seawater, 80%. Incubation was at 18 C, and after growth the stock cultures were stored at 4 C.

Liquid cultures used as inocula for the various tests performed were grown in Brain Heart Infusion (Difco) containing sodium chloride at a concentration of 1.5% (BHI broth).

Morphological characteristics. After 24 hr of incubation at 18 C in BHI broth, the organisms were stained by the Hucker modification of the Gram stain (17). Colony appearance on BHI agar (BHI broth containing 1.5% Difco agar) was examined after 3 to 4 days of incubation at 18 C. Cell morphology and motility were determined by phase-contrast examination of 24-hr broth cultures grown in casein hydrolysate medium. The casein hydrolysate medium consisted of 0.1% $(NH_4)H_2PO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 1.5% NaCl, and 0.2% vitamin-free acid-hydrolyzed casein; the *p*H was 7.0.

Media and tests. The requirement for salt was established by streaking cultures on agar plates prepared with distilled water, 0.2% tryptone, 2.0% sodium chloride, and 1.5% Difco agar, and on plates of the same medium without sodium chloride. A standard inoculum was one loopful of a 24-hr BHI broth culture. All plates were incubated at 18 C.

The ability of the vibrios to grow at 5, 18, 25, 30, and 37 C was tested by inoculating a drop of a 24-hr broth culture onto BHI agar plates which had been preincubated at the temperature of incubation. The plates were examined for 1 week for the presence of growth.

The reaction to antibiotics was determined by placing Sensidiscs of the antibiotics (BBL) on BHI agar plates streaked with the test organism. The strains were tested for susceptibility to chlortetracycline (30 μg), bacitracin (10 units), chloramphenicol (30 μg), dihydrostreptomycin (10 μg), erythromycin (15 μg), nitrofurazone (100 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 units), polymyxin B (300 units), sulfadiazine (1.0 mg), oxytetracycline (30 μg), and tetracycline (30 μg).

Starch hydrolysis was carried out by growing cultures on starch-agar (Difco) containing 1.5% sodium chloride. The cultures were incubated for 1 week and then flooded with a dilute iodine solution.

Gelatin hydrolysis was tested by inoculating Nutrient Agar containing 0.4% gelatin and 1.5% sodium chloride. After incubation, the plates were flooded with acid mercuric chloride.

Nitrate reduction and acetylmethylcarbinol production were tested by the method outlined in the *Manual of Microbiological Methods* (17). The media used for these tests consisted of nitrate broth (Difco) and MR-VP medium (Difco) containing 1.5% sodium chloride.

Indole production was determined by use of 1.0% tryptone (Difco) broth containing 1.5% sodium chloride.

TABLE 1. *Source of isolates*

Isolate	Source	Received from
Northwest U.S. vibrios		
2.1, 4.3, 14, 16, 53	Chinook salmon	E. J. Ordal
8, 17, 30, 78, 83	Steelhead trout	E. J. Ordal
61-1	Sockeye salmon	R. E. Pacha
56, 60	Pacific herring	E. J. Ordal
European vibrios (*V. anguillarum*)		
NCMB6	Cod	J. M. Shewan
V-2911, V-2916	Finnock	I. W. Smith

Hydrogen sulfide formation was detected by inoculation of SIM Medium (Difco) containing 1.5% sodium chloride.

The Hugh-Leifson procedure was used to test for the production of acid from carbohydrates (9). The carbohydrate solution was filter-sterilized and added to the basal medium at a final concentration of 1.0%. The basal medium was supplemented with 1.5% NaCl.

Nutritional requirements of the organisms were tested by use of a casein hydrolysate medium. The composition of this medium was the same as that used in studying the morphological chracteristics of the organisms. Utilization of citrate as a source of carbon was tested by use of Simmons citrate agar (Difco).

Catalase was detected by adding growth from a BHI agar plate to a drop of 2% hydrogen peroxide.

Serological analyses. Immune sera were obtained from rabbits injected intravenously with saline-washed suspensions of cells in successive doses of 0.2, 0.4, 0.8, and 1.0 ml every third day. The rabbits were bled from the marginal ear vein 2 weeks after the last injection. The sera were stored in the frozen state.

A slide agglutination procedure was used to examine the serological relationships among the isolates. The antigen used in these studies consisted of a suspension of saline-washed cells which had been heated in boiling water for 2 hr to destroy the flagellar antigens. Adsorbed antisera were prepared by adding 1 ml of a dense suspension of antigen to 1 ml of antiserum. The antigen was prepared in the same way as that used in the agglutination studies. Adsorption was carried out at 50 C for 30 min and for several hours at 4 C. The antigen and adsorbed antibody were removed by centrifugation.

RESULTS

Cell morphology. The organisms were all small gram-negative rods (average size, 1 × 2.5 μm) displaying a slight curvature. Occasionally the organisms existed in pairs or short chains which gave them a spiral appearance. Round or oval bodies similar to those described by Liston (11) for the gut group vibrios were often visible. All of the organisms were polarly flagellated and actively motile.

Colonial morphology. Colonies of the organisms on BHI agar were 1 to 4 mm in diameter, tan in color, translucent, slightly raised, and mucoid with an entire edge. The internal structure of the colonies was finely granular at 15 times magnification. Strain 53 was the only isolate observed to produce a yellowish-green water-soluble pigment.

Growth requirements. All of the organisms except strains 17, 56, and 60 were found to grow throughout the temperature range of 5 to 37 C. However, as would be expected, growth at 5 C was much slower than at higher temperatures. Strains 17, 56, and 60 failed to grow at 37 C, but grew well between 5 and 30 C.

All of the isolates grew abundantly on tryptone-agar containing 2% sodium chloride and, except for strain 53, failed to grow on the same medium from which the sodium chloride had been omitted. An attempt was made to substitute other salts for the sodium chloride with strains 2.1, 4.3, V-2911, V-2916, and NCMB6 as test organisms. It was found that 0.07% KCl, 0.1% $CaCl_2$, or 0.7% $MgSO_4 \cdot 7H_2O$ could not replace the requirement for sodium chloride in the strains tested.

The nutritional requirements for the organisms were not complex. All of the organisms were able to grow on vitamin-free casein hydrolysate medium containing sodium chloride.

Susceptibility to antibiotics. The 16 strains studied were sensitive to chlortetracycline, chloramphenicol, neomycin, oxytetracycline, and tetracycline. The majority of the isolates also were sensitive to dihydrostreptomycin, erythromycin, nitrofurazone, novobiocin, and polymyxin B, whereas none of the strains was sensitive to bacitracin or penicillin. The antibiotic resistance of the 16 isolates is summarized in Table 2.

Physiological properties. All of the isolates reduced nitrate to nitrite, and were weakly catalase-positive. The majority of the organisms also hydrolyzed gelatin and produced both indole and acetylmethylcarbinol. None of the strains produced hydrogen sulfide in SIM medium. A detailed comparison of the strains can be made from the data presented in Table 2. It should be noted that the strains of vibrio isolated from salmonids from the Pacific Northwest (strains 2.1, 4.3, 8, 14, 15, 17, 30, 78, 83, and 61-1) and the cultures of *V. anguillarum* (strains NCMB6, V-2911, and V-2916) were identical in these tests, except that strain 78 did not produce acetylmethylcarbinol.

Carbohydrate utilization. With the exception of strains V-2916 and 60, all of the isolates were able to utilize citrate as a sole source of carbon. Aside from strain 53, all of the isolates hydrolyzed starch and were capable of fermenting a variety of carbohydrates. Strain 53 oxidized the majority of the carbohydrates tested, but did not ferment these compounds. All of the fermentative strains produced acid anaerobically from glucose, mannitol, maltose, galactose, levulose, and mannose. The majority of the strains fermented glycerol. Lactose was fermented only by strain 60, and none of the isolates attacked xylose anaerobically.

The cultural characteristics of the vibrios examined in this study are summarized in Table 2.

Serological analyses. Antisera prepared against two of the vibrio isolates were used to test the antigenic relationships among the organisms. Strains 2.1 and NCMB6 were used in the preparation of the antisera. The homologous titers of antisera ranged from 1:640 to 1:1,280. Each of the isolates employed in this investigation was tested against these antisera by use of the slide agglutination technique (Table 3). It was found that all of the strains reacted with one or more of the antisera. Strain 53 was the only isolate which failed to react with antiserum prepared against culture NCMB6.

Agglutinin-adsorption studies were carried out in order to examine further the antigenic relationships among the isolates. Each of the antisera was adsorbed with the heterologous strains which cross-reacted with them. These adsorbed sera were then tested against each of the antigens by use of the slide agglutination technique. The results of selected agglutinin-adsorption experiments are shown in Table 4. When antiserum 2.1 was adsorbed with strain 4.3, 8, 14, 16, 17, 30, 78, 83, or 61-1, the adsorbed antisera failed to agglutinate the homologous strain. Similarly, the ability of antiserum NCMB6 to agglutinate the homologous strain was lost after adsorption with either strain V-2911 or V-2916. The results obtained with these adsorbed sera in which homologous strains failed to react are not included in Table 4.

By use of the data presented in Table 4, it is possible to assign arbitrary symbols to represent the antigens detected in the isolates (Table 5). The antigens detected in strain 2.1 also can be assigned to strains 4.3, 8, 14, 16, 17, 30, 78, 83, and 61-1, since these organisms completely adsorb antiserum 2.1. Similarly, the antigens detected in strain NCMB6 can be assigned to strains V-2911 and V-2916. It is interesting to note from the data presented in Table 5 that the majority of the strains possess a common antigen, antigen 1, and are thus interrelated. It will be noted that a close relationship exists between the

TABLE 2. *Cultural and physiological properties of marine vibrios*[a]

Characteristic	Strain														
	2.1	4.3	8	14	16	17	30	78	83	61-1	53[b]	56	60	NCMB6	V-2911	V-2916
Growth at																
5 C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30 C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 C	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
NaCl tolerance																
Growth in 0%	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Growth in 2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrate utilization																
Glucose oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Sucrose oxidized	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
Sucrose fermented	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
Lactose oxidized	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−
Lactose fermented	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Mannitol oxidized	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Mannitol fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Maltose oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Galactose oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Levulose oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levulose fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Xylose oxidized	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Xylose fermented	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannose oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose fermented	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Arabinose oxidized	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−
Arabinose fermented	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−
Glycerol oxidized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol fermented	+	+	−	+	−	−	+	−	−	−	−	+	+	+	−	+
Nitrate reduced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetylmethylcarbinol produced	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	+
Indole produced	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
Hydrogen sulfide produced	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gelatin hydrolyzed	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Starch hydrolyzed	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Citrate utilized	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−
Casein hydrolysate utilized	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Susceptible to																
Chlortetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bacitracin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dihydrostreptomycin	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+
Erythromycin	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Nitrofurazone	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Neomycin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Novobiocin	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Penicillin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Polymyxin B	+	−	−	+	+	+	+	−	+	−	+	+	+	+	+	+
Sulfadiazine	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−
Oxytetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Symbols + = organisms possess characteristic; − = organisms lack characteristic.
[b] Strain 53 is probably a pseudomonad rather than a vibrio.

TABLE 3. *Homologous and heterologous agglutination reactions of antisera 2.1 and NCMB6*

Strain	Antiserum	
	2.1	NCMB6
2.1	+	+
4.3	+	+
8	+	+
14	+	+
16	+	+
17	+	+
30	+	+
78	+	+
83	+	+
61-1	+	+
53	+	−
56	+	+
60	+	+
V-2911	+	+
V-2916	+	+
NCMB6	+	+

TABLE 4. *Slide agglutination reactions of antisera 2.1 and NCMB6 after adsorption with heterologous cells*

Serum	Adsorbing strain	Antigen				
		2.1	NCMB6	53	56	60
2.1	NCMB6	+	−	+	−	−
	53	+	+	−	+	+
	56	+	+	+	−	−
	60	+	+	+	+	−
NCMB6	2.1	−	+	−	−	−
	56	+	+	−	−	−
	60	+	+	−	+	−

TABLE 5. *Antigenic composition of vibrio isolates*

Strains	Antigens				
	1	2	3	4	5
2.1, 4.3, 8, 14, 16, 17, 30, 78, 83, 61-1	+	+	+	+	−
53	−	+	−	−	−
56	+	−	+	−	−
60	+	−	−	−	−
2911, 2916, NCMB6	+	−	+	+	+

strains of *Vibrio* isolated from salmonid fishes in the Pacific Northwest (strains 2.1, 4.3, 8, 14, 16, 17, 30, 78, 83, and 61-1) and *V. anguillarum* (strains NCMB6, V-2911, and V-2916) because these organisms have three antigens in common. Nevertheless, these two groups of organisms can be distinguished from each other and are not identical. The vibrios isolated from herring (strains 56 and 60) are more distantly related to the strains of *V. anguillarum* and to the strains isolated from salmonids from the Pacific Northwest. Strain 53 is the only isolate which did not possess antigen 1, and it appears to be serologically distinct. This organism, however, does possess an antigen in common with strain 2.1.

DISCUSSION

When studied morphologically and physiologically, the strains of *V. anguillarum* were found to be very similar to the majority of the vibrios isolated from fish in the Pacific Northwest. Some variation existed among the cultures with regard to antibiotic susceptibility, indole production, acetylmethylcarbinol production, gelatin hydrolysis, citrate utilization, and the ability to ferment arabinose, sucrose, lactose, and glycerol. These variations were not consistent between the two groups of vibrios and probably represent slight strain differences. Strain 53 was the only isolate found to be significantly different from the other organisms studied. This isolate was oxidative rather than fermentative, produced a yellowish-green water soluble pigment, and was not obligately halophilic. Most likely this organism is a pseudomonad rather than a vibrio.

In his description of *V. anguillarum*, Nybelin (12) subdivided the species into two types. The variety *typica* produced acid but no gas from sucrose and mannitol and produced indole. The variety *anguillicida* was differentiated by its inability to ferment these sugars and to produce indole. Recently, Smith (16) identified a third variant isolated from finnock. This organism was referred to as type C and was intermediate between the *typica* and *anguillicida* varieties described by Nybelin. Type C differed from the variety *typica* by its inability to produce indole. Of the three types of *V. anguillarum* reported in the literature, the majority of the vibrios isolated from fish in the Pacific Northwest seem to be most closely related to the variety *typica*. All of the Northwest isolates fermented mannitol, and all but strain 60 fermented sucrose and produced indole. *V. anguillarum* var. *typica* was recently identified as the causative agent of an epizootic of vibriosis in juvenile chinook salmon reared in a salt-water impoundment on the Oregon coast (8).

Although the strains of vibrio isolated from fish in the Pacific Northwest were morphologically and biochemically similar to the cultures of *V. anguillarum* studied, these organisms could

be distinguished serologically. On the basis of the cross-adsorption studies of the thermostable antigens, the vibrios could be placed into three serotypes. These are shown in Table 6. The distribution of antigens in the strains within each serotype shows that the Northwest salmonid vibrios (serotype 1) and the European vibrios (serotype 2) are closely related and that both of these groups are more distantly related to the Pacific herring vibrios (serotype 3). Of the Pacific herring vibrios, strain 56 is most closely related to the European and Northwest salmonid vibrios because the organism has 2 antigens in common with each of these groups. This relationship is supported by the cultural characteristics. Strain 53 was not included in a serotype as this isolate is not considered to be a vibrio. The isolate does have an antigen in common with the organisms in serotype 1, but this is not considered to be evidence of a close relationship between the organisms because they are physiologically distinct.

The organisms in serotype 1 were isolated from two different hatcheries in the state of Washington. One group (strains 2.1, 4.3, 14, 16, and 61-1) was obtained from chinook and sockeye salmon at the Deception Pass Marine Station of the Washington State Department of Fisheries. The other group of organisms (strains 8, 17, 30, 78, and 83) was isolated from steelhead trout at the Vancouver Hatchery of the Washington State Department of Game. Since the isolates from these two sources are nearly identical, it is possible that a common source of infection was responsible for the outbreaks of disease at these two hatcheries. Another possibility is that the disease was transmitted by some means from one hatchery to the other. In this regard, it is of interest to note that, prior to the initial outbreak of disease, fish at the Vancouver Hatchery were fed marine scrapfish and salmon viscera obtained from the vicinity of the Deception Pass Marine Station. As a result, the disease may have been introduced into the Vancouver Hatchery in contaminated food.

TABLE 6. *Serotypes of pathogenic marine vibrios based on cross-adsorption studies*

Serotype	Vibrio strains	Thermostable antigens
1	2.1, 4.3, 8, 14, 16, 17, 30, 78, 83, and 61-1	1, 2, 3, 4
2	NCMB6, V-2911, and V-2916	1, 3, 4, 5
3	56 and 60	1, (3)[a]

[a] Present in some members of serotype 3 but absent in others.

The natural reservoir of vibrio disease is still uncertain. However, it is possible that herring or other natural populations of fish may harbor the organism and serve as a source of infection for fish in salt-water hatcheries. In view of the findings from the present study, it would seem that serological procedures could provide a useful tool for studying the epidemiology of the disease among fish populations.

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