INFECTION AND IMMUNITY, Mar. 1983, p. 1220–1227
0019-9567/83/031220-08$02.00/0

Vol. 39, No. 3

Molecular Factors Associated with Virulence of Marine Vibrios Isolated from Striped Bass in Chesapeake Bay

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Received 4 October 1982/Accepted 13 December 1982

On the basis of cultural and biochemical properties as well as DNA homology assays, 81 *Vibrio* strains isolated from diseased striped bass and from Chesapeake Bay water were assigned to eight distinct groups. All organisms belonging to two of the groups were pathogenic for striped bass and were identified as *Vibrio anguillarum*, whereas organisms classified in the other six groups were nonpathogenic and were designated as *Vibrio* spp. Unlike the pathogenic *V. anguillarum* strain 775 isolated in the Pacific Northwest, strains pathogenic for striped bass did not contain any plasmids; however, they were similar to the Northwest isolates in that virulence was correlated with their ability to grow in the presence of nonimmune striped bass serum or under conditions of iron limitation. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of outer membranes showed that additional proteins were induced in those organisms capable of growth under conditions of iron limitation. It was of interest that 22 of the nonpathogenic isolates harbored one or more plasmids which, by restriction endonuclease analyses, were shown to be clearly different from the virulence plasmid pJM1.

Vibriosis has been a particularly devastating disease in the marine environment, affecting salmonids and many other species of anadromous and catadromous fish (1, 16–18, 20, 24, 32, 36, 37). Although most studies have focused on *Vibrio anguillarum* as an etiological agent, other members of the genus *Vibrio* have also been implicated in epizootics of cultured and wild marine fish throughout the world (20, 23, 25, 27, 28, 31, 34, 40) and with disease in larval and juvenile bivalve molluscs and crustaceans (3, 5, 15, 39).

Virulent strains of *V. anguillarum* isolated from salmonids in the Pacific Northwest have been reported to harbor a specific plasmid class, pJM1, and a correlation between carriage of pJM1 and virulence has been substantiated by plasmid segregation studies in *V. anguillarum* strain 775 (13, 14). It has also been reported that this plasmid specifies an efficient iron-sequestering system which enables bacteria to survive under conditions of limited iron availability, such as those imposed by the iron-binding proteins, transferrin and lactoferrin, in the host fish system (8, 9). Later studies indicated that new outer membrane proteins are induced under conditions of iron limitation, and at least one of these proteins is produced when the virulence plasmid pJM1 is present (12). In addition, the ability to withstand the bactericidal action of nonimmune fish serum, a factor under chromosomal control, appears to be another component of virulence (38).

In this study, we report the first molecular characterization of marine vibrios isolated from striped bass held in Chesapeake Bay water. Of particular interest is a comparison of the molecular factors associated with virulence of these isolates with those reported for the Pacific Northwest strains.

MATERIALS AND METHODS

Bacterial isolation and characterization. The marine *Vibrio* strains used in this study were isolated from moribund striped bass (*Morone saxatilis*) held in tanks supplied with Chesapeake Bay water at Solomons Island or Shady Side, Md. The fish showed symptoms characteristic of vibriosis, including hemorrhaging of the fins, eyes, and ventral surface as well as internal petechiae. In addition, vibrios isolated directly from water were examined for comparative purposes.

Morphological and biochemical characteristics of the isolates were determined with the procedures described by Shotts and Bullock (35). Gram stain,

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oxidase test, motility and morphology, fermentation of glucose, and sensitivity to the vibriostatic agent 0/129 (Sigma Chemical Co.) and novobiocin (10 μg/ml) (Difco Laboratories) were used to identify the isolates as members of the genus *Vibrio*. Additional biochemical tests were conducted to group the different strains. *V. anguillarum* reference strains (ATCC no. 14181, 19105, 19109, and 19264) and *V. anguillarum* strain 775 were included as controls.

The marine vibrios were routinely cultivated in nutrient broth (Difco) supplemented with 0.5% yeast extract and 1.5% NaCl and were maintained at 15°C under mineral oil on slants of tryptic soy agar (Difco) with 1.5% NaCl. To test the capacity of marine vibrios to grow under iron-limiting conditions, the strains were grown in M-9 minimal medium (33) containing the iron-chelating agent ethylenediaminodiacetate (EDDA) (Sigma) at a concentration of 3 μM.

Infectivity assays. The pathogenicity of a representative number of *Vibrio* strains from each biochemical group was tested in juvenile striped bass (4 to 8 g) held in estuarine water at a salinity of 10 to 13 mg/liter and a temperature range of 17 to 20°C. Bacterial cultures grown at 22°C for 24 h were centrifuged, and the cells were resuspended in phosphate-buffered saline (pH 7.4) to 10^8 cells per ml as measured by the optical density at 540 nm. Fish were anesthetized with tricaine methane sulfonate and inoculated intraperitoneally with bacterial doses ranging from 10^2 to 10^7 cells, with 8 to 10 fish being used per dose. Viable bacteria in the inocula were determined by the spread-plate method. Mortalities were recorded daily for 10 days and were considered to be due to the inoculated *Vibrio* strains only when the strain was recovered from the kidney in pure culture. Virulence is reported as the number of bacteria needed to kill 50% of the animals inoculated (50% lethal dose [LD_{50}]) (30).

Serum susceptibility assay. Blood from juvenile striped bass was collected aseptically by severing the caudal peduncle. Sera were pooled and stored at −20°C. Overnight cultures of the marine vibrios were diluted to contain 10^4 to 10^5 cells per ml, and 0.1 ml was inoculated into either 0.9 ml of serum or 0.9 ml of medium as the control. Cultures were incubated at 20°C, and growth was determined by plate counts on samples withdrawn after 0, 5, 10, and 18 h of incubation. *V. anguillarum* strain 775 (serum resistant) and strain 1800 (serum sensitive) served as controls.

DNA hybridizations. Whole-cell DNA, for use in DNA-DNA hybridizations, was prepared essentially as described by Brenner et al. (4). Purified DNA was dialyzed against distilled water and sheared by ultrasonic treatment to an approximate molecular weight of 2.5×10^5. The DNA preparations were then adjusted to 0.42 M NaCl and stored at −20°C until use. Labeled whole-cell DNA from *Vibrio* strains 43-F, 89-F, and 104-F, representatives of three of the biochemical groups, was prepared from cells grown overnight at 22°C in M-9 minimal salts medium (33) in the presence of 20 μCi of [^{3}H]thymidine per ml (New England Nuclear Corp.; specific activity, 82 Ci/mmol). DNA-DNA hybridizations were carried out as described previously (33). Approximately 0.1 to 0.2 μg of ^{3}H-labeled, sheared, denatured whole-cell DNA was incubated with a 1,500-fold excess of unlabeled, sheared, denatured whole-cell DNA from each strain to be tested. DNA reassociations were performed in 0.42 M NaCl at 63°C for 23 h. The degree of homology was assessed by use of the S_1 endonuclease method of Crosa et al. (10).

Plasmid screening. The marine *Vibrio* strains were grown overnight in nutrient broth with 0.5% yeast extract and 1.5% NaCl and were screened for the presence of plasmid DNA by the method described by Crosa and Falkow (11) using TS buffer (50 mM Tris-hydrochloride, 100 mM NaCl [pH 8.0]) and by the Kado and Liu method (21). DNA samples (20 to 25 μl) were electrophoresed through 0.7% agarose (Sigma; type I) in Tris-borate buffer (TBE) (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8.0]) at 150 V and 60 mA for 5.5 h or in Tris-acetate buffer (40 mM Tris-acetate, 2 mM disodium EDTA [pH 7.9]) at 150 V and 110 mA for 4 h.

Electrophoresis was performed on a horizontal apparatus fitted with a water-cooling plate. The gels were stained for 2 h in a 0.5-μg/ml ethidium bromide solution, destained in water, and photographed under short-wave UV light with a Polaroid MP4 camera with type 55 P/N film and 23A and 2B filters. Molecular weights of plasmids were estimated by comparison with DNA standards included with each group of strains examined.

Restriction endonuclease cleavage analysis. Plasmid DNA from selected *Vibrio* strains was isolated by the method of Hansen and Olsen (19), avoiding the washing step and omitting disodium EDTA in the Tris-sucrose buffer. The preparations were purified by centrifugation in CsCl-ethidium bromide density gradients in a Beckman L8-70 ultracentrifuge at 45,000 rpm for 14 h at 15°C. Plasmid DNA bands were collected, and the ethidium bromide was extracted with CsCl-saturated isopropanol followed by dialysis against 7 mM Tris-hydrochloride–0.1 mM disodium EDTA (pH 7.5).

Cleavage of purified plasmid DNA with *Eco*RI, *Bam*HI, and *Hin*dIII restriction endonucleases was performed following the specifications of the supplier (Bethesda Research Laboratories, Bethesda, Md.). Controls included λ phage DNA and pJM1 plasmid DNA. Reactions were stopped by the addition of 5 μl of a mixture containing 7% sodium dodecyl sulfate (SDS), 33% glycerol, and 0.07% bromophenol blue. The samples were electrophoresed in 0.7% agarose gels at 60 V and 35 mA for 17 h, stained with ethidium bromide, destained in water, and photographed as described above.

Analysis of membrane proteins. Total cell envelopes, as well as outer membranes from marine *Vibrio* strains, were prepared as previously described (12). Basically, bacterial cultures (10 ml) were grown overnight at 20°C in M-9 minimal medium containing either 20 μM $FeCl_3$ or the chelating agent EDDA at a concentration of 3 μM.

Cells were resuspended in 1.5 ml of 10 mM Tris-hydrochloride–0.3% NaCl (pH 8.0) and sonically treated. After centrifugation in a microcentrifuge (Eppendorf), the supernatant, which contained the cell envelopes, was centrifuged for 1 h at 20,000 × *g* in a Beckman J2-21 centrifuge. To prepare outer membranes, the cell envelopes were treated with 1.5% Sarkosyl (wt/vol) in 10 mM Tris-hydrochloride (pH 8.0) at room temperature for 20 min to dissolve the inner membrane. Outer membrane material was collected by centrifugation at 20,000 × *g* for 1 h. The

TABLE 1. Biochemical groupings and virulence of marine *Vibrio* strains isolated from diseased striped bass[a]

Group no.	No. of strains	Growth at 37°C	Growth in 5% NaCl	Citrate utilization	Gelatin hydrolysis	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Acid from:			Virulence (LD_{50})[b]
									Arabinose	Sucrose	Mannitol	
1	17	+	+	−	+	+	−	−	−	+	+	3×10^2
2	9	+	+	+	+	+	−	−	+	+	+	8×10^3
3	6	−	+	−	+	(+)[c]	−	−	−	+	+	$>1 \times 10^6$
4	3	−	+	+	+	(+)	−	−	+	+	+	$>3 \times 10^7$
5	15	−	+	+	+	−	−	−	+	+	+	$>1.3 \times 10^6$
6	9	−	+	−	+	(+)	−	−	−	−	+	$>1 \times 10^6$
7	13	+	+	−	+	−	+	+	−	−	−	5.6×10^6
8	9	V[d]	+	V	−	−	−	−	V	+	+	$>2 \times 10^7$

[a] None of the 81 isolates grew at 42°C or in 8% NaCl, and all produced indole.
[b] LD_{50} was determined by the Reed and Muench method (30). The values shown are an average from two or more strains tested in each group.
[c] A weak or delayed positive reaction.
[d] V, Variable.

preparation was washed twice with distilled water and stored at −20°C until use.

Pellets from total cell envelopes or outer membranes were dissolved in 50 μl of a buffer consisting of 62.5 mM Tris-hydrochloride (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, 0.001% bromophenol blue, and 5% beta-mercaptoethanol. Suspensions were boiled for 5 min, and samples were applied to a 12.5% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 44:0.8) in 375 mM Tris-hydrochloride (pH 8.8) containing 0.2% SDS, with a 3% stacking gel in 125 mM Tris-hydrochloride (pH 6.8) containing 0.1% SDS. The electrophoresis buffer was 2.5 mM Tris base–200 mM glycine–0.1% SDS. After electrophoresis (100 V for 16 h), gels were stained with 0.05% Coomassie blue in 25% isopropanol–10% acetic acid. Gels were destained with 5% acetic acid and photographed.

RESULTS

Biochemical characteristics and virulence for striped bass. A total of 81 bacterial strains were isolated from kidney, liver, and skin lesions of diseased striped bass and from water in the fish holding tanks. None of these strains were able to grow in the absence of salt and, therefore, were characterized as marine vibrios, based on cultural and biochemical properties. The marine vibrios were divided into eight major groups, with arabinose and sucrose utilization, growth at 37°C, and arginine dihydrolase reaction serving as differentiating traits (Table 1). According to their characteristics, the organisms in groups 1 and 2 were identified as *V. anguillarum*, whereas the organisms in all other groups were designated as *Vibrio* spp. It was found that groups 3 and 6 are closely related, with sucrose utilization being a differentiating test. Also, the differentiating feature for groups 4 and 5 was the arginine dihydrolase reaction. Group 7 contained vibrios showing a close relationship to the human pathogen *V. vulnificus* (lactose-positive vibrios). Group 8 (gelatin-negative vibrios) was the most heterogeneous of the groups, with several reactions being variable among the strains.

A representative number of strains from each group was tested for infectivity in striped bass. Table 1 shows that members of groups 1 and 2 were the most virulent, with mean LD_{50}s of 3×10^2 and 8×10^3, respectively. Strains of the other six groups also tested were either avirulent or required concentrations 4 to 5 logs higher than for groups 1 and 2. *V. anguillarum* strain 775 and the ATCC strain 19264 were compared for infectivity and were found to be equally virulent, with LD_{50}s of approximately 10^3 cells.

DNA relationships among the vibrios. To confirm the phenetic classification, DNA homology tests, following the S1 endonuclease method, were done (10), results of which, expressed as percent homology, are shown in Table 2. Clearly, the results supported the grouping of strains based on phenetic analysis. Using labeled DNA from vibrios 43-F (group 1), 104-F (group 2), and 89-F (group 4), it was found that all of the pathogenic strains isolated from striped bass, as well as the European reference strain of *V. anguillarum* (ATCC 19264) and the Pacific Northwest strain 775, showed a high degree of relatedness (>80% homology) and were only distantly related to the nonpathogenic vibrios tested (groups 3, 4, 5, and 6). Conversely, the high degree of polynucleotide sequence homology between *Vibrio* spp. strains from groups 4 and 5 confirms the phenotypic similarity indicated by phenetic analysis (Table 1).

Plasmid content and restriction endonuclease cleavage patterns. It was recently shown that a high-virulence phenotype is correlated with the presence of a specific plasmid class, pJM1, in *V. anguillarum* strains isolated in the Pacific Northwest (13, 14).

Consequently, all of the *Vibrio* strains were examined for plasmids, as described above. Un-

TABLE 2. Polynucleotide sequence relationships among strains of marine vibrios isolated from striped bass

Source of unlabeled DNA[a]	% Homology[b] with labeled DNA from the following strains:		
	43-F	104-F	89-F
Group 1			
43-F	(100)[c]	(94.2)	6.3
56-F	(100)	(80)	7.3
Group 2			
96-F	(86)	(80)	5
99-F	(100)	(83)	28
104-F	(85)	(100)	10
Group 3			
61-F	10	9.4	12
SS-1	3.6	11.4	21.5
Group 4			
67-F	12	7	(100)
89-F	8.3	5.2	(100)
Group 5 strain 63-F	7.3	5.4	(100)
Group 6 strain 39-W	5.3	9.5	28.4
Reference strains			
V. anguillarum 775	(100)	(80)	7
V. anguillarum ATCC 19264	(100)	(90)	4
V. cholerae M-17151	11	29	6
Escherichia coli WP2	12	4.2	3.3

[a] Strain numbers are given for the biochemical groups defined in Table 1.

[b] Percent homology was determined by analysis with the single-strand-specific nuclease S1.

[c] Numbers in parentheses represent >80% homology, indicating a high degree of relatedness.

expectedly, plasmids were not detected in strains belonging to the pathogenic groups 1 and 2, although 22 of the nonpathogenic strains (27%) harbored one or more plasmids. Plasmids were present mainly in strains of groups 3 and 6. In these groups, all strains carried one high-molecular-weight plasmid (molecular weight ranging between 70×10^6 and 100×10^6) (Fig. 1).

Possible relationships between the plasmids, as well as with the pJM1 plasmid, were assessed by restriction endonuclease analysis in which the cleavage patterns obtained after digestion of the plasmids with the enzymes *Eco*RI, *Bam*HI, and *Hin*dIII were compared. Results of restriction analysis for plasmids of groups 3 and 6 are shown in Fig. 2. Plasmids from group 3, i.e., strains 44-F and 61-F, showed very similar cleavage patterns (Fig. 2, lanes D and E, J and K, and P and Q). However, this was by no means the rule, since other group 3 and group 6 plasmids had different restriction cleavage patterns (Fig. 2, lanes B and C, H and I, and N and O). The restriction cleavage patterns of all of the plasmids were clearly different from those of the virulence plasmid pJM1 (Fig. 2, lanes A, G, and M).

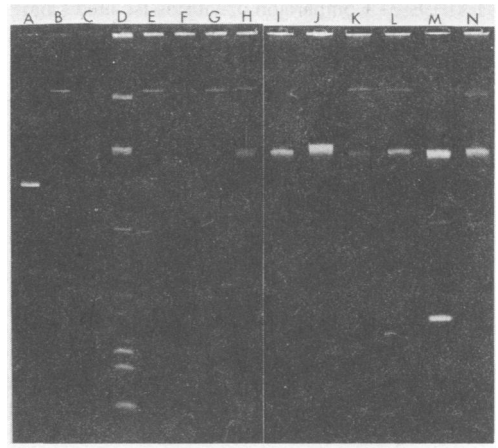

FIG. 1. Detection of plasmids in marine vibrios isolated from striped bass by agarose gel electrophoresis. Lane: A, *Vibrio* spp. 21-F (group 7); B, *Vibrio* spp. 44-F (group 3); C, *V. anguillarum* ATCC 14181; D, *E. coli* V517; E, *Vibrio* spp. 40-F (group 3); F, *Vibrio* spp. 61-F (group 3); G, *Vibrio* spp. 33-W (group 6); H, *Vibrio* spp. 32-W (group 3); I, *V. anguillarum* 35-F (group 1); J, *V. anguillarum* 96-F (group 2); K, *Vibrio* spp. 38-F (group 6); L, *Vibrio* spp. 66-F (group 6); M, *V. anguillarum* ATCC 19264; N, *V. anguillarum* 775.

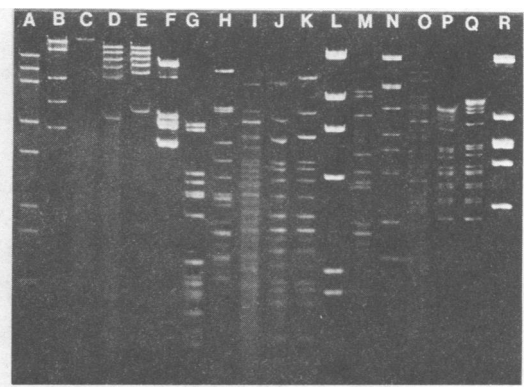

FIG. 2. Restriction endonuclease cleavage patterns of selected *Vibrio* plasmids. *Bam*HI cleaved: lane A, pJM1; B, 66-F plasmid; C, SS-1 plasmid; D, 44-F plasmid; E, 61-F plasmid; F, λ DNA. *Hin*dIII cleaved: lane G, pJM1; H, 66-F plasmid; I, SS-1 plasmid; J, 44-F plasmid; K, 61-F plasmid; L, λ DNA. *Eco*RI cleaved: lane M, pJM1; N, 66-F plasmid; O, SS-1 plasmid; P, 44-F plasmid; Q, 61-F plasmid; R, λ DNA.

TABLE 3. Comparison of vibrios isolated from striped bass as to their virulence, serum resistance, and ability to grow under conditions of iron limitation

Biochemical groups or bacterial strains	Growth in minimal medium containing 3 μM EDDA[a]	Serum resistance[b]	LD_{50}[c]
1	13/13	+	3×10^2
2	6/6	+	8×10^3
3	2/6(s)[d]	−	$>1 \times 10^6$
4	3/3	−	$>3 \times 10^7$
5	5/5	ND[e]	$>1.3 \times 10^6$
6	4/5(s)[d]	−	$>1 \times 10^6$
7	4/4	−	5.6×10^6
8	0/4	−	$>2 \times 10^7$
Controls			
775 (pJM1)	+	+	1×10^3
H775-3[f]	−	+	1×10^7

[a] Shown as the number of strains growing/number of strains tested. +, Indicates ability to grow in the presence of 3 μM EDDA.

[b] +, Indicates ability to grow in the presence of striped bass serum.

[c] Number of organisms needed to kill 50% of the fingerling striped bass inoculated intraperitoneally. Values shown for the biochemical groups are an average of two or more strains from each group.

[d] Slow or poor growth.

[e] ND, Not done.

[f] This strain is a low-virulence, plasmidless derivative of 775 (pJM1) obtained by curing of the pJM1 plasmid as previously described (13).

Growth under iron limitation and induction of outer membrane proteins. Results of previous studies (12) indicated that in Pacific Northwest strains of *V. anguillarum*, new outer membrane proteins are induced under conditions of iron limitation and at least one of these proteins is produced only when the virulence plasmid pJM1 is present.

To determine whether the marine *Vibrio* strains isolated from striped bass behaved in a similar fashion under conditions of iron limitation, the growth kinetics and induction of outer membrane proteins in the presence of iron-chelating agents were examined for strains selected from each of the groups.

From Table 3 it can be seen that growth of strains belonging to the pathogenic groups 1 and 2 was not affected by EDDA which acts to bind trace amounts of iron present in the minimal medium. Unexpectedly, strains belonging to the nonpathogenic groups 4, 5, and 7 had the capacity to grow under iron limitation conditions, although strains from groups 3, 6, and 8 showed weak or no growth at all under these conditions.

Total cell envelopes, as well as outer membranes prepared from cells grown under either high or low iron concentrations, were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 3a shows that the total membrane protein patterns of two representative strains of the pathogenic group 1 were identical (lanes B_1 and B_2 and C_1 and C_2). In fact, all members of this group showed the same pattern (data not shown). Pathogenic strains of group 2 showed total membrane protein patterns different from those of group 1 strains. At least two protein patterns were detected (Fig. 3a, cf. lanes D_1 and D_2 and E_1 and E_2 and F_1 and F_2 and G_1 and G_2).

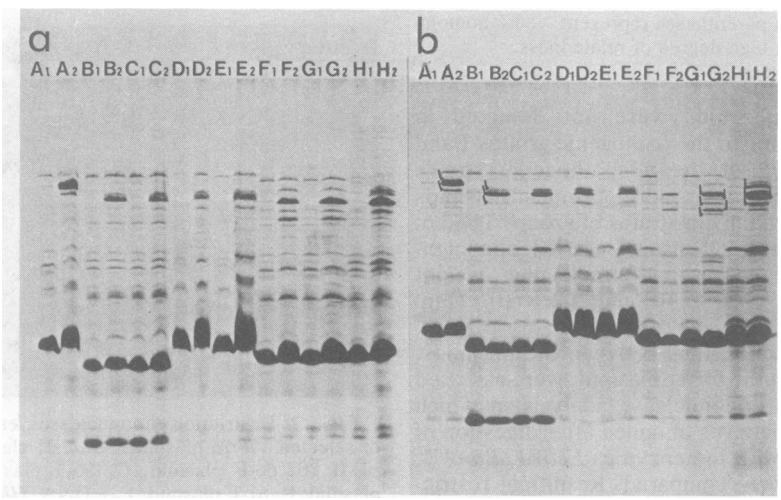

FIG. 3. Total cell envelope (a) and outer membrane (b) proteins of selected pathogenic strains of *V. anguillarum*. Lane: A, *V. Anguillarum* 775; B, strain 43-F; C, strain 94-F; D, strain 96-F; E, strain 103-F; F, strain 100-F; G, strain 104-F; H, *V. anguillarum* ATCC 19264. Subscript: 1, cells grown with 20 μM $FeCl_3$; 2, cells grown under conditions of iron limitation.

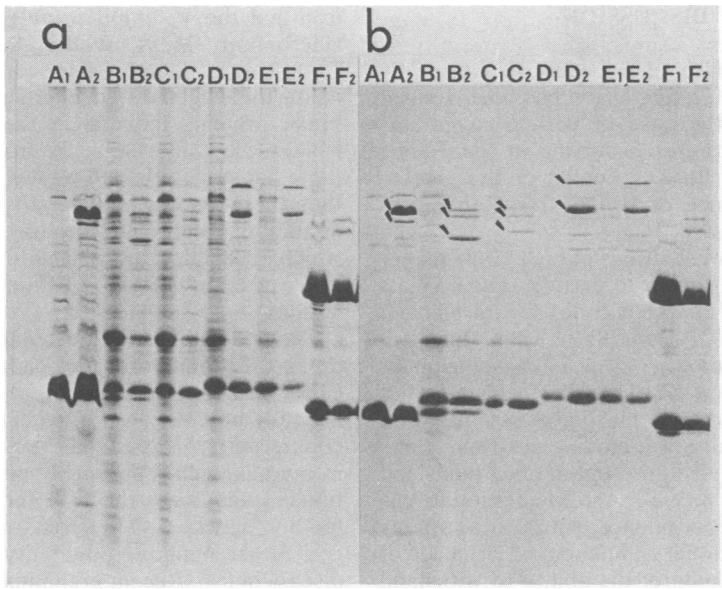

FIG. 4. Total cell envelope (a) and outer membrane (b) proteins of selected nonpathogenic *Vibrio* spp. strains. Lane: A, (control) *V. anguillarum* 775; B, strain SS-1; C, strain 66-F; D, strain 89-F; E, strain 63-F; F, strain 21-F. Subscript: 1, cells grown with 20 μM $FeCl_3$; 2, cells grown under conditions of iron limitation.

Analysis of the membrane proteins induced under conditions of iron limitation showed that at least three novel outer membrane proteins (molecular weights of 80,000, 75,000, and 73,000, respectively; identified by arrows) are apparent in strains of both groups 1 and 2 (Fig. 3b, lanes B_1 and B_2, C_1 and C_2, D_1 and D_2, and E_1 and E_2). However, in some strains of group 2, induction of five proteins ranging in size from 75,000 to 67,000 as well as several lower-molecular-weight proteins also occurred (Fig. 3b, lanes F_1 and F_2 and G_1 and G_2). Strain ATCC 19264 shows induction of four proteins ranging in size from 88,000 to 73,000 daltons.

All of the outer membrane proteins induced in these pathogenic strains from groups 1 and 2 are different in molecular weight from the OM2 protein (86,000, molecular weight) mediated by the virulence plasmid pJM1 (or the chromosome-mediated OM3 [79,000, molecular weight]) in *V. anguillarum* 775 (Fig. 3b, lanes A_1 and A_2).

The total membrane protein patterns of selected nonpathogenic *Vibrio* spp. (Fig. 4a) are completely different from those of *V. anguillarum* strains, although intergroup similarities are detectable which support the DNA hybridization and phenetic groupings (cf. group 4 and 5 strains in lanes D_1 and D_2 and E_1 and E_2).

Groups 4 and 5, which grow under iron limitation conditions (Table 3), showed induction of at least two specific outer membrane proteins (100,000 and 87,000 daltons, respectively; identified by arrows) (Fig. 4b, lanes D_1 and D_2 and E_1 and E_2). The other groups, which grew poorly under iron limitation conditions, showed induction of three proteins of about 87,000, 84,000, and 73,000 daltons for group 3 strains (Fig. 4b, lanes B_1 and B_2) and 88,000, 84,000, and 74,000 daltons for group 6 strains (Fig. 4b, lanes C_1 and C_2).

Group 7 strains, which resemble the human pathogen *V. vulnificus* showed a peculiar total membrane protein pattern, with two very large bands of major outer membrane proteins and no apparent induction of novel outer membrane proteins (Fig. 4a and b, lanes F_1 and F_2).

Group 8 strains did not grow under conditions of iron limitation, and no protein patterns are shown.

Serum susceptibility. Resistance to the bactericidal action of nonimmune serum of the host fish species appears to play an important role in the virulence of Pacific Northwest strains of *V. anguillarum* (38). To determine whether this property was also present in the striped bass isolates, the capacity to grow in the presence of nonimmune striped bass serum was tested. Results given in Table 3 show that all members of groups 1 and 2 were resistant to the lytic activity of unheated fish serum, whereas strains from nonpathogenic groups were sensitive, suggesting that this factor may play an important role as a component of virulence of *V. anguillarum* for striped bass.

DISCUSSION

The genetic basis of fish vibriosis caused by infection with *V. anguillarum* has been studied extensively in the case of pathogenic strains isolated in epizootics occurring in the Pacific Northwest, but little is known of the mechanisms of virulence of strains from other geographical locales.

Pacific Northwest strains of high virulence for salmonids were shown to carry a specific plasmid class (13, 14) which codes for an efficient iron-sequestering system (8, 9). Thus, this attribute enables microorganisms establishing an infection to evade host nonspecific mechanisms of defense, posed by complexing of essential iron by the high-affinity iron-binding proteins, transferrin and lactoferrin, present in body fluids and secretions (2, 6, 8, 9, 29, 41). In addition to this plasmid-mediated virulence factor, these strains possess an additional component of virulence of a chromosomal nature: the ability to withstand the bactericidal activity of normal (nonimmune) serum of the host fish species (38), which is also an important component of virulence in human pathogenic vibrios (7) as well as other fish pathogens (26).

As part of a program to determine whether these mechanisms of virulence are found in *V. anguillarum* strains from other ecological niches, we report here the analysis of properties of pathogenic *V. anguillarum* strains isolated from striped bass reared in the Atlantic Coast. The results indicate that these organisms are highly related to pathogenic strains isolated in the Pacific Northwest, as assessed by cultural and biochemical, i.e., phenetic, properties, as well as by the DNA homology assay, substantiating previous work suggesting that pathogenic *V. anguillarum* strains from different geographical locations possess a high degree of homology (22, 33).

However, unlike the pathogenic strains of *V. anguillarum* isolated in the Pacific Northwest, strains pathogenic for striped bass did not contain any plasmids. Nonetheless, these Atlantic Coast strains were similar to the Northwest isolates in that virulence was correlated with their ability to grow under conditions of iron limitation and to withstand the bactericidal activity of fish serum.

Growth under iron limitation conditions induced the appearance of specific outer membrane proteins in these strains, although, at least in molecular size, they do not appear to be related to the OM2 outer membrane protein induced under conditions of iron limitation in *V. anguillarum* strains containing the virulence plasmid, pJM1. Recent evidence indicates that OM2 may be a surface receptor for a complex of iron and the *V. anguillarum* plasmid-mediated siderophore (M. A. Walter, S. A. Potter, and J. H. Crosa, submitted for publication). It remains to be determined whether the outer membrane proteins induced in the Atlantic Coast isolates play any role in the iron metabolism of these bacteria, although we have recently found that these strains, when growing under iron limitation conditions, produce a siderophore which resembles the plasmid-mediated siderophore of *V. anguillarum* 775 (unpublished observations).

The finding of a chromosomally mediated iron uptake system(s) in the pathogenic Atlantic Coast strains of *V. anguillarum*, which are genetically homologous to their Pacific Northwest counterparts, suggests the possibility that the iron uptake genes are highly mobile recombinationally and, as is the case for other virulence factors, may be part of a transposition unit. This is a rather ominous possibility with regard to disease in fish. Recent preliminary hybridization experiments support this idea in the sense that apparently pJM1 plasmid sequences are indeed present in the chromosome of some of these strains (unpublished results). Knowledge of whether these common sequences correspond to the iron transport genes awaits further dissection of the pJM1 plasmid DNA regions by molecular cloning. Experiments to prove this hypothesis are in progress.

It was of interest that 22 of the nonpathogenic isolates harbored one or more plasmids, which by restriction endonuclease analysis, were shown to be clearly different from the virulence plasmid pJM1. These strains, not *V. anguillarum* but identified as *Vibrio* spp., did not grow, or grew poorly, under conditions of iron limitation and were sensitive to the bactericidal action of nonimmune striped bass serum.

The study of the relationships between the iron transport and serum resistance genetic determinants present in isolates from striped bass and salmon will be important in assessing the contribution of host range to the virulence repertoire of *V. anguillarum*.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant no. AI 19108 from the National Institutes of Health to J.H.C., by National Oceanic and Atmospheric Administration grant NA 81 AA-D-00030 (project R/A 20) to J.H.C. and grant NA 81AA-D-00040 to R.R.C., and by grant P84-81-04 to F.M.H. from the Maryland Department of Natural Resources. A.E.T. and J.L.B. were supported by fellowships from the Juan March Foundation of Spain and by National Science Foundation grant DEB 77-14646.

LITERATURE CITED

1. **Anderson, J. I. W., and D. A. Conroy.** 1970. Vibrio disease in marine fish. Am. Fish. Soc. Spec. Publ. 5:266–272.
2. **Bezkorovainy, A.** 1980. Microbial iron uptake and the

antimicrobial properties of the transferrins, p. 305–342. *In* A. Bezkorovainy (ed.), Biochemistry of nonheme iron. Plenum Publishing Corp., New York.
3. **Bowser, P. R., R. Rosemar, and C. R. Reiner.** 1981. A preliminary report of vibriosis in cultured American lobster, *Homarus americanus*. J. Invertebr. Pathol. **37**:80–85.
4. **Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citarella, and S. Falkow.** 1969. Polynucleotide sequence relationships among members of *Enterobacteriaceae*. J. Bacteriol. **98**:637–650.
5. **Brown, C., and E. Losee.** 1978. Observations on natural and induced epizootics of vibriosis in *Crassostrea virginica* larvae. J. Invertebr. Pathol. **31**:41–47.
6. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infections. Curr. Top. Microbiol. Immunol. **80**:1–35.
7. **Carruthers, M. M., and W. J. Kabat.** 1981. *Vibrio vulnificus* (lactose-positive vibrio) and *Vibrio parahaemolyticus* differ in their susceptibilities to human serum. Infect. Immun. **32**:964–966.
8. **Crosa, J. H.** 1979. *Vibrio anguillarum*: an iron uptake mechanism as a factor of virulence. Fish Health News **8**:7–9.
9. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **284**:566–568.
10. **Crosa, J. H., D. J. Brenner, and S. Falkow.** 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. J. Bacteriol. **115**:904–911.
11. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, Chapter 15, p. 266–282. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
12. **Crosa, J. H., and L. L. Hodges.** 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum* 775. Infect. Immun. **31**:223:227.
13. **Crosa, J. H., L. L. Hodges, and M. H. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infect. Immun. **27**:897–902.
14. **Crosa, J. H., M. H. Schiewe, and S. Falkow.** 1977. Evidence for a plasmid contribution to the virulence of the marine fish pathogen *Vibrio anguillarum*. Infect. Immun. **18**:509–513.
15. **Disalvo, L. H., J. Blecka, and R. Zebal.** 1978. *Vibrio anguillarum* and larval mortality in a California coastal shellfish hatchery. Appl. Environ. Microbiol. **35**:219–221.
16. **Egidius, E., and K. Andersen.** 1977. Norwegian reference strains of *Vibrio anguillarum*. Aquaculture **10**:215–219.
17. **Evelyn, T. P. T.** 1971. First records of vibriosis in Pacific salmon cultured in Canada, and taxonomic status of the responsible bacterium, *Vibrio anguillarum*. J. Fish. Res. Board Can. **28**:517–525.
18. **Fryer, J. L., J. S. Nelson, and R. L. Garrison.** 1972. Vibriosis in fish. Prog. Fish Food Sci. **5**:129–131.
19. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
20. **Harrell, L. W., A. J. Novotny, M. H. Schiewe, and H. O. Hodgins.** 1976. Isolation and description of two vibrios pathogenic to Pacific salmon in Puget Sound, Washington. Fish. Bull. **74**:447–449.
21. **Kado, C. I., and S. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
22. **Kiehn, E. D., and R. E. Pacha.** 1969. Characterization and relatedness of marine vibrios pathogenic to fish: deoxyribonucleic acid homology and base composition. J. Bacteriol. **100**:1248–1255.
23. **Kusuda, R., H. Sako, and K. Kawai.** 1979. Classification of vibrios isolated from diseased fish. I. On the morphological, biological and biochemical properties. Fish Pathol. **13**:123–137.
24. **Levin, M. A., R. E. Wolke, and V. J. Cabelli.** 1972. *Vibrio anguillarum* as a cause of disease in winter flounder (*Pseudopleuronectes americanus*). Can. J. Microbiol. **18**:1585–1592.
25. **McCarthy, D. H.** 1976. Vibrio disease in eels. J. Fish Biol. **8**:317–320.
26. **Mittal, K. R., G. Lalonde, D. LeBlanc, G. Oliver, and R. Lallier.** 1980. *Aeromonas hydrophila* in rainbow trout: relation between virulence and surface characteristics. Can. J. Microbiol. **26**:1501–1503.
27. **Muroga, K., S. Takahashi, and H. Yamanoi.** 1979. Non-cholera vibrio isolated from diseased Ayu. Bull. Jap. Soc. Sci. Fish. **45**:829–834.
28. **Muroga, K., J. Yasuhiko, and R. M. Nisibuchi.** 1976. Pathogenic vibrio isolated from cultured eels. I. Characteristics and taxonomic status. Fish. Pathol. **11**:141–145.
29. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
30. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27**:493–497.
31. **Robohm, R. A., and C. Brown.** 1978. A new bacterium (presumptive *Vibrio* species) causing ulcers in flat fish. Mar. Fish Rev. **40**:5–7.
32. **Rucker, R. R., B. J. Earp, and E. J. Ordal.** 1953. Infectious diseases of Pacific salmon. Trans. Am. Fish. Soc. **83**:307–312.
33. **Schiewe, M. H., J. H. Crosa, and E. J. Ordal.** 1977. Deoxyribonucleic acid relationships among marine vibrios pathogenic to fish. Can. J. Microbiol. **23**:954–958.
34. **Schiewe, M. H., T. J. Trust, and J. H. Crosa.** 1981. *Vibrio ordalii* sp. nov.: a causative agent of vibriosis in fish. Curr. Microbiol. **6**:343–348.
35. **Shotts, E. B., and G. L. Bullock.** 1975. Bacterial diseases of fish: diagnostic procedures for gram negative pathogens. J. Fish. Res. Board Can. **32**:1243–1247.
36. **Strout, R. G., E. S. Sawyer, and B. A. Countermarsh.** 1978. Pathogenic vibrios in confinement-reared and feral fishes of the Maine-New Hamsphire Coast. J. Fish. Res. Board Can. **35**:403–408.
37. **Tajima, K., M. Yoshimizu, Y. Ezura, and T. Kimura.** 1981. Studies on the causative organisms of vibriosis among the pen-cultured coho salmon *Oncorhynchus kisutch* in Japan. Bull. Jap. Soc. Sci. Fish. **47**:35–42.
38. **Trust, T. J., I. D. Courtice, A. G. Khouri, J. H. Crosa, and M. H. Schiewe.** 1981. Serum resistance and hemagglutination ability of marine vibrios pathogenic for fish. Infect. Immun. **34**:702–707.
39. **Tubiash, H. S., R. R. Colwell, and R. R. Sakazaki.** 1970. Marine vibrios associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. J. Bacteriol. **103**:271–272.
40. **Vanderzant, C., and R. Nickelson.** 1973. *Vibrio parahaemolyticus*: a problem for mariculture. J. Milk Food Technol. **36**:135–139.
41. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.