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Evidence for Plasmid Contribution to the Virulence of the Fish Pathogen *Vibrio anguillarum*

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Analysis of the plasmid deoxyribonucleic acid complement of high- and low-virulent strains of the fish pathogen *Vibrio anguillarum* showed a correlation between enhanced virulence and the presence of a 50-megadalton plasmid class. All 50-megadalton plasmids isolated from different high-virulent *V. anguillarum* strains were homologous as judged by the analysis of plasmid deoxyribonucleic acid-deoxyribonucleic acid hybridization. The 50-megadalton plasmid class did not have polynucleotide sequences in common with plasmids of different incompatibility groups.

Recurrent bacterial epizootics of marine fish have emerged as a major limiting factor in the growth of commercial aquaculture. Vibriosis caused by infection with *Vibrio anguillarum* has been particularly devastating in the marine culture of salmonid fishes in the Pacific Northwest (10, 19, 26).

The etiological agent, *V. anguillarum*, was first described by Bergman in 1909 (6) as the cause of "red pest of eels" in the Baltic Sea. Three years later (7), Bergman isolated this same bacterium from the eye of a diseased cod (*Gadus morhua*). Before these two reports, however, Canestrini (9) published accounts dating back to 1718 of epizootics in eels from Denmark, Germany, and Holland that implicated a bacterium described as *Bacillus anguillarum*. The pathology of the disease and the characteristics of bacteria described by both authors suggest that the causative organisms were the same.

Vibriosis was not reported in North America until 1953, when Rucker et al. (26) isolated *V. anguillarum* from chum salmon (*Oncorhynchus keta*) being reared at Deception Pass (Puget Sound). The disease was characterized by hemorrhaging at the base of the fins, internal inflammation, and a generalized septicemia.

In the Pacific Northwest, vibriosis was reported as the cause of an epizootic in cultured chinook salmon (*O. tshawytscha*) being reared in a saltwater slough near Waldport, Ore. (10). This report and subsequent experimental work (19) catalyzed the intensive research effort to develop effective vibrio vaccines.

Thus far, most of the studies dealing with marine vibrios pathogenic to fish have focused upon vaccine trials, cultural characteristics, and virulence properties of a number of different strains (1, 2, 6-10, 19, 20, 22, 24, 26; R. D. Gunnels, M.S. thesis, University of Washington, Seattle, 1974; J. Rohevec, Ph.D. thesis, Oregon State University, Corvallis, 1975). There has not been, to our knowledge, any attempt to specifically examine the precise mechanisms of pathogenesis of vibriosis or to examine its genetic basis. With this goal in mind, we initiated an analysis of the molecular relationships among pathogenic marine vibrios (27) that eventually led to the determination of their plasmid complement. As an extension of this study, we report here a possible relationship between the presence of a particular plasmid class in *V. anguillarum* and an enhanced capacity to produce overt disease in salmonid fishes.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Fish infectivity assays. The virulence assays were performed on juvenile coho salmon (*O. kisutch*) weighing about 11 g. For each bacterial dilution tested, five fish were anesthetized with tricaine methane sulfonate (100 ppm [1 mg/liter]) and inoculated subcutaneously at the posterior base of the dorsal fin with 0.1-ml dilutions of a bacterial suspension from a 24-h broth culture. Bacteria were grown at 25°C in Trypticase soy broth (BBL) plus 1% sodium chloride. After bacterial challenge, the test fish were fin clipped for identification and maintained in freshwater at 15 ± 1°C for 7 days. The container was checked daily for dead fish, and mortalities were examined by bacteriological culture techniques. Kidney material was streaked, and bacteria were reisolated and tested for purity. Mortalities were considered to be due to *V. anguillarum* only when the bacterium was reisolated in pure culture.

TABLE 1. *Comparison of geographic source, virulence, and plasmid content of strains of V. anguillarum*[a]

V. anguillarum strain	Geographic source	Virulence[b]	Plasmid content (megadaltons)[c]
NCMB-572	Japan	2×10^3	50
LS-173	Oregon, U.S.	2×10^2	50
775	Washington, U.S.	1.2×10^2	50
ES-1	Maine, U.S.	3×10^2	50
133-S	Washington, U.S.	1.2×10^3	50
RG 75-834	Alaska, U.S.	4×10^2	50; 23, 20
V1	Canada	6×10^4	50; 3.5
V2	Canada	6×10^6	3.5
2911	Scotland	3×10^5	No plasmid
1800	Washington, U.S.	$>2.3 \times 10^8$	No plasmid
700-14	Arizona, U.S.	$>4 \times 10^7$	No plasmid
286-D	Washington, U.S.	$>3 \times 10^6$	No plasmid
NCMB-1291	Scotland	$>1 \times 10^7$	No plasmid

[a] All the strains with or without plasmids were resistant to three antibiotics: ampicillin, streptomycin, and kanamycin. Minimal inhibitory concentrations, as determined by the method of Bauer et al. (5), were as follows: ampicillin, 60 μg/ml; streptomycin, 30 μg/ml; kanamycin, 30 μg/ml.

[b] Figures shown are mean lethal dose values (number of microorganisms that will kill 50% of the animals inoculated, as determined by the Reed-Muench method [25]). Fish infectivity assays were performed as described in Materials and Methods.

[c] Determined from the agarose gel shown in Fig. 1 by the use of molecular weight standards.

Determination of plasmid DNA content. The analysis of plasmid deoxyribonucleic acid (DNA) in cesium chloride-ethidium bromide (CsCl-EtBr) density gradients and the preparation of ^{3}H-labeled plasmid DNA and unlabeled whole-cell DNA were described previously (12, 13).

The presence of plasmid DNA was also determined by an agarose electrophoretic method (13). Strains were grown overnight in 30 ml of brain heart infusion broth (Difco). Lysates were prepared (13), and, after digestion with ribonuclease and extraction with phenol (once) and chloroform (twice), the clear aqueous phase was adjusted to 0.3 M sodium acetate and precipitated with twice the volume of precooled ethanol (−20°C) overnight at −20°C. After centrifugation at −10°C at 12,000 × *g* for 20 min, the precipitate was suspended in 0.2 ml of TES buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-5 mM ethylenediaminetetraacetate-50 mM NaCl, pH 8]. A sample was electrophoresed in a 0.7% agarose gel in Tris-borate buffer (89 mM Tris base-2.5 mM disodium ethylenediaminetetraacetate-89 mM boric acid). DNA was visualized by staining with ethidium bromide and viewing the fluorescence produced by the intercalated dye when illuminated with an ultraviolet light source.

DNA hybridization. DNA-DNA hybridizations were carried out essentially as described previously (11). Approximately 0.01 μg of ^{3}H-labeled, sheared, denatured plasmid DNA was incubated with 150 μg of unlabeled, sheared, denatured whole-cell DNA from each of the indicated organisms. DNA reassociation was performed in 0.42 M NaCl at 63°C for 14 h. The degree of homology was assessed by the S1 nuclease method of Crosa et al. (11).

RESULTS

Molecular characterization of plasmids in *V. anguillarum*. Initially, we began our examination of the plasmids of *V. anguillarum* with a view toward determining whether the strains were homogeneous in their plasmid content or whether some differences could be detected in strains from several distinct geographical sources. We included in this analysis strains that were recently reported to be of low virulence (8). Figure 1 shows CsCl-EtBr density gradient profiles of ^{3}H-labeled DNA from lysates of one of the high-virulent and one of the low-virulent strains. The presence of extrachromosomal DNA could only be detected in the lysate of the high-virulent strain. This result prompted us to analyze the plasmid DNA complement of many high- and low-virulent strains, this time by an agarose electrophoresis method (23).

Figure 2 shows the agarose electrophoresis pattern obtained for lysates of *V. anguillarum*, and Table 1 compares geographic source, virulence, and plasmids present in the strains. All of the strains, independent of whether they have plasmids, showed resistance to several antibiotics (Table 1). All highly virulent strains showed the presence of a 50-megadalton (Mdal) plasmid species, whereas the low-virulent strains were plasmid-free. The results obtained with the V1 and V2 strains isolated in Canada in 1972 (17) were particularly dramatic. Both strains were initially characterized as highly virulent. During in vitro passage, however, V1 has continued to show a high-virulent phenotype, whereas strain V2 has consistently been classified as possessing a low-virulent phenotype. The two strains possess identical cultural characteristics. The analysis of their plasmid content shows that they share a 3.5-Mdal plasmid class in common, but

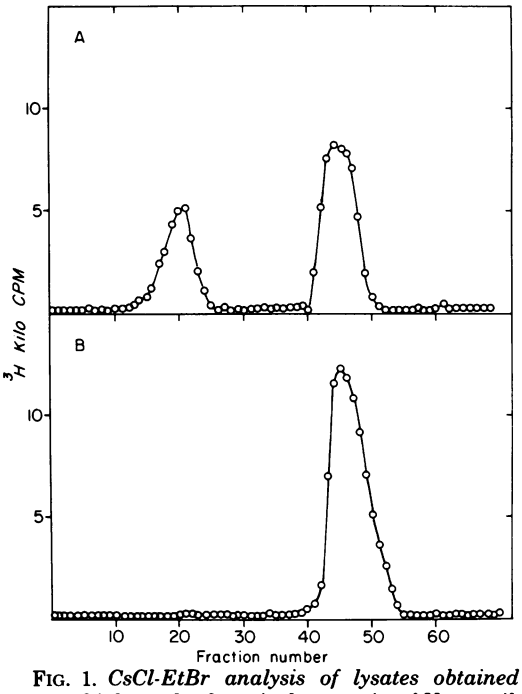

FIG. 1. *CsCl-EtBr analysis of lysates obtained from a high- and a low-virulent strain of V. anguillarum. Strains 775 and 1800 were grown at 25°C in a minimal salt medium (12) containing, in addition, Casamino Acids (Difco) and glucose. Labeling was carried out by adding [^{3}H]thymine (New England Nuclear Corp.) and 250 μg of deoxyadenosine per ml. At a cell concentration of about 2 × 10^8 cells per ml, cells were pelleted and, after washing with TES buffer, lysed by the Triton X-100 method. Lysates were centrifuged in CsCl-EtBr gradients, as described previously (13). (A) High-virulent V. anguillarum 775; (B) low-virulent V. anguillarum 1800.*

that the high-virulent V1 strain carries a 50-Mdal plasmid species, while the low-virulent V2 strain does not.

Polynucleotide sequence relationships among *V. anguillarum* plasmids. We have used the S1 nuclease method (11) to assess the DNA homology among the 50-Mdal plasmids isolated from different sources. We hybridized ^{3}H-labeled, 50-Mdal plasmid DNA (purified through several CsCl-EtBr gradients) isolated from the highly virulent *V. anguillarum* strain 775 with unlabeled total DNA extracted from *V. anguillarum* strains of high and low virulence. DNA extracted from high-virulent *V. anguillarum* strains containing a 50-Mdal plasmid species shared a high level of homology with the ^{3}H-labeled, 50-Mdal plasmid DNA isolated from the high-virulent 775 strain, independent of the geographic source. DNA extracted from low-virulent *V. anguillarum* strains (including V2) showed only a low percentage of DNA relatedness with ^{3}H-labeled, 50-Mdal plasmid DNA (Table 2).

DNA-DNA hybridization experiments with plasmids from different incompatibility groups were also carried out (Table 2). Results indicate that the *V. anguillarum* 50-Mdal plasmid class does not share an appreciable amount of nucleotide sequence relatedness with any of the plasmids of the most common incompatibility groups.

DISCUSSION

It has become increasingly apparent that a variety of important properties of microorganisms are plasmid mediated. The best-known example of the plasmid pool of bacteria are the plasmid-mediated antibiotic resistance determinants, the so-called R plasmids.

Most medically important microorganisms of man and domestic animals have been shown to be able to acquire R plasmids, and this has often caused considerable difficulty in the treatment of infectious diseases. Antibiotics have also been employed in the treatment of various diseases of fish, and R plasmids have made their presence known in commercial aquaculture (3, 4). Plasmids also often encode for traits that directly contribute to the pathogenicity of microorganisms. These plasmid-mediated properties include the biosynthesis of enterotoxins and hemolysins, and also antigenic determinants that enhance the colonization of specific animal or human hosts (28–32).

In the analysis of high-virulent strains of *V. anguillarum*, we have consistently found the presence of a 50-Mdal plasmid class. This plasmid class was not present in the low-virulent strains. DNA-DNA hybridization studies indicated that all 50-Mdal plasmids were highly related, independent of the geographic source. The case with the Canadian strains V1 and V2 (17) was of some interest. The 50-Mdal plasmid present in the highly virulent V1 strain seems to have been "naturally" cured, giving rise to the lower-virulent V2 strains.

The data presented in this paper strongly suggest that virulence of *V. anguillarum* is enhanced by the presence of the 50-Mdal plasmid class, although the lack of a suitable phenotypic marker has so far made the detection of plasmid loss difficult to demonstrate in the laboratory.

From other studies in other plasmid systems (28–32), we can anticipate that a plasmid-mediated determinant of virulence in *V. anguillarum* could operate by either the biosynthesis of a potent enterotoxin or the biosynthesis of a specific surface antigen that promotes attachment of the organism to the fish at either a specific tissue target or by overcoming local de-

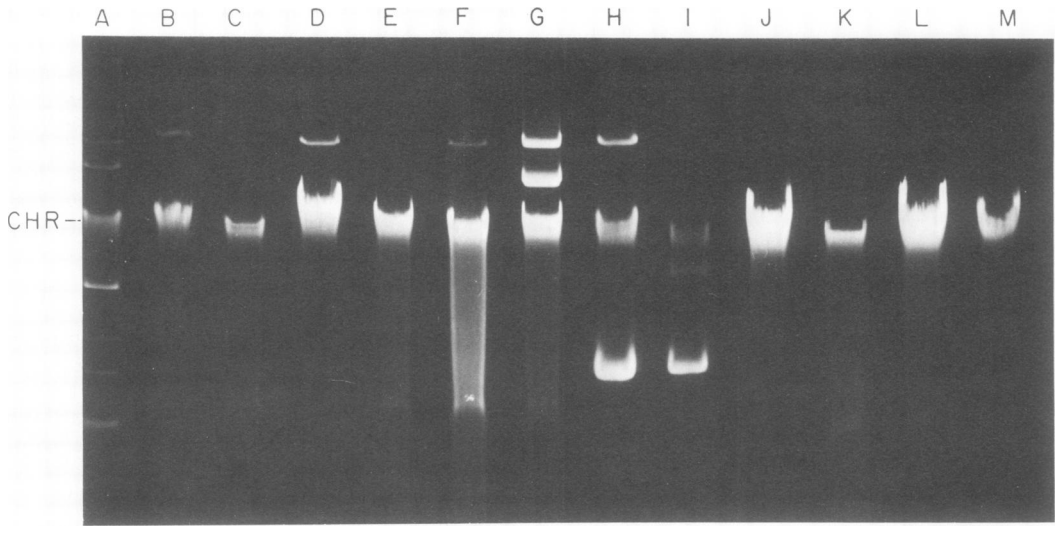

FIG. 2. *Agarose gel electrophoresis of DNA from high-virulent (HV) and low-virulent (LV) strains of V. anguillarum. (A) Standard plasmid DNAs, ranging in molecular weights from 62 Mdal (uppermost band) to 1.8 Mdal (lowest band). CHR indicates the position of the chromosomal DNA. (B) Strain NCMB-572, HV; (C) strain LS-173, HV; (D) strain 775, HV; (E) strain ES-1, HV; (F) strain 133-S, HV; (G) strain RG 75-834, HV; (H) strain V1, HV; (I) strain V2, LV; (J) strain 2911, LV; (K) strain 1800, LV; (L) strain 700-14, LV; (M) strain NCMB-1291, LV.*

TABLE 2. *Hybridization between ^{3}H-labeled 775 plasmid and whole-cell DNA*

Source of unlabeled DNA	Relative DNA sequence homology with ^{3}H-labeled 775 plasmid DNA (%)[a]
V. anguillarum strain:	
775	100
NCMB-572	100
LS-173	98
ES-1	95
133-S	98
RG 75-834	93
V1	86
V2	5
2911	5
1800	5
700-14	5
NCMB-1291	5
286-D	5
E. coli J5-3 containing:	
F (FI)	1
R1 (FII)	1
RP4 (PI)	1
R6K (X)	1
N3 (N)	1
Sa (W)	1
R144 (I)	1
R16 (O)	1

[a] Degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (11). The actual extent of reassociation for the homologous reaction was an average of 85%. All other reactions were normalized to this value set at 100%. Each value shown is an average of three separate determinations.

fense mechanisms. We are presently investigating these possibilities.

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