APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1991, p. 2956–2962
0099-2240/91/102956-07$02.00/0

Vol. 57, No. 10

Distribution of Plasmid- and Chromosome-Mediated Iron Uptake Systems in *Vibrio anguillarum* Strains of Different Origins

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Received 25 March 1991/Accepted 26 July 1991

We investigated the incidence of plasmid-mediated and chromosome-mediated iron uptake systems in strains of *Vibrio anguillarum* that belong to serotypes O1 and O2 and were isolated from different fish species and in different geographic areas. All of the strains gave positive reactions in CAS agar medium and in the Arnow test, which indicated that catechol types of siderophores were produced. The majority of *V. anguillarum* serotype O1 strains harbored a 65-kb plasmid similar to plasmid pJM1 from strain 775, which encodes the siderophore anguibactin and its outer membrane receptor, protein OM2. All of the isolates harboring this plasmid promoted the growth of an anguibactin-deficient receptor-proficient mutant derived from strain 775, but none of these isolates promoted the growth of mutants lacking receptor OM2. Furthermore, under iron-limiting conditions all of these strains induced outer membrane proteins that were identical in size to protein OM2 of strain 775. In contrast, none of the serotype O2 strains contained a high-molecular-weight plasmid, but all of them induced the growth of mutants defective in the anguibactin-mediated system regardless of the presence or absence of receptor OM2. The serotype O2 strains, but not the plasmid-bearing serotype O1 strains, also induced the growth of *Salmonella typhimurium enb*-1 which utilizes only enterobactin as a siderophore. Moreover, the pattern of iron-regulated outer membrane proteins in serotype O2 isolates was totally different from the pattern in the serotype O1 strains harboring the 65-kb plasmid. Interestingly, several plasmidless strains belonging to serotype O1 behaved in the same way as serotype O2 strains in cross-feeding experiments and produced the same iron-regulated outer membrane protein patterns. These results indicate that regardless of their origins all *V. anguillarum* strains belonging to serotype O2 contain similar chromosome-mediated iron-sequestering systems. However, in the serotype O1 strains both chromosome- and plasmid-encoded iron uptake systems occur, but there is a significant predominance of the plasmid-mediated system.

Vibrio anguillarum is an important bacterial pathogen that causes a septicemic disease known as vibriosis, which affects a great number of mainly marine fish species (13, 25; see reference 30 for a review). Although there are 10 *V. anguillarum* serotypes (designated O1 to O10 in the European system) that are distinguished on the basis of different somatic antigens (27), serotypes O1 and O2 are the two main groups that are responsible for vibriosis outbreaks throughout the world (27, 30, 33). Within serotype O2 there are two subgroups (subgroups O2a and O2b [5] or subgroups O2α and O2β [24]) that are distinguished by several immunological tests, such as immunoelectrophoresis tests, dot blot tests, and enzyme-linked immunosorbent assays.

For most pathogenic bacteria iron is an essential nutrient (21) that is not readily available in the host organism, since it is tightly bound by several proteins (mainly serum transferrins). Thus, the ability to acquire iron from these proteins is an important virulence factor for most pathogens (15). Iron acquisition is accomplished by secreting low-molecular-weight highly specific iron chelators called siderophores (20), which are taken up into the cells in the chelate form by means of a specific outer membrane protein complex.

In *V. anguillarum* it has been demonstrated that the ability to obtain iron from a host also plays a crucial role in the virulence of the organism (6, 10, 11, 34, 37). In strain 775 (serotype O1), which was isolated from the Pacific Northwest coast of United States, the iron uptake system is encoded by a 65-kb plasmid (pJM1) (6, 8). This plasmid encodes the synthesis of the diffusible siderophore anguibactin, a catechol type of siderophore with a unique structure (1, 16), and the receptor for the ferric siderophore identified as 86-kilodalton (kDa) outer membrane protein OM2 (2, 7). Other pathogenic strains of *V. anguillarum* belonging to serotype O1 that were isolated from cultured fish on the northwestern coast of Spain also harbor a plasmid which exhibits structural and functional homology with pJM1, as assessed by hybridization analysis and curing experiments (28). This 65-kb plasmid seems to be widespread in many strains belonging to serotype O1 (22, 35).

In several *V. anguillarum* strains belonging to serotype O2 and in some serotype O1 strains which do not harbor the 65-kb plasmid, workers have described an iron uptake system which is encoded by chromosomal genes and does not exhibit any homology with the plasmid-mediated system (18, 31). This chromosomally encoded system consists of a catechol type of siderophore of unknown structure which resembles enterobactin (produced by several enterobacteria), in its biological properties (18) and several iron-regulated outer membrane proteins with molecular weights ranging between 70,000 and 79,000. In addition, another iron-regulated protein, which has a molecular weight of 150,000, has been detected in total membrane fractions. None of these proteins has been shown to be involved in the iron uptake process mediated by the siderophore.

Since both iron uptake systems (the plasmid-mediated system and the chromosome-mediated system) have been studied only in representative strains, our aim was to investigate the distribution of the two systems in strains of *V. anguillarum* belonging to serotypes O1 and O2 that were

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TABLE 1. Growth under iron limiting-conditions and production of siderophores in *V. anguillarum* strains obtained from different sources[a]

Strain	Serotype	Origin	pJM1-like plasmid	MIC of EDDA (μM)	CAS agar test[b]	Arnow test (A_{550})
SO102.1	O1	Pacific salmon (Spain)	+	150	++	+ (0.198)
SO86-3	O1	Pacific salmon (Spain)	+	150	++	+ (0.234)
775	O1	Pacific salmon (United States)	+	40	++	+ (0.091)
6018/1	O1	Rainbow trout (Denmark)	+	150	++	+ (0.147)
NCMB 2129	O1	Rainbow trout (Norway)	+	150	++	+ (0.194)
NCMB 571	O1	Rainbow trout (Japan)	+	150	++	+ (0.274)
TM-10.1	O1	Rainbow trout (Spain)	+	150	+	+ (0.165)
TM14	O1	Rainbow trout (Spain)	+	150	++	+ (0.091)
DH11	O1	Striped bass (France)	+	150	++	+ (0.219)
96F	O1	Striped bass (United States)	−	300	+++	+ (0.152)
103F	O1	Striped bass (United States)	−	300	+	+ (0.171)
RP13	O1	Turbot (Spain)	+	150	+	+ (0.188)
RM81	O1	Turbot (Spain)	+	150	+	+ (0.188)
R82	O1	Turbot (Spain)	+	150	++	+ (0.176)
RH-81	O1	Turbot (Spain)	+	150	+	+ (0.101)
RP63	O1	Turbot (Spain)	+	150	++	+ (0.150)
RC16.1	O1	Turbot (Spain)	+	150	++	+ (0.176)
RC91	O1	Turbot (Spain)	+	150	+	+ (0.124)
PW1	O1	Water of tank (Spain)	+	200	++	+ (0.124)
T268	O2α	Atlantic salmon (England)	−	300	++	+ (0.169)
N1801	O2α	Atlantic salmon (Norway)	−	300	+++	+ (0.124)
PT-24	O2α	Ayu (Japan)	−	300	++	+ (0.130)
ATCC 14181	O2α	Brown trout (Scotland)	−	300	++	+ (0.186)
ATCC 19264	O2α	Cod (Denmark)	−	300	+++	+ (0.182)
1049/2	O2α	Cod (Denmark)	−	300	+	+ (0.091)
1466/1	O2α	Cod (Denmark)	−	350	+	+ (0.206)
1474/1	O2α	Cod (Denmark)	−	300	+	+ (0.151)
1570/1	O2α	Eel (Denmark)	−	300	+	+ (0.168)
F4-1/8	O2α	Feces (Denmark)	−	300	++	+ (0.169)
8087-D/2	O2α	Rainbow trout (Denmark)	−	300	++	+ (0.138)
840817-2/6	O2α	Rainbow trout (Denmark)	−	350	+	+ (0.146)
2887	O2α	Rainbow trout (Italy)	−	300	+	+ (0.112)
SE1/5	O2α	Sediment (Denmark)	−	300	++	+ (0.205)
RC71	O2α	Turbot (Spain)	−	300	+++	+ (0.172)
RG111	O2α	Turbot (Spain)	−	300	++	+ (0.155)
RG181	O2α	Turbot (Spain)	−	300	++	+ (0.185)
91079	O2α	Turbot (Scotland)	−	300	+++	+ (0.147)
YL8	O2α	Turbot (Norway)	−	300	+++	+ (0.134)
V2-1/2	O2α	Water (Denmark)	−	300	++	+ (0.147)
840814-1/10	O2β	Eel (Denmark)	−	300	+	+ (0.174)
820723-2/8	O2β	Rainbow trout (Denmark)	−	300	+	+ (0.170)
860619-3/2	O2β	Rainbow trout (Denmark)	−	300	+	+ (0.229)
NCMB 2133	O2β	Saithe (Norway)	−	300	+	+ (0.226)
43F	O2β	Striped bass (United States)	−	300	+	+ (0.140)
100F	O2β	Striped bass (United States)	−	300	+++	+ (0.163)
6828C	O2β	Turbot (Denmark)	−	350	++	+ (0.100)
RV22	O2β	Turbot (Spain)	−	300	++	+ (0.116)

[a] All strains were negative in the Csáky test (A_{520}).
[b] Ratio of the diameter of the halo to the diameter of the colony: +++, >4; ++, 3 to 4; +, <3.

isolated in different geographic areas and from different fish species.

MATERIALS AND METHODS

Bacterial strains. A total of 47 *V. anguillarum* strains isolated from vibriosis outbreaks that occurred in different geographic areas and affected several fish species were studied. Slide agglutination and dot blot assays (5, 32) were used to assign the strains to serotypes O1 and O2 in the European scheme of Sørensen and Larsen (27). The strains were routinely cultured on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 1% NaCl and were stored frozen at −70°C in tryptic soy broth (Difco) containing 15% (vol/vol) glycerol. The origins of the strains and their serotypes are shown in Table 1.

Growth under iron-limiting conditions. *V. anguillarum* strains were cultured in M9 minimal medium (19) supplemented with 0.2% (wt/vol) Casamino Acids (Difco). The nonassimilatable iron chelator ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA) was added at a concentration of 10 μM to achieve iron-limiting conditions. The MICs of EDDA were determined on plates of M9 medium containing different concentrations of the chelator. The MIC of

EDDA was defined as the lowest concentration at which no bacterial growth was observed.

Siderophore production. The production of compounds with siderophore activity by the *V. anguillarum* strains was investigated by using the method of Schwyn and Neilands (26) and chromoazurol S (CAS) agar plates. CAS agar contains a dye which is blue when it is chelated with iron and orange when a stronger chelator, such as a siderophore, removes the iron. The ratio of orange halo diameter to colony diameter after 48 h of incubation at 25°C was considered to be an indicator of the siderophore level produced. The presence of phenolic compounds was detected in supernatants by using the colorimetric test of Arnow (4), and the presence of hydroxamic acids was determined by using a modification of the Csáky method (3, 12). Both tests were performed by using cell-free supernatants from cultures in M9 medium containing 10 μM EDDA after 24 h of incubation at 25°C.

Bioassays. Siderophore activity was also determined by performing bioassays as previously described (34); in these bioassays we tested the ability of cell-free supernatants from cultures of each strain to cross-feed different indicator strains that were defective in the iron uptake system (Table 2). *V. anguillarum* H775-3, a plasmidless strain that was obtained from strain 775 by curing of pJM1 and lacks a siderophore and the receptor, and *V. anguillarum* 775::Tn*1-5* (pJHC-91), which was receptor positive and anguibactin negative, were used to detect anguibactin-like siderophores. Two mutants of *Salmonella typhimurium* LT2 that were deficient in the biosynthesis of enterobactin (strain *enb*-1, which can use only enterobactin, and strain *enb*-7, which can use enterobactin and the intermediate 2,3-dihydroxybenzoic acid) (23) were used for enterobactin detection. *Escherichia coli* LG1522, a mutant that is deficient in aerobactin biosynthesis but has an intact receptor for this siderophore, was used as an indicator of aerobactin production (36). *V. anguillarum* wild-type strain 775, *S. typhimurium* LT2, and *E. coli* LG1315 were used as positive controls for the detection of anguibactin, enterobactin, and aerobactin siderophores, respectively. All indicator strains used in the bioassays were kindly provided by J. H. Crosa (Oregon Health Sciences University, Portland).

In addition, cross-feeding experiments with different *V. anguillarum* strains were performed by using selected strains belonging to different groups as indicators. Each indicator strain was inoculated into M9 minimal medium containing a concentration of EDDA that was higher than the MIC of EDDA for that strain. The results were scored as positive when cell-free supernatants from cultures of each strain promoted the growth of indicator strains.

Plasmid analysis. The presence of plasmids was screened by agarose gel electrophoresis, using the rapid extraction method of Kado and Liu (17).

Analysis of membrane proteins. Total membrane proteins from *V. anguillarum* strains were prepared as described previously (9), and the outer membrane fractions were obtained by the method of Filip et al. (14) by differentially solubilizing the inner membranes with 1.5% sodium lauryl sarcosinate. Pellets from total and outer membrane fractions were suspended in 40 μl of a buffer containing 12.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.002% bromophenol blue, and 5% 2-mercaptoethanol. The suspensions were boiled for 5 min, and samples were applied to an SDS-polyacrylamide gel, which contained 12.5% acrylamide in the separating gel and 3% acrylamide in the stacking gel. Induction of new proteins

TABLE 2. Results of cross-feeding experiments performed with different indicator strains[a]

Strain	Serotype	Ability to cross-feed			
		V. anguillarum strains[b]		*S. typhimurium* strains[c]	
		H775-3	775::Tn*1-5*	*enb*-1	*enb*-7
SO102.1	O1	−	+	−	+
SO86-3	O1	−	+	−	+
775	O1	−	+	−	+
6018/1	O1	−	+	−	+
NCMB 2129	O1	−	+	−	+
NCMB 571	O1	−	+	−	+
TM-10.1	O1	−	+	−	+
TM14	O1	−	+	−	+
DH11	O1	−	+	−	+
96F	O1	+	+	+	+
103F	O1	+	+	+	+
RP13	O1	−	+	−	+
RM81	O1	−	+	−	+
R82	O1	−	+	−	+
RH-81	O1	−	+	−	+
RP63	O1	−	+	−	+
RC91	O1	−	+	−	+
RC16.1	O1	−	+	−	+
PW1	O1	−	+	−	+
T268	O2α	+	+	+	+
N1801	O2α	+	+	+	+
PT-24	O2α	+	+	+	+
ATCC 14181	O2α	+	+	+	+
ATCC 19264	O2α	+	+	+	+
1049/2	O2α	+	+	+	+
1466/1	O2α	+	+	+	+
1474/1	O2α	+	+	+	+
1570/1	O2α	+	+	+	+
F4-1/8	O2α	+	+	+	+
8087-D/2	O2α	+	+	+	+
840817-2/6	O2α	+	+	+	+
2887	O2α	+	+	+	+
SE1/5	O2α	+	+	+	+
RC71	O2α	+	+	+	+
RG111	O2α	+	+	+	+
RG181	O2α	+	+	+	+
91079	O2α	+	+	+	+
YL8	O2α	+	+	+	+
V2-1/2	O2α	+	+	+	+
840814-1/10	O2β	+	+	+	+
820723-2/8	O2β	+	+	+	+
860619-3/2	O2β	+	+	+	+
NCMB 2133	O2β	+	+	+	+
43F	O2β	+	+	+	+
100F	O2β	+	+	+	+
6828C	O2β	+	+	+	+
RV22	O2β	+	+	+	+

[a] All strains were negative in cross-feeding experiments performed with *E. coli* LG1522. This strain is an *E. coli* mutant that is deficient in the biosynthesis of aerobactin and the enterobactin receptor and uses only aerobactin as a siderophore because it has an intact receptor for iron-aerobactin complexes.

[b] Strains H775-3 and 775::Tn*1-5* are *V. anguillarum* mutants that are deficient in the iron uptake system; strain H775-3 is a plasmidless strain that lacks plasmid pJM1, and strain 775::Tn*1-5*(pJHC-91) is a strain 775 derivative that is receptor positive and siderophore negative.

[c] Strains *enb*-1 and *enb*-7 are *S. typhimurium* LT2 mutants that are deficient in enterobactin biosynthesis; strain *enb*-1 uses only enterobactin, and strain *enb*-7 uses enterobactin and 2,3-dihydroxybenzoic acid.

under iron-limiting conditions was examined by growing the strains in M9 minimal medium in the presence of 10 μM EDDA and comparing the protein patterns with the patterns obtained when strains were grown in M9 medium supplemented with 10 μM $FeCl_3$.

RESULTS

Growth under iron-limiting conditions. Different concentrations of the iron chelator EDDA were used to determine the MICs of this chelator for all of the *V. anguillarum* strains studied. All of the strains grew in the presence of 10 μM EDDA in M9 medium. When greater concentrations of EDDA were used, we observed clear differences between serotype O1 and O2 strains (Table 1). Whereas for serotype O1 strains the MIC of EDDA was 150 μM, for serotype O2 strains the MIC of EDDA ranged between 300 and 350 μM. For serotype O1 strains 96F and 103F the MIC was more similar to the MIC for serotype O2 strains (300 μM).

Plasmid profiles. The majority of the *V. anguillarum* strains belonging to serotype O1 harbored a 65-kb plasmid that was similar to plasmid pJM1 of wild-type strain 775. In strain DH11 a plasmid larger than pJM1 (75 to 80 kb) was detected. Only two serotype O1 strains (strains 96F and 103F), which were isolated from striped bass (*Morone saxatilis*) in the United States, did not contain any plasmid. In the *V. anguillarum* serotype O2 strains no pJM1-like plasmids were observed (Table 1). In four serotype O2 strains several low-molecular-weight plasmids (<20 kb) were detected.

Siderophore detection and bioassays. All *V. anguillarum* strains gave positive results on CAS agar plates, since all of them produced clear orange halos in this medium. In general, the *V. anguillarum* serotype O2 strains produced larger halos than the serotype O1 strains, but it was not possible to correlate size of halo and MIC of EDDA.

The biological activities of the siderophores produced by the strains were determined by performing cross-feeding experiments, in which we tested the capacity of cell-free supernatants from cultures of each strain to induce the growth of the other *V. anguillarum* isolates, as well as the growth of several indicator strains that were deficient in the iron uptake mechanisms. The results of the cross-feeding experiments performed with serotype O1 and O2 *V. anguillarum* strains showed that all of the strains tested promoted the growth of the other strains, regardless of serotype. However, the results of bioassays performed with two *V. anguillarum* indicator strains, strain 775::Tn*1-5* (a derivative strain that possesses the OM2 receptor for anguibactin, but does not produce the siderophore) and strain H775-3 (a strain 775 derivative, which also lacks an anguibactin receptor) (34), indicated that only the strains that harbored the pJM1-like plasmids, and hence produced anguibactin, stimulated the growth of mutant 775::Tn*1-5* but that these strains did not induce the growth of cured strain H775-3. Thus, we concluded that these strains produced the siderophore anguibactin. However, the remaining strains tested, strains 96F and 103F (plasmidless strains belonging to serotype O1) and all of the *V. anguillarum* serotype O2 strains, cross-fed both the receptor-proficient and the receptor-deficient derivatives of *V. anguillarum* 775, which indicated that these strains produced another siderophore that was different from anguibactin and was coded for by chromosomal genes.

S. typhimurium enb-1 and *enb*-7, which are mutants blocked in the biosynthesis of enterobactin, and *E. coli* LG1522, which possesses the aerobactin receptor, were used to determine the biological activities of the siderophores. All of the strains tested were negative with strain LG1522, indicating that the *V. anguillarum* serotype O1 and O2 strains did not produce aerobactin-related siderophores (Table 2). The bioassay with *S. typhimurium* mutants revealed differences between the plasmidless strains and the strains harboring pJM1-like plasmids. All of the serotype O2 strains and the plasmidless serotype O1 strains (strains 103F and 96F) induced the growth of both strain *enb*-1 (which used only enterobactin) and strain *enb*-7 (which used enterobactin and 2,3-dihydroxybenzoic acid). In contrast, the serotype O1 strains that harbored pJM1-like plasmids promoted the growth of only strain *enb*-7, since it has been shown that there is chromosome-mediated production of 2,3-dihydroxybenzoic acid (1) (Table 2).

The chemical tests confirmed the results described above. The Arnow assay demonstrated that all of the strains produced phenolic compounds, whereas the Csáky test confirmed that the culture supernatants from the *V. anguillarum* strains did not contain hydroxamate compounds (Table 1).

Induction of outer membrane proteins under iron-limiting conditions. *V. anguillarum* 775 shows the induction of two new outer membrane proteins when it was grown under iron-limiting conditions. One of these proteins, a 86-kDa protein (OM2), was encoded by pJM1 (2), but the other, a 79-kDa protein (OM3), was chromosomally encoded (9). We studied the induction of the outer membrane proteins under iron-limiting conditions in all of the strains analyzed. Each strain was grown with and without available iron, and outer membrane proteins were examined by SDS-polyacrylamide gel electrophoresis. Two outer membrane proteins with mobilities identical to those of the OM2 and OM3 proteins present in *V. anguillarum* 775 were induced in all of the serotype O1 strains carrying pJM1-like plasmids when strains were cultured under iron-limiting conditions (Fig. 1a, lanes A through L). In addition, another protein (73 kDa) was detected in these strains. In serotype O1 plasmidless strains 96F and 103F (Fig. 1a, lanes M and N) and in all of the serotype O2 strains (Fig. 1b, lanes A to N) the pattern of protein induction was very different from that in the strains bearing the pJM1-like plasmids. Two proteins with molecular weights lower than that of protein OM2 were detected in all of the plasmidless strains. One of these was a 75-kDa protein, and the other was a 70-kDa protein. In some cases other induced proteins appeared; these ranged in size from 79 to 73 kDa. In addition, a protein with a molecular weight of about 150,000 was detected in the majority of the plasmidless strains growing under iron-limiting conditions when the total membrane proteins were analyzed (Fig. 2, lane B); this protein was never observed in the plasmid-bearing strains (Fig. 2, lane D). This iron-regulated protein was not observed in any of the outer membrane preparations.

DISCUSSION

Two different iron uptake systems have been described in *V. anguillarum*. One is encoded by plasmid pJM1, and the other is chromosome encoded. The plasmid-encoded system is an essential factor for pathogenicity in *V. anguillarum* 775 (6, 34). However, the role of the chromosome-mediated iron uptake system in virulence is still unknown. In this work we studied the incidence of both systems in *V. anguillarum* strains belonging to serotypes O1 and O2 that were responsible for vibriosis outbreaks in several fish species in different geographical areas.

It has been demonstrated that *V. anguillarum* strains

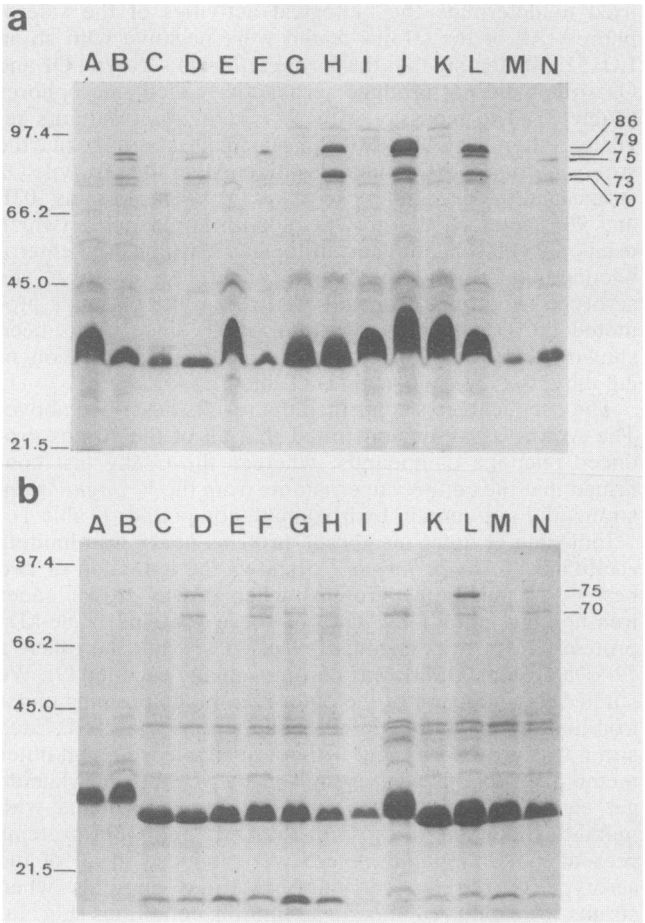

FIG. 1. SDS-polyacrylamide gel electrophoresis of the outer membrane proteins of *V. anguillarum* strains. (a) Serotype O1 strains. Lanes A and B, strain 775; lanes C and D, strain 6018/1; lanes E and F, strain DH11; lanes G and H, strain TM14; lanes I and J, strain SO86-3; lanes K and L, strain PW1; lanes M and N, strain 96F. Lanes B, D, F, H, J, I, and N contained outer membrane proteins from cells cultured under iron-limiting conditions. (b) Serotype O2 strains. Lanes A and B, strain 2887; lanes C and D, strain PT24; lanes E and F, strain RG111; lanes G and H, strain RC71; lanes I and J, strain 1474/1; lanes K and L, strain NCMB 2133; lanes M and N, strain RV22. The numbers on the left indicate the molecular weights (10^3) of the standard markers, and the numbers on the right indicate the molecular weights (10^3) of the iron-regulated proteins.

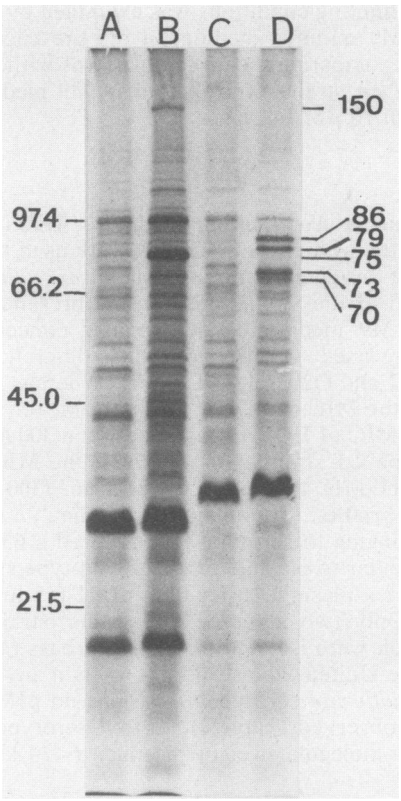

FIG. 2. SDS-polyacrylamide gel electrophoresis of the total membrane proteins of *V. anguillarum* strains. Lanes A and B, strain 43F (serotype O2); lanes C and D, strain 775 (serotype O1). Lanes B and D contained total membrane proteins from cells cultured under iron-limiting conditions. The numbers on the left indicate the molecular weights (10^3) of the standard markers, and the numbers on the right indicate the molecular weights (10^3) of the iron-regulated proteins.

belonging to serotype O1 harbor a 65-kb plasmid similar to pJM1 of wild-type strain 775 (35). Our results confirm that the majority of serotype O1 strains carry pJM1-like plasmids, and only two strains of this serotype (strains 96F and 103F, which were isolated from striped bass in the United States) were plasmidless. In all of the *V. anguillarum* serotype O2 strains tested no high-molecular-weight plasmids were observed, indicating that the iron uptake systems are chromosome encoded.

All of the strains grew under iron-limiting conditions, but the MICs of EDDA were higher for the plasmidless strains than for the plasmid-bearing strains, which indicated that there were greater levels of siderophore activity or higher iron affinities in the plasmidless strains. Strain 775, which was isolated on the Pacific Northwest coast of the United States, had the lowest MIC of EDDA, which confirmed previous observations that the strains originating from diseased fish from the Atlantic coast (Table 1) had an increased siderophore production phenotype (29). The results of cross-feeding experiments, chemical tests for siderophore detection, and tests for induction of new proteins under iron-limiting conditions allowed us to determine the incidence of both systems within each serotype.

All of the *V. anguillarum* serotype O1 strains tested that harbored pJM1-like plasmids had the anguibactin-mediated system. All of the *V. anguillarum* serotype O2 strains and strains 96F and 103F (serotype O1 plasmidless strains) had similar chromosome-mediated iron uptake systems. Previously, Lemos et al. (18), using six *V. anguillarum* strains from Spain and the United States, demonstrated that the chromosome-mediated iron uptake system is not related to the anguibactin-mediated system. Our results confirmed this finding, since the siderophores produced by all of the virulent plasmidless strains induced the growth of *V. anguillarum* H775-3, which lacks plasmid pJM1 and hence does not possess membrane receptor OM2. Since *V. anguillarum* strains can use the siderophore enterobactin independent of the presence of receptor OM2 (1), we decided to check whether the *V. anguillarum* strains produced a siderophore related to enterobactin. The results of the bioassays per-

formed with *S. typhimurium* mutants confirmed that all of the plasmidless strains produced a siderophore that is functionally related to enterobactin, which was not detected in any of the plasmid-bearing serotype O1 strains. In addition, the pattern of induced proteins under iron-limiting conditions in the serotype O1 plasmid-bearing strains was very different from the patterns induced in plasmidless strains in which protein OM2 was never detected. In some strains of this group, a 79-kDa protein that was similar in size to protein OM3 was observed. The results of preliminary immunological assays (data not shown) suggested that protein OM3 may also be present in the serotype O2 strains.

In conclusion, our results indicate that regardless of the host species and geographical origin, all of the *V. anguillarum* strains belonging to serotype O2 have similar chromosome-mediated iron uptake systems. However, in the serotype O1 strains both chromosome- and plasmid-coded iron-sequestering systems are present, although there is a significant predominance of the anguibactin-mediated system.

Further genetic and biochemical studies are in progress to assess the importance of the chromosome-encoded iron uptake mechanism in the pathogenicity of plasmidless strains of *V. anguillarum*.

ACKNOWLEDGMENTS

This work was supported by grant PB87-1027 from the Dirección General de Investigación Científica y Técnica to A.E.T. and by grant 29904A90 from Xunta de Galicia to M.L.L. R.F.C. acknowledges the Ministerio de Educación y Ciencia (Spain) for a research fellowship.

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