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Chromosome-Mediated Iron Uptake System in Pathogenic Strains of *Vibrio anguillarum*

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We describe in this work a new iron uptake system encoded by chromosomal genes in pathogenic strains of *Vibrio anguillarum*. This iron uptake system differs from the plasmid-encoded anguibactin-mediated system present in certain strains of *V. anguillarum* in several properties. The siderophore anguibactin is not utilized as an external siderophore, and although characteristic outer membrane proteins are synthesized under iron-limiting conditions, these are not related to the plasmid-mediated outer membrane protein OM2 associated with ferric anguibactin transport. Furthermore, the siderophore produced by the plasmidless strains may be functionally related to enterobactin as demonstrated by bioassays with enterobactin-deficient mutants, although its behavior under various chemical treatments suggested major differences from that siderophore. Hybridization experiments suggested that the *V. anguillarum* chromosome-mediated iron uptake system is unrelated genetically to either the anguibactin or enterobactin-associated iron assimilation systems.

One of the most important virulence factors in many pathogenic bacteria is the ability to utilize iron from the host fluids by means of an efficient iron-sequestering system (5, 8, 18, 22, 34). *Vibrio anguillarum* is a bacterium highly pathogenic for different species of marine fish, causing the disease known as vibriosis (6, 15, 25). In strain 775, isolated from the Pacific Northwest coast of the United States, an iron uptake system is mediated by a 65-kilobase plasmid designated pJM1 (7, 11, 13). This system allows bacteria to grow at low concentrations of available iron imposed by the high-affinity iron-binding proteins present in the host fluids. Experimental infections demonstrated that possession of the pJM1 iron uptake genetic determinants is essential to cause disease (7, 11, 33, 35).

Genetic characterization of the pJM1-mediated iron uptake system allowed the location of the iron uptake genes to a 25-kilobase region of the pJM1 plasmid (12, 29, 33). These genes determine the synthesis of the two essential components involved in the iron uptake process: the diffusible siderophore anguibactin (1, 33) and a component of the receptor for the iron-siderophore complex identified as the 86-kilodalton outer membrane protein OM2 (2, 10). Other pathogenic strains of *V. anguillarum* isolated from cultured turbot on the northwestern Atlantic coast of Spain also harbor a plasmid which exhibits a high structural and functional homology with pJM1 (28). However, in recent studies with other virulent strains of *V. anguillarum* that were also iron uptake proficient, no plasmids have been detected, suggesting that the iron uptake system of these strains must be chromosomally encoded (30).

In the present work we report the characterization of this chromosome-mediated iron uptake system present in certain plasmidless strains of *V. anguillarum*. Our results indicate that this new system is different from the one encoded by the pJM1-like plasmids.

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MATERIALS AND METHODS

Bacterial strains. The *V. anguillarum* strains used in this work were isolated from vibriosis outbreaks that occurred in turbot (*Scophthalmus maximus*) reared in intensive culture systems on the Atlantic coast of northwestern Spain (31a). Bacterial isolation from internal organs of moribund fish as well as identification of the strains were achieved as previously described (30, 31a). The serotype of the isolates was determined as before (31). Strains 43F and 96F previously isolated from striped bass (*Morone saxatilis*) in Chesapeake Bay (American Atlantic coast) (30) were also studied. Pathogenic *V. anguillarum* R72 previously isolated in northwestern Spain (28) as well as strain 775, containing the pJM1 plasmid and isolated from the American Pacific coast, were also included for comparative purposes. The origins of the strains with their corresponding serotypes are listed in Table 1.

Experimental infections. The degree of pathogenicity of the different *V. anguillarum* strains was comparatively tested in fingerling rainbow trout (mean weight, 5 g), using mean lethal dose values (23). Fish were inoculated intraperitoneally as previously described (30).

Growth under iron-limiting conditions. *V. anguillarum* strains were cultured in M9 minimal medium (21) supplemented with 0.2% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.). The iron chelator ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA) was added at a concentration of 10 μM to achieve the iron limitation conditions. The MICs of EDDA were determined on plates of M9 with increasing concentrations of the chelator.

Production of siderophores. Production of siderophore compounds by the *V. anguillarum* strains was tested in the medium described by Schwyn and Neilands (26). The method is based on the fact that the dye chrome-azurol S incorporated into the medium can form stable complexes with iron, resulting in a blue solution. When a chelator, such as a siderophore, removes the iron from the complex, the

TABLE 1. Characteristics of *V. anguillarum* strains described in this work

Strain	Origin	Serotype	Virulence (LD_{50})[a]	pJM1-like plasmid	Presence of OM2[b]	Siderophore production[c]	MIC of EDDA (μM)[d]
RV22	Turbot (Spain)	O2	1×10^2	−	−	+	500
RV15	Turbot (Spain)	O2	2×10^2	−	−	+	500
43F	Striped bass (United States)	O2	3×10^2	−	−	+	500
96F	Striped bass (United States)	O1	8×10^3	−	−	+	500
775	Pacific salmon (United States)	O1	1×10^3	+	+	+	40
R72	Turbot (Spain)	O1	7×10^2	+	+	+	150
H775-3	From 775 by curing of pJM1	O1	1×10^8	−	−	−	2

[a] Mean lethal dose (LD_{50}) values were determined as described in the text.
[b] The presence of OM2 protein was tested by immunoblot analysis of sodium dodecyl sulfate-polyacrylamide gels of outer membrane proteins obtained from bacteria cultured under iron-limiting conditions (10 μM EDDA).
[c] Production of siderophore compounds was tested by the method described by Schwyn and Neilands (26).
[d] Determined on plates containing M9 minimal medium on 0.7% agarose with increasing concentrations of EDDA.

color turns to orange. This method was used to detect the presence of siderophore activity in plates and supernatants.

Siderophore activity was also determined in bioassays performed as previously described (12, 33) by testing the ability of cell-free supernatants from cultures of each strain to cross-feed different mutants defective in the iron uptake system. Mutants of *V. anguillarum* lacking production of siderophore, receptor, or both were used (Table 2). In addition, two different mutants of *Salmonella typhimurium* LT-2 (*enb-1* and *enb-7* strains) deficient in the biosynthesis of enterobactin and *Escherichia coli* LG1522, a mutant deficient in aerobactin biosynthesis, but with an intact aerobactin receptor, and the enterobactin receptor (provided by P. H. Williams, University of Leicester, Leicester, U.K.), were included in these bioassays as indicator strains.

The ability of the plasmidless *V. anguillarum* isolates to promote their own growth in iron-limiting conditions as well as their ability to utilize different purified siderophores were also evaluated.

The presence of phenolic compounds was examined by the Arnow test (3), and that of hydroxamates was determined by the method of Csáky (14). Paper chromatography of crude preparations of siderophores was done by the method of Rogers (24), using ammonium formate (5%) in formic acid (0.5%) as solvent. Chromatograms were developed with the reagent of Schwyn and Neilands (26).

TABLE 2. Results of cross-feeding experiments with various indicator strains

Strain	Ability to cross-feed					
	V. anguillarum[a]			*S. typhimurium*[b]		*E. coli* LG1522[c]
	H775-3	775::Tn*1-5*	775::Tn*1-6*	*enb-1*	*enb-7*	
RV22	+	+	+	+	+	−
RV15	+	+	+	+	+	−
43F	+	+	+	+	+	−
96F	+	+	+	+	+	−
775	−	+	−	−	+	−
R72	−	+	−	−	+	−
H775-3	−	−	−	−	+	−

[a] *V. anguillarum* mutants deficient in the iron uptake system: H775-3, plasmidless strain lacking plasmid pJM1; 775::Tn*1-5*(pJHC-91) (receptor proficient, siderophore deficient); 775::Tn*1-6*(pJHC9-8) (deficient in the production of both receptor and siderophore activities).
[b] Mutants of *S. typhimurium* LT-2 deficient in the biosynthesis of enterobactin: *enb-1* can use only enterobactin; *enb-7* can use enterobactin as well as 2,3-dihydroxybenzoic acid.
[c] Mutant of *E. coli* deficient in the biosynthesis of aerobactin and the enterobactin receptor but possessing an intact receptor for iron-aerobactin complexes.

Plasmid and total DNA analysis. The presence of plasmids was screened by the rapid method of Kado and Liu (19) and also by the method of Birnboim and Doly (4).

Total DNA was obtained by the method described by Meade et al. (20). Southern blot hybridizations (27) were done as described before (32). Radiolabeling of the various DNA probes was performed by the oligolabeling method of Feinberg and Vogelstein (16), using [^{32}P]ATP.

Analysis of membrane proteins. Total membrane proteins were prepared as previously described (10), and the outer membrane fractions were obtained by the method of Filip et al. (17) by differential solubilization of the bacterial cell envelopes, using 1.5% sodium lauroyl sarcosinate. Total and outer membrane proteins were examined by sodium dodecyl sulfate-polyacrylamide gels, using 12.5% acrylamide in the separating gel and 3% in the stacking gel. Induction of new proteins under iron limitation was examined by growing the strains in M9 medium in the presence of 10 μM EDDA and comparing the protein patterns with those appearing when strains were grown in M9 minimal medium with 10 μM $FeCl_3$.

Preparation of anti-OM2 serum and immunoblot analysis of the outer membrane proteins were carried out as previously described (2).

RESULTS

Isolation and identification of *V. anguillarum* strains. Samples obtained from different organs of moribund fish showing typical signs of an acute bacterial hemorrhagic septicemia yielded pure-culture colonies on tryptic soy agar-NaCl medium which were identified as *V. anguillarum* by means of a range of biochemical and physiological tests (Toranzo et al., in press). Two strains, RV22 and RV15, were chosen to represent the group. This group of strains is very similar phenotypically to previous isolates (43F and 96F as representatives) from striped bass from the American Atlantic coast (30) (Table 1). Plasmid analysis indicated that strains RV22 and RV15 were plasmidless, a feature previously demonstrated for strains 43F and 96F (30). The restriction pattern of total chromosomal DNA (DNA fingerprints) of these American and Spanish isolates demonstrated that they are very similar, but not identical (data not shown).

Agglutination assays with antisera from reference strains allowed us to classify strains RV22, RV15, and 43F as serotype O2 and strain 96F as serotype O1.

Experimental infections, performed with either fingerling trout or turbot, showed that all of these strains were highly virulent, with mean lethal doses ranging from 1×10^2 to 8×10^3 (Table 1).

Growth under iron-limiting conditions. It has been demonstrated that the ability to grow under iron-limiting conditions is an important virulence factor in *V. anguillarum* (7). All strains described here were able to grow in M9 medium containing the iron chelator EDDA at a concentration of 10 μM. Increasing concentrations of EDDA were tested to determine the MIC of this chelator. Table 1 shows that strain 775 harboring pJM1 and strain R72 containing a pJM1-like plasmid had EDDA MICs of 40 and 150 μM, respectively. However, the virulent plasmidless strains RV22 and RV15 from turbot and 43F and 96F from striped bass had a higher EDDA MICs (500 μM).

Siderophore activity and utilization of siderophores. The production of siderophores was tested by the method of Schwyn and Neilands and by bioassays. By the chemical method, all strains gave positive results, showing an orange halo on the blue agar plates and a change of color in the liquid reagent. However, strains RV22, RV15, 43F, and 96F gave stronger positive results than strains with pJM1-like plasmids, producing a very large orange halo on the plates and a strong color change in supernatants. This observation agrees with the wide difference in MICs of EDDA obtained for each group of strains and could indicate the production of a siderophore with a high iron affinity.

The production of a diffusible siderophore was also demonstrated by the ability of the supernatants from each of the strains to stimulate the growth of not only the producing strain but also each of the other strains in M9 medium with 600 μM EDDA. Also, growth of the strains bearing pJM1-like plasmids (775 and R72) was stimulated.

The plasmid pJM1-encoded siderophore anguibactin could promote growth of the derivative strain 775::Tn*1-5* containing the receptor OM2, which is specific for anguibactin (1, 33), but could not induce the growth of the 775 type strains lacking the OM2 receptor (Table 2). However, strains RV22, RV15, 43F, and 96F produce a siderophore which can cross-feed both the receptor-proficient and the receptor-deficient derivatives of *V. anguillarum* 775, indicating that a novel siderophore must be produced by these strains and that it can be used by *V. anguillarum* 775 independently of the presence of the anguibactin receptor.

Since *V. anguillarum* strains can utilize the siderophore enterobactin independently of the presence of the OM2 receptor (1), we decided to check whether the novel siderophore was functionally related to the enterobactin-mediated system. Bioassays for enterobactinlike activities were carried out with two different mutants of *S. typhimurium* deficient in the enterobactin system. It was of interest that all virulent plasmidless strains could induce the growth of both the *enb-1* strain, which can only use enterobactin, and strain *enb-7*, which can use enterobactin and the metabolic intermediate 2,3-dihydroxybenzoic acid. In contrast, strains harboring pJM1-like plasmids could only promote the growth of *enb-7* due to the chromosome-mediated production of 2,3-dihydroxybenzoic acid (1). Testing of supernatants by the Arnow assay confirmed that these strains excreted phenolic compounds.

Determination of hydroxamates by Csáky's test, as well as bioassays for aerobactin production, demonstrated that these supernatants did not contain hydroxamates or aerobactin-related compounds. Attempts to purify the novel siderophore by using procedures developed for enterobactin isolation failed. Also, paper chromatography analysis of concentrated supernatants gave R_f values slightly different from those of supernatants containing enterobactin, suggesting that the chromosome-mediated siderophore of the *V. anguillarum* strains, although functionally related to the siderophore enterobactin, may possess different physicochemical properties.

The utilization of siderophores by the virulent plasmidless strains was evaluated by bioassays in M9 containing an EDDA concentration sufficiently high to inhibit growth (Table 3). Purified siderophores containing phenolate and hydroxamate structures were tested. The only catechol siderophore able to stimulate growth of the strains in these conditions was enterobactin, as well as 2,3-dihydroxybenzoic acid. The siderophore anguibactin from *V. anguillarum* 775 could not be used by any of the strains, which indicates that they lack the receptor for anguibactin. The hydroxamate-type siderophores, ferrichrome and rhodotorulic acid, were also utilized by the four strains (Table 3), probably in an unspecific way since we previously observed that *V. anguillarum* 775 can also utilize ferrichrome as siderophore (1).

Hybridization with enterobactin and anguibactin system-specific probes. Since the virulent plasmidless *V. anguillarum* strains possess a chromosome-mediated iron uptake system apparently related to the enterobactin system as assessed by bioassays, hybridization experiments with *E. coli* enterobactin-associated genes were also carried out. Clones pCPIII (containing genes *fepC*, *febB*, and *entF*) and pCP410 (containing genes *entA*, *entC*, *entG*, *entB*, and *entE*) (both kindly provided by C. F. Earhart, University of Texas, Austin) were used as probes for hybridization with RV22 and 43F chromosomal DNA digested with *Eco*RI and *Bam*HI, using *E. coli* LE392 as a positive control.

Results show that there was no appreciable homology between the two systems. Hybridization with the pCP410 probe is shown in Fig. 1b. While the *E. coli* strain gives a clear signal, no hybridization was detected with RV22 or 43F DNA, even after a very long exposure, at which time only a weak signal was noted. Hybridization with the pCPIII probe was also negative (data not shown). These results demonstrated that there is no homology between the enterobactin-mediated iron uptake system and the chromosome-encoded system of *V. anguillarum*.

Similarly, no homology could be found with a probe containing sequences of the anguibactin biosynthetic genes (Fig. 1a).

Induction of outer membrane proteins under iron-limiting conditions. The induction of outer membrane proteins in iron-limited media was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each strain was grown in iron-rich and iron-limited media, and their total and outer membrane proteins were compared (Fig. 2a and b).

TABLE 3. Utilization of various siderophores by plasmidless strains of *V. anguillarum*

Siderophores	*V. anguillarum* strain			
	RV22	RV15	43F	96F
Anguibactin	–	–	–	–
Enterobactin	+	+	+	+
Amonabactin	–	–	–	–
Pyochelin	–	–	–	–
Pyoverdin	–	–	–	–
2,3-Dihydroxybenzoic acid	+	+	+	+
Ferrichrome	+	+	+	+
Rhodotorulic acid	+	+	+	+
Aerobactin	–	–	–	–

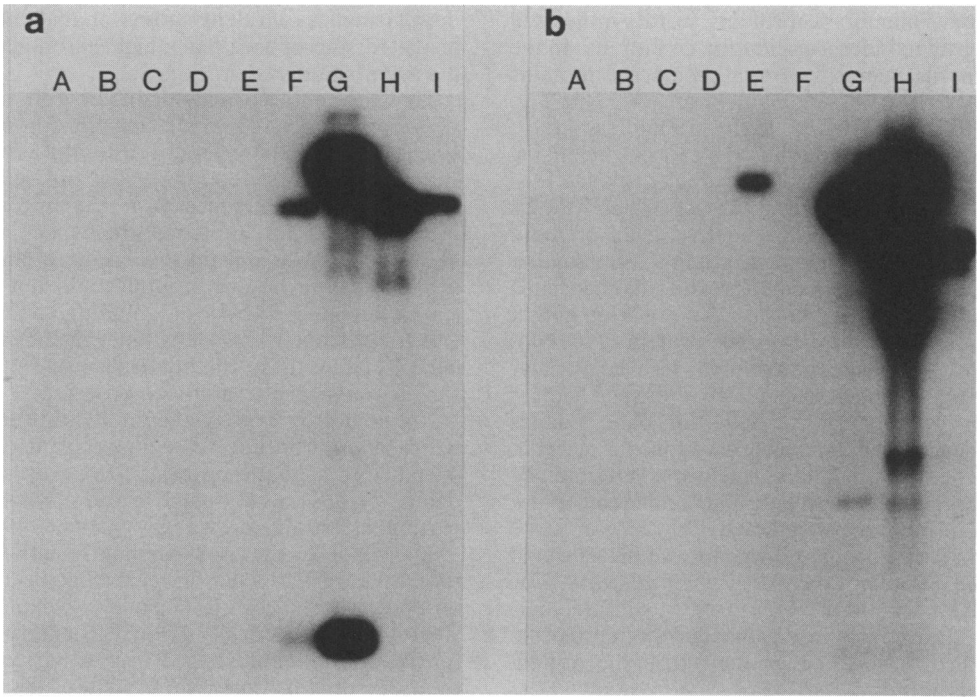

FIG. 1. Southern blot hybridizations of digested total DNA of plasmidless strains with anguibactin (a) and enterobactin (b) genes. Lanes: A and B, 43F; C and D, RV22; E, *E. coli* LE392; F, pJM1; G, pJHCS300; H, pCP410; I, pCPIII. DNA in lanes A, C, E, F, G, H, and I was digested with *Eco*RI. DNA in lanes B and D was digested with *Bam*HI. In panel a, the ^{32}P-labeled probe is pJHCS300, which is a pBR325 clone of a pJM1 *Eco*RI/*Pst*I fragment containing part of anguibactin biosynthetic genes, and in panel b, the probe is pCP410, containing enterobactin genes as described in the text cloned in pACYC184. Hybridization observed between the different probes is due to the homology of vectors pACYC184 and pBR325.

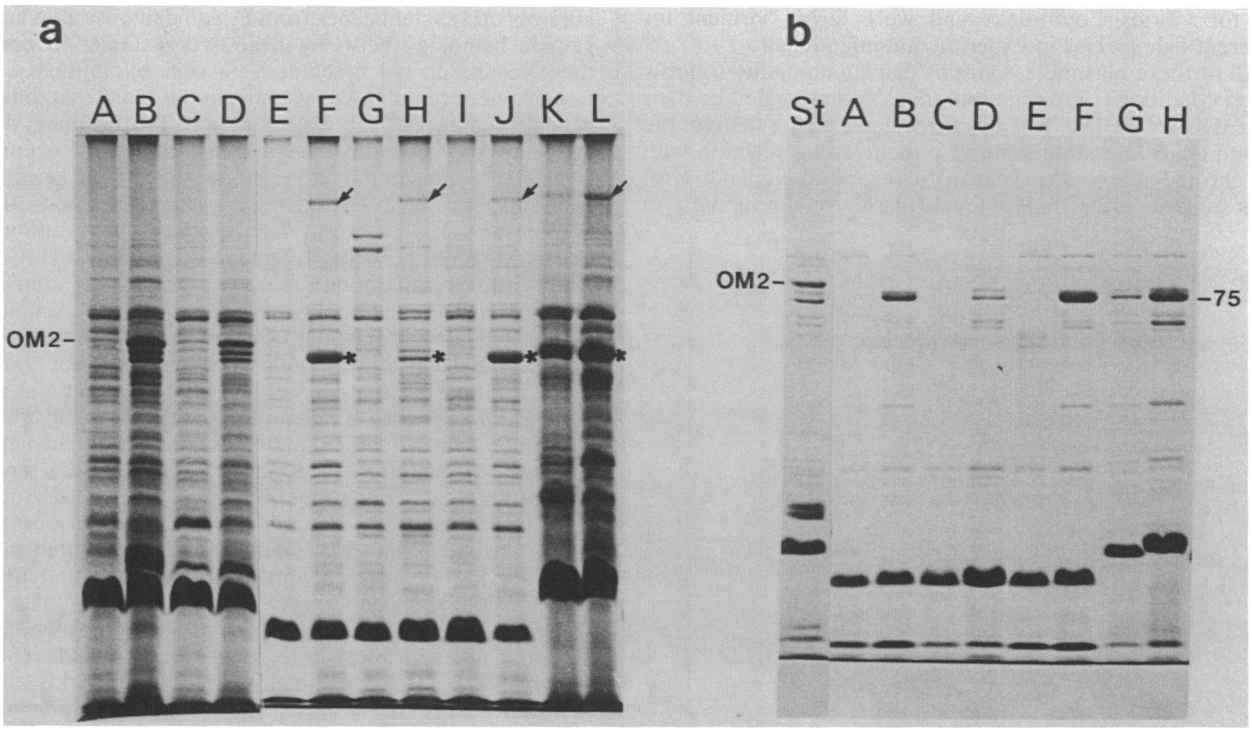

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total and outer membrane proteins of *V. anguillarum* strains. (a) Total membrane proteins of all strains. Lanes: A and B, 775; C and D, R72; E and F, RV22; G and H, RV15; I and J, 43F; K and L, 96F. Lanes B, D, F, H, J, and L are total membrane proteins from cells cultured under iron-limiting conditions. The position of OM2 of strain 775 is indicated. The arrows and asterisks indicate the main proteins induced in iron-limiting conditions in the plasmidless strains. (b) Outer membrane proteins from strains RV22 (lanes A and B), RV15 (C and D), 43F (E and F), and 96F (G and H). The standard (St) strain 775 is shown as reference. The number indicates the molecular weight (10^3) of the iron-regulated proteins.

In all strains new membrane proteins were synthesized when cells were grown under iron-limiting conditions. In the plasmidless strains the patterns of total membrane proteins were different from those obtained with strains 775 and R72, which contain pJM1-like plasmids. In the plasmidless strains two patterns were observed: one shared by strains belonging to serotype O2 (RV22, RV15, and 43F) (Fig. 2a, lanes E to J), and the other pattern shown by strain 96F which belongs to serotype O1 (Fig. 2a, lanes K and L). In all of these plasmidless strains the iron-regulated proteins were smaller in size than the OM2 protein encoded by pJM1-like plasmids in strains 775 and R72, and all of them have in common at least one iron-regulated outer membrane protein of 75 kilodaltons (Fig. 2b). In addition, a new protein with a molecular weight of about 150,000 was detected in all of the plasmidless strains growing in conditions of iron limitation (Fig. 2a, lanes F, H, J, and L), which was never observed in the plasmid-bearing strains. The iron-regulated protein was not obtained in any of the outer membrane preparations, indicating that it might be located in the inner membrane.

To determine the relationship between these proteins and the iron-regulated protein OM2 of *V. anguillarum* 775, immunoblot analysis with anti-OM2 serum was carried out. Results (Fig. 3) showed no immunological homology between OM2 and the iron-regulated proteins from the virulent plasmidless strains.

DISCUSSION

The presence of plasmid pJM1 encoding an iron uptake system is an essential factor for pathogenicity in *V. anguillarum* 775 (7, 33, 34). However, in this work we report that certain plasmidless strains of *V. anguillarum* were responsible for vibriosis outbreaks and were highly virulent for different fish species in experimental infections.

All of these plasmidless strains display an ability to grow under conditions of iron limitation, as demonstrated by their high MICs of EDDA, which is three times higher than for the plasmid-carrying strains, and by their strong reaction with the chrome-azurol S reagent of Schwyn and Neilands (26). This suggests either higher levels of siderophore activity in the plasmidless virulent strains or that their chromosome-mediated siderophore has a higher iron affinity than that of the anguibactin system.

FIG. 3. Immunoblot analysis of the outer membrane proteins from *V. anguillarum* strains. Lanes: A and B, 775; C and D, R72; E and F, RV22; G and H, 43F; I and J, 96F. The second lanes of each strain are the outer membrane proteins from cells cultured in iron-limiting conditions.

The new chromosome-mediated iron uptake system is apparently unrelated to the anguibactin-mediated system, since the siderophore produced by the virulent plasmidless strains could cross-feed derivatives of *V. anguillarum* 775 regardless of the presence of the anguibactin receptor. Moreover, the OM2 protein normally mediated by the pJM1-like plasmids was not present in these plasmidless strains, and purified ferric anguibactin could not release the iron starvation imposed by iron limitation conditions. Instead of OM2, other outer as well as inner membrane proteins were induced under these conditions. These proteins could play a similar role in the iron uptake process.

No homology was found in hybridization experiments between the iron uptake region of pJM1 and the chromosomal DNA from these strains. However, sequences homologous to pJM1 were found in the chromosome of certain plasmidless strains previously examined (9).

According to the cross-feeding results with *S. typhimurium* mutants affected in enterobactin biosynthesis, the virulent plasmidless strains of *V. anguillarum* produce a siderophore functionally related to enterobactin, which so far has only been described in members of the *Enterobacteriaceae* (8, 18). Likewise, growth of the virulent plasmidless *V. anguillarum* strains in highly iron-depleted media was induced by the addition of enterobactin or by the presence of an enterobactin producer strain.

All of this suggests that a siderophore analogous to enterobactin may be produced by the virulent plasmidless strains of *V. anguillarum*. However, hybridization experiments with two different clones spanning a region with most of the enterobactin system genes from *E. coli* demonstrated lack of genetic homology between these two systems. Of course, these results do not preclude a possible evolutionary relationship between the two systems, in the sense that different genes could encode the same function. For instance, divergence in the genes for the aerobactin system occurs in certain enteric bacteria, although the siderophore aerobactin is identical to that encoded by plasmid pColV-K30 (V. Waters and J. H. Crosa, manuscript in preparation; L. Crosa, M. Wolf, L. Actis, J. Sander-Loehr, and J. H. Crosa, manuscript in preparation). However, preliminary studies on the nature of the siderophore produced by the plasmidless strains indicate that it actually differs from enterobactin in its chemical and chromatographic behavior (data not shown). Thus, it is possible that the virulent plasmidless strains of *V. anguillarum* produce a siderophore physiologically related to enterobactin but with a modified structure, explainable in terms of an evolutionary divergence between the families *Vibrionaceae* and *Enterobacteriaceae*. We have also found that other strains of *V. anguillarum* that do not possess a pJM1 plasmid, such as NCMB6 and Z911, isolated in Europe, do harbor an iron uptake system mediated by the enterobactinlike siderophore described here (data not shown). Thus, the presence of the new chromosome-mediated iron uptake system must be of common occurrence in *V. anguillarum* isolates in many parts of the world.

In conclusion, in pathogenic strains of *V. anguillarum* two different iron uptake systems can occur. One of them, responsible for virulence in plasmid-bearing strains, is encoded by the pJM1-type plasmids and is mediated by the siderophore anguibactin. The other, coded for by chromosomal genes, may be functionally related to the enterobactin-mediated iron transport system. Further genetic and bio-

chemical characterization of this new iron uptake system, currently under way, will allow us to assess its actual relationship to both the enterobactin- and the anguibactin-mediated systems as well as its role in pathogenicity.

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