Vibrio strains isolated from diseased turbot in an experimental fish farm on the Atlantic coast of northwest Spain were identified as Vibrio anguillarum. The isolates shared many biochemical characteristics with V. anguillarum strains obtained from other sources, and harboured a plasmid species that showed extensive homology with plasmid pJM1, carried by V. anguillarum strain 775 isolated from an epizootic in North America. Restriction endonuclease analysis showed that the two plasmids were very similar albeit not identical. The presence of the plasmid in the turbot isolates was associated with their ability to cause disease in fish. Plasmid-carrying bacteria could also grow under conditions of iron limitation. Two outer membrane proteins, of 86 and 79 kDal, were induced, and a similar siderophore activity to that produced by V. anguillarum 775 was also detected under these conditions. The 86 kDal outer membrane protein cross-reacted immunologically with antiserum raised against the outer membrane protein OM2 produced by strain 775. Nonvirulent plasmidless derivatives were unable to grow under iron-limiting conditions, and were also unable to produce either siderophore activity or the 86 kDal outer membrane protein, suggesting the plasmid-mediated nature of these components.

INTRODUCTION

Bacterial epizootics of marine fish are a major limiting factor in their commercial aquaculture. Vibrio anguillarum and other members of the genus Vibrio are the aetiopathological agents of the devastating fish disease vibriosis (Canestrini, 1893; Evelyn, 1971; Harbell et al., 1979; Rucker et al., 1953; Schiewe et al., 1981). Virulent strains of V. anguillarum isolated from diseased Pacific salmon harbour a 65 kb plasmid, pJM1 (Crosa et al., 1977, 1980), which confers the high virulence phenotype by mediating a very efficient iron-sequestering system (Crosa, 1979, 1980, 1984). By means of this mechanism bacteria can grow in media with low concentrations of available iron, conditions which mimic those found in host fluids and secretions, imposed by the iron-binding proteins transferrin and lactoferrin (Bullen et al., 1978; Weinberg, 1978; Neilands, 1982). This system, composed of a siderophore and its receptor, both encoded by an approximately 20 kb stretch of DNA in the pJM1 plasmid, has recently been mapped and cloned (Crosa, 1984; Crosa et al., 1983; Walter et al., 1983; Tolmasky & Crosa, 1984). The iron-regulated 86 kDal outer membrane protein, OM2, encoded by pJM1 may function as the siderophore receptor (Crosa, 1981; Crosa & Hodges, 1981).

In this study we report the molecular and physiological characterization of V. anguillarum strains isolated from diseased turbot (Scophthalmus maximus) on the Atlantic coast of northwest Spain.

Abbreviation: EDDA, ethylenediamine di(o-hydroxyphenylacetic acid).
**METHODS**

**Bacterial strains.** Bacterial isolation from liver and muscle of moribund turbot (mean weight 24 g) obtained from an epizootic was made on thiosulphate/citrate/bile/sucrose agar and Trypticase soy agar supplemented with 2% (w/v) NaCl (Toranzo et al., 1983). Strains R72 and R722 were isolated from liver; strain R722 was isolated after experimental infection of turbot with strain R72. Strain R61 was isolated from muscle.

Other *V. anguillarum* strains used were the wild-type strain 775, carrying plasmid pJM1, and the mutant 775::Tn7-5, carrying plasmid pHJC-91 (a derivative of pJM1 having a Tn7 insertion), which produces receptor but not siderophore activity (Walter et al., 1983). *V. anguillarum* NCMB 6 was from the laboratory stock and 91079 (Agius et al., 1983) was provided by Dr M. T. Horne (University of Sterling, Scotland).

**Isolation of plasmid DNA, restriction endonuclease analysis and Southern blot hybridizations.** Plasmid DNA was isolated by the method of Birnboim & Doly (1979) followed by ultracentrifugation in a caesium chloride/ethidium bromide density gradient as previously described (Walter et al., 1983). Restriction endonuclease cleavage of plasmid DNA was done under the conditions recommended by the supplier (BRL). Electrophoresis of cleaved DNA was done in a horizontal 0-7% (w/v) agarose gel as previously described (Walter et al., 1983). Whole DNA was obtained as described by Brenner et al. (1969) and the mean mol % G + C was calculated based on the midpoint of thermal denaturation curves according to Mandel et al. (1970). Southern blot hybridizations (Southern, 1975) between 32P-labelled pJM1 DNA and the plasmid DNA from the turbot strains were done as before (Walter et al., 1984).

**Growth under iron-limiting conditions.** *V. anguillarum* was cultured in M9 minimal medium (Miller, 1972). Iron-limiting conditions were achieved by the addition of the nonassimilable iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDA) to a concentration of 10 μM.

**Analysis of outer membrane proteins.** Outer membrane proteins were prepared and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Crosa & Hodges (1981).

**Immunoblot analysis.** Antiserum against OM2 was raised in 6-month-old rabbits by injecting crushed polyacrylamide gel fragments containing the OM2 protein band. The material, emulsified in 1 ml of Freund's complete adjuvant, was injected at multiple subcutaneous sites on the upper back of the rabbits three times at 15 d intervals. One week after the last injection, the rabbits received a booster dose with the same material emulsified in Freund's incomplete adjuvant. Serum was obtained at 10 d intervals after the initial dose and the immunoglobulin fraction was prepared by (NH4)2SO4 precipitation. Outer membrane proteins were prepared for SDS-PAGE and separated on 12.5% (w/v) acrylamide gels. After electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose paper (0.45 pm BA85) by Towbin et al. (1979), using a constant current of 250 mA at 10 °C in a Trans-Blot cell (Bio-Rad) with 25 mM-Tris base, 192 mM-glycine, 20% (v/v) methanol. After electrophoresis, the nitrocellulose paper was soaked for 30 min at room temperature in Tris-buffered saline (10 mM-Tris/HCl, pH 7-4, 0-9% NaCl) containing 3% (w/v) bovine serum albumin, followed by incubation with anti-OM2 serum diluted 1/500 in NIBB solution (50 mM-Tris/HCl, pH 7-4, 150 mM-NaCl, 5 mM-EDTA, 0-05% Triton X-100) for 3 h at room temperature. The blots were washed twice in NIBB, incubated in a 1/1000 dilution of horseradish peroxidase conjugated Protein A (0-5 mg ml⁻¹) in NIBB for 3 h at room temperature. The nitrocellulose paper was stained with 0-022% H2O2 and 0-075% HRP Color Development Reagent (Bio-Rad) for 5–10 min at room temperature.

**Experimental infections.** Pathogenicity was assayed by intraperitoneal inoculation using either cultured or wild turbot (mean weight 12 g) or rainbow trout (*Salmo gairdneri*; mean weight 5 g) maintained in seawater at 15°C. Fish were inoculated with bacterial doses ranging from 102 to 107 cells, following the experimental conditions described previously (Toranzo et al., 1983). Virulence was quantified as the mean lethal dose (LD50) as described by Reed & Muench (1938).

**RESULTS**

**Isolation and biochemical characterization of *V. anguillarum* strains from turbot and comparison with other isolates**

A vibriosis outbreak occurred in juvenile turbot reared in an experimental intensive marine culture station in Galicia (Atlantic coast of northwest Spain). This epizootic started during the spring of 1983, a month after the fish were shipped from Scotland. The moribund turbot showed clinical signs typical of an acute bacterial septicaemia, with haemorrhages mainly centred at the base of the fins, exophthalmia and corneal opacity. Internal examination showed a very pale liver and haemorrhages in the muscle with oedematous lesions on the hypodermis. Material obtained from different organs of moribund fish yielded large numbers of rapidly-growing bacteria of similar colonial morphology upon culturing on Trypticase soy agar. The isolates were Gram-negative rods, motile by polar flagella, cytochrome oxidase positive, able to ferment
Plasmids and iron uptake in *V. anguillarum*

Table 1. Properties of *V. anguillarum* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Siderophore activity*</th>
<th>Presence of OM2†</th>
<th>Growth in medium with 10 μM-EDDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>R72H10</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R61</td>
<td>pTA83A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R72</td>
<td>pTA83B</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R722</td>
<td>pTA83C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>775</td>
<td>pJM1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>775::TnI-5</td>
<td>pJHC-91</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Siderophore activity was determined by testing the ability of cell free supernate from cultures of each strain to cross-feed the indicator strain 775::TnI-5 in bioassays performed as previously described (Crosa et al., 1983).
† The presence of OM2 was tested for by immunoblot analysis of SDS-PAGE of outer membrane proteins obtained from bacteria cultured under iron-limiting conditions (10 μM-EDDA, with the exception of strain 775::TnI-5(pJHC-91), for which 2 μM-EDDA was used). The anti-OM2 serum was obtained as described in Methods.

Glucose and arabinose, and sensitive to the vibriostatic agent O/129 and to novobiocin (10 μg per disk); they produced haemolysin on sheep blood. The isolated micro-organisms were identified as *Vibrio anguillarum* by a range of physiological and biochemical tests (Furniss et al., 1978), using the Pacific Northwest strain *V. anguillarum* 775, the European reference strain *V. anguillarum* NCMB 6, and *V. anguillarum* 91079, isolated in Scotland from turbot, as controls (Agius et al., 1983). Our turbot isolates, R61, R72 and R722, showed a positive indole reaction, and the only difference from the reference strain NCMB 6 was their failure to give a positive methyl red reaction. In addition, the turbot isolates differed from strain 91079 in their ability to grow at 37°C and to utilize arabinose. The mean G + C content was 46.5 mol%, which is similar to the value for other *V. anguillarum* strains (Schiewe et al., 1977).

Since in many *V. anguillarum* strains there is a correlation between high virulence and an ability to grow under iron-limiting conditions (Crosa, 1979, 1980), we examined the turbot isolates for their ability to grow under those conditions (Table 1). The three strains were able to grow in the presence of 10 μM-EDDA, as did *V. anguillarum* strain 775. Strain 775::TnI-5, which carries a mutant derivative of pJM1, pJHC-91 (Walter et al., 1983), and is unable to produce a siderophore, did not grow under these conditions.

Detection of plasmids and restriction endonuclease analysis

The analysis of the plasmid content of the three *V. anguillarum* strains isolated from turbot revealed that they all carried a plasmid which had a mobility close to that of the 65 kb virulence plasmid pJM1 (Crosa et al., 1977). The plasmid from strain R72 could be cured with a frequency of 3-3% after several passages of the bacteria at 37°C. A plasmidless derivative of strain R72, *V. anguillarum* R72H10, was unable to grow under iron-limiting conditions (Table 1).

Plasmid DNA was purified and subjected to restriction endonuclease analysis. The plasmids were designated pTA83A (from strain R61), pTA83B (from strain R72) and pTA83C (from strain R72). These three plasmids showed extensive homology with the virulence plasmid pJM1 in Southern blot hybridizations. Results for pTA83A and pTA83B are shown in Fig. 1 (a, b). An identical result was obtained by hybridization of pTA83C with pJM1 (not shown). Digestion of each of the three plasmids with BamHI or EcoRI produced an identical restriction pattern; this differed somewhat from that obtained with plasmid pJM1 cleaved with the same enzymes (Fig. 2). The pJM1 BamHI fragments 4 and 5 (Fig. 1a, lane A) were isolated from the agarose gel, labelled with 32P-dATP and hybridized against BamHI-digested DNA from plasmids pJM1, pTA83A, pTA83B and pTA83C. Fig. 1 shows the results obtained with pJM1, pTA83A and pTA83B. Identical results were obtained with pTA83C (not shown). The pJM1 BamHI fragments 4 and 5 showed strong homology with the pTA83A and pTA83B BamHI fragments 4* and 5* respectively (Fig. 1a, c, d). The pJM1 BamHI fragment 5 also showed weak homology with fragments 1 and 2 (Fig. 1d). This result was expected since it was known that...
Fig. 1. Restriction endonuclease cleavage and hybridization of V. anguillarum plasmids. (a) Ethidium bromide stained 0.7% agarose gel of BamHI digestion of: A, pJM1; B, pTA83A; C, pTA83B. (b, c, d) Autoradiographs of Southern blot hybridizations of gels identical to that in (a), using the following 32P-labelled probes: (b), pJM1; (c), pJM1 BamHI fragment 4; (d), pJM1 BamHI fragment 5. The pJM1 BamHI fragments 4 and 5 were isolated from the gel as described by Benson (1984). The DNA fragments were labelled numerically in decreasing order of size.
Plasmids and iron uptake in *V. anguillarum*

**Fig. 2.** Restriction endonuclease cleavage of *V. anguillarum* plasmids. (a) *BamHI*-cleaved DNA: lane A, pJM1; B, pTA83A; C, pTA83B; D, pTA83C. (b) *EcoRI*-cleaved DNA: lane E, pJM1; F, pTA83A; G, pTA83B; H, pTA83C. DNA fragments were numbered numerically in decreasing order of size.

*BamHI* fragments 1, 2 and 5 contain short DNA repeated sequences (Crosa *et al.*, 1985). Weak homology was also apparent in the high molecular weight region, due to pJM1 fragments that were not totally digested. This experiment confirmed that the *BamHI* fragments 4 and 5 from pJM1 correspond to the larger *BamHI* fragments 4* and 5*, respectively, from pTA83A, pTA83B and pTA83C. The increase in size was estimated to be 1.9 kb in the case of *BamHI* fragment 4* and 0.4 kb for the *BamHI* fragment 5*. Similarly, using pJM1 *EcoRI* fragments as 32P-labelled probes we were able to demonstrate that *EcoRI* fragments 4 and 7 from pJM1 were homologous to *EcoRI* fragments 4* and 7* respectively from pTA83A (not shown).

**Induction of specific outer membrane proteins under iron-limiting conditions**

*V. anguillarum* 775 growing under conditions of iron limitation shows the induction of two novel outer membrane proteins (Crosa & Hodges, 1981). One, an 86 kDal protein (OM2), is encoded by pJM1 but the other, a 79 kDal protein (OM3), is chromosomally encoded. We examined by SDS-PAGE the outer membrane proteins produced by the turbot isolates cultured under iron-rich and iron-limiting conditions. Two outer membrane proteins with identical mobility to the OM2 and OM3 proteins present in *V. anguillarum* 775 were induced when strains R72, R722, and R61 were cultured under iron-limiting conditions (Fig. 3, Table 1). In the case of the plasmidless derivative R72H10 only a 79 kDal outer membrane protein was induced under these conditions (Fig. 3), indicating that an 86 kDal outer membrane protein is plasmid mediated and iron regulated in the turbot isolates. Immunoblot analysis of a gel similar to that shown in Fig. 3, using antiserum against the OM2 protein of *V. anguillarum* 775, showed that the 86 kDal protein produced by the turbot isolates under conditions of iron limitation was immunologically related to the OM2 protein of *V. anguillarum* 775 (Fig. 4, Table 1).
Synthesis of siderophores

Since the *V. anguillarum* isolates were able to grow under iron-limiting conditions we also tested their ability to produce siderophore activity. Culture supernates were scored for their ability to cross-feed the indicator strain *V. anguillarum* 775::TnI-5, which harbours the plasmid pJHC-91, a pJM1 derivative with a TnI insertion. Under iron-limiting conditions this strain synthesizes the receptor for iron-siderophore complexes but not siderophore activity. Culture supernates from the turbot isolates that harboured a plasmid were able to cross-feed *V. anguillarum* 775::TnI-5, while the supernate obtained from the cured derivative R72H10 could not cross-feed the indicator strain (Table 1). This result indicates that the plasmid carried by the turbot isolates must mediate the production of a siderophore that is capable of cross-feeding the indicator strain.

Experimental infections

Intraperitoneal inoculation of turbot with the three turbot isolates reproduced the clinical signs of vibriosis. The mean LD$_{50}$ was 7.0 \times 10^{4} (range 6.9–7.1 \times 10^{4} in four experiments) for cultured turbot and of 1.0 \times 10^{3} (range 0.7–2.0 \times 10^{3}) for wild turbot. The virulence of strain R72 and its cured derivative R72H10 were also tested using rainbow trout. While the parental strain had an LD$_{50}$ of 1.8 \times 10^{4}, the plasmidless derivative had an LD$_{50}$ of 2.8 \times 10^{6}, indicating that the presence of the plasmid was associated with the high-virulence phenotype of this bacterium.
Fig. 4. Immunoblot analysis of the outer membrane proteins from *V. anguillarum* strains. Outer membrane proteins from *V. anguillarum* strains cultured under iron-limiting conditions were subjected to SDS-PAGE, electrophoretically transferred to a nitrocellulose paper and incubated with anti-OM2 serum and developed as described in Methods. A, *V. anguillarum* R72H10; B, R61; C, R72; D, R722; E, 775.

**DISCUSSION**

*V. anguillarum* 775, isolated from salmon in epizootics occurring in the Pacific Northwest of the United States of America, carries a plasmid, pJM1, associated with the virulence of this strain (Crosa et al., 1977, 1980). This plasmid mediates a very efficient iron-sequestering system which enables the bacteria to compete for the iron bound by high-affinity iron-binding proteins (Crosa, 1979, 1980). However, this is not the case for all *V. anguillarum* strains examined. For example, no plasmids were detected in strains obtained from striped bass off the Atlantic coast of the United States of America although there was a perfect correlation between the high-virulence phenotype and the ability to grow under conditions of iron limitation (Toranzo et al., 1983).

In this work we describe the isolation and characterization of new *V. anguillarum* strains from cultured turbot on the Atlantic coast of northwest Spain. Two strains isolated from liver (R72 and R722) and one from muscle (R61) were scored for their iron uptake characteristics and for the presence of plasmids. These isolates harboured a plasmid which, although showing minor differences, exhibited extensive homology with pJM1, as demonstrated by restriction endonuclease analysis and Southern blot hybridization. All three strains were able to grow under iron-limiting conditions and, as is the case for *V. anguillarum* 775 (Crosa et al., 1980), loss of the plasmid was associated with both a substantial attenuation of virulence and the loss of ability to grow under iron-limiting conditions. These results indicated that the plasmids carried by the turbot strains may be directly involved in the ability of these bacteria to take up iron efficiently and also in their high-virulence phenotype. The three strains also showed induction of an 86 kDal outer membrane protein, immunologically related to the OM2 protein of *V. anguillarum* strain 775, when cultured under iron-limiting conditions. Genes encoding the OM2 protein have
recently been cloned (Tolmasky & Crosa, 1984), and it has been demonstrated that this protein plays a role in the iron transport process in V. anguillarum 775 (Actis et al., 1985). It is therefore likely that the immunologically related protein found in strains R61, R72 and R722 plays a similar role. A 79 kDal chromosomally-encoded outer membrane protein was also induced in these strains under iron-limiting conditions. A protein of a similar molecular weight, OM3, is produced by V. anguillarum 775, and this is also coded for by the chromosome (Crosha & Hodges, 1981). Supernates from the turbot isolates contained siderophore activity. It remains to be seen if the siderophore produced by the turbot isolates is the same as or is related to that produced by V. anguillarum 775. The finding of the iron uptake genes of pJM1 in the turbot isolates, carried by pJM1-like plasmids, suggests that these genes may be highly mobile and distributed widely as a virulence factor in strains from different fish and in different geographical regions. The presence of the V. anguillarum iron uptake genes in plasmids and in chromosomes (Crosha et al., 1985), together with the fact that in pJM1 those genes are flanked by repeated sequences (Crosha et al., 1985), suggests that these genes may have spread by recombinational processes, possibly involving transposition. The study of the relationship between the genes mediating iron-sequestering systems in different strains will be of importance in assessing the evolution of this important virulence factor of V. anguillarum.

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