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Iron Uptake System Mediated by *Vibrio anguillarum* Plasmid pJM1

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Plasmid pJM1 from an invasive strain of *Vibrio anguillarum* mediates an iron-sequestering system that is associated with the ability of this bacterium to cause septicemia in marine fishes. This plasmid-mediated iron uptake system was analyzed by using mutations caused by transposon Tn*1*. Restriction endonuclease analysis of iron uptake-deficient and -proficient derivatives generated by insertion of Tn*1* and molecular cloning experiments permitted us to localize the plasmid regions involved in the process of iron sequestration to a stretch of about 20 kilobase pairs. In addition, the existence of two plasmid-mediated components involved in the process of iron uptake in *V. anguillarum* was defined: a diffusible substance which functions as a siderophore and a nondiffusible receptor for complexes of iron-siderophore, which we have tentatively identified as the pJM1 plasmid-mediated outer membrane protein OM2 of *V. anguillarum*.

Vibrio anguillarum 775 is an invasive pathogen of marine fish which causes a terminal hemorrhagic septicemia. This prevalent marine pathogen has been particularly devastating in the marine culture of salmonid fishes (3, 13, 16, 22). It causes the characteristic vibriosis bloody lesions in the musculature and hemorrhaging at the base of the fins, with internal inflammation continuing into a generalized septicemia; death ensues from hypoxia and dysfunction of various organs (16; D. P. Ransom, C. N. Lannan, J. S. Rohovec, and J. L. Fryer, in press). Crosa et al. (9) found a relationship between the presence of the pJM1 plasmid in *V. anguillarum* and an enhanced capacity to produce overt disease. Curing of the pJM1 plasmid was correlated with a decreased virulence (7). This virulence plasmid specifies a very efficient iron uptake system (4, 5), which is required for the bacteria to be able to utilize the otherwise unavailable host iron which is bound by high-affinity iron-binding proteins like transferrin in serum or lactoferrin in secretions (2, 19, 20, 23). Concomitant with an efficient plasmid-mediated uptake of iron into the cell cytosol during growth under conditions of iron limitation (5, 6), the synthesis of two novel outer membrane proteins is induced: OM2, an 86,000-dalton protein associated with the presence of the pJM1 plasmid, and OM3, a 79,000-dalton protein of chromosomal origin (6).

In this paper, we describe Tn*1* transposon-induced mutations (17) in the pJM1 iron uptake system. By using a combination of restriction endonuclease cleavage analysis and molecular cloning we were able to construct a physical map of the pJM1 plasmid and localize a region involved in the production of essential components of the pJM1 iron uptake system. Preliminary reports of these findings have been recently published (10, 11).

MATERIALS AND METHODS

Bacterial strains. *V. anguillarum* 775 carrying the virulence plasmid pJM1 has been previously described (7, 9). Tn*1* insertion derivatives of this strain are described in Table 1. *Escherichia coli* K-12 J53(RP4) Ap^r Km^r Nm^r Tc^r (resistance to ampicillin, kanamycin, neomycin, and tetracycline, respectively) was used in conjugation experiments to transfer RP4 containing the transposition sequence Tn*1* (Ap^r) to *V. anguillarum* 775(pJM1) Rif^r Str^r (chromosomal resistance to rifampin and streptomycin, respectively). *E. coli* K-12 RR1 F^- *hsdS20 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* was used in transformation experiments.

Conjugation and transposition experiments. Plasmid RP4 was conjugated from *E. coli* J53(RP4) to *V. anguillarum* 775(pJM1) by using a filter method. The donor and recipient cells were separately diluted into 10 ml of brain heart infusion (BHI) broth (supplemented with 1% NaCl in the case of *V. anguillarum*) and allowed to grow to log phase (10^8 cells per ml) at 37°C for the *E. coli* culture and at 20°C for the *V. anguillarum* culture. A mixture containing 1.5 ml of each culture (1.5×10^8 cells of each strain) was passed through a membrane filter (0.2-μm pore diameter; Bio-Rad Laboratories, Richmond, Calif.). The filter was then placed on Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% NaCl and incubated at 20°C for 6, 24, and 48

h. After incubation, the filters were placed in 2 ml of BHI broth supplemented with 1% NaCl and mixed gently to remove the cells. Samples of the mating mixtures were plated on Trypticase soy agar plus 1% NaCl plates containing ampicillin (1.5 mg/ml), streptomycin (20 μg/ml), and rifampin (20 μg/ml) to select for *V. anguillarum* exconjugants. Each colony appearing on the selection plates was transferred next to two series of plates: one series was supplemented with ampicillin, streptomycin, and rifampin as above and the other was supplemented with kanamycin (1 mg/ml), streptomycin, and rifampin. Incubation was again carried out at 20°C for 48 h. Ap^r Km^r and Ap^r Km^s exconjugant clones were obtained and stored at −70°C in BHI broth plus 1% NaCl and containing 40% glycerol until further use. Ap^r Km^s exconjugants were likely to be transposition derivatives in which RP4 was no longer present (loss of kanamycin resistance due to the loss of RP4 but retention of the Ap^r phenotype of the Tn*1* sequence). Both types of exconjugants were then tested for the ability to grow under conditions of iron limitation in M9 minimal medium (5) containing the nonassimilable iron chelator ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA) at 10 μM and also analyzed for the presence of plasmid DNA. Clones were grown overnight in 3 ml of BHI broth supplemented in 1% NaCl, and lysates were prepared by a quick lysis procedure (1). Determination of the presence of transposition sequences on plasmid DNA was carried out by electrophoresis in 0.7% agarose gels in Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid, pH 8.2). The gels were electrophoresed, stained, and photographed as previously described (9).

Bioassays for detection of siderophore activity. The ability of supernatant fluid to support the growth of iron uptake-deficient mutants in iron-depleted medium was tested by impregnating a sterile disk with 10 μl of supernatant fluid from the growth of iron-proficient wild-type *V. anguillarum* 775(pJM1) strains or Tn*1* derivatives that were still iron proficient. Strains were grown in M9 minimal medium containing 10 μM EDDA for 48 h at 22°C. After centrifugation of the cells, the supernatants were removed and filter sterilized. The disks were placed on a minimal agar plate containing 10 μM EDDA that had been seeded with a lawn of 0.1 ml (10^8 cells) of an iron uptake-deficient strain (either 775::Tn*1*-5, 775::Tn*1*-6, 775::Tn*1*-7, or H775-3). For the iron uptake-deficient mutants, which were grown in about 1 μM EDDA, the sterile, filtered supernatant fluids were made to 10 μM EDDA before addition to the test disk.

Isolation of plasmid DNA. Large-scale purification of plasmid DNA was performed by the method of Hansen and Olsen (15) or Birnboim and Doly (1), modified as described previously (18). Further purification was achieved by two successive centrifugations in cesium chloride-ethidium bromide density gradients at 45,000 rpm for 16 h at 15°C in the VTi 65 rotor in a Beckman ultracentrifuge (L8-70). The rapid alkaline procedure of Birnboim and Doly (1) was used to screen for plasmid DNA in transposition and recombinant DNA experiments.

Restriction endonuclease cleavage analysis. Restriction endonucleases were used under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of cleaved DNA was performed in a horizontal 0.5 or 0.6% agarose gel, using a Tris-borate buffer system (9). For mapping experiments, restriction fragment bands were cut out of a 0.5% gel and placed into separate large dialysis bags. Electroelution of the DNA from the agarose into the dialysis bag was carried out using a Tris-borate buffer (1/10 the concentration specified above) for 3 h at 200 V (20 mA) (18). After reversing the current for 10 s, the dialysis bag contents were placed into Nalgene centrifuge tubes, and agarose was sedimented by centrifugation at 17,000 × *g* at 4°C for 10 min. The DNA-containing supernatant was made 0.3 M in sodium acetate, and the DNA was precipitated by adding 2.5 volumes of absolute ethanol at −20°C. The DNA was then sedimented by centrifugation for 1 h at 29,000 × *g* at −10°C. The precipitated DNA was washed with 70% ethanol (−20°C), and the dried pellet was suspended in the desired volume of 6 mM Tris-hydrochloride, pH 7.5. DNA restriction fragments isolated in this manner were used in ligation experiments or for further treatments with other restriction endonucleases.

Molecular cloning into the plasmid vector pBR325. Partial and total restriction endonuclease-cleaved pJM1 plasmid DNA was cloned into the cloning vector pBR325 (18). Phosphatase-treated vector was used in all the recombinant DNA experiments performed. Calf alkaline phosphatase was used according to the directions of the supplier (New England Nuclear Corp., Boston, Mass.). Ligation experiments were carried out at 15°C for 24 h at a ratio of 2 pmol of pBR325 phosphatase-treated ends to 0.5 pmol of *Bam*HI-cleaved pJM1 plasmid DNA and 0.05 U of T4 DNA ligase (Bethesda Research Laboratories). Transformation of *E. coli* RR1 with the ligation mixture was carried out as described (8).

Analysis of membrane proteins. Total cell envelopes as well as outer membranes from *V. anguillarum* were prepared essentially as described previously (6). Bacterial cultures (10 ml) were grown overnight at 20°C in M-9 minimal medium containing either 20 μM $FeCl_3$ (iron sufficiency conditions) or 10 μM EDDA for iron-proficient derivatives or 2 μM EDDA for iron uptake-deficient mutants (iron limitation conditions). Cells were harvested by centrifugation and suspended in 1.5 ml of 10 mM Tris-hydrochloride–0.3% NaCl (pH 8.0) and sonically treated. After a 60-s centrifugation in a microcentrifuge (Eppendorf), the supernatant fluid, which contained the cell envelopes, was centrifuged for 1 h at 37,000 × *g* in a Beckman J2-21 centrifuge. To prepare outer membranes, the cell envelopes were treated with 1.5% (wt/vol) Sarkosyl (final concentration 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 37,000 × *g* for 1 h, and the preparation was washed twice with distilled water and stored at −20°C until ready to use. Pellets from total cell envelopes or outer membranes were suspended in 50 μl of a buffer consisting of 62.5 mM Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 0.001% bromphenol blue, and 5% β-mercaptoethanol. The 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-

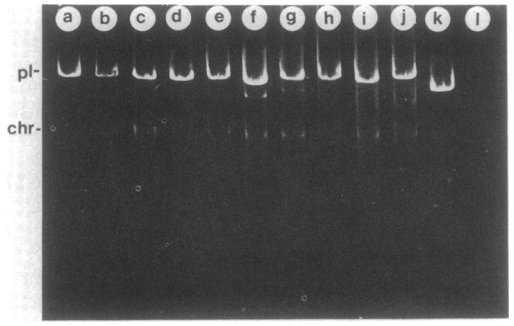

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from *V. anguillarum*. Plasmid DNA was prepared by an alkaline lysis procedure (1) and analyzed as described in the text. Lanes a through k, plasmid DNA: (a) pJHC-W3; (b) pJHC-W4; (c) pJHC-W5; (d) pJHC-W10; (e) pJHC-W11; (f) pJHC-W6, plate transferred; (g) pJHC-W7, plate transferred; (h) pJHC-W7; (i) pJM1; (j) pJHC-91; and (k) pJHC9-8. Lane l, plasmidless strain H775-3. pl, Plasmid DNA; chr, chromosomal DNA.

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.

Experimental infections. Virulence tests were carried out on juvenile coho salmon (*Oncorhynchus kisutch*), each weighing about 5 g. For each bacterial dilution tested, five fish were anesthetized with tricaine methane sulfonate (0.1 g/liter) and inoculated subcutaneously at the posterior base of the dorsal fin with 0.1 ml of various dilutions of bacterial suspensions from a 24-h culture grown with shaking in M9 minimal medium plus glucose and Casamino Acids (Difco Laboratories, Detroit, Mich.) at 20°C. The range of cell concentrations tested was from 10^2 to 10^8 cells per ml. After bacterial challenge, test fish were maintained in freshwater at 12 ± 1°C for at least 7 days. Mortalities were collected daily, and the kidney material was examined by bacteriological culture techniques. Mortalities were considered to be due to *V. anguillarum* only when the bacterium was reisolated in pure culture. Virulence was expressed as the 50% lethal dose (LD_{50}) as determined by the method of Reed and Muench (21).

RESULTS

Transposition mutagenesis of the pJM1 plasmid. RP4 (12, 14), a P1 incompatibility group plasmid which possesses the transposition sequence Tn*1* and carries ampicillin resistance determinants (17), was used to initiate a mutagenic analysis of the pJM1 plasmid iron uptake region. We were able to obtain two classes of exconjugants: Ap^r Km^s and Ap^r Km^r. The phenotype Ap^r Km^s indicated that these exconjugants must have lost RP4 while retaining ampicillin resistance, suggesting that they must have been generated by Tn*1* transposition to either the pJM1 plasmid or the *V. anguillarum* chromosome. The Ap^r Km^r exconjugants must still contain RP4, although they could also have suffered a transposition event. Consequently, all of the exconjugants, regardless of their phenotype, were analyzed for their ability to grow under conditions of iron limitation by using minimal medium containing 10 μM EDDA. A series of clones was identified in which the molecular weight of the pJM1 plasmid (Fig. 1, lane i) had been increased by about 4.9 kilobase pairs (kb), due to insertion of Tn*1* into the plasmid genome (Fig. 1, lanes a through e, h, and j, and Table 1). In one of the strains, 775::Tn*1*-6(pJHC9-8), the plasmid actually has a deletion of approximately 19 kb, possibly in-

TABLE 1. Tn*1* insertion derivatives of *V. anguillarum*

Strain	Plasmid	Molecular size (kb)	Growth in 10 μM EDDA[a]	Able to[b]:		Virulence (LD_{50})[c]
				Cross-feed	Be cross-fed	
775	pJM1	65	+	+	NA	1.2×10^3
H775-3	Cured		−	−	−	2.1×10^7
775::Tn*1*-5	pJHC-91	70	−	−	+	7.0×10^8
775::Tn*1*-6	pJHC9-8	51	−	−	−	5.0×10^8
775::Tn*1*-7	pJHC-W7	70	−	−	+	9.0×10^8
775::Tn*1*-3	pJHC-W3	70	+	+	NA	2.2×10^3
775::Tn*1*-10	pJHC-W10	70	+	+	NA	1.0×10^3
775::Tn*1*-4	pJHC-W4	70	+	+	NA	1.5×10^3
775::Tn*1*-55	pJHC-W5	70	+	+	NA	0.9×10^3
775::Tn*1*-11	pJHC-W11	70	+	+	NA	1.0×10^3
H775-3	RP4	54.5	−	NT	NT	2.1×10^8

[a] Growth in minimal medium containing 10 μM EDDA was determined by measuring the optical density at 590 nm at 21, 48, and 72 h.

[b] Cross-feeding experiments were carried out as described in the text. NA, Not applicable; NT, not tested.

[c] Virulence was determined as LD_{50} (number of bacteria killing 50% of the fish inoculated) by using the method of Reed and Muench (21).

duced by a Tn*1* integration and deletion process (Fig. 1, lane k, and Table 1). In other transposed derivatives, insertions appeared to be quite unstable and kept generating deleted plasmids after about 50 successive plate transfers (Fig. 1, lane f and g). Most of the strains containing the pJM1 insertion derivatives were iron proficient, indicating that insertion of Tn*1* took place in a plasmid region nonessential for the iron uptake process. However, the deletion mutant 775::Tn*1*-6(pJHC9-8) and insertion derivatives 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7) showed an impairment in their ability to grow under conditions of iron limitation (Table 1). Although not seen in Fig. 1 (due to its low copy number), strains containing the pJM1::Tn*1* insertion derivatives in lanes a through d and h also carried plasmid RP4 as determined by both the antibiotic resistance phenotypes and the restriction endonuclease cleavage patterns in Fig. 3 (cleaved RP4 DNA can be more readily detected due to the increased fluorescence of the intercalated ethidium bromide, which is higher than in covalently closed circular DNA). The presence of RP4 did not affect either the iron uptake properties or the virulence of *V. anguillarum* (Table 1).

Nature of the iron uptake deficiency mutations. To investigate whether the iron uptake-deficient mutations could be assigned to a deficiency in siderophore activity or to a lesion resulting in either the absence of an active membrane receptor or a modification that renders it unable to recognize the iron-siderophore complex, we performed cross-feeding experiments. Table 1 shows that under conditions of iron limitation, the iron uptake-deficient mutants 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7) were able to be cross-fed by supernatant fluids from strains carrying an intact pJM1 plasmid or Tn*1* derivatives that were still iron proficient. Growth did not occur when the disks in the bioassay contained supernatant fluids from iron uptake-deficient mutants or from a plasmidless strain of *V. anguillarum*. This cross-feeding effect was specific for the aforementioned mutants, since wild-type plasmid-carrying strains could not cross-feed deletion mutant 775::Tn*1*-6(pJHC9-8) or the plasmidless strain H775-3 (Table 1). The ability to utilize the supernatant fluids from the growth of iron-limited cultures of wild-type plasmid-carrying strains suggests the presence of a siderophore-like substance in these supernatant fluids, which is absent in supernatant fluids from either the plasmidless strain, the deletion mutant 775::Tn*1*-6(pJHC9-8), or from two insertion derivatives, 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7). The ability to utilize a diffusible siderophore produced only by wild-type plasmid-carrying strains indicates that the plasmid-mediated outer membrane receptors in the iron uptake-deficient mutants 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7) are still functional. Supernatant fluids from the deletion mutant 775::Tn*1*-6(pJHC9-8) could not cross-feed the insertion mutants 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7), suggesting that the deletion process that generated the plasmid pJHC9-8 also affected the plasmid genes involved in siderophore biosynthesis or activity. This deletion mutant cannot be cross-fed by wild-type plasmid-carrying strains, indicating that the 19-kb deletion affected not only the putative siderophore genes or a regulatory region involved with siderophore activity but also the pJM1 plasmid genes for the cognate membrane receptor or a regulatory region involved with receptor activity.

Virulence and plasmid-mediated iron transport. The Tn*1*-generated iron-deficient mutants were no longer virulent. The LD_{50} of these mutants increased by approximately 10^4 (Table 1).

Analysis of membrane proteins. Analysis of the membrane proteins produced under iron limitation at 10 μM EDDA showed that the OM2 protein was induced in all the iron uptake-proficient insertion derivatives (Fig. 2) but was not detected in those cultures grown in high iron concentration. Since the iron uptake-deficient transposition derivatives could not grow at EDDA concentrations higher than 2 μM, we used this concentration rather than 10 μM to investigate the induction of OM2 in these mutants. Figure 2, panels a and b, lanes C_2 and D_2, show that the OM2 protein was induced in the iron uptake-deficient insertion mutants 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7), respectively, but was absent from the deletion mutant 775::Tn*1*-6(pJHC9-8) shown in lane B_2. In this case a novel protein, ΔOM2, of about 69,000 daltons was induced under condition of iron limitation. In all the iron uptake-proficient and iron uptake-deficient derivatives the chromosomally mediated OM3 protein was induced.

Construction of a physical map of the pJM1 plasmid. pJM1 DNA was cleaved by the *Bam*HI restriction endonuclease into eight fragments of 19.9, 14.4, 10.9, 6.5, 4.9, 3.2, 2.7, and 2.1 kb (Fig. 3, lane d), whereas *Sal*I cleaved pJM1 DNA into four fragments of 29.7, 20.3, 13.2, and 1.4 kb (data not shown). In Fig. 3 we also show the *Bam*HI cleavage of the plasmid DNAs from the deletion derivative (lane c), and seven pJM1::Tn*1* derivatives (lanes b, e, f, g, h, i, and j). Transposition sequence Tn*1* possesses one site susceptible to *Bam*HI at a position located at 1.4 kb from one of its ends, close to the gene for beta-lactamase. Tn*1* is not cleaved by *Sal*I.

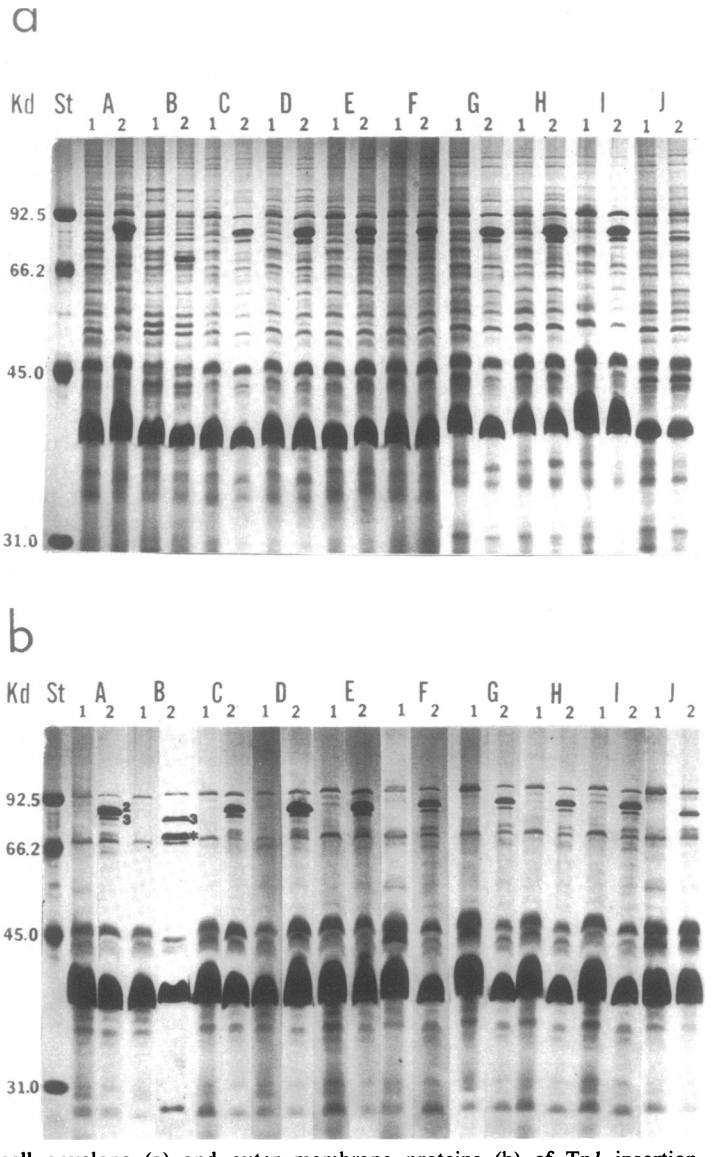

FIG. 2. Total cell envelope (a) and outer membrane proteins (b) of Tn*1* insertion derivatives of *V. anguillarum*. Kd, Kilodaltons. Lane St, molecular weight standards; A, strain 775(pJM1); B, 775::Tn*1*-6(pJHC9-8); C, 775::Tn*1*-5(pJHC-91); D, 775::Tn*1*-7(pJHC-W7); E, 775::Tn*1*-3(pJHC-W3); F, 775::Tn*1*-10(pJHC-W10); G, 775::Tn*1*-55(pJHC-W5); H, 775::Tn*1*-4(pJHC-W4); I, 775::Tn*1*-11(pJHC-W11); J, plasmidless H775-3. Subscript 1 corresponds to membranes obtained from cells grown under iron sufficiency (20 μM $FeCl_3$), whereas subscript 2 corresponds to membranes from cells grown under iron limitation conditions (10 μM EDDA for iron uptake-proficient derivatives and 2 μM EDDA for iron uptake-deficient derivatives). The following symbols are used on panel b to identify specific outer membrane proteins: lane A_2, 2, OM2 protein; lane A_2, 3, OM3 protein; lane B_2, 3, OM3 protein; and lane B_2, *, ΔOM2 protein. The positions of the OM2 and OM3 proteins on the other lanes (C_2 through I_2) are identical to those shown in lane A_2. In lane J_2 (plasmidless strain), the only outer membrane protein induced under conditions of iron limitation is the chromosomally mediated OM3.

Hence, digestions with these two enzymes permitted us to locate the sites of Tn*1* insertions on the plasmid DNA obtained from the iron uptake-proficient and -deficient transposition derivatives. By performing double digestions with both *Bam*HI and *Sal*I on pJM1 DNA, we were able to obtain a preliminary order for these restriction fragments. This order was confirmed by molecularly cloning partial digests obtained from cleavage of pJM1 with *Bam*HI into the pBR325 vec-

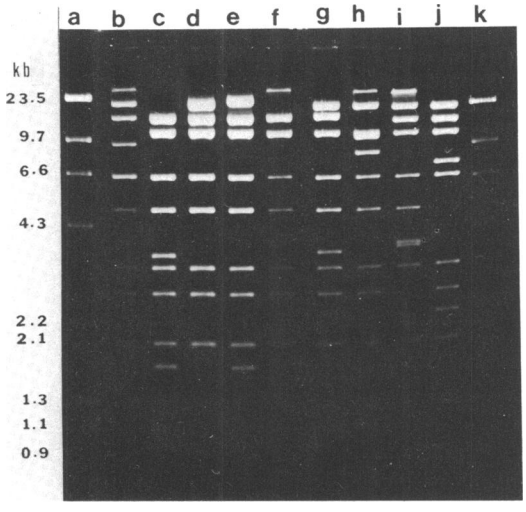

FIG. 3. Restriction endonuclease cleavage analysis of Tn*1* insertion derivatives of the pJM1 plasmid. Restriction endonuclease reactions were carried out as described in the text. Lanes a and k, molecular weight markers (*Hin*dIII-cleaved lambda DNA plus *Hae*III-cleaved ϕX174 DNA). Lanes b through j are *Bam*HI-cleaved DNA from the plasmids: b, pJHC-W3; c, pJHC9-8; d, pJM1; e, pJHC-91; f, pJHC-W7; g, pJHC-W10; h, pJHC-W4; i, pJHC-W5; j, pJHC-W11. The top band in lanes b, f, g (light), h, and i is RP4 that has been cleaved at its only *Bam*HI site.

tor. By using a combination of these two methods with all the transposition derivatives, we were able to obtain a physical map of the pJM1 plasmid with the sites of Tn*1* insertions (Fig. 4). Further studies with other restriction endonucleases (*Xho*I and *Kpn*I) confirmed the assigned locations and orientations of the Tn*1* insertions (data not shown). In Fig. 4, the location of the deletion which generated pJHC9-8 is also shown. This plasmid is harbored by a strain that is iron uptake deficient and was categorized in the bioassay to be both siderophore and membrane receptor activity negative. Analysis of this deletion plasmid as well as of those plasmids containing Tn*1* insertions which generated iron uptake-deficient mutants which did not produce a functional siderophore but that have an operating membrane receptor, allowed us to localize the iron uptake region of pJM1 within a 20-kb region of the plasmid, which includes possibly all of *Bam*HI fragment 1 and parts of *Bam*HI fragments 5 and 6.

DISCUSSION

In recent years the virulence of invasive strains of *E. coli* causing bacteremias in humans and animals and *V. anguillarum* causing septicemic disease in fish has been attributed to the presence of efficient plasmid-mediated iron transport systems (4, 5, 24, 25). In this paper we describe the molecular characterization of the pJM1 system by using the mutagenic capability of the transposition sequence Tn*1*. The R plasmid RP4 was conjugated to *V. anguillarum* and used as the donor of Tn*1*, generating a series of iron uptake-proficient and -deficient derivatives. By using a physiological bioassay we were able to classify the iron uptake-deficient mutants into two classes. Some mutants were able to be cross-fed by supernatant fluids from wild-type plasmid-carrying strains of *V. anguillarum* and thus lacked an active putative plasmid-mediated siderophore. Other mutants could not be cross-fed by supernatant fluids from wild-type plasmid-carrying strains and could not cross-feed members of the other class of iron uptake-deficient mutants. Mutants in this second class must have not only a lesion in genes involved in siderophore production or activity but also in DNA regions associated with the activity or biosynthesis of a membrane receptor. Plasmid DNA from mutants belonging to these two classes was analyzed by restriction endonuclease cleavage analysis, and the sites of Tn*1* insertions or deletions were located. The mutants behaving as siderophore negative receptor positive were identified as possessing insertions in the *Bam*HI fragment 1, which is 19.9 kb. A deletion affecting portions of this fragment resulted in a siderophore-negative receptor-negative phenotype. Analysis of the outer membrane proteins of the iron uptake-deficient mutants 775::Tn*1*-5(pJHC-91) and 775::Tn*1*-7(pJHC-W7), which were able to be cross-fed, indicated that these Tn*1* insertions on the pJM1 plasmid which affected production of siderophore did not affect the receptor activity or the biosynthesis of the OM2 protein. The insertion-deletion process that generated pJHC9-8 from pJM1 affected both the functional siderophore and receptor activity. In this case, concomitant with the loss of receptor activity there was a disappearance of the outer membrane protein OM2. Under conditions of iron limitation, a novel protein of about 69,000 daltons was induced in this deletion derivative. We hypothesize that this novel protein, which we called ΔOM2, is a truncated OM2 protein, possibly generated by Tn*1*-mediated deletion of a portion of *Bam*HI fragment 1 carrying the carboxy terminal end of the gene for the OM2 protein. If so, OM2 would be the presumed plasmid-mediated outer membrane receptor for the iron uptake system encoded by pJM1. Of course, this contention depends upon the demonstration that ΔOM2 is actually derived from OM2 and that the OM2 protein can specifically bind complexes of *V. anguillarum* siderophore with iron. Radioimmunological experiments to prove the relatedness between OM2 and ΔOM2

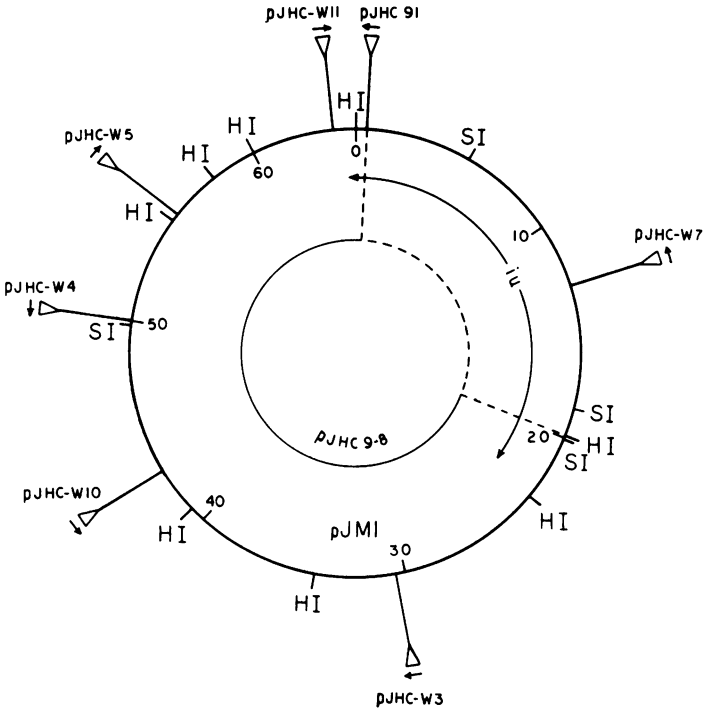

FIG. 4. Genetic and physical map of the pJM1 genome. The ordering of restriction endonuclease fragments was obtained by a combination of double digestions and molecular cloning of partial digests of the pJM1 plasmid. The iron uptake region is indicated by a line ending in two arrows and marked iu. The ends of this region have not been determined but may possibly be somewhere in *Bam*HI fragments 5 and 6. The symbol ▽ indicates the sites of Tn*1* insertions, and the arrow over this symbol indicates the orientation of the Tn*1* sequence with respect to beta-lactamase gene transcription. Each different Tn*1* insertion is identified by the initials corresponding to the plasmid generated by the insertion, for example, pJHC-W3. The deletion plasmid pJHC9-8 is also shown as a smaller circle within the pJM1 map. The dotted lines on pJHC9-8 indicate the extent of the deletion of pJM1 material. The restriction endonuclease cleavage sites are given as: BI, *Bam*HI; SI, *Sal*I. The numbers inside the map are the coordinates in kb.

are in progress. To date we have been unable to generate by Tn*1* insertion mutagenesis derivatives of a siderophore-positive receptor-negative phenotype, although both siderophore-negative receptor-positive and siderophore-negative receptor-negative phenotypes were obtained. Whether this is due to a regulatory problem, for instance the existence of only one promoter for the genes for siderophore and receptor, awaits further analysis. Nonetheless, we have found that there is an intimate relationship between plasmid-mediated iron transport and bacterial virulence, since any mutation leading to an iron uptake-deficient phenotype was also reflected in an attenuation of virulence.

The construction of a physical map of pJM1 DNA, on the basis of specific cleavage of pJM1 DNA by *Bam*HI and *Sal*I restriction endonucleases and by molecular cloning techniques, allowed us to determine the location of the iron transport region on the pJM1 plasmid to about 20 kb of pJM1 DNA (Fig. 4), which includes possibly the entire *Bam*HI fragment 1 and parts of *Bam*HI fragments 5 and 6. Further dissection and molecular cloning of restriction fragments within this region is currently being carried out.

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LITERATURE CITED

1. **Birnboim, H., and J. Doly.** 1979. A rapid extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1525.
2. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infections. Curr. Top. Microbiol. Immunol. **80**:1–35.
3. **Cisar, J. O., and J. L. Fryer.** 1969. An epizootic of vibriosis in chinook salmon. Bull. Wildl. Dis. Assoc. **5**:73–76.
4. **Crosa, J. H.** 1979. *Vibrio anguillarum*: an iron uptake mechanism as a factor of virulence. Fish Health News **8**:7–9.

5. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **283**:566–568.
6. **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.
7. **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.
8. **Crosa, J. H., L. K. Luttropp, and S. Falkow.** 1978. Molecular cloning of replication and incompatibility regions from the R-plasmid R6K. J. Mol. Biol. **124**:443–460.
9. **Crosa, J. H., M. H. Schiewe, and S. Falkow.** 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum*. J. Bacteriol. **18**:509–513.
10. **Crosa, J. H., M. A. Walter, and S. A. Potter.** 1983. Iron uptake-deficient mutants of *Vibrio anguillarum* 775 generated by insertional inactivation of the virulence plasmid pJM1, p. 354–358. *In* D. Schlessinger (ed.), Microbiology—1983. American Society for Microbiology, Washington, D.C.
11. **Crosa, J. H., M. A. Walter, and S. A. Potter.** 1983. The genetics of plasmid-mediated virulence in the marine fish pathogen *Vibrio anguillarum*, p. 21–30. *In* J. H. Crosa (ed.), Bacterial and viral diseases of fish: molecular studies. Washington Sea Grant, Seattle.
12. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1244–1249.
13. **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.
14. **Grinsted, J., J. R. Saunders, L. C. Ingram, R. B. Sykes, and M. H. Richmond.** 1972. Properties of an R factor which originated in *Pseudomonas aeuroginosa* 1822. J. Bacteriol. **110**:529–537.
15. **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.
16. **Harbell, S. O., H. O. Hodgins, and M. H. Schiewe.** 1979. Studies on the pathology of vibriosis in coho salmon (*Oncorhynchus kisutch*). J. Fish Dis. **2**:527–535.
17. **Hedges, R. W., and A. E. Jacob.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132**:31–40.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Large-scale isolation of plasmid DNA, p. 90–91. *In* Molecular cloning—a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
20. **Payne, S. M., and R. A. Finkelstein.** 1978. The critical role of iron in host-bacterial interactions. J. Clin. Invest. **61**:1428–1440.
21. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
22. **Rucker, R. R., B. J. Earp, and E. J. Ordal.** 1953. Infectious diseases of Pacific salmon. Trans. Am. Fish. Soc. **83**:297–312.
23. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
24. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. Infect. Immun. **26**:925–932.
25. **Williams, P. H., and P. J. Warner.** 1980. ColV plasmid-mediated, colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. Infect. Immun. **29**:411–416.