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Characterization of the *Vibrio anguillarum fur* Gene: Role in Regulation of Expression of the FatA Outer Membrane Protein and Catechols

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The chromosomally encoded *Vibrio anguillarum fur* gene was characterized. The amino acid sequence of the Fur protein showed a very high degree of homology with those of *V. cholerae* and *V. vulnificus*. The degree of homology was lower, although still high, with the *Escherichia coli* and *Yersinia pestis* Fur amino acid sequences, while the lowest degree of homology was found with the *Pseudomonas aeruginosa* Fur protein. The C-terminal portion of Fur is the least conserved region among these Fur proteins. Within this portion, two regions spanning amino acids 105 to 121 and 132 to the end are the least conserved. A certain degree of variation is also present in the N termini spanning amino acids 28 to 46. Regulation of expression of the *V. anguillarum fur* gene by iron was not detected by immunoblot analysis. Mutations in the cloned *fur* gene were generated either by site-directed mutagenesis (the Lys-77 was changed to a Gly to generate the derivative FurG77) or by insertion of a DNA fragment harboring the *aph* gene in the same position. FurG77 was impaired in its ability to regulate a reporter gene with the Fur box in its promoter, while the insertion mutant was completely inactive. *V. anguillarum fur* mutants were obtained by isolating manganese-resistant derivatives. In one of these mutants, which encoded a Fur protein with an apparent lower molecular weight, the regulation of the production of catechols and synthesis of the outer membrane protein FatA were partially lost. In the case of another mutant, no protein was detected by anti-Fur serum. This derivative showed a total lack of regulation of biosynthesis of catechols and FatA protein by iron.

The ability of a bacterial pathogen to scavenge iron from its host's fluids is an important virulence factor (34, 60). Most bacteria possess efficient iron uptake systems that are expressed when the bacterial cell enters a mammalian host, which is a low-iron environment, allowing it to capture iron from the high-affinity host's iron binding proteins. In *Escherichia coli*, the iron regulation of the iron uptake gene expression depends on a single regulatory gene, *fur* (ferric uptake regulator), which acts as a classical repressor, blocking transcription in the presence of high concentrations of iron (5, 28, 44). The C-terminal portion of the *E. coli* Fur protein binds Fe^{2+}, inducing a conformational change in the N-terminal region of the protein which allows binding to the operator of the Fur-regulated genes (13). This operator consists of a 21-bp dyad symmetric consensus sequence (10, 11, 20–22). Fur was subsequently found in other bacteria: *Salmonella typhimurium* (25), *Yersinia pestis* (45), *Vibrio cholerae* (33), *V. vulnificus* (35), and *Pseudomonas aeruginosa* (37). It was demonstrated that Fur also controls the expression of toxins and other virulence factors apparently unrelated to iron metabolism, e.g., hemolysin in *V. cholerae* (46), Shiga-like toxin of *E. coli* (10), and pH-regulated proteins in *S. typhimurium* (25).

V. anguillarum is the causative agent of the fish disease vibriosis (40). Some virulent strains carry a virulence plasmid, such as pJM1 in strain 775 (14–16, 53), which encodes a very efficient iron uptake system composed of anguibactin, a siderophore that has hydroxamate and catechol moieties in its molecule (1, 30), and membrane components that play a role in internalization of iron(III)-anguibactin complexes inside the cell (3, 32). This is one of the first systems in which a perfect correlation of virulence and iron uptake was demonstrated (14, 19, 52, 61). The expression of many genes of this system was shown to be regulated by the concentration of iron in the medium (2, 17, 49). Studies of the regulation of expression of genes encoding different components of this iron-uptake system demonstrated that, in the *V. anguillarum* pJM1-encoded system, plasmid-encoded factors such as the AngR protein, the *trans*-acting factor(s) (TAF), and an antisense RNA, RNAα, play roles in regulation (41, 42, 49, 52, 55). However, we recently demonstrated the presence of a chromosomally encoded Fur-like activity in *V. anguillarum* (58) and identified the presence of a putative Fur binding site within the promoter region of the gene encoding the bifunctional protein AngR (24), a protein that plays a role as a regulator and can also complement an *entE* mutant of *E. coli* (50). These findings suggested a role for Fur in the pJM1-mediated iron uptake system. Hence, we conducted this study of the *V. anguillarum fur* gene and present in this report its characterization and nucleotide sequence, as well as evidence of Fur regulation of expression of the outer membrane protein FatA and synthesis of catechols, which are presumed to be intermediates in the biosynthesis of anguibactin. In addition, the isolation and characterization of *V. anguillarum fur* mutants by the manganese resistance selection method (28) are described.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The genotypes and sources of strains and plasmids used in this study are shown in Table 1. *E. coli* HB101 or JM107 was used as the bacterial host.

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
HB101	F⁻ *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY1 tonA21 l* mutant *supE44*	8
JM107	*thiD(lac-proAB) gyrA96 endA1 hsdR17 relA1 supE44* F'(*traD36 proAB lacI*^{q} *lacZDM15*)	62
BL21(DE3)(pLysE)	*entE* derivative of AB1515	47
BN4020	*fur*::Tn*5*	6
RRJC1	Derivative of *E. coli* BN4020 (*fur* mutant) with a *lacZ* reporter under the control of the *fhuF* Fur box	39
V. anguillarum		
775	Natural isolate, prototype (pJM1)	14
H775-3	Plasmidless derivative of strain 775	18
775MET9	*fur* mutant isolated in the presence of 10 mM $MnCl_2$	This work
775MET11	*fur* mutant isolated in the presence of 10 mM $MnCl_2$	This work
Plasmids		
pBCSK⁺	Cloning vector	Stratagene
pT7-5	Expression vector	48
pUC4K	Contains the Tn*903 aph* in a restriction site mobilizing element	57
pSC27.1	*fur* reporter gene. β-galactosidase is under the control of Fur	11
pMH15	*E. coli fur* gene cloned in pACYC184	27
pTAW1.8	*fur V. anguillarum* gene cloned in pBCSK⁺	This work
pTAW3.12	Site-directed mutagenized *fur* mutant derivative of pTAW1.8. Nucleotides 229 and 230 were changed from AA to GG	This work
pTAW4.1	Km^r fragment from pUC4K inserted into the *Bam*HI site in pTAW3.12	This work
pTAW2.1	*V. anguillarum fur* gene cloned in pT7-5	This work
pMET31	Insertionally mutated *fur* gene from pTAW4.1 cloned in pT7-5	This work
pMET67	Recombinant clone carrying the *V. anguillarum fur* gene	58

Plasmid pBCSK⁺ (Stratagene, La Jolla, Calif.) was used as vector for DNA sequencing and site-directed mutagenesis. Plasmid pT7-5 was used for overexpression of Fur with *E. coli* BL21(DE3)(pLysE) as the host. The uses of the other plasmids and strains are described below. *V. anguillarum* was grown in either Trypticase soy broth or agar supplemented with 1% NaCl or M9 minimal medium containing either 50 μM $FeCl_3$ (iron rich) or 2.5 μM ethylenediaminedi-(*o*-hydroyphenyl) acetic acid (EDDHA [iron limiting]).

Chemical and enzymatic determinations. Levels of 2,3-dihydroxybenzoic acid in culture supernatants were determined with the Arnow phenolic assay (4). β-Galactosidase levels were measured by the method described by Putnam and Koch (38). Units were determined as described by Miller (36). All experiments to determine 2,3-dihydroxybenzoic acid and β-galactosidase levels were performed at least four times. The ratios between the values among the different strains were constant. Protein concentrations were determined as described by Bradford (9).

General DNA procedures. Plasmid DNA was purified according to the method of Birnboim and Doly (7) with the modifications described by Weickert and Chambliss (59). Transformations were performed by the method of Cohen et al. (12). Sequencing of double-stranded DNA was performed by the dideoxy chain termination method (43) with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio), with T7 and T3, and, in some cases, with specific synthetic primers. Site-directed mutagenesis of pTAW1.8 was carried out with the Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) and the synthetic mutagenic oligonucleotide AAGGTGGAGGATCCGTTT, following the recommendations of the manufacturer with modifications previously described (26), to generate plasmid pMETAW3.12. The mutation was confirmed by the fast DNA sequencing method (51) with the appropriate primers. Basically, the method consisted of electrophoresis of the sequencing reaction mixtures obtained as described above (43) in a small gel 18 cm long run at 1,000 V for 20 min. Plasmid pMETAW4.1 was generated by insertion mutagenesis performed by ligating the kanamycin resistance (Km^r) fragment from pUC4K into the *Bam*HI site generated after site-directed mutagenesis. Hybridization experiments were carried out at 37°C under low-stringency conditions (0.75 M sodium chloride, 0.075 M sodium citrate, Denhardt's solution, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 25% formamide). After hybridization, the filters were washed at 50°C in a solution containing 0.3 M sodium chloride, 0.03 M sodium citrate, and 0.1% SDS.

Immunoblot analysis. Antiserum against Fur was raised in 6-month-old rabbits as previously described for FatA (2) by using purified *E. coli* Fur protein (a gift from J. Neilands, University of California, Berkeley). Antiserum against FatA was prepared as described before (2). Proteins from cytosol, total cell extracts, or outer membrane preparations were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Crosa and Hodges (17), with prestained high-range protein molecular weight standards (Bio-Rad). After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper (0.2-μm pore size, BAS 83, reinforced NC [Schleicher & Schuell]) essentially as described by Towbin et al. (56) with the Genie electrophoretic blotter (Idea Scientific Co., Minneapolis, Minn.) under the conditions recommended by the supplier. The blots were incubated in the presence of the anti-Fur or anti-FatA serum and developed by reaction with peroxidase and staining with H_2O_2 and horseradish peroxidase color development reagent (2).

Isolation of Fur mutants by manganese selection. Isolation of Fur mutants in the presence of manganese was carried out

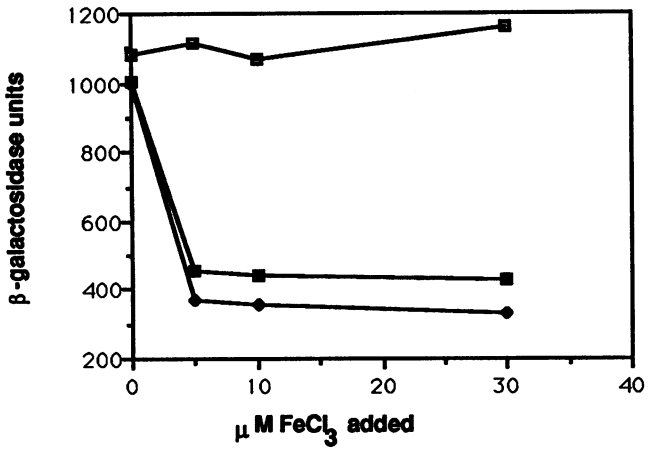

FIG. 1. β-Galactosidase activities of *E. coli* derivatives. β-Galactosidase activities, expressed as Miller units (36), were determined for *E. coli* BN4020 harboring pSC27.1 (□), pSC27.1 and pMH15 (◆), or pSC27.1 and pMET67 (■) grown in minimal medium with the addition of 1 μM EDDHA and the $FeCl_3$ concentrations indicated.

essentially as described by Hantke (28) for *E. coli*, *Serratia* spp., and *Klebsiella* spp. with the modifications of Prince et al. (37) for *P. aeruginosa*. *V. anguillarum* 775 was cultured in Trypticase soy broth supplemented with 1% NaCl at 24°C overnight. Aliquots (100 μl) of this culture were spread onto Trypticase soy agar plates supplemented with 1% NaCl and 10 mM $MnCl_2$. After 5 days of incubation at 24°C, the Fur proteins in growing colonies (about five per plate) were analyzed by immunoblotting.

Overexpression of Fur in *E. coli* BL21(DE3)(pLysE). Both intact and interrupted *fur* genes from recombinant clones pTAW1.8 and pTAW4.1, respectively, were cloned under the control of the φ10 promoter with the vector pT7-5 (48). Ligation of *Xba*I- and *Eco*RI-digested pT7-5 with the *Xba*I-*Eco*RI fragment containing the *fur* gene in pTAW1.8 generated pTAW2.1, and ligation with the *Xba*I-*Eco*RI fragment containing the truncated *fur* gene in pTAW4.1 generated pMET31. Recombinant clones pTAW2.1 and pMET31 were transformed into *E. coli* BL21(DE3)(pLysE). Cells were grown in Luria broth containing ampicillin at 37°C until the optical density at 600 nm reached 0.7. Protein expression was then induced by addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were incubated for an additional 3 h and collected by centrifugation. After washing with 1 volume of 10 mM magnesium sulfate, the cells were lysed with SDS-PAGE sample buffer at 100°C for 10 min and analyzed by SDS-PAGE.

Nucleotide sequence accession number. The nucleotide and predicted amino acid sequences of the *V. anguillarum fur* gene will appear in the EMBL and GenBank sequence libraries under accession no. L19717.

RESULTS

Cloning of the *V. anguillarum fur* gene. A recombinant clone was isolated from the plasmidless *V. anguillarum* H775-3 library (54) by screening pools of recombinant plasmid DNA by Southern blot hybridization under low-stringency conditions with a *Hin*dIII-*Bgl*I DNA fragment encompassing most of the *fur E. coli* gene (27) used as a probe. Recombinant plasmids in a hybridizing pool were then screened individually. Recombinant clone pMET67 was isolated in this manner, and its

```
          10                  30                  50
          .                   .                   .
1   ATGTCAGATAATAACCAAGCGCTCAAGGATGCAGGTCTTAAAGTTACCCTTCCTAGGCTA   60
1   M  S  D  N  N  Q  A  L  K  D  A  G  L  K  V  T  L  P  R  L    20

          70                  90                  110
          .                   .                   .
61  AAAATTTTAGAAGTGCTACAGCAGCCTGAATGCCAACATATCAGCGCTGAAGAACTGTAT  120
21  K  I  L  E  V  L  Q  Q  P  E  C  Q  H  I  S  A  E  E  L  Y    40

          130                 150                 170
          .                   .                   .
121 AAGAAATTGATTGATCTTGGTGAAGAAATCGGTCTTGCGACTGTTTATCGAGTATTAAAC  180
41  K  K  L  I  D  L  G  E  E  I  G  L  A  T  V  Y  R  V  L  N    60

          190                 210                 230
          .                   .                   .
181 CAATTTGATGATGCGGGTATTGTCACTCGTCACCATTTTGAAGGTGGAAAATCCGTTTTT  240
61  Q  F  D  D  A  G  I  V  T  R  H  H  F  E  G  G  K  S  V  F    80

          250                 270                 290
          .                   .                   .
241 GAACTTTCAACACAACACCACCACGACCACTTAGTGTGCTTAGATTGTGGTGAAGTGATT  300
81  E  L  S  T  Q  H  H  H  D  H  L  V  C  L  D  C  G  E  V  I   100

          310                 330                 350
          .                   .                   .
301 GAGTTTTCAGATGAGGTGATAGAACAACGCCAAAGAGAGATTGCCGAGCAATATAATGTA  360
101 E  F  S  D  E  V  I  E  Q  R  Q  R  E  I  A  E  Q  Y  N  V   120

          370                 390                 410
          .                   .                   .
361 CAGCTCACCAATCACAGCCTTTATCTATATGGTAAATGTGCCGACGGCAGTTGCAAGCAG  420
121 Q  L  T  N  H  S  L  Y  L  Y  G  K  C  A  D  G  S  C  K  Q   140

          430                 450
          .                   .
421 AACCCTAACGCGCACAAGTCAAAAAGATAG  450
141 N  P  N  A  H  K  S  K  R  *    149
```

FIG. 2. Nucleotide and predicted amino acid sequences of the *V. anguillarum fur* gene.

biological activity was tested by introduction of this plasmid and the Fur reporter plasmid pSC27.1 (11) into the *fur* mutant *E. coli* BN4020. The activity of β-galactosidase was inhibited in the presence of high concentrations of iron, indicating that pMET67 produced a Fur-like activity (Fig. 1). The Fur activity elicited by pMET67 was comparable to that of the *E. coli* Fur protein coded for by pMH15 (Fig. 1).

The *V. anguillarum fur* gene was subcloned by performing a *Sau*3AI partial restriction endonuclease treatment of pMET67 followed by ligation to *Bam*HI-digested pBCSK$^+$. Transformation of the Fur reporter *E. coli* RRJC1 produced a few white colonies (which represented about 1% of the total transformants) when plated on McConkey's agar supplemented with 100 μM ferric chloride. One of these white colonies was further analyzed and was shown to carry a recombinant plasmid, pTAW1.8, which had a 1.6-kbp insert.

Nucleotide sequence and expression of the *V. anguillarum fur* gene. The *V. anguillarum fur* gene included in pTAW1.8 was sequenced. The nucleotide sequence (Fig. 2) showed homology to the nucleotide sequences of *fur* genes from other bacterial species. Homologies to the *fur* coding sequences of *V. cholerae* (33), *V. vulnificus* (35), *Y. pestis* (45), *E. coli* (44), and *P. aeruginosa* (37) were 81.6, 80.4, 70.1, 70.0, and 60.9%, respectively. An open reading frame of 149 amino acids was present which had a high degree of homology with the amino acid sequences of the Fur proteins of the other bacteria (Fig. 2 and 3), especially *V. cholerae* and *V. vulnificus*. To identify the *V. anguillarum* Fur protein, the pTAW1.8 insert was recloned into vector pT7-5 to generate pTAW2.1, which was transformed into *E. coli* BL21(DE3)(pLysE). Upon induction with IPTG, a protein with a mass of about 20 kDa was detected, which is the approximate mass of the predicted *V. anguillarum* Fur protein (Fig. 4). The identity of this band was confirmed by analysis of the protein extract of *E. coli* BL21(DE3)(pLysE) harboring a derivative consisting of the insertionally mutated *fur* gene (see below) cloned in pT7-5 (plasmid pMET31). Figure 4 (lane F) shows that the 20-kDa band is indeed Fur, since pMET31 did not express this protein. Immunoblot

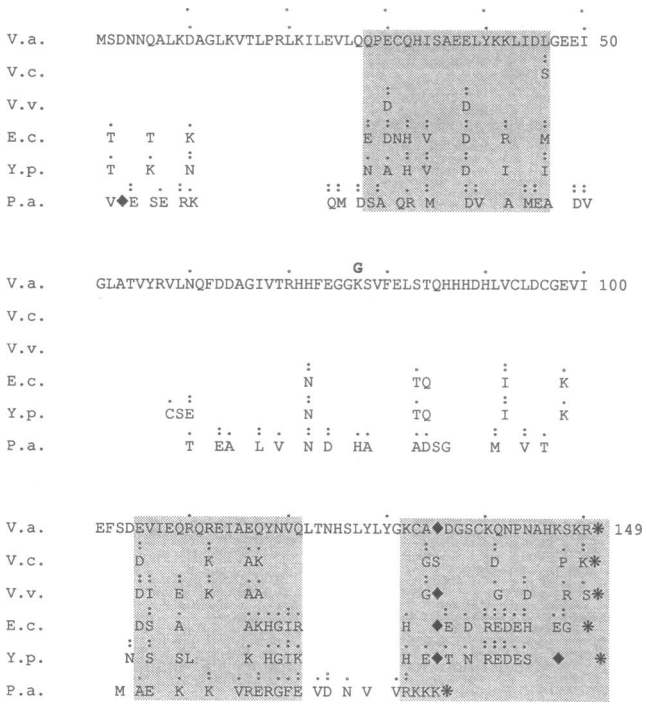

FIG. 3. Alignment of deduced amino acid sequences of Fur proteins from a variety of bacterial species. The *V. anguillarum* (V.a.) Fur protein amino acid sequence is compared with the Fur protein sequences from *V. cholerae* (V. c.), *V. vulnificus* (V. v.), *E. coli* (E. c.), *Y. pestis* (Y. p.), and *P. aeruginosa* (P. a.). Differences are shown by letters, and blank spaces represent identity with the *V. anguillarum* Fur sequence. Solid diamonds indicate spacing changes to maximize alignment. The percentages of identity and similarity, respectively, of the *V. anguillarum* Fur sequence with the other sequences are as follows: *V. cholerae*, 98 and 94; *V. vulnificus*, 97 and 92; *E. coli*, 88 and 76; *Y. pestis*, 84 and 76; and *P. aeruginosa*, 76 and 56. Dots show conserved amino acid substitutions; the presence of one or two dots indicates the degree of similarity.

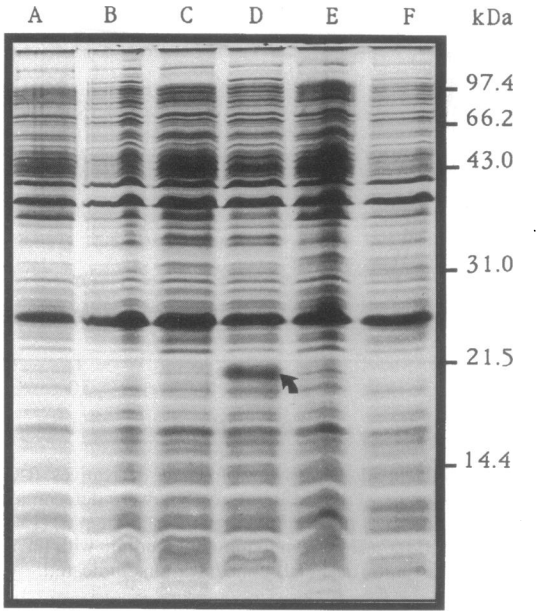

FIG. 4. Expression of Fur in *E. coli* BL21(DE3)(pLysE). The wild-type and mutated *fur* genes were subcloned in pT7-5 and transformed in *E. coli* BL21(DE3)(pLysE). Proteins were analyzed by SDS-PAGE and immunoblotting as described in Materials and Methods. Lanes show *E. coli* BL21(DE3)(pLysE) with pT7-5 (A and B), pTAW2.1 (C and D [wild-type *fur*]), or pMET31 (E and F [*fur* mutated by insertion]). Lanes A, C, and E were noninduced; lanes B, D, and F were induced with 0.4 mM IPTG. The arrow indicates the band corresponding to Fur.

analysis of a gel identical to that in Fig. 4 with anti-Fur serum further confirmed that the 20-kDa protein overexpressed by *E. coli* BL21(DE3)(pLysE, pTAW2.1) corresponds to Fur (data not shown).

Regulation of expression of the *V. anguillarum fur* gene. To determine whether the biosynthesis of the *V. anguillarum* Fur protein was iron regulated, cells were grown under iron-rich and iron-limiting conditions and the cytosolic extracts were subjected to immunoblotting. Immunoblot analysis (Fig. 5) demonstrates that the same amount of Fur protein was expressed in both conditions, indicating that its expression is constitutive in *V. anguillarum*. The production of 2,3-dihydroxybenzoic acid was used as a control for the expression of iron-regulated products. The optical densities at 510 nm obtained when the Arnow reaction was carried out, as described in Materials and Methods for the cultures under iron-rich and iron-limiting conditions, were 0.030 and 0.353, respectively, showing that 2,3-dihydroxybenzoic acid production is iron regulated.

Mutagenesis of the *V. anguillarum fur* gene. The *V. anguillarum fur* gene in pTAW1.8 was mutated by site-directed mutagenesis; nucleotides 229 and 230 were changed from AA to GG (Fig. 2) to generate plasmid pTAW3.12. As a result of this mutation, a *Bam*HI site was generated and the amino acid at position 77 was changed from Lys to Gly (this mutant derivative was called FurG77) (Fig. 2 and 3). Another mutant was generated by inserting the Km^r fragment from pUC4K into the newly generated *Bam*HI site to create plasmid pTAW4.1. The mutated recombinant clones were transferred to the *E. coli fur* mutant RRJC1 and plated on McConkey's agar plates supplemented with 100 μM $FeCl_3$. *E. coli* RRJC1 with no plasmid or with pTAW4.1 produced red colonies, indicating that β-galactosidase was being produced. Colonies of *E. coli* RRJC1(pTAW1.8) were white as a result of the inhibition of production of β-galactosidase in the presence of Fur and iron. *E. coli* RRJC1(pTAW3.12) generated pink colonies, indicating that the FurG77 encoded by this mutant is leaky. Next, β-galactosidase levels were determined for these strains under iron-rich and iron-limiting conditions (Table 2). The results indicate that the insertion mutant pTAW4.1 is a null mutant while pTAW3.12 encodes a protein, FurG77, which has some Fur activity (Table 2).

The plasmid pTAW4.1 was used to attempt to construct a *V. anguillarum fur* mutant by marker exchange. However, as was already described for *P. aeruginosa* (37), we were unable to isolate a *V. anguillarum fur* mutant by this technique. Therefore, we attempted to select *V. anguillarum fur* mutants by using the positive selection method first described by Hantke (28). *V. anguillarum* cells were spread onto plates containing 10 mM manganese chloride, and the plates were incubated for 5 days at 24°C. The colonies that grew in these conditions were analyzed by immunoblotting to identify proteins that run differently from the wild-type Fur protein in SDS-PAGE. Two types of mutants were obtained—one that showed the presence of a Fur-related protein but migrates with an apparently lower molecular mass (*V. anguillarum* 775MET9) and another

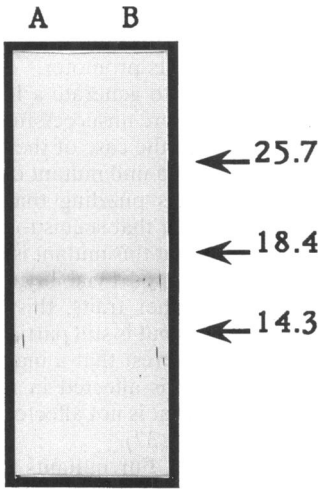

FIG. 5. Regulation of expression of the *V. anguillarum fur* gene. Immunoblot analysis of the cytosolic extracts of wild-type *V. anguillarum* grown in minimal medium under iron-rich (lane A) or iron-limiting (lane B) conditions was performed as described in Materials and Methods with anti-Fur serum. Total extracts (50 μg of protein), obtained by sonication and ultracentrifuged at 100,000 × *g* for 2 h, and the cytosolic soluble protein were subjected to SDS-PAGE and immunoblotting with anti-Fur serum, as described in Materials and Methods. Molecular masses (in kilodaltons) are shown to the right.

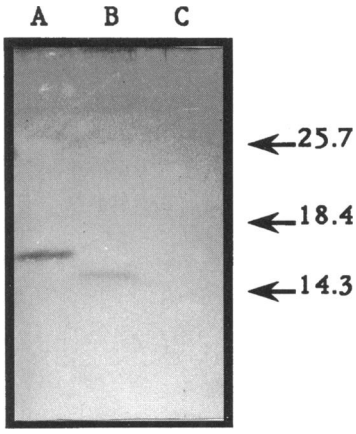

FIG. 6. Immunoblot analysis of *V. anguillarum fur* mutants. The Fur proteins present in total protein extracts of the *V. anguillarum fur* mutants selected in the presence of 10 mM $MnCl_2$ were analyzed by SDS-PAGE and immunoblotting with anti-Fur serum, as described in Materials and Methods. *V. anguillarum* 775 (lane A), 775MET9 (lane B), and 775MET11 (lane C) were the strains analyzed. Molecular masses (in kilodaltons) are shown to the right.

which showed no reaction against the anti-Fur serum (*V. anguillarum* 775MET11) (Fig. 6).

These mutants were used to analyze whether components of the *V. anguillarum* iron-uptake system are under the control of Fur. For this purpose, the strains were cultured in iron-rich or iron-limiting minimal medium and the biosynthesis of both the catechols and the FatA outer membrane protein was analyzed. As shown in Table 3, although in wild-type strain 775 the production of catechols is iron regulated, in *V. anguillarum* 775MET11 all regulation by the iron concentration in the milieu was lost. A degree of regulation can still be seen in *V. anguillarum* 775MET9. This could indicate that *V. anguillarum* 775MET11 is a null or nearly null mutant while strain 775MET9 is leaky.

The 86-kDa outer membrane protein FatA (2, 53) is an important component of the receptor for the ferric anguibactin complexes (2). Expression of the *fatA* gene is highly regulated by the concentration of iron in the medium, and at least two regulatory factors have already been identified, TAF and RNAα (42, 49, 58). The results from experiments analyzing regulation of expression of *fatA* with an *E. coli* Fur mutant and a recombinant clone harboring the *E. coli fur* gene were inconclusive (58). Therefore, the *V. anguillarum* manganese-resistant *fur* mutants were used to study regulation of expression of *fatA* by Fur. Wild-type *V. anguillarum* and the mutants 775MET9 and 775MET11 were cultured under iron-rich and iron-limiting conditions, and outer membrane proteins were purified. The presence of FatA was detected by immunoblotting with anti-FatA serum. Figure 7 shows that, while no FatA was detected in the wild-type strain grown under iron-rich conditions, this protein was present in the outer membrane preparations of both mutants. However, it is also clear from Fig. 7 that, in the case of mutant 775MET9, there is still a certain degree of regulation, while the amounts of FatA detected in mutant 775MET11 grown under iron-rich and iron-limiting conditions are virtually the same. This is likely due to the fact that *V. anguillarum* 775MET9 behaves as a leaky mutant, while the mutation in derivative 775MET11 is null or nearly null.

DISCUSSION

The pJM1-mediated iron uptake system of *V. anguillarum* is highly regulated by the concentration of iron in the surrounding medium. Recently, several plasmid-encoded regulators of this system have been identified: TAF (49); the bifunctional protein AngR, which besides its function as a regulator can complement an *entE* mutant of *E. coli* (50, 55), and RNAα, an antisense RNA that regulates the expression of FatA and FatB (42, 58). Besides these plasmid-mediated regulators, a chromosomally encoded protein with a Fur-like activity was also detected (58). Here we report the cloning and sequencing of

TABLE 2. β-Galactosidase activities

Plasmid in *E. coli* RRJC1	β-Galactosidase activity under[a]:		Ratio of iron-limiting to iron-rich activity
	Iron-rich conditions	Iron-limiting conditions	
None	1,762	1,486	0.84
pTAW1.8	155	1,304	8.41
pTAW3.12	352	1,679	4.76
pTAW4.1	2,081	2,243	1.07

[a] Activity is measured in Miller units (36).

TABLE 3. Production of catechols by *V. anguillarum* strains

Strain	Catechol production (OD_{510}) under[a]:	
	Iron-rich conditions	Iron-limiting conditions
775	0.019	0.260
775MET9	0.118	0.323
775MET11	0.458	0.471

[a] Catechol production was determined by the method of Arnow (4) on overnight cultures as described previously (23). OD_{510}, optical density at 510 nm.

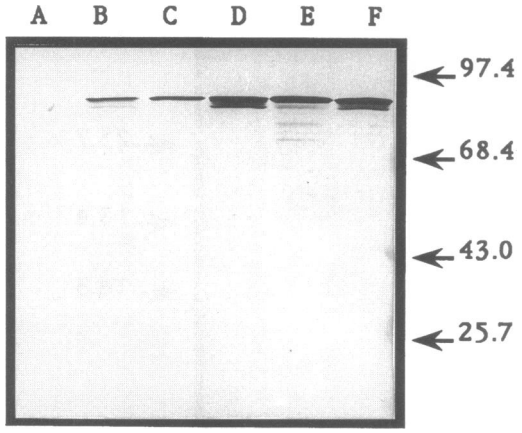

FIG. 7. Regulation of expression of FatA in *V. anguillarum* mutants. Outer membrane proteins of *V. anguillarum* 775 (lanes A and B), 775MET9 (lanes C and D), and 775MET11 (lanes E and F), cultured under iron-rich (lanes A, C, and E) or iron-limiting (lanes B, D, and F) conditions, were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, incubated with anti-FatA serum, and developed as described in Materials and Methods. Molecular masses (in kilodaltons) are shown to the right.

the gene encoding that protein. The highest degree of homology of the *V. anguillarum* amino acid sequence of the Fur protein was found with the Fur amino acid sequences reported for *V. cholerae* and *V. vulnificus* (33, 35). The degree of homology was lower (although still high) with the *E. coli* and *Y. pestis* Fur amino acid sequences (44, 45). The lowest degree of homology was found with the *P. aeruginosa* Fur protein, which seems to be the most distant from the rest of the Fur amino acid sequences reported so far. The C-terminal portion of the protein seems to be the less conserved region of the Fur proteins. The regions spanning amino acids 105 to 121, and 132 to the end (shaded in Fig. 3) are the least conserved within this C-terminal portion, which has been described as playing a role in the binding of metal ions (13, 29, 31). A certain degree of heterogeneity is also found in the N termini spanning amino acids 28 to 46 (shaded in Fig. 3).

It has been suggested previously that expression of the *E. coli fur* gene is autoregulated by its product (22); however, no iron regulation of the *fur* genes was detected in *V. cholerae* or *V. vulnificus* (33, 35). Our results obtained by immunoblotting of extracts of *V. anguillarum* cultured under iron-rich or iron-limiting conditions indicate that, in this bacterium, regulation of expression of *fur* by iron either does not exist or is at a very low level. We are presently performing transcription and operon fusion analyses to confirm these results.

Studies of metal ion activation of Fur proteolysis demonstrated that, in the presence of $MnCl_2$, the *E. coli* Fur protein is digested by trypsin between Lys-76 and Ser-77, suggesting that, upon binding the metal ion, the protein undergoes a conformational change that makes this region available to trypsin (13). As a result of this conformational change, the N terminus of the Fur protein could bind the DNA. This region has been postulated as one probable boundary between the DNA-binding N terminus and the metal ion-binding C terminus (13) and, with the exception of the *P. aeruginosa* Fur protein, its amino acid sequence is conserved in all Fur proteins sequenced so far (Fig. 3). We generated a mutant by site-directed mutagenesis in this region (pTAW3.12). Lys-77 (equivalent to Lys-76 of the *E. coli* Fur protein) was changed to Gly, generating the derivative FurG77. Analysis of this mutant showed that FurG77 was impaired in its ability to regulate a gene that has the Fur box in its promoter.

Attempts to use pTAW4.1 to generate a *V. anguillarum fur* mutant by marker exchange were unsuccessful. A similar result has been recently reported in the case of the *P. aeruginosa fur* gene (37). A possibility is that a null mutant of Fur is lethal for some bacteria. However, it is puzzling that, by manganese selection, we isolated a mutant that seems to be null or nearly null. Explanations could be that this mutant is not null and that the activity left is enough to support viability, or that, since it is known that Fur regulates other traits, this mutant lost the ability to regulate some genes but is still partially or totally able to regulate others. It is of interest that a manganese-resistant Fur mutant of *P. aeruginosa* is affected in regulation of the biosynthesis of siderophores but is not affected in regulation of the production of exotoxin A (37).

Of the manganese-resistant Fur mutants isolated by these techniques, two were further analyzed in this work. One of them, *V. anguillarum* 775MET9, partially lost its ability to regulate the production of catechols as determined by the Arnow reaction. Precursors for biosynthesis of the siderophore anguibactin, as well as anguibactin itself, are detected by the Arnow reaction. Other factors have also been implicated in regulation of the biosynthesis of anguibactin (41, 49, 55). Therefore, we cannot yet discriminate the step that is Fur regulated in the synthesis of anguibactin. The mutant *V. anguillarum* 775MET9 produced a Fur protein with an apparently lower molecular weight, as determined by immunoblotting. In the case of the other mutant, *V. anguillarum* 775MET11, no protein was detected by anti-Fur serum. This could be due to a total loss of Fur production or to the production of a very small Fur derivative or a protein that lost all recognizable epitopes. Since the anti-Fur serum is polyclonal, this last possibility seems improbable. Iron regulation of biosynthesis of catechols in this mutant was completely lost.

In the case of FatA, a protein that is part of the receptor that recognizes ferric-anguibactin complexes, regulation by iron was impaired in mutant 775MET9 and almost lost in mutant 775MET11, as determined by immunoblot analysis. The presence of higher levels of FatA in the mutants than in the wild-type strain, when cultured under iron-limiting conditions, may be due to the existence of background levels of Fur in the wild type that can exert some negative regulation even at the low concentrations of iron present under those conditions. Regulation of *fatA* by Fur in an *E. coli* background was studied before with a clone of the *E. coli* Fur protein, with inconclusive results (58). We showed before that expression of *fatA* is under the control of other regulatory factors such as TAF (49) or RNAα (42, 49). The results in this paper prove that Fur plays an important role in the regulation of *fatA*, adding a new factor to the regulatory circuit of this gene. Experiments are being carried out to assess the degree of contribution of TAF, RNAα, and Fur to the regulation of *fatA*.

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REFERENCES

1. **Actis, L. A., W. Fish, J. H. Crosa, K. Kellerman, S. R. Ellenberger, F. M. Hauser, and J. Sanders-Loehr.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). J. Bacteriol. **167**:57–65.
2. **Actis, L. A., S. A. Potter, and J. H. Crosa.** 1985. Iron-regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by

virulence plasmid pJM1. J. Bacteriol. **161:**736–742.
3. **Actis, L. A., M. E. Tolmasky, D. Farrell, and J. H. Crosa.** 1988. Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid-mediated iron-transport system. J. Biol. Chem. **263:**2853–2860.
4. **Arnow, L.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118:** 531–537.
5. **Bagg, A., and J. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry **26:**5471–5477.
6. **Bagg, A., and J. B. Neilands.** 1985. Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. **161:**450–453.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
8. **Boyer, H. W., and D. Roulland-Doussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**249–252.
10. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
11. **Calderwood, S. B., and J. J. Mekalanos.** 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015–1017.
12. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
13. **Coy, M., and J. Neilands.** 1991. Structural dynamics and functional domains of the fur protein. Biochemistry **30:**8201–8210.
14. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **284:**566–568.
15. **Crosa, J. H.** 1984. The relationship of plasmid mediated iron transport and bacterial virulence. Annu. Rev. Microbiol. **38:**69–89.
16. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
17. **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.
18. **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.
19. **Crosa, J. H., M. H. Schiewe, and S. Falkow.** 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum*. Infect. Immun. **18:**509–513.
20. **De Grandis, S., J. Ginsberg, M. Toone, S. Climie, J. Friesen, and J. Brunton.** 1987. Nucleotide sequence and promoter mapping of the *Escherichia coli* Shiga-like toxin operon of bacteriophage H-19B. J. Bacteriol. **169:**4313–4319.
21. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid pColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:**2624–2630.
22. **de Lorenzo, V. L., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur (ferric uptake regulator) protein and CAP (catabolic-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. Eur. J. Biochem. **173:**537–546.
23. **Echenique, J. R., H. Arienti, M. E. Tolmasky, R. R. Read, R. J. Staneloni, J. H. Crosa, and L. A. Actis.** 1992. Characterization of a high-affinity iron transport system in *Acinetobacter baumannii*. J. Bacteriol. **174:**7670–7679.
24. **Farrell, D., P. Mikesell, L. Actis, and J. H. Crosa.** 1990. A regulatory gene, *angR*, of the iron uptake system of *Vibrio anguillarum*: similarity with phage P22 *cro* and regulation by iron. Gene **86:**45–51.
25. **Foster, J. W., and H. K. Hall.** 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174:**4317–4323.
26. **Gammie, A. E., M. E. Tolmasky, and J. H. Crosa.** 1993. Functional characterization of a replication initiator protein. J. Bacteriol. **175:**3563–3569.
27. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol. Gen. Genet. **197:**337–341.
28. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12. Mol. Gen. Genet. **210:**135–139.
29. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol. Microbiol. **4:**1621–1628.
30. **Jalal, M., D. Hossain, J. van der Helm, J. Sanders-Loehr, L. A. Actis, and J. H. Crosa.** 1989. Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. J. Am. Chem. Soc. **111:**292–296.
31. **Klug, A., and D. Rhodes.** 1987. Zinc fingers: a novel protein motif for nucleic acid recognition. Trends Biochem. Sci. **12:**464–469.
32. **Koster, W. L., L. A. Actis, L. Waldbeser, M. E. Tolmasky, and J. H. Crosa.** 1991. Molecular characterization of the iron transport system mediated by the pJM1 plasmid in *Vibrio anguillarum* 775. J. Biol. Chem. **266:**23829–23833.
33. **Litwin, C. M., S. A. Boyko, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897–1903.
34. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
35. **Litwin, C. M., and S. B. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by in vivo marker exchange. J. Bacteriol. **175:**706–715.
36. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589–2598.
38. **Putnam, S., and A. Koch.** 1975. Complications in the simplest cellular enzyme assay: lysis of *Escherichia coli* for the assay of β-galactosidase. Anal. Biochem. **63:**350–360.
39. **Read, R., and J. H. Crosa.** Unpublished data.
40. **Rucker, R., B. J. Earp, and E. J. Ordal.** 1953. Infectious diseases of Pacific salmon. Trans. Am. Fish. Soc. **83:**297–312.
41. **Salinas, P., M. E. Tolmasky, and J. H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: evidence for a cooperative effect between two transcriptional activators. Proc. Natl. Acad. Sci. USA **86:**3529–3533.
42. **Salinas, P., L. Waldbeser, and J. H. Crosa.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as repressor. Gene **123:**33–38.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
44. **Schaffer, S., K. Hantke, and V. Braun.** 1985. Nucleotide sequence of the iron regulatory gene *fur*. Mol. Gen. Genet. **200:**110–113.
45. **Staggs, T. M., and R. D. Perry.** 1991. Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. J. Bacteriol. **173:**417–425.
46. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891–2895.
47. **Studier, F., A. Rosenberg, J. Dunn, and J. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
48. **Tabor, S., and C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
49. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1988. Genetic analysis of the iron uptake region of the *Vibrio anguillarum* plasmid pJM1: molecular cloning of genetic determinants encoding a novel *trans* activator of siderophore biosynthesis. J. Bacteriol. **170:**1913–1919.
50. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence

plasmids, results in hyperproduction of anguibactin. Infect. Immun. **61**:3228–3233.

51. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. Virulence plasmids. *In* D. Rickwood and B. Hame (ed.), Plasmids: a practical approach, p. 95–118. IRL Press, Oxford.
52. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. J. Bacteriol. **160**:860–866.
53. **Tolmasky, M. E., and J. H. Crosa.** 1991. Regulation of plasmid-mediated iron transport and virulence in *Vibrio anguillarum*. Biol. Metals **4**:33–35.
54. **Tolmasky, M. E., A. Gammie, and J. H. Crosa.** 1992. Characterization of the *recA* gene of *Vibrio anguillarum*. Gene **110**:41–48.
55. **Tolmasky, M. E., P. C. Salinas, L. A. Actis, and J. H. Crosa.** 1988. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum*. Infect. Immun. **56**:1608–1614.
56. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
57. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
58. **Waldbeser, L., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1993. Mechanisms for negative regulation by iron of the *fatA* outer membrane protein gene expression in *Vibrio anguillarum* 775. J. Biol. Chem. **268**:10433–10439.
59. **Weickert, M., and G. Chambliss.** 1989. Acid-phenol minipreps make excellent sequencing templates. United States Biochemical Corp. Ed. Comments **16**:5–6.
60. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
61. **Wolf, M., and J. H. Crosa.** 1986. Evidence for the role of a siderophore in promoting *Vibrio anguillarum* infections. J. Gen. Microbiol. **132**:2949–2952.
62. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.