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A Single Amino Acid Change in AngR, a Protein Encoded by pJM1-Like Virulence Plasmids, Results in Hyperproduction of Anguibactin

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The siderophore anguibactin is produced in vivo in a diffusible form and is an important factor in the virulence of *Vibrio anguillarum*. The natural isolate *V. anguillarum* 531A is a hyperproducer of anguibactin when compared with the prototype strain *V. anguillarum* 775. The *angR* gene was found to be responsible for this difference in levels of anguibactin produced. Nucleotide sequence analysis showed that the $angR_{531A}$ differed in a single nucleotide from the $angR_{775}$ present in the prototype plasmid pJM1. This nucleotide substitution resulted in a change in amino acid 267 from His in strain 775 to Asn in strain 531A. This amino acid is located in a region between one of the two helix-turn-helix domains and the neighboring leucine zipper. Mutations to replace His with either Leu or Gln, generated by site-directed mutagenesis, in amino acid 267 resulted in strains for which the MIC of the iron chelator ethylenediamine di(*o*-hydroxyphenyl) acetic acid were lower than for the proptotype 775 but higher than for iron uptake-deficient strains. In addition to its transcriptional activating function, AngR also complemented a mutation in the *Escherichia coli entE* gene, which encodes the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP ligase. Therefore, AngR may also function in *V. anguillarum* as an EntE-like enzyme for the biosynthesis of anguibactin.

A major virulence factor of the pathogenic bacterium *Vibrio anguillarum* 775 is the pJM1-mediated iron uptake system composed of the siderophore anguibactin (1, 14, 19) and a receptor complex that recognizes ferric-anguibactin (1–3, 11–13, 20). Anguibactin is an important virulence factor that is produced in vivo in a diffusible form, and the levels present in blood or kidney of infected fish are sufficient to provide iron for growth of a *V. anguillarum* strain impaired in anguibactin biosynthesis (42). Many *V. anguillarum* strains from various geographical locations were recently studied, and it was found that most of them harbored pJM1-like plasmids (33, 35). Several of these strains showed a higher activity of anguibactin when compared with the prototype *V. anguillarum* 775 strain (37). For one of these strains, *V. anguillarum* 531A, it was determined that the *angR* gene, $angR_{531A}$, encoded by its pJM1-like plasmid, pJHC1, was responsible for this higher anguibactin activity (37). The *angR* gene encodes a 110-kDa protein, AngR, that acts as a *trans*-activator of other gene(s) of the iron uptake system (25, 32). A region of AngR has a helix-turn-helix motif typical of prokaryotic DNA binding proteins with homology to the DNA binding domain of the P22 bacteriophage protein Cro (15). It was also recently shown that AngR shares homology in a specific domain with several proteins of three groups of ATP-utilizing enzymes: the acid-thiol ligases, the activating enzymes for the biosynthesis of enterobactin, and the synthetases for tyrocidine, gramicidine S, and penicillin, also known as the firefly luciferase family. Among these enzymes are the 57-kDa peptide of the 4-chlorobenzoate dehalogenase from *Pseudomonas* sp., the gramicidin S synthetase and the tyrocidine synthetase from *Bacillus brevis*, the 4-coumarate:coenzyme A ligase from *Petroselinum crispum*, the luciferase from *Photinus pyralis*, the 2,3-dihydroxybenzoate-AMP ligase (EntE) from *Escherichia coli* (29, 31), and the D-alanine-activating enzyme from *Lactobacillus casei* (17).

In this report, we present the nucleotide sequence of $angR_{531A}$ and compare it with the sequence of $angR_{775}$ and show that they differ in only one nucleotide. In addition, we demonstrate that AngR has an enzymatic activity which can complement a mutation in the *E. coli entE* gene involved in the biosynthesis of the siderophore enterobactin.

MATERIALS AND METHODS

Bacterial strains and plasmids. 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. Plasmid pBluescript SK+ (Stratagene, La Jolla, Calif.) was used as the vector for site-directed mutagenesis and DNA sequencing. Plasmid pKK223-3 was used as the expression vector of AngR for the complementation experiments of *E. coli* AN93, an *entE* mutant derivative of *E. coli* AB1515 (28). Uses of other plasmids and strains are described below.

General DNA procedures. Plasmid DNA was purified by the method of Birnboim and Doly (5). Transformation was done by the method of Cohen et al. (10). Double-stranded DNA was sequenced by the dideoxy chain termination method (27) with the Sequenase kit (U.S. Biochemical, Cleveland, Ohio) with the T7 and T3 and, in some cases, specific synthetic primers.

Site-directed mutagenesis of *angR*. The *Sal*I-*Eco*RI fragment containing the *angR* gene from pJM1 was cloned in pBluescript SK+ to generate pMETAngR and site directed mutagenized by using the Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) and the synthetic mutagenic oligonucleotides TGGGGGCTT CAAAAATA (for pMET775HL), GGGAATCAAAAATACC (for pMET775HN), GGGCAACAAAAATACC (for pMET775

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype	Source or reference
Bacterial strains		
E. coli		
HB101	F^- *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY1 tonA21* λ^- *supE44*	6
JM107	*thiD(lac-proAB) gyrA96 endA1 hsdR17 relA1 supE44* F' (*traD36 proAB lacI^q lacZDM15*)	43
AN93	*entE* derivative of AB1515	28
V. anguillarum		
531A	Natural isolate, enhanced production of anguibactin, pJHC1	37
531A(pJHC1#4, pPH1JI)	Generated by marker exchange, harbors pJHC1#4 and pPH1JI	This work
531A(pJHC1, pPH1JI)	Control strain carrying pJHC1 and pPH1HI	This work
775::Tn1-6B#4	Iron uptake deficient pJHC-T2612#4, pJHC9-8	32
Plasmids		
pBluescriptSK+	Cloning vector	Stratagene, La Jolla, Calif.
pKK223-3	Expression vector	Pharmacia LKB, Piscataway, N.J.
pJHC1#4	pJHC1 with a Tn*3*-HoHo1 insertion in *angR* generated by marker exchange	This work
pJHC-T2612#4	Recombinant clone carrying the pJM1 iron uptake region using as vector pVK102 with a Tn*3*-HoHo1 insertion in *angR*; encodes resistance to tetracycline and ampicillin	32
pJHC9-8	Deletion derivative of pJM1 lacking all of the iron uptake region but carrying the regulator TAF needed for full expression of the system	32, 41
pJHC-S100	Cloning vector, pBR325 with the pUC4K Km^r fragment inserted in *Pst*I site	25
pJHCS531A*ptac*	$angR_{531A}$ cloned in pKK223-3 (formerly called pJHC-S2572)	25
pJHCS531A*ptacNco*	Derivative of pJHCS531A*ptac* generated by digestion with *Nco*I followed by filling the cohesive ends with Klenow enzyme (formerly called pJHC-S2570)	25
pJHCS531A	$angR_{531A}$ cloned in pJHC-S100 (formerly called pJHC-S2571)	25
pJHCS775	$angR_{775}$ cloned in pJHC-S100 (formerly called pJHC-S2771)	25
pMETAngR	pJM1 *Sal*I-*Eco*RI fragment cloned in pBluescriptSK+ used for site-directed mutagenesis	This work
pMET775	pJM1 *Sal*I-*Eco*RI fragment cloned in pJHC-S100 and used to conjugate into *V. anguillarum*(pJHC9-8, pJHC-T2612#4)	This work
pMET775HN	*Sal*I-*Eco*RI fragment with the His-267 substituted with Asn, cloned in pJHC-S100, and used to conjugate into *V. anguillarum*(pJHC9-8, pJHC-T2612#4)	This work
pMET775HL	*Sal*I-*Eco*RI fragment with the His-267 substituted with Gln, cloned in pJHC-S100, and used to conjugate into *V. anguillarum*(pJHC9-8, pJHC-T2612#4)	This work
pMET775HQ	*Sal*I-*Eco*RI fragment with the His-267 substituted with Gln, cloned in pJHC-S100, and used to conjugate into *V. janguillarum* (pJHC-T2612#4)	
pMET775Tr	*Sal*I-*Eco*RI fragment with the insertion of a nucleotide at amino acid location 888, cloned in pJHC-S100, and used to conjugate into *V. anguillarum*(pJHC9-8, pJHC-T2612#4)	This work
pJHC1	Natural iron uptake plasmid in *V. anguillarum* 531A	37
pPH1JI	Incompatible with pJHC-T2612#4; encodes resistance to gentamicin	18

HQ), and GACAAACCTCTAGCA (for pMET775Tr). Mutations were confirmed by DNA sequencing with the appropriate primers. Once mutated, the *Sal*I-*Eco*RI fragments from the different derivatives were recloned into pJHC-S100 to generate the plasmids carrying the AngR mutants (listed in Table 1) and transferred by conjugation to *V. anguillarum* 775::Tn*1-6*(pJHC-T2612#4). Conjugations were done as described before (34). The transconjugant strains were tested for the MIC of the iron chelator ethylenediamine di(*o*-hydroxyphenyl) acetic acid (EDDA) as described previously (34).

Generation of an *angR*-deficient mutant by marker exchange. The *angR*-deficient mutant *V. anguillarum* 531A(pJHC1#4) was generated by the marker exchange technique (24). Plasmid pJHC-2612#4 was transferred to *V. anguillarum* 531A by conjugation with *E. coli*(pRK2073) as the helper as described before (34). Next, in a second conjugation, plasmid pPH1JI (which encodes resistance to gentamicin) (18) was conjugated to *V. anguillarum* 531A(pJHC-2612#4), and cells were plated in the presence of 50 μg of gentamicin per ml and 1 mg of ampicillin per ml. Colonies growing in these conditions were then tested for susceptibility to tetracycline. Those colonies resistant to gentamicin and ampicillin and susceptible to tetracycline were analyzed for their plasmid content, and it was proved that they carried pPH1JI and a derivative of pJHC1, pJHC1#4, which had a Tn*3*-HoHo1 insertion in *angR* product of homologous recombination between the wild-type *angR* in pJHC1 and the mutated *angR* present in pJHC-T2612#4.

Complementation of the *entE* mutant *E. coli* AN93. *E. coli* AN93 harboring either pKK223-3, pJHCS531A*ptac*, or pJHCS531A*ptacNco* was cultured overnight and used to inoculate 2 ml of L broth containing ampicillin (50 μg/ml) and increasing amounts of EDDA. Bacterial growth was recorded spectrophotometrically at 600 nm, after incubation at 37°C for 18 h.

Protein analysis. The proteins encoded by the *angR* derivatives were analyzed by coupled cell-free transcription-translation (Amersham Corp., Arlington Heights, Ill.) and then sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the $[^{35}S]$methionine-labeled proteins and fluorography (2, 36). Immunoblot analysis to detect FatA was done with anti-FatA serum as describe previously

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1 MNQNEHPFAFPETKLPLTSNQNWQLSTQRQRTEKKSITNFTYQEFDYENISRDTLERCLTTIIKHHPI

69 FGAKLSDDFYLHFPSKTHIETFAVNDLSNALKQDIDKQLADTRSAVTKSRSQAIISIMFSI

130 L P K N I I R L H V R F N S V V V D N P S V T

153 L F F E Q L

159 TQLLSGSPLSFLNQEQTISAYNHKVNNELLSVDLESARWNEYILTLPSSANLPTICEPEKLDETDITR

227 RCITLSQRKWQQLVTVSKKHNVTPEITLASIFSTVLSLWG H  QKYLMMRFDITKINDYTGIIGQ
                                         CAT
                         531A and 16.9 N
                                         AAT
                                    16.1 L
                                         CTT
                                   16.10 Q
                                         CAA

290 F T E P L L V G M S G F E Q S F L S L V K N

312 NQKKFEEAYHYDVKVPVFQCVNKLSNISDSHRYPANITFSSELLNTNHSKKAVWGCRQSANTWLSLHA

380 VIEQEQLVLQWDSQDAIFPKDMIKDMLHSYTDLLDLLSQKDVNWAQPLPTLLPKHQESIRNKINQQGD

448 LELTKELLHQRFFKNVESTPNALAIIHGQESLDYITLASYAKSCAGALTEAGVKSGDRVAVTMNKGIG

516 QIVAVLGILYAGAIYVPVSLDQPQERRESIYQGAGINVILINESDSKNSPSNDLFFFLDWQTAIKSEP

584 MRSPQDVAPSQPAYHYTSGSTGLPKGVVISHQGALNTCIAINRRYQIGKNDRVLALSALHFDLSVYD
                    4
652 IFGLLSAGGTIVLVSELERRDPIAWCQAIEEHNVTMWNSVPALFDMLLTYATCFNSIAPSKLRLTMLS

720 GDWIGLDLPQRYRNYRVDGQFIAMGGATEASIWSNVFDVEKVPMEWRSIPYGYPLPRQQYRVVDDLGR

788 DCPDWVAGELWIGGDGIALGYFDDELKTQAQFLHIDGHAWYRTGDMGCYWPDTLEF

845 L G R R D K Q V K V G G Y R I E L G E I E V A

868 LNNIP

873 G V Q R A V A I A V G N K D K T L A A P I V

896 MDSEQAPIVTAPLDAEEVQLLLNKQLPNYMVPKRIIFLETFPLTANGKVDHKALTRMTNREKKTSQSI

963 NKPIITASEDRVAKIWNDVLGPTELYKSSDFFLSGGDAYNAIEVVKRCHKAGYLIKLSMLYRYSTIEA

1031 FAIIMDRCRLAPQEEAEL
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FIG. 1. Comparison of the deduced amino acid sequence of $AngR_{775}$ and $AngR_{531A}$. In amino acid 267 where they differ, the codons are also shown as well as codon and corresponding amino acids for mutants pMET775HL, pMET775HN, and pMET775HQ. Leu, Ile, and Val residues in the leucine zippers as well as helixes in the helix-turn-helix motifs are shaded. Amino acids 598 to 610 (boldface and shaded) form the region that shares homology with fragments of enzymes belonging to the firefly luciferase family. The symbol ⫯ represents the location of the Tn*3*-HoHo1 insertion in pJHC-T2612#4 (32). The sequence of *$angR_{531A}$* will appear in the EMBL/GenBank nucleotide sequence libraries under the accession number Z12000.

(2). Helix-turn-helix motifs were analyzed by Chou-Fasman algorithms (9) as well as by the method of Brennan et al. (7).

RESULTS

Nucleotide sequence of *$angR_{531A}$*. *V. anguillarum* 531A, which carries the pJM1-like plasmid pJHC1, produces higher levels of anguibactin than *V. anguillarum* 775 (37). Since previous experiments showed that the pJHC1-encoded *$angR_{531A}$* was responsible for this enhanced anguibactin production (25), we sequenced and compared this gene with the reported DNA sequence of *$angR_{775}$* (15). The only difference found between the two nucleotide sequences was a single change in base 1159 from C, in *$angR_{775}$*, to A, in *$angR_{531A}$*, with a concomitant change from His to Asn at the amino acid residue 267 (Fig. 1). To confirm the importance of amino acid 267, the basic, positively charged amino acid His in *$angR_{775}$* was changed to either Asn, the amino acid found naturally in *$angR_{531A}$* which has an uncharged polar group (pMET 775HN), Gln, which has the same uncharged polar group as Asn but has an extra methylene group (pMET775HQ), or the nonpolar amino acid Leu (pMET775HL) (Fig. 1). All these three derivatives as well as recombinant clones harboring either the prototype *$angR_{775}$* (pJHCS775) or *$angR_{531A}$* (pJHCS531A) were transferred by conjugation to *V. anguillarum* 775::Tn*1*-*6*B#4. This strain harbors pJHC-T2612#4, which is a clone containing all the pJM1 iron uptake region with a Tn*3*-HoHo1 insertion in *$angR_{775}$* (Fig. 1), and the plasmid pJHC9-8, which carries the *trans*-acting factor (TAF), necessary for full production of anguibactin (34). Table 2 shows the MICs of the iron chelator EDDA for all transconjugants. MICs for strains carrying pJHCS775 or pJHCS531A were 10 or 20 μM, respectively. The His-to-Asn mutation (pMET16.9) resulted in an MIC of EDDA of 20 μM (the same increase observed for the natural *$angR_{531A}$*), confirming that the difference found between the iron uptake systems encoded by pJM1 and pJHC1 was actually due to the amino acid change from His to Asn. It was of interest that replacing His with Gln (pMET775HQ), which is an amino acid similar to Asn but with a longer R group, resulted in an MIC of EDDA lower than that for the wild-type control (pJHCS775). The replacement of Asn with the nonpolar amino acid Leu (pMET775HL) resulted in an even further reduction of MIC of EDDA to the levels found in the iron uptake-deficient control.

TABLE 2. Properties of *angR* mutants

Recombinant plasmid[a]	Mutation	Amino acid 267	MIC of EDDA (μM)[b]
None			2.5
pJHCS531A		N	20
pJHCS775		H	10
pMET775HL	CAT to CTT	L	2.5
pMET775HN (identical to *angR* pJHC1)	CAT to AAT	N	20
pMET775HQ	CAT to CAA	Q	5

[a] *V. anguillarum* strains harbor pJHC9-8 and pJHC-T2612#4 in addition to the indicated recombinant clones.

[b] MIC of EDDA was determined in CM9 minimal medium cultures containing increasing amounts of EDDA. Determinations of MIC were performed three times with identical results.

Analysis of protein synthesis by in vitro-coupled transcription-translation showed that AngR was produced at comparatively the same levels, using as DNA templates plasmids pMET775HL, pMET775HN, and pMET775HQ, as well as the controls pJHCS775 and pJHCS531A (Fig. 2). The band corresponding to AngR was confirmed by including in the experiment the transcription-translation products of plasmid pMET 775Tr. In this plasmid, a nucleotide was inserted at the location of amino acid 888 which generated a change of phase and concomitantly a truncated version of AngR. As shown in Fig. 2, in this case the AngR band is absent and instead a new band with the predicted size for the truncated product is present.

AngR possesses two helix-turn-helix and leucine zipper domains. It was reported previously that the $AngR_{775}$ protein has a helix-turn-helix motif typical of DNA binding proteins (15). In this work, we further analyzed the sequence of this protein and identified another potential helix-turn-helix motif located between amino acids 290 and 311 of $AngR_{775}$ (Fig. 1). It was of interest that both helix-turn-helix domains were preceded by leucine zipper motifs (Fig. 1). The presence of

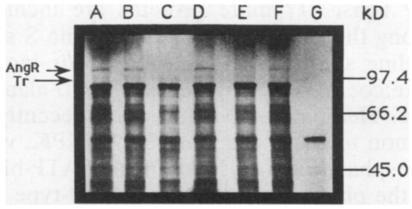

FIG. 2. Transcription-translation analysis of AngR mutants. SDS-PAGE of polypeptides synthesizes by coupled cell-free transcription-translation assays of recombinant clones harboring the mutated *angR* gene derivatives. Lanes: A, pMET775HL; B, pMET775HN; C, pMET775Tr; D, pMET775HQ; E, pJHCS531A; F, pJHCS775; G, pJHC-S100 (25). Tr indicates the position of the truncated AngR in lane F. The electrophoretic mobilities (in kilodaltons) of rabbit muscle phosphorylase *b* (97.4), bovine serum albumin (66.2), and hen egg white ovalbumin (45) used as markers are indicated on the right margin.

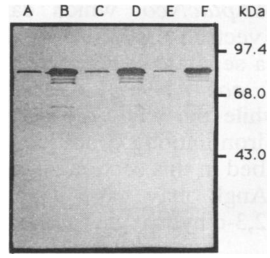

FIG. 3. Immunoblot analysis of outer membrane proteins of *V. anguillarum* derivatives. Outer membrane proteins from *V. anguillarum* strains cultured under either iron-rich (CM9 minimal medium supplemented with 50 μM ferric chloride, lanes A, C, and E) and iron-limiting (CM9 minimal medium with the addition of 5 μM EDDA, lanes B, D, and F) conditions were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, incubated with anti-FatA serum, and developed by reaction with peroxidase and staining with H_2O_2 and horseradish peroxidase color development reagent (2). Molecular masses are shown to the right. Lanes: A and B, *V. anguillarium* 531A(pJHC1); C and D, *V. anguillarum* 531A(pJHC1, pPH1JI); E and F, *V. anguillarum* 531A(pJHC1#4, pPH1JI).

these motifs is in keeping with the possibility of AngR interacting with other proteins or itself and binding DNA to regulate expression of some genes.

Construction of an AngR$_{531A}$ mutant by marker exchange. To analyze the role played in regulation of expression of components of the iron uptake system by AngR$_{531A}$ and discard any possible contribution of the copy number of the recombinant clones used in the experiments, we generated a mutation of *angR* in pJHC1 by marker exchange. Plasmid pJHC-T2612#4 (32) was transferred to *V. anguillarum* 531A by conjugation. In a second conjugation, the plasmid pPH1JI (which is incompatible with pJHC-T2612#4) was transferred to this strain and was plated in the presence of gentamicin and ampicillin to select for those cells in which the mutated *angR* replaced the *angR* gene in pJHC1. Colonies growing in the presence of gentamicin and ampicillin were susceptible to tetracycline, confirming that pJHC-T2612#4 was lost by incompatibility with pPH1JI. Restriction endonuclease analysis of the plasmid content showed a pJHC1 derivative which had a Tn*3*-HoHo1 insertion in *angR*. The mutated strain, *V. anguillarum* 531A(pJHC1#4, pPH1JI), was analyzed for its ability to grow under conditions of iron limitation. The MIC of EDDA for *V. anguillarum* 531A(pJHC1#4, pPH1JI) was 2.5 μM, while that for the control strain *V. anguillarum* 531A(pJHC1, pPH1JI) was 20 μM. Therefore, the mutated strain became iron uptake deficient.

It was shown before that *V. anguillarum* strains carrying recombinant clones harboring the iron uptake region of pJM1 with an insertion in *angR* were able to transport iron when anguibactin was supplemented externally (32). However, it was also shown that iron was transported in these conditions even in the presence of very low levels of the outer membrane protein FatA (2, 3, 34). This 86-kDa protein encoded by *fatA* (2, 3, 35) is a highly regulated (26, 40) important component of the receptor for the ferric anguibactin complexes (2). Comparison of FatA levels in strains carrying recombinant clones was not accurate because the copy number of the recombinant clones was not the same as the natural plasmid. Therefore, regulation of FatA expression by AngR could not be discarded. *V. anguillarum* 531A(pJHC1#4, pPH1JI) was an ideal strain to test this possibility because it carried the natural plasmid with a mutation in *angR*. Anti-FatA immunoblot analysis was done after isolation and electrophoresis of outer membrane proteins from *V. anguillarum* 531A(pJHC1#4, pPH1JI) and the controls *V. anguillarum* 531A(pJHC1, pPH1JI) and *V. anguillarum* 531A. Figure 3 shows that there are no significant differences in the levels of FatA expressed in *V. anguillarum* 531A(pJHC1#4, pPH1JI), *V. anguillarum* 531A(pJHC1, pPH1JI), and *V. anguillarum* 531A in iron-rich or iron-limiting conditions. These results indicate that AngR is not involved in regulation of biosynthesis of FatA.

AngR complements an *entE E. coli* mutant. The AngR and EntE proteins share a motif (Fig. 1) which is also conserved among other enzymes of the firefly luciferase family (15a, 17, 29, 31). The EntE protein plays an essential role in the biosynthesis of the siderophore enterobactin, as a 2,3-dihydroxybenzoate-AMP ligase. Therefore, it was of interest to determine whether AngR could complement the *entE* mutation in *E. coli* AN93. Figure 4 shows that transformation of this enterobactin-deficient mutant with plasmid pJHCS531A*ptac*, carrying the *angR*$_{531A}$ gene, led to restoration of the iron uptake-proficient phenotype measured by the ability of this complemented mutant strain to grow in the presence of high concentrations of EDDA. Conversely,

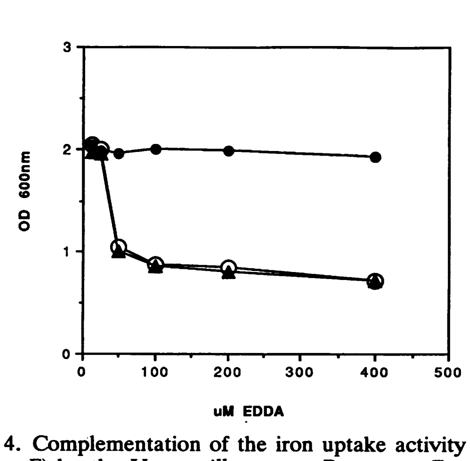

FIG. 4. Complementation of the iron uptake activity of *E. coli* AN93 (*entE*) by the *V. anguillarum angR*$_{775}$ gene. *E. coli* strains harboring pJHCS531A*ptac* (●), pJHCS531A*ptacNco* (▲), or the vector pKK223-3 (○) were cultured overnight in L broth containing different EDDA concentrations. Cell growth was recorded spectrophotometrically at 660 nm. OD, optical density.

neither pJHCS531A*ptacNco*, which carries a truncated $angR_{531A}$, nor the vector pKK223-3 was able to complement this mutation. In a separate experiment, *E. coli* AN93 was compared with *E. coli* AN93(pKK223-3), and the curves were identical, while the wild-type parent strain AB1515 grew in the same iron-limiting conditions (data not shown). The results described in this section suggest that besides its regulatory role, AngR may have an enzymatic function related to that of 2,3-dihydroxybenzoate-AMP ligase.

DISCUSSION

V. anguillarum owes its high-virulence phenotype to the presence of the plasmid-mediated iron uptake system composed of the siderophore anguibactin and a receptor for iron-anguibactin complexes. It was previously shown that the *angR* gene was required for anguibactin production (32). Therefore, AngR is an important factor in the pathogenicity of *V. anguillarum*.

The *V. anguillarum angR* gene encodes a regulatory protein, AngR, of 1,048 amino acids (15). Inspection of the amino acid sequence of AngR demonstrated that it has two leucine zippers each followed by a helix-turn-helix motif. In eukaryotic regulators, a leucine zipper is often followed by a basic DNA binding region to form the so-called bZIP. These proteins interact with another regulatory protein molecule and bind DNA (4, 22, 23, 30, 38, 39). Therefore, one or both of the leucine zipper–helix-turn-helix domains found in AngR might play a role in protein and DNA recognition, and specifically, one of them may be involved in the synergistic action found between AngR and the regulator TAF (25, 37). The presence of leucine zippers in prokaryotic proteins other than AngR was also recently reported (8, 16, 21).

We recently identified natural isolates carrying pJM1-like plasmids, several of them showing a more effective iron uptake system than the prototype strain *V. anguillarum* 775, which possesses the plasmid pJM1 (25, 37). The *angR* gene was identified as responsible for the difference in at least one strain, *V. anguillarum* 531A. In this study, we sequenced $angR_{531A}$, and the only difference found was a substitution of His (in $AngR_{775}$) for Asn (in $AngR_{531A}$) in amino acid 267. It was of interest that the substituted amino acid is located between the first leucine zipper and helix-turn-helix motif (Fig. 1), a structure that is often found in eukaryotic regulators (see above). Other amino acid changes were engineered by site-directed mutagenesis in position 267 of the protein. Different MICs of EDDA were obtained for the different mutations (see Results). Substitution of the His with Leu or Gln generated AngR derivatives that conferred to the *V. anguillarum* strain carrying them MICs of EDDA lower than that for the wild type, indicating that this is an important location for AngR activity. Whether this region is essential for either its regulatory, enzymatic, or both activities is presently being investigated. The location of the mutation between a leucine zipper and a helix-turn-helix motif could indicate that its effect is on AngR's regulatory role by modifying protein-protein or DNA-protein interactions involving AngR.

Besides its regulatory function, an enzymatic activity was identified for AngR by complementation of an *E. coli entE* mutant with a recombinant clone harboring *angR*. Our results showed that the activity was related to the *E. coli* 2,3-dihydroxybenzoate-AMP ligase. These results are in agreement with recent reports that placed AngR within a group of enzymes that have a common motif (15a, 17, 29, 31). Together with AngR and the *E. coli* 2,3-dihydroxybenzoate-AMP ligase, 17 more proteins are included in this group, among them the *B. brevis* gramicidin S synthetase I and tyrocidine synthetase I, the *P. pyralis* luciferase, the 4-coumarate:coenzyme A ligase, and the D-alanine-activating enzyme. Heaton and Neuhaus (17) recently defined a motif common to all 19 proteins, GXXGXPK, which could be the phosphate-binding loop of the ATP-binding site. Studies of the physical parameters of wild-type and mutant derivatives of AngR will establish the specific domains associated with the regulatory and enzymatic activities.

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