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# Chromosome-Mediated 2,3-Dihydroxybenzoic Acid Is a Precursor in the Biosynthesis of the Plasmid-Mediated Siderophore Anguibactin in *Vibrio anguillarum*

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**We have isolated a recombinant clone harboring the chromosomal *aroC* gene, encoding chorismate synthase, from *Vibrio anguillarum* 775 by complementation of the *Escherichia coli aroC* mutant AB2849 which was transfected with a cosmid gene bank of the plasmidless *V. anguillarum* H775-3. The nucleotide sequence was determined, and an open reading frame that corresponds to a protein of 372 amino acids was found. The calculated mass of 40,417 Da was correlated with the size of the *V. anguillarum aroC* product detected in vitro. The homology of the *V. anguillarum aroC* gene to the *aroC* genes of *E. coli* and *Salmonella typhi* is 68% at the nucleotide level and 78% at the protein level. The expression of the *aroC* transcript is not regulated by iron, as determined by Northern (RNA) blot hybridization analysis. After insertion of an antibiotic resistance gene cassette within the cloned *aroC* gene, an *aroC* mutant of *V. anguillarum* was generated by allelic exchange. This mutant is deficient in the production of 2,3-dihydroxybenzoic acid (2,3-DHBA). Our bioassay and complementation experiments with this mutant demonstrate that the chromosome-mediated 2,3-DHBA is a precursor of the pJM1 plasmid-mediated siderophore anguibactin.**

*Vibrio anguillarum* is an important fish pathogen which causes a highly fatal hemorrhagic septicemic disease in salmonid fish (10). One important component of the virulence of *V. anguillarum* 775 is the iron uptake system encoded by the 65-kb pJM1 plasmid. This system consists of the siderophore anguibactin and a specific iron transport system that includes a membrane receptor for the ferric iron-anguibactin complex (2, 3, 11–13, 18, 20).

Anguibactin has been purified from culture supernatant of *V. anguillarum* 775 (1) and identified as ω-*N*-hydroxy-ω [[2′-(2″,3″-dihydroxy-phenyl) thiazolin-4′-yl]-carboxy]histamine (18). The molecule of anguibactin possesses a 2,3-dihydroxybenzoic acid (2,3-DHBA) moiety, a compound that in *V. anguillarum* is produced independently of the presence of the pJM1 plasmid (1). The biosynthetic pathway for anguibactin in *V. anguillarum* is still unknown. However, since the *aroC* gene encoding chorismate synthase, which catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate to chorismic acid, is a central precursor for aromatic compound biosynthesis, including that of 2,3-DHBA (42), we cloned and sequenced this gene from *V. anguillarum* to identify whether the chromosome-mediated 2,3-DHBA is a precursor for anguibactin biosynthesis. The *aroC* gene has also been cloned and characterized from *Escherichia coli*, *Bacillus subtilis*, *Neurospora crassa*, and *Salmonella typhi* (9, 22, 43) and from the higher plant *Corydalis sempervirens* (30). In this study, we generated a 2,3-DHBA-deficient mutant of *V. anguillarum* by inserting an antibiotic resistance gene cassette, interrupting the cloned *aroC* gene. Analysis of the *V. anguillarum aroC* mutant demonstrated that it was impaired in the biosynthesis of anguibactin. This deficiency could be complemented by the cloned *aroC* gene, showing that the chromosome-mediated 2,3-DHBA is a precursor in the biosynthesis of this plasmid-mediated siderophore.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria broth or on Luria broth solidified with 1.5% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) (23). *V. anguillarum* strains were grown at 26°C in Trypticase soy broth supplemented with 1% (wt/vol) sodium chloride (TSBS), in Trypticase soy agar supplemented with 1% (wt/vol) sodium chloride (TSAS) or in M9 minimal medium with no extra sodium chloride (23) supplemented with 0.2% (wt/vol) Casamino acids (Difco Laboratories). Iron-limiting conditions were established by adding the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic) acid (EDDA) (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 100 μM for TSBS or 2 μM for M9 minimal medium. Iron-rich conditions were obtained by adding $FeCl_3$ up to 50 μM to M9 minimal medium or by using TSBS without the addition of $FeCl_3$ as the growth medium. Antibiotics were added to the culture medium at the following concentrations: ampicillin (Ap), 500 μg/ml; kanamycin (Km), 200 μg/ml; tetracycline (Tc), 20 μg/ml; and gentamicin (Gm), 50 μg/ml. Phenylalanine, tryptophan, tyrosine, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid (all from Sigma Chemical Co.) were all added to the M9 minimal medium at 20 μg/ml.

**Isolation of plasmid and chromosomal DNAs, restriction endonuclease analysis, and Southern blot hybridization.** Plasmid DNA was prepared by the method of Birnboim and Doly (5). Chromosomal DNA was isolated as described by Hull et al. (17). Restriction endonuclease digestion of DNA was carried out under the conditions recommended by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Southern blot hybridizations were done under conditions previously described (41). A 2.4-kb *Eco*RI-*Pst*I fragment of

TABLE 1. Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant genotype | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| HB101 | F⁻ *thr-1 leuB6 dam-4 thi-1 hsdS1 lacY1 tonA21* λ⁻ *supE44* | 7 |
| JM109 | *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_k^-$ $m_k^+$) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*$^q$ *lacZ*ΔM15] | 45 |
| AB2849 | *tsx-357 supE42* λ⁻ *aroC355* | 25 |
| *S. typhimurium enb7* | Deficient in the biosynthesis of enterobactin; can use enterobactin as well as 2,3-DHBA | 26 |
| *V. anguillarum* | | |
| 775 | Natural isolate, prototype (pJM1) | 11 |
| H775-3 | Plasmidless derivative of 775 | 13 |
| 775::Tn*1*-5 | Iron-anguibactin receptor proficient; anguibactin deficient (pJHC9-16) | 41 |
| 775::Tn*1*-6 | Iron-anguibactin receptor; anguibactin deficient (pJHC9-8) | 41 |
| 531A | Natural isolate, prototype (pJHC1) | 38 |
| 531A-QC5 | *aroC* mutant isolated by allelic exchange | This work |
| Plasmids | | |
| pBCSK+ | Cloning vector | Stratagene[a] |
| M13mp18 | Cloning vector | 24 |
| M13mp19 | Cloning vector | 24 |
| pBR325 | Cloning vector | 6 |
| pBR325* | Modified pBR325, with *Cla*I and *Sal*I sites inactivated by blunt ligation of *Cla*I-*Sal*I fragment of pBR325 | This work |
| pVK100 | Cosmid vector | 19 |
| pVK102 | Cosmid vector | 19 |
| pUC4K | Contains Tn*903 aph* in a restriction-site-mobilizing element | 40 |
| pPH1JI | Incompatible with pQC2.3; encodes Gm$^r$ | 16 |
| pATC1 | ca. 30-kb genomic insert of *V. anguillarum* harboring *aroC* gene in cosmid pVK100 | This work |
| pQC2 | 4.2-kb *Eco*RI fragment harboring *V. anguillarum aroC* gene cloned in pBR325 | This work |
| pQC2.1 | 4.2-kb *Eco*RI fragment harboring *V. anguillarum aroC* gene cloned in pBR325* | This work |
| pQC2.2 | Fragment carrying Km$^r$ gene from pUC4K inserted in the *Sal*I site of pQC2.1 | This work |
| pQC2.3 | Insertionally mutated *aroC* from pQC2.2 cloned in pVK100 | This work |
| pQC3 | 2.4-kb *Eco*RI-*Pst*II fragment harboring *V. anguillarum aroC* gene cloned in pBR325 | This work |
| pQC4 | *aroC* deletion mutant | This work |

[a] Stratagene, La Jolla, Calif.

pQC3 and the 1.3-kb *Bam*HI fragment carrying the *aph* gene from pUC4K were used as probes and labeled with [$^{32}$P]dATP as described by Feinberg and Vogelstein (15). DNA fragments were gel purified by using a Geneclean kit, following the supplier's instructions (Bio 101, Inc., La Jolla, Calif.).

**Construction of a cosmid gene bank and transductions.** A cosmid gene bank of *Hin*dIII-cleaved *V. anguillarum* H775-3 genomic DNA was constructed by using the cosmid vector pVK102 (37). Transduction of the *E. coli aroC* mutant AB2849 with the gene bank was performed as previously described (37).

**In vitro transcription-translation and sequencing analysis.** In vitro transcription-translation was performed by following the supplier's recommendation with a prokaryotic DNA-directed translation kit from Amersham (Arlington Heights, Ill.). The labeled protein products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) (2). Autoradiography was carried out at −70°C for 24 h. A series of deletion clones from the 2.4-kb *Eco*RI-*Pst*I fragment of pQC3 were constructed in pBCSK+, M13mp18, and M13mp19 vectors. Sequencing of double- or single-stranded DNA was performed by the dideoxy chain termination method (29) with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) with the T3 and T7 sequencing primers for pBCSK+ clones and the universal primer for M13 clones, as well as specific synthetic primers. DNA sequencing was analyzed with the Genetics Computer Group (Madison, Wis.) computer software, version 7.

**Allelic exchange.** The 1.3-kb fragment carrying the *aph* (Km$^r$) gene from plasmid pUC4K was inserted into the *Sal*I site of pQC2.1 to produce pQC2.2 (Fig. 1). A 4.2-kb *Eco*RI fragment of pQC2.2 was cloned into the incompatibility P vector pVK100. The resulting plasmid pQC2.3 was introduced into *V. anguillarum* 531A by conjugation as described previously (35). Then, plasmid pQC2.3 was segregated by the introduction of plasmid pPH1JI and selection on TSAS plates containing Gm and Km. Gm$^r$ and Km$^r$ colonies were further screened by using two sets of TSAS plates. One set contained Km and Gm, and the other contained Tc. Those colonies that had a Gm$^r$ Km$^r$ Tc$^s$ phenotype were selected for further analysis.

**Arnow test and bioassay.** The Arnow test was used to determine the amount of extracellular phenolic compounds such as 2,3-DHBA (4). The presence of 2,3-DHBA was also determined by a bioassay using the *enb7* strain of *Salmonella typhimurium* LT-2 deficient in the biosynthesis of 2,3-DHBA and enterobactin. The bioassay was carried out as described previously (21).

**Bioassays for detection of siderophore activity.** The siderophore activity was detected by testing the abilities of

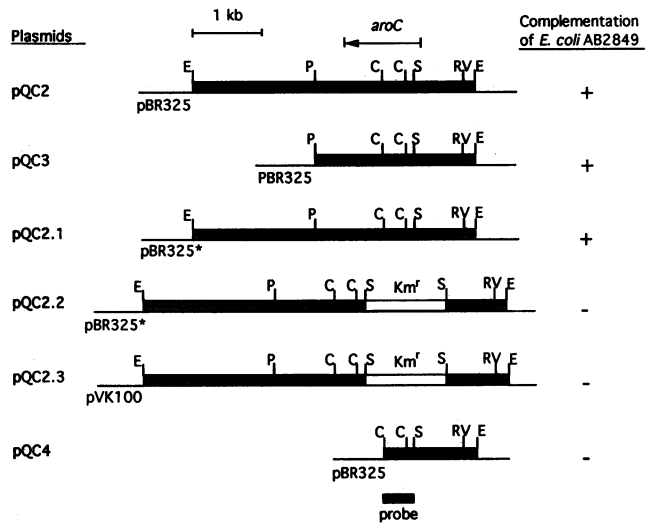


FIG. 1. Subcloning of the *V. anguillarum aroC* gene and construction of *aroC* mutants in vitro. Only the cloned DNA is shown in detail. *V. anguillarum* DNA (hatched boxes), the Km$^r$ cassette from pUC4K (open box), and vector DNA (solid lines) are represented. Restriction enzyme sites include *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Eco*RV (RV), and *Sal*I (S). The location and orientation of the cloned *V. anguillarum aroC* gene are indicated by the arrow. The probe used in Northern blot hybridization analysis is indicated by the solid bar. The abilities of recombinant clones to complement *E. coli aroC* mutant AB2849 to allow iron uptake proficiency and growth in M9 minimal medium without adding aromatic compounds are indicated (+, present; −, not present).

supernatants or cell cultures from *V. anguillarum* strains to cross-feed either indicator strain 775::Tn*1*-5, which is anguibactin deficient and receptor proficient, or strain 775::Tn*1*-6, which is both anguibactin and receptor deficient. *V. anguillarum aroC* mutant 531A-QC5 was cultured in TSBS medium containing 100 μM EDDA or 100 μM EDDA plus 100 μM 2,3-DHBA for 24 h at 26°C. Supernatants were obtained from equal amounts of cell cultures. Cell density was determined by the optical density at 600 nm. An overnight culture of the indicator strains (either 775::Tn*1*-6 or 775::Tn*1*-5) was inoculated (1:100) into 1 ml of M9 minimal medium containing 200 μl of supernatant and a final concentration of 25 μM EDDA. Cells were incubated at 26°C for 20 h and the optical densities at 600 nm were determined. Alternatively, cells were cultured overnight at 26°C in M9 minimal medium containing 5 μg of Tc per ml for strain 531A-QC5(pQC3) or 200 μg of Ap per ml for 775::Tn*1*-5 and 775::Tn*1*-6. The same amount of cells from the overnight culture of 531A-QC5(pQC3), along with either 775::Tn*1*-5 or 775::Tn*1*-6, was added to 3 ml of M9 minimal medium containing 300 μM EDDA, without adding any antibiotics. Cells were incubated at 26°C for 24 h, and 0.1 ml of diluted cell culture ($10^3$ cells per ml) was plated on TSAS plates. Colonies from these plates were further screened by using two sets of TSAS plates. One set contained Tc (5 μg/ml), selected for 531A-QC5(pQC3); the other contained Ap (1 mg/ml), selected for 775::Tn*1*-5 or 775::Tn*1*-6. Colonies from each plate were counted, and the percentage of each kind of cells was calculated.

**Northern (RNA) blot analysis.** *V. anguillarum* strains were grown in M9 minimal medium containing either 50 μM $FeCl_3$ or 2 μM EDDA at 26°C. Total RNA was isolated as described by Summers (32). RNA samples were electrophoresed on 1.2% (wt/vol) formaldehyde–agarose gels and transferred to Nytran membranes as described by Thomas (33). Equal loading and transfer of RNA were assessed by methylene blue staining of membranes. Blots were prehybridized at 42°C for 2 h and hybridized overnight at 42°C with a 420-bp *Cla*I-*Sal*I fragment of pQC3, located within the *aroC* coding region. Blots were washed twice for 15 min at room temperature in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, twice for 15 min in 0.1× SSC–0.1% SDS, and finally once for 30 min in 0.1× SSC–0.1% SDS at 65°C. Membranes were exposed to Kodak XAR film at −70°C for 6 to 24 h. As an internal control, the same blots were washed with 50% formamide in 6× SSPE (1× SSPE is 0.18 NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7]) at 65°C for 2 h to remove the original probe and reprobed with the 5.7-kb *Eco*RI-*Pst*I fragment carrying the *fatA* gene (2), as described above. Probes were prepared as described by Feinberg and Vogelstein (15).

**Nucleotide sequence accession number.** The nucleotide and predicted amino acid sequences of the *V. anguillarum aroC* gene will appear in the EMBL and GenBank sequence libraries under accession number L29562.

## RESULTS

**Cloning of the *V. anguillarum aroC* gene.** The *E. coli aroC* mutant AB2849, which could not grow under iron-limiting conditions, was transfected with a *V. anguillarum* H775-3 cosmid gene library. Therefore, recombinants which complemented the *aroC* lesion of AB2849 to iron uptake proficiency were selected by plating infected cells on L-agar plates containing 300 μM EDDA and 25 μg of Tc per ml. Complementation of the *E. coli aroC* mutant AB2849 with recombinant clones was further confirmed by testing infected cells for growth in M9 minimal medium without adding any aromatic compounds. The recombinant plasmid from one of the growing colonies, pATC1, was isolated and analyzed. It had a ca. 30-kb insert of the *V. anguillarum* genomic DNA. Its complementation ability was confirmed by retransformation of *E. coli* AB2849. Random subcloning of *Eco*RI fragments of pATC1 into the plasmid vector pBR325 yielded the recombinant plasmid pQC2 with a 4.2-kb insert that was still capable of complementing *E. coli* AB2849 (Fig. 1). After the restriction endonuclease mapping of pQC2, further subcloning from the *Pst*I and *Eco*RI sites of pQC2 generated recombinant plasmid pQC3 with a 2.4-kb genomic insert that could also complement the *E. coli aroC* mutant AB2849 (Fig. 1) and thus must contain the *V. anguillarum aroC* gene.

**Sequencing analysis of the *V. anguillarum aroC* gene.** To obtain the nucleotide sequence of the *aroC* gene from *V. anguillarum*, a series of deletion plasmids from the 2.4-kb *Eco*RI-*Pst*I fragment of pQC3 were constructed in pBCSK+ and M13 vectors. By using these derivatives of pQC3, we sequenced 1.8 kb of DNA including the *aroC* gene. Both DNA strands were sequenced to confirm the nucleotide sequence in both directions. Analysis of the DNA sequence of the 1.8-kb stretch of DNA revealed an open reading frame encoding a protein of 372 amino acids (Fig. 2) with a calculated mass of 40,417 Da. The open reading frame of the *V. anguillarum aroC* gene has two possible ATG start codons at positions 598 and 631 and the TAA stop codon at position 1714. The putative −10 promoter element from positions 557 to 562 has 66.7% identity with the −10 consensus sequences (27). The putative −35 element from positions 531 to 536 has 83.3% identity with the −35 consensus sequences (27). Two sequences close to the consensus *E. coli* ribosome binding sites GGAGG (31) were

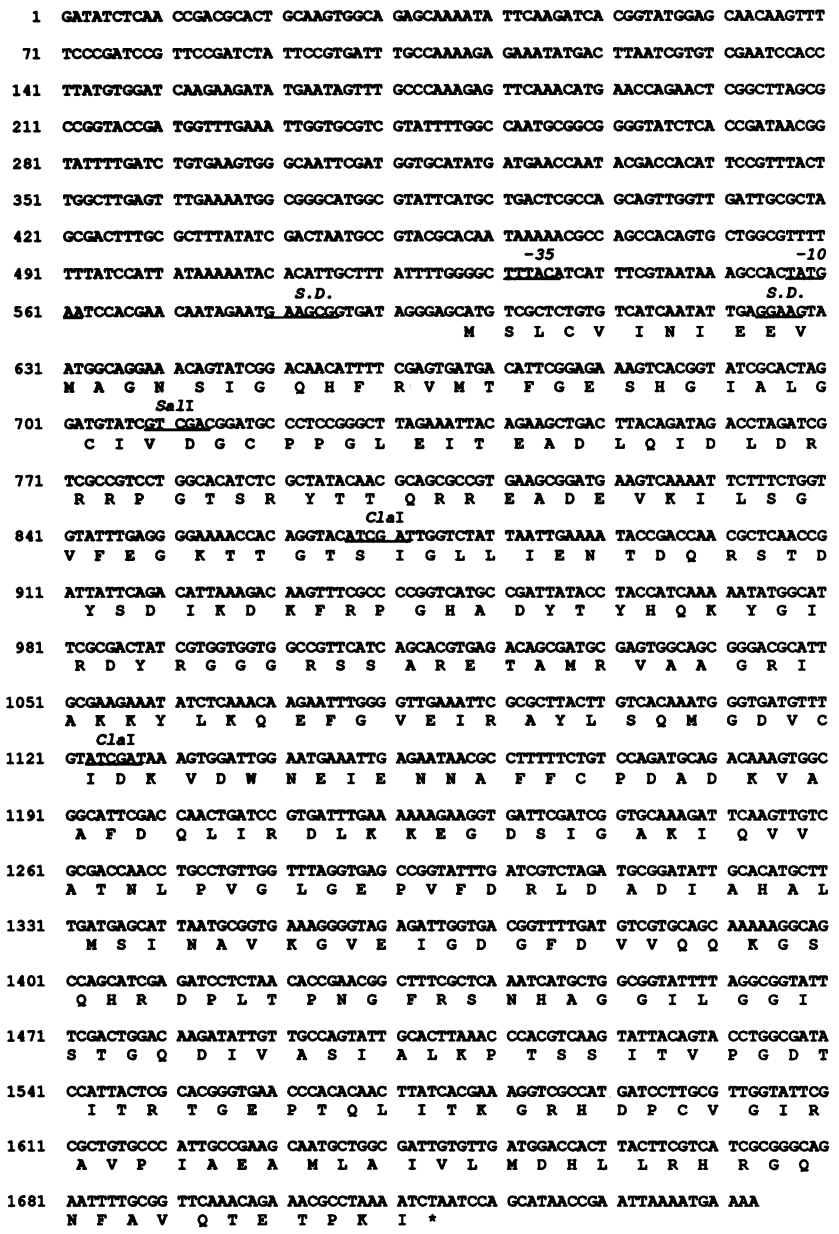


FIG. 2. Nucleotide and amino acid sequences of the *V. anguillarum aroC* gene. The putative −10 and −35 promoter elements, the Shine-Dalgarno-like sequences (S.D.), the *Sal*I site used to construct the insertion mutant, and *Cla*I sites are underlined.

found at positions 579 to 586 and 624 to 628. The *Sal*I site used to construct the *aroC* insertion mutant, as shown in Fig. 1, was located 112 bp downstream from the first possible start codon of the open reading frame (Fig. 2).

The sequences of the *V. anguillarum aroC* gene and other *aroC* genes were compared for homology. Figure 3 shows the comparison of the deduced amino acid sequence of the *V. anguillarum* AroC protein with the *E. coli* and *S. typhi* AroC amino acid sequences. There is a 78% identity at the amino acid level, while there is a 68% identity at the nucleotide level in the entire coding region including the upstream region.

**Mutagenesis and polypeptide expression from *aroC* recombinants in vitro.** An insertion mutation of the cloned *V. anguillarum aroC* gene was constructed in vitro (Fig. 1). A *Sal*I fragment including the $Km^r$ cassette from plasmid pUC4K was inserted into the *Sal*I site of plasmid pQC2.1 obtaining pQC2.2. A deletion derivative, pQC4, was also generated by cloning the *Cla*I-*Eco*RI fragment from pQC2 into plasmid pBR325 (Fig. 1). The polypeptide encoded by the *aroC* recombinants was detected by in vitro transcription-translation and SDS-PAGE (Fig. 4). The *aroC*-complementing plasmids pQC3 and pQC2.1 each produced a 40-kDa polypeptide (Fig. 4, lanes 2 and 3), which was correlated with the predicted size from the sequence analysis (Fig. 2) and the size of the *E. coli aroC* product (9). This polypeptide was absent from the transcription-translation mixture of the mutants pQC2.2 and pQC4 (Fig. 4, lanes 4 and 5) and the control plasmid vector pBR325 (Fig. 4, lane 1). Both derivatives pQC2.2 and pQC4

```
     1                                                            50
EC   .......... .MAGNTIGQL FRVTTFGESH GLALGCIVDG VPPGIPLTEA
ST   .......... .MAGNTIGQL FRVTTFGESH GLAVGGIVDG VPPGIPLTEA
VA   MSLCVINIEE VMAGNSIGQH FRVMTFGESH GIALGCIVDG CPPGLEITEA
                 **** ***  *** ****** * * * ****  ***   ***
     51
                                                             100
EC   DLQHDLDRRR PGTSRYTTQR REPDQVKILS GVFEGVTTGT SIGLLIENTD
ST   DLQHDLDRRR PGTSRYTTQR REPDQVKILS GVFDGVTTGT SIGLLIENTD
VA   DLQIDLDRRR PGTSRYTTQR READEVKILS GVFEGKTTGT SIGLLIENTD
     *** ****** ********** ** * ***** *** * **** **********
     101
                                                             150
EC   QRSQDYSAIK DVFRPGHADY TYEQKYGLRD YRGGGRSSAR ETAMRVAAGA
ST   QRSQDYSAIK DVFRPGHADY TYEQKYGLRD YRGGGRSSAR ETAMRVAAGA
VA   QRSTDYSDIK DKFRPGHADY TYHQKYGIRD YRGGGRSSAR ETAMRVAAGA
     *** *** ** * ******** ** **** ** ********** **********
     151
                                                             200
EC   IAKKYLAEKF GIEIRGCLTQ MGDIPLDIKD WSQVEQNPFF CPDPDKIDAL
ST   IAKKYLAEKF GIEIRGCLTQ MGDIPLEIKD WRQVELNPFF CPDADKLDAL
VA   IAKKYLKQEF GVEIRAYLSQ MGDVCIDKVD WNEIENNAFF CPDADKVAAF
     ******   * * ***  * * ***       * *   * * ** *** **  *
     201
                                                             250
EC   DELMRALKKE GDSIGAKVTV VASGVPAGLG EPVFDRLDAD IAHALMSINA
ST   DELMRALKKE GDSIGAKVTV MASGVPAGLG EPVFDRLDAD IAHALMSINA
VA   DQLIRDLKKE GDSIGAKIQV VATNLPVGLG EPVFDRLDAD IAHALMSINA
     * * * **** *******  *  *   * *** ********** **********
     251
                                                             300
EC   VKGVEIGDGF DVVALRGSQN RDEITKDGFQ SNHAGGILGG ISSGQQIIAH
ST   VKGVEIGEGF NVVALRGSQN RDEITAQGFQ SNHAGGILGG ISSGQHIVAH
VA   VKGVEIGDGF DVVQQKGSQH RDPLTPNGFR SNHAGGILGG ISTGQDIVAS
     ******* **  **   ***  **  *  **  ********** ** ** * *
     301
                                                             350
EC   MALKPTSSIT VPGRTINRFG EEVEMITKGR HDPCVGIRAV PIAEAMLAIV
ST   MALKPTSSIT VPGRTINRMG EEVEMITKGR HDPCVGIRAV PIAEAMLAIV
VA   IALKPTSSIT VPGDTITRTG EPTQLITKGR HDPCVGIRAV PIAEAMLAIV
      ********** *** ** * * *    ***** ********** **********

     351                  373
EC   LMDHLLRQRA QNADVKTDIP RW.
ST   LMDHLLRHRA QNADVKTEIP RW.
VA   LMDHLLRHRG QNFAVQTETP KI.
     ******* *  **  * *  *
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FIG. 3. Alignment of the deduced amino acids encoded by the *aroC* genes from *E. coli*, *S. typhi*, and *V. anguillarum*. *, amino acid residues conserved across all species. EC, *E. coli* (9); ST, *S. typhi* (9); VA, *V. anguillarum* (this study).

lost both the ability to complement the *E. coli aroC* mutant AB2849 to iron uptake proficiency and to grow in M9 minimal medium without the addition of any aromatic compounds (Fig. 1).

**Generation of a *V. anguillarum aroC* mutant.** In order to get a 2,3-DHBA-deficient *V. anguillarum* strain, a *V. anguillarum* chromosomal *aroC* mutant was isolated by allelic exchange. The *Eco*RI fragment from plasmid pQC2.2 containing the insertionally inactivated *aroC* gene was recloned into the cosmid pVK100 to produce recombinant plasmid pQC2.3 (Fig. 1). Plasmid pQC2.3 was introduced by conjugation into *V. anguillarum* 531A. We used strain 531A because it has a more active recombination system than strain 775 (37). The plasmid pQC2.3 was then segregated by the introduction of the incompatible plasmid pPH1JI in the presence of Gm for selection of the incoming plasmid pPH1JI and of Km for selection of the mutant gene. This procedure selects all those bacteria in which the plasmid-carried mutated *aroC* gene was exchanged into the chromosome by homologous recombination with the wild-type gene. $Km^r$ $Gm^r$ colonies were further screened for sensitivity to Tc. Several of the $Km^r$ $Gm^r$ $Tc^s$ colonies were tested for their abilities to grow in M9 minimal medium without the addition of any aromatic compounds. Like the *E. coli aroC* mutant, none of them could grow in M9 minimal medium without aromatic compounds (Table 2), and this deficiency

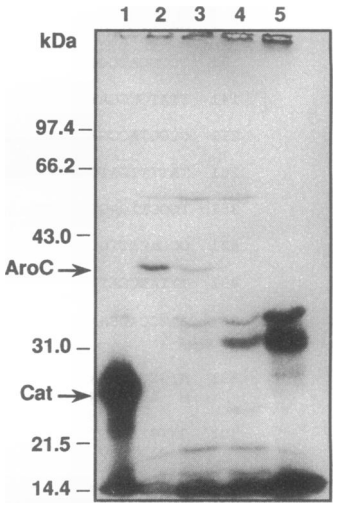


FIG. 4. In vitro transcription-translation and SDS-PAGE of plasmid-encoded proteins by *aroC* recombinants. The expression of the *aroC*-encoded polypeptide was investigated by in vitro transcription-translation. Lanes: 1, pBR325; 2, pQC3; 3, pQC2.1; 4, pQC2.2; 5, pQC4. The protein bands corresponding to the *V. anguillarum* chorismate synthase (AroC) and pBR325-encoded chloramphenicol acetyl transferase (Cat) are indicated. SDS-PAGE was carried out with a 12.5% (wt/vol) gel, and the autoradiography was carried out at −70°C for 24 h.

could be complemented by adding the mixture of aromatic compounds to M9 minimal medium (Table 2).

To prove that the correct double crossover recombinational event occurred between pQC2.3 and the chromosome of *V. anguillarum* 531A, one of the mutants, 531A-QC5, was analyzed by Southern blot hybridization by using the 2.4-kb *Eco*RI-*Pst*I fragment of pQC3 harboring the *aroC* gene (Fig. 5A) and the 1.3-kb *Bam*HI fragment of pUC4K harboring the $Km^r$ cassette as probes (Fig. 5B). In the blot probed against the *Eco*RI-*Pst*I fragment of pQC3, the hybridizing fragment from the *aroC* mutants pQC2.3 and 531A-QC5 (Fig. 5A, lanes 2 and 4) migrated more slowly than that from the wild-type pQC2.1 and H775-3 (Fig. 5A, lanes 1 and 3), since the *aroC* mutants carried the $Km^r$ cassette. In the blot probed with the *Bam*HI fragment of pUC4K, hybridization was detected in only the *aroC* mutants pQC2.3 and 531A-QC5 (Fig. 5B, lanes 2 and 4). The upper band in plasmid pQC2.3 (Fig. 5B, lane 2) is due to the $Km^r$ gene present in the vector pVK100 (19). Therefore,

TABLE 2. Catechol, 2,3-DHBA production, and growth of *V. anguillarum* strains[a]

| Strain | Growth in[b]: | | Catechol production[c] | 2,3-DHBA production[d] |
|---|---|---|---|---|
| | Fe-limiting M9 | M9 with aromatics | | |
| 531A | + | + | + | + |
| 531A-QC5 | − | + | − | − |
| 531A-QC5(pQC3) | + | + | + | ND |

[a] +, positive; −, negative.
[b] The strains were cultured in either M9 minimal medium without aromatic compounds under iron-limiting conditions or M9 minimal medium with a mixture of aromatic compounds (phenylalanine, tryptophan, tyrosine, *p*-aminobenzoic acid, and *p*-hydrobenzoic acid [all at 20 μg/ml]).
[c] Determined by Arnow test.
[d] Determined by ability to cross-feed *S. typhimurium enb7*. ND, not determined.

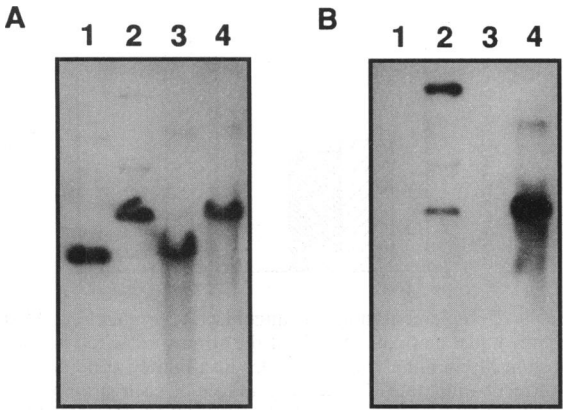


FIG. 5. Southern blot hybridization analysis of *V. anguillarum aroC* mutants. Plasmid and chromosomal DNAs were cleaved with *Eco*RI and then electrophoresed on 0.8% (wt/vol) agarose gel. Lanes (blot A and blot B): 1, pQC2.1; 2, pQC2.3; 3, *V. anguillarum* H775-3; 4, *V. anguillarum* 531A-QC5. Blot A was probed with the 2.4-kb *Eco*RI-*Pst*I fragment of pQC3 which harbored the *V. anguillarum aroC* gene, and blot B was probed with the 1.3-kb *Bam*HI fragment of pUC4K which carries the $Km^r$ cassette.

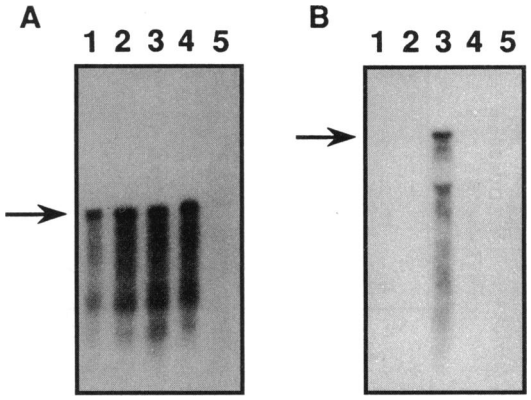


FIG. 6. Northern blot hybridization analysis of *aroC* transcript. Total RNAs were isolated from *V. anguillarum* strains cultured in M9 minimal medium containing either 50 μM $FeCl_3$ or 2 μM EDDA, except that 531A-QC5 was cultured in TSBS. Lanes: 1, H775-3 (EDDA); 2, H775-3 ($FeCl_3$); 3, 775(pJM1) (EDDA); 4, 775(pJM1) ($FeCl_3$); 5, 531A-QC5. (A) Blot probed with the 420-bp *Cla*I-*Sal*I fragment of pQC3. The 1.4-kb *aroC* transcript is indicated by the arrow. (B) Blot A washed to remove the *aroC* probe and reprobed with the 5.7-kb *Eco*RI-*Pst*I fragment harboring the *fatA* gene. The arrow indicates the 2.4-kb *fatA* transcript.

these results indicated that a correct allelic exchange between pQC2.3 and the chromosome of *V. anguillarum* 531A had taken place.

**Regulation of transcription of the *aroC* gene by iron.** It is known that the pJM1-encoded genes involved in the biosynthesis and transport of anguibactin in *V. anguillarum* are iron regulated (34). To investigate whether the chromosomal *aroC* gene is also regulated by iron, the expression of the *V. anguillarum aroC* transcript was analyzed by Northern blot hybridization. Total RNAs were isolated from *V. anguillarum* strains cultured in M9 minimal medium under iron-limiting or iron-rich conditions. A Northern blot of total RNA was probed with the 420-bp *Cla*I-*Sal*I fragment of pQC3 located within the *aroC* coding region (Fig. 1). Figure 6A shows that there are no differences between the levels of the 1.4-kb *aroC* transcript in *V. anguillarum* H775-3 and 775 strains grown under iron-limiting and iron-rich conditions (compare lane 1 with lane 2 and lane 3 with lane 4), while the *aroC* transcript was not detected in cells of the *V. anguillarum aroC* mutant 531A-QC5 generated by allelic exchange (Fig. 6A, lane 5). As a control for iron regulation, the same blot was washed to remove the *aroC* probe and reprobed with the 5.7-kb *Eco*RI-*Pst*I fragment harboring the *fatA* gene which has been shown to be iron regulated (3). The abundant 2.4-kb *fatA* transcript was detected only under iron-limiting conditions in the plasmid-containing 775 strain (Fig. 6B, lane 3). This result suggested that transcription of the *V. anguillarum aroC* gene is not regulated by iron.

**Lack of 2,3-DHBA production by the *V. anguillarum aroC* mutant.** To determine whether the *aroC* mutant is deficient in the production of 2,3-DHBA, the presence of phenolic compounds by the Arnow test and the presence of 2,3-DHBA by bioassays were examined. Cell-free supernatants obtained from cultures of the *aroC* mutant 531A-QC5 grown in TSBS containing 100 μM EDDA were negative by the Arnow test (Table 2), suggesting that this mutant did not produce phenolic compounds. Bioassays determined that this result was due to a deficiency in 2,3-DHBA production. The *enb7* mutant strain of *S. typhimurium* can use both enterobactin and 2,3-DHBA to support its growth under iron-limiting conditions (26). It has been shown that *V. anguillarum* 531A produces both anguibactin and 2,3-DHBA and that anguibactin does not cross-feed *S. typhimurium enb7* (1, 14). Our results clearly showed that the *V. anguillarum aroC* mutant was deficient in 2,3-DHBA production, because the supernatants from the wild-type strain 531A promoted the growth of *enb7*, whereas the supernatants from the *aroC* mutant 531A-QC5 did not (Table 2). To determine whether the cloned *aroC* could complement the 2,3-DHBA deficiency of *V. anguillarum* 531A-QC5, pQC3 was introduced into 531A-QC5 by conjugation. The presence of pQC3 in 531A-QC5 enabled the transconjugant cells to grow in M9 minimal medium under iron-limiting conditions (Table 2). Furthermore, cell-free supernatants of 531A-QC5(pQC3) cultured in M9 minimal medium were positive by the Arnow test (Table 2) indicating that the 2,3-DHBA deficiency of the *aroC* mutant could be complemented with the wild-type *V. anguillarum aroC* clone.

**2,3-DHBA is a precursor in anguibactin biosynthesis.** It is known that 2,3-DHBA is a precursor of enterobactin in *E. coli* (8), and it has been shown that the *V. anguillarum* plasmidless H775-3 strain produces abundant 2,3-DHBA (1). It is thus possible that 2,3-DHBA is also a precursor in anguibactin biosynthesis. Since the *V. anguillarum aroC* mutant lost the ability to grow under iron-limiting conditions, we investigated whether adding 2,3-DHBA would reverse this deficiency. The *aroC* mutant 531A-QC5 was cultured in TSBS medium supplemented with enough EDDA (200 μM) to inhibit cell growth. As shown in Fig. 7, the *aroC* mutant grew poorly under iron-limiting conditions, whereas it grew well when 2,3-DHBA was added to the iron-limited medium. The wild-type strain 531A harboring pPH1JI also grew well under both conditions (data not shown). This suggested that 2,3-DHBA may serve as a precursor to produce anguibactin and thus promote cell growth under iron-limiting conditions.

We next determined whether the addition of 2,3-DHBA to iron-limited medium enables the *V. anguillarum aroC* mutant to produce anguibactin. We measured siderophore activity by testing the abilities of cell-free supernatants from cultures of the *V. anguillarum aroC* mutant supplemented with EDDA

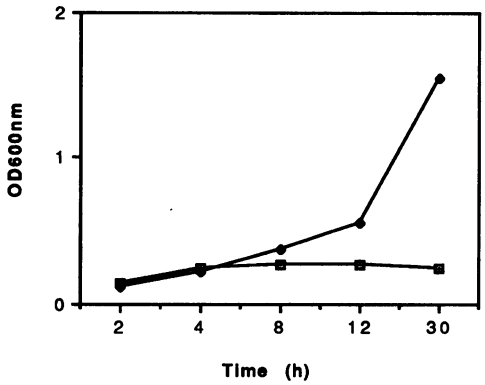


FIG. 7. Growth curve of *V. anguillarum aroC* mutant. The *V. anguillarum aroC* mutant 531A-QC5 was cultured in TSBS medium supplemented with 200 μM EDDA (dotted square) or supplemented with 200 μM EDDA plus 100 μM 2,3-DHBA (solid diamond). OD, optical density.

alone or with EDDA plus 2,3-DHBA to cross-feed different *V. anguillarum* mutants defective in the pJM1-mediated iron uptake system. Mutants deficient in either the production of anguibactin, the receptor complex, or both were used. Figure 8 shows that supernatants from the *aroC* mutant 531A-QC5 cultured in TSBS medium supplemented with 100 μM EDDA did not promote growth of either mutant under iron-limiting conditions, whereas supernatants from 531A-QC5 cultured in TSBS medium supplemented with 100 μM EDDA plus 100 μM 2,3-DHBA promoted only the growth of the receptor-proficient mutant 775::Tn*1*-5 (Fig. 8), as did the wild-type strain 531A harboring pPH1JI (data not shown). This demonstrated that 2,3-DHBA can be used by the *V. anguillarum aroC* mutant 531A-QC5 to produce anguibactin. Furthermore, we tested whether the presence of the wild-type *aroC* clone pQC3 in the *aroC* mutant 531A-QC5 enabled the cell to produce anguibactin. In this case, we performed the experiment by preparing a mixture (1:1 ratio) of the *aroC* mutant strain 531A-QC5 harboring pQC3 which carried the cloned *aroC* gene with either of the indicator strains 775::Tn*1*-5 (receptor proficient) or 775::Tn*1*-6 (receptor deficient). The results, shown in Fig. 9, demonstrated that the original ratio of 1:1 was well conserved when 531A-QC5(pQC3) and the receptor-

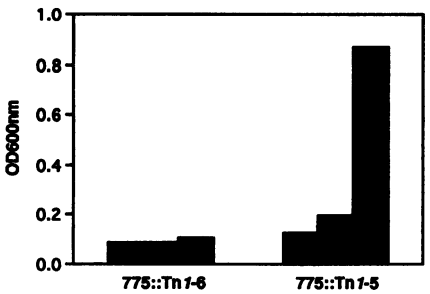


FIG. 8. Bioassay of anguibactin production. Anguibactin activity was determined by testing the abilities of cell-free supernatants from cultures of *V. anguillarum aroC* mutant 531A-QC5 to cross-feed different mutants defective in the iron uptake system. ■, TSBS medium as supernatants; ▨, supernatants from 531A-QC5 cultured in TSBS medium supplemented with 100 μM EDDA; ▩, supernatants from 531A-QC5 cultured in TSBS medium supplemented with 100 μM EDDA plus 100 μM 2,3-DHBA. OD, optical density.

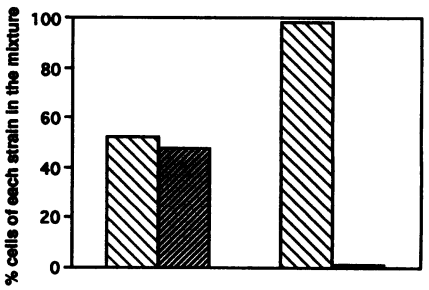


FIG. 9. Complementation of anguibactin production. Mixtures containing equal amounts of cells of the strains indicated below were cultured in M9 minimal medium under iron-limited conditions. After 24 h of incubation at 26°C, the percentages of each strain of cells in each mixture were determined. ▧, 531A-QC5(pQC3); ▨, 775::Tn*1*-5; ■, 775::Tn*1*-6.

proficient 775::Tn*1*-5 were cocultured in M9 minimal medium under iron-limiting conditions. Conversely, almost 100% of the cells were 531A-QC5(pQC3) when the mixture contained the receptor-deficient 775::Tn*1*-6. The control alone, either 775::Tn*1*-5 or 775::Tn*1*-6, could not grow under these conditions. This result further supported the hypothesis that 2,3-DHBA must be a precursor for anguibactin biosynthesis.

## DISCUSSION

The siderophore anguibactin mediated by the pJM1 plasmid of the fish pathogen *V. anguillarum* 775 is an important virulence factor (44). Transposition mutagenesis analysis identified genetic regions encoding products involved in the biosynthesis of anguibactin (34, 36). However, the specific biosynthetic genes and the pathway of anguibactin biosynthesis have not been identified as yet. Our studies showed that both hydroxamate and catechol groups are present in the anguibactin molecule (1). Later, physical and chemical studies not only confirmed these results but also led to the elucidation of the structure of this siderophore as ω-*N*-hydroxy-ω [[2′-(2″,3″-dihydroxy-phenyl) thiazolin-4′-yl]-carboxy]histamine (1, 18). It is thus possible that its backbone is derived from ω-*N*-hydroxy-histamine, cysteine, and 2,3-DHBA. Both the presence of 2,3-DHBA in the anguibactin molecule and the fact that *V. anguillarum* 775 produces abundant chromosome-mediated 2,3-DHBA, which did not show any siderophore activity (1), suggested that 2,3-DHBA was an intermediary in the biosynthesis of anguibactin. Therefore, to initiate the characterization of the anguibactin biosynthetic pathway, we first investigated whether 2,3-DHBA is a precursor of anguibactin, as it is in the case of enterobactin biosynthesis in *E. coli* (8).

Our strategy to achieve this goal was to generate *V. anguillarum* chromosomal mutants deficient in the production of 2,3-DHBA and assess whether the mutation resulted in a concomitant loss of their ability to produce anguibactin. To perform our mutagenesis analysis, we chose the *aroC* gene analog in *V. anguillarum*, since chorismate synthase encoded by this gene catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate to chorismic acid, which is a common precursor of aromatic compounds such as 2,3-DHBA (42). In order to obtain a *V. anguillarum aroC* mutant, we first cloned the *V. anguillarum aroC* gene as part of a 2.4-kb *Eco*RI-*Pst*I fragment from a *V. anguillarum* chromosomal library by complementation of an *E. coli aroC* mutant. Sequencing analysis of the *V. anguillarum aroC* gene revealed one open reading frame with two possible start sites, 30 nucleotides apart, encoding a

protein of 372 or 361 amino acids. There are Shine-Dalgarno sequences in front of both start codons (31). Comparison of the deduced amino acid sequence of the *V. anguillarum aroC* gene with those of the *E. coli* and *S. typhi* analogs suggests that the second start site in the open reading frame is used (Fig. 3). However, this prediction can only be determined by primer extension analysis and amino acid sequencing, which are being carried out. The cloned *V. anguillarum aroC* gene encoded a polypeptide of 40 kDa, which correlated with the predicted size from sequence analysis and is similar to that of the *E. coli aroC* product (9). Northern blot hybridization demonstrated that transcription of the chromosomal *aroC* gene in *V. anguillarum* is not regulated by the iron status of the cell.

By using the cloned *aroC* gene, we generated two mutants. One mutant was obtained by insertion of a fragment containing the $Km^r$ gene into the *SalI* site mapped within the *aroC* gene, while the other was a deletion derivative. Both modified derivatives lost the ability to complement the *aroC* lesion of *E. coli* AB2849 and did not produce the 40-kDa polypeptide (Fig. 1 and 4). We then obtained a chromosomal *aroC* mutant (531A-QC5) of *V. anguillarum* by allelic exchange, using the clone containing the insertionally inactivated *aroC* gene. This *aroC* mutant did not produce 2,3-DHBA, and this deficiency could only be complemented with the wild-type clone. However, it was noteworthy that this mutation also affected dramatically the ability of this derivative to grow under conditions of iron limitation, which was in turn associated with a deficiency in anguibactin production. The addition of 2,3-DHBA to the culture medium or introduction of the cloned *aroC* gene not only allowed for growth under iron-limiting conditions but also resulted in production of anguibactin, as determined by siderophore utilization bioassays. Therefore, these results demonstrate that 2,3-DHBA is a precursor of anguibactin.

Our previous genetic analysis of the *V. anguillarum* anguibactin-mediated iron uptake system identified various iron-regulated genetic units on the pJM1 plasmid that were responsible for anguibactin biosynthesis (34). Our results in this work are therefore consistent with the existence in *V. anguillarum* of a plasmid-mediated biosynthetic system which uses as a raw material the chromosome-mediated 2,3-DHBA, to build the molecule of anguibactin.

The mechanism by which 2,3-DHBA is incorporated into anguibactin is still unknown; however, we have recently found that AngR, a transactivator for anguibactin biosynthesis encoded by the pJM1 plasmid has, in addition to its regulatory function, an enzymatic activity related to the *E. coli* 2,3-dihydroxybenzoate-adenosine monophosphate ligase (35). This enzyme participates in the activation of 2,3-DHBA for use in the biosynthesis of enterobactin in *E. coli* (28). Therefore, an attractive possibility, which we are currently investigating, is that one of the roles of the plasmid-mediated AngR protein in *V. anguillarum* is the activation of 2,3-DHBA for its use in the biosynthesis of anguibactin.

It has been reported that an aromatic-dependent *aroA* mutant of *Aeromonas salmonicida*, constructed by allelic replacement and whose virulence to fish was attenuated, was effective as a live vaccine against the salmonid disease furunculosis (39). Since mutants in the production of anguibactin have already been proven avirulent (36, 41), the *V. anguillarum aroC* mutant is expected to have lost the high-virulence phenotype. Therefore, its potential utilization for the development of a vaccine to prevent fish vibriosis is currently under investigation.

## ACKNOWLEDGMENT

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