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Plasmid- and Chromosome-Encoded Redundant and Specific Functions Are Involved in Biosynthesis of the Siderophore Anguibactin in *Vibrio anguillarum* 775: a Case of Chance and Necessity?

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We report the identification of a novel chromosome cluster of genes in *Vibrio anguillarum* 775 that includes redundant functional homologues of the pJM1 plasmid-harbored genes *angE* and *angC* that are involved in anguibactin biosynthesis. We also identified in this cluster a chromosomal *angA* gene that is essential in anguibactin biosynthesis.

The fish pathogen *Vibrio anguillarum* causes a fatal hemorrhagic septicemic disease in salmonids (1). Some of the virulent serotype O1 strains of this bacterium harbor the pJM1 plasmid essential for pathogenicity, encoding an iron-sequestering system that includes almost all the biosynthetic genes for the siderophore anguibactin (Fig. 1A) (12, 13, 14, 15, 29, 31) as well as those for the cognate transport proteins (2, 3, 4, 5, 18, 19).

The existence of additional chromosome- and plasmidborne genes for synthesis and activation of 2,3-dihydroxybenzoic acid (DHBA), a precursor of anguibactin, and 2,3-dihydro-2,3-DHBA, a precursor of DHBA, has also been suggested (17, 21, 31). We report here the identification and analysis of these novel chromosome- and plasmidborne gene clusters.

Bacterial strains, plasmids, and growth conditions. Strains and plasmids are listed in Table 1. *V. anguillarum* was grown at 25°C, while *Escherichia coli* and *Salmonella enterica* serovar Typhimurium were grown at 37°C (31). For iron-restricted conditions, *V. anguillarum* was grown in chemically defined minimal medium CM9 (12) supplemented with different concentrations of the iron chelator ethylenediamine-di-(*o*-hydroxyphenylacetic) acid (EDDA), while for iron-rich growth, ferric ammonium citrate (FAC) at 4 μg/ml was used.

Identification of a chromosomeborne gene cluster with homology to anguibactin biosynthetic genes. The sequence of the pJM1 plasmid revealed an open reading frame 42 (ORF42), named *angE*, that could be involved in the activation of DHBA (15). To determine the functionality of this gene, we constructed the *angE* mutant AC1 by generating an unmarked, in-frame deletion as described previously (22). Bioassays listed in Table 2 and the halo observed on chrome azurol S (CAS) plates (27) (Fig. 2A) demonstrated that the siderophore anguibactin was synthesized in this mutant. Therefore, either the *angE* gene is not involved in anguibactin biosynthesis, or there is an additional gene harbored in the chromosome, complementing this function. Using partial *angE* sequence information obtained from the analysis of the *V. anguillarum* 775 genome (unpublished data), we amplified by inverse PCR an ORF with a predicted translated protein, a 2,3-dihydroxybenzoate-AMP ligase, comparable to VibE from *V. cholerae* (EMBL accession no. AAC45927; 80% similarity and 60% identity) (6). Sequencing the upstream region of this gene revealed two other complete ORFs (Fig. 1B). One of them, named *angA*, showed similarity to proteins with a 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity, such as the VibA protein from *V. cholerae* (EMBL accession no. AAC4924; 73% similarity and 54% identity). The other ORF, named *angC*, would encode a predicted protein with similarity to the isochorismate synthase VibC from *V. cholerae* (EMBL accession no. AAC45925; 83% similarity and 62% identity).

The proteins encoded by this chromosomal cluster show similarities to the homologues encoded on the pJM1 plasmid; however, they are not identical. The chromosome-encoded AngC protein shows 86% similarity and 70% identity to the plasmid-encoded AngC protein, while the chromosome-encoded AngE presents 78% similarity and 60% identity to the plasmid-encoded AngE protein that is 6 amino acids shorter. Although the chromosomal *angA* gene is complete, there is a frameshift mutation in the plasmid homologue caused by a single nucleotide insertion (15). If this mutation is theoretically reversed, the identity of the putative encoded protein with the chromosomal AngA is 32%.

The *angE*, *angC*, and *angA* genes borne on the chromosome and the pJM1 plasmid will be identified by the subscripts ch and p, respectively.

Comparison of the genetic arrangements of the chromosome and pJM1 plasmid clusters. The genetic organization of the novel chromosomal cluster resembles that described for *V. cholerae* (32). However, it shows differences with respect to the version encoded on the pJM1-like plasmids (15, 31) (Fig. 1B). For example, there is divergence in the upstream regions of both *angC* genes: while in the $angC_p$ upstream region there is a transposase gene, in the corresponding region of $angC_{ch}$ the

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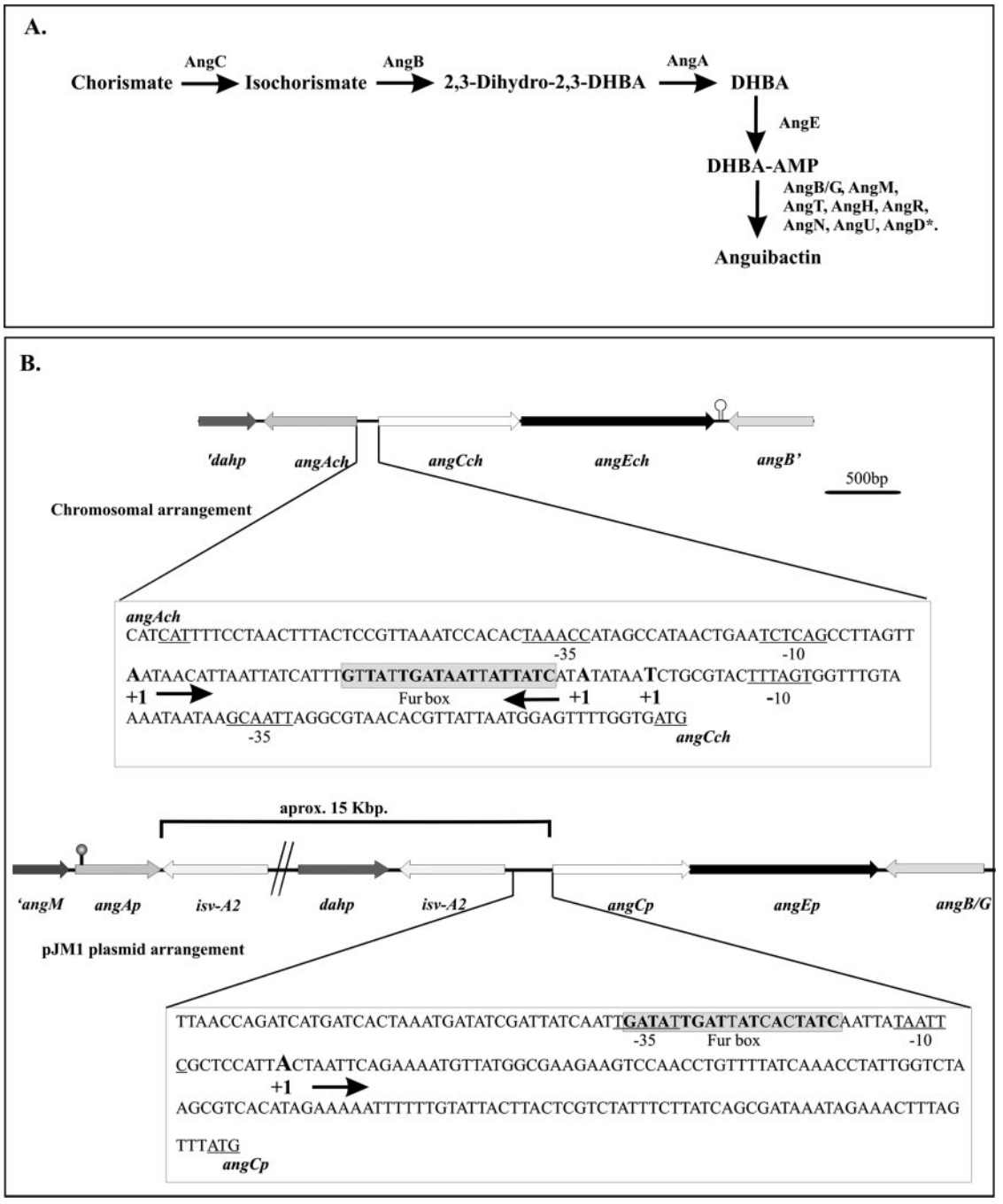

FIG. 1. Scheme of the anguibactin biosynthesis pathway and arrangement of biosynthetic genes in the plasmid and chromosome clusters. (A) Anguibactin biosynthesis pathway. The AngE, AngA, and AngC enzymes are described in the text. The asterisk beside AngD means that this is a putative enzyme whose activity has yet to be proven. For a more detailed pathway, see Crosa and Walsh (13). (B) Genetic arrangement of the *angC*, *angE*, and *angA* genes in the pJM1 plasmid and chromosomal DNA. The intergenic region located between $angA_{ch}$ and $angC_{ch}$ and the $angC_{p}$ promoter are also shown. In this work we identified a chromosomal *dahp* gene, a homologue to that encoded on the pJM1 plasmid (15), that encodes a predicted translated protein similar to 3-deoxy-D-arabino-heptulosonate-7-phosphate synthases. The first codon and the −10 and −35 regions of each gene are underlined; +1 indicates the transcription start sites of each gene determined using primer extension analysis (26). The direction of transcription is denoted by horizontal arrows. For the primer extension analysis, the RNAWiz (Ambion) was used to extract total RNA from *V. anguillarum* strain 775 grown in CM9 supplemented with 2.5 μM EDDA. The primers used in these experiments were as follows: C2 (5′ TAGCTGATTAGCCATTTTTGAAAACCC 3′) located 45 bp downstream from the start codon of the $angC_{ch}$ gene, CPB (5′ GGATCCAAA AAAGAACGGTGATTTTAA 3′) located 118 bp downstream from the start codon of the $angC_{p}$ gene, and A3 (5′ ATTTTTATCCGTCGCTA CAACTCG 3′) and CCK (5′ GGTACCATTTTCCTAACTTTACTCCGTT 3′) located 114 and 8 bp downstream from the start codon of the $angA_{ch}$ gene, respectively. The symbols shown with the $angA_{p}$ gene and downstream of the $angE_{ch}$ gene indicate a frame shift and a transcriptional terminator, respectively. The double diagonal lines between the *isv-A2* and *dahp* genes in the plasmid cluster indicate approximately 12 kbp. The putative Fur boxes are shaded, and nucleotides identical to the *E. coli* Fur consensus (16) are shown in bold characters.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
V. anguillarum strains		
775 (pJM1)	Wild-type Pacific Ocean prototype	Laboratory stock
H775-3	Plasmidless derivative of 775	Laboratory stock
775::Tn*1-5* (pJHC-91)	Anguibactin production deficient	29
775::Tn*1-6* (pJHC9-8)	Anguibactin production and transport deficient	29
775 MET11	*fur*	30
AC1	775 Δ*angE*$_p$	This work
AC2	775 Δ*angE*$_{ch}$	This work
AC3	775 Δ*angE*$_p$ Δ*angE*$_{ch}$	This work
AC4	AC3 complemented with the *angE*$_p$ gene	This work
AC5	AC3 complemented with the *angE*$_{ch}$ gene	This work
AC6	AC3 harboring the vector pMMB208	This work
AC7	775 Δ*angA*$_{ch}$	This work
AC8	AC7 complemented with the *angA*$_{ch}$ gene	This work
AC9	AC7 complemented with the *angA*$_p$ gene	This work
AC10	AC7 harboring the vector pMMB208	This work
AC11	775Δ*angC*$_p$	This work
AC12	775 Δ*angC*$_{ch}$	This work
AC13	775 Δ*angC*$_p$ Δ*angC*$_{ch}$	This work
AC14	AC13 complemented with the *angC*$_p$ gene	This work
AC15	AC13 complemented with the *angC*$_{ch}$ gene	This work
AC16	AC13 harboring the vector pMMB208	This work
AC17	775 Δ*angC*$_p$ Δ*angC*$_{ch}$ Δ*menF*	This work
E. coli strains		
DH5α	*supE44 hsdR17 recA1 gyrA96 thi-1* Δ*lacU169 relA1* (ϕ80*lacZ*ΔM15)	Laboratory stock
MM294	F$^-$ *endA1 hsdR17 supE44 thi-1* λ$^-$ harboring plasmid pRK201	Laboratory stock
S17-1 λpir	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7 λpir	28
S. enterica serovar *Typhimurium* strains		
enb1	Enterobactin production deficient (uses enterobactin as iron source)	25
enb7	Enterobactin production deficient (uses enterobactin and DHBA as iron sources)	25
Plasmids		
pCR2.1-TOPO	Km^r Amp^r	Invitrogen
pMMB208	*incQ lac1*^{q} Ω*cat* Ω*Ptac* Cm^r	24
pDM4	Suicide plasmid R6K origin; Cm^r	22
pALE1	pDM4 harboring Δ*angE*$_p$	This work
pALE2	pDM4 harboring Δ*angE*$_{ch}$	This work
pALE3	pDM4 harboring Δ*angA*$_{ch}$	This work
pALE4	pMMB208 harboring *angE*$_p$	This work
pALE5	pMMB208 harboring *angE*$_{ch}$	This work
pALE6	pMMB208 harboring *angA*$_p$	This work
pALE7	pMMB208 harboring *angA*$_{ch}$	This work
pALE8	pDM4 harboring Δ*angC*$_p$	This work
pALE9	pDM4 harboring Δ*angC*$_{ch}$	This work
pALE10	pMMB208 harboring *angC*$_p$	This work
pALE11	pMMB208 harboring *angC*$_{ch}$	This work
pALE12	pDM4 harboring Δ*menF*	This work
p32	199-bp fragment of the *angE*$_{ch}$ gene cloned into pCR2.1 Topo	This work
pQSH6	420-bp SaI-ClaI fragment of the *aroC* gene cloned in pBluescript-II SK+	14

angA$_{ch}$ gene is found (Fig. 1B). In contrast, downstream of *angE*$_{ch}$ we identified an ORF that encodes a predicted protein with similarity to the isochorismate lyase AngB, located in the same region as that in the pJM1-like plasmids (Fig. 1B) (15, 31). It is thus possible that transposition events might have occurred on the plasmid, resulting in genetic rearrangements. Since the chromosomal and plasmid homologues differ at both the nucleotide and amino acid levels, it is possible that they evolved independently and that the latter were acquired by horizontal transfer. One other alternative is that they are paralogue genes whose original duplication event was not recently accomplished.

Involvement of *angE* and *angC* chromosome and plasmid genes in the biosynthesis of anguibactin. Since the *angE*$_p$ null mutant AC1 is able to synthesize anguibactin, the following new strains were constructed: the single *angE*$_{ch}$ (AC2) and double *angE*$_p$ *angE*$_{ch}$ (AC3) mutants. The CAS assays and bioassays showed that the double mutant cannot synthesize anguibactin, while the single *angE*$_{ch}$ mutant is as proficient in siderophore production as the *angE*$_p$ mutant (Fig. 2A and

TABLE 2. Bioassay experiments with various indicator strains[a]

Strain tested and relevant genotype	Growth of *V. anguillarum* indicator strain[b]:		
	H775-3	775::Tn*1-5* (pJHC-91)	775::Tn*1-6* (pJHC9-8)
AC1 Δ*angE*$_p$	−	+	−
AC2 Δ*angE*$_{ch}$	−	+	−
AC3 Δ*angE*$_p$ Δ*angE*$_{ch}$	−	−	−
AC4 Δ*angE*$_p$ Δ*angE*$_{ch}$ pALE4 (*angE*$_p$)	−	+	−
AC5 Δ*angE*$_p$ Δ*angE*$_{ch}$ pALE5 (*angE*$_{ch}$)	−	+	−
AC6 Δ*angE*$_p$ Δ*angE*$_{ch}$ pMMB208	−	−	−
AC7 Δ*angA*$_{ch}$	−	−	−
AC8 Δ*angA*$_{ch}$ pALE7 (*angA*$_{ch}$)	−	+	−
AC9 Δ*angA*$_{ch}$ pALE6 (*angA*$_p$)	−	−	−
AC10 Δ*angA*$_{ch}$ pMMB208	−	−	−
AC11 Δ*angC*$_p$	−	+	−
AC12 Δ*angC*$_{ch}$	−	+	−
AC13 Δ*angC*$_p$ Δ*angC*$_{ch}$	−	+/−	−
AC14 Δ*angC*$_p$ Δ*angC*$_{ch}$ pALE10 (*angC*$_p$)	−	+	−
AC15 Δ*angC*$_p$ Δ*angC*$_{ch}$ pALE11 (*angC*$_{ch}$)	−	+	−
AC16 Δ*angC*$_p$ Δ*angC*$_{ch}$ pMMB208	−	+/−	−
AC17 Δ*angC*$_p$ Δ*angC*$_{ch}$ Δ*menF*	−	−	−

[a] Supernatants were obtained from cultures of the various strains to be tested and grown under iron-limiting conditions for 16 to 20 h. Five microliters of each supernatant was spotted onto CM9 agar minimal media plates supplemented with 2.5 μM EDDA that had previously been seeded with the indicator strains of *V. anguillarum.* The plates were examined for halos of growth after 24 to 72 h.

[b] *V. anguillarum* mutants deficient in the iron uptake system included 775::Tn*1-6* (pJHC9-8), deficient in the production of the anguibactin siderophore and its receptor; and 775::Tn*1-5*(pJHC-91), receptor proficient and siderophore deficient. Symbols: +, presence of growth halo; +/−, presence of a small growth halo after 48 h; −, no growth.

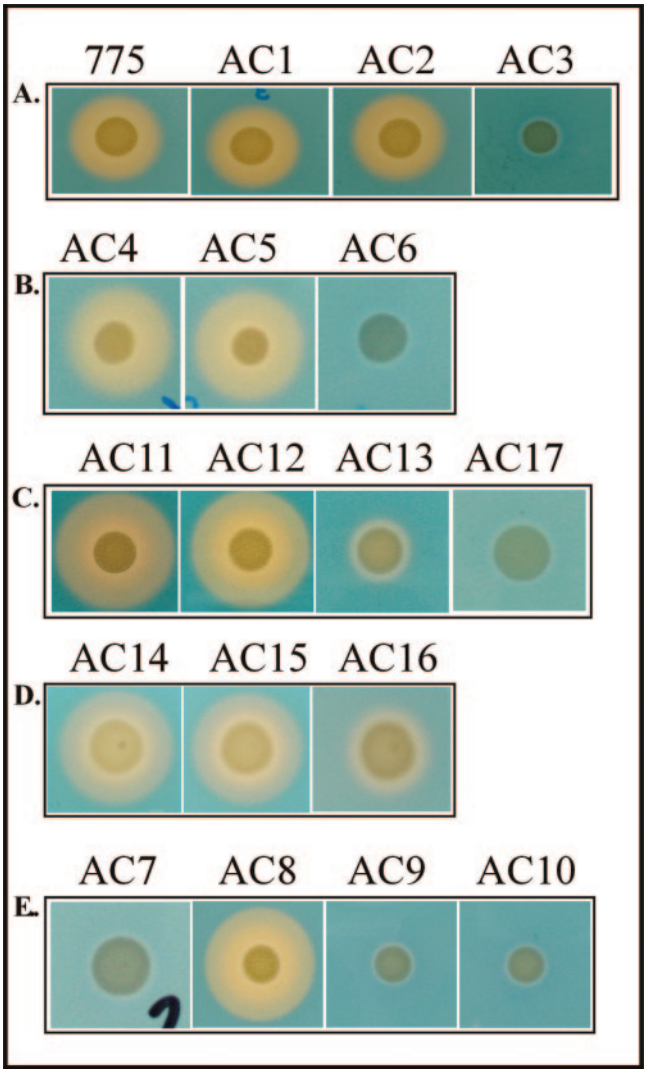

FIG. 2. Detection of siderophore production on CAS agar plates in *V. anguillarum* strains. (A) From left to right: wild-type *V. anguillarum* strain 775, AC1 (Δ*angE*$_p$), AC2 (Δ*angE*$_{ch}$), and AC3 (Δ*angE*$_p$ Δ*angE*$_{ch}$). (B) Genetic complementation of the double Δ*angE*$_p$ Δ*angE*$_{ch}$ (AC3) mutant strain with each wild-type *angE* gene. From left to right: the AC3 strain complemented with the *angE*$_p$ gene (AC4), complemented with the *angE*$_{ch}$ gene (AC5), and harboring the empty vector pMMB208 (AC6). (C) From left to right: AC11 (Δ*angC*$_p$), AC12 (Δ*angC*$_{ch}$), AC13 (Δ*angC*$_p$ Δ*angC*$_{ch}$), and AC17 (Δ*angC*$_p$ Δ*angC*$_{ch}$ Δ*menF*). (D) Genetic complementation of the double Δ*angC*$_p$ Δ*angC*$_{ch}$ (AC13) mutant strain with each wild-type *angC* gene. From left to right: the AC13 strain complemented with the *angC*$_p$ gene (AC14), complemented with the *angC*$_{ch}$ gene (AC15), and harboring the empty vector pMMB208 (AC16). (E) From left to right: AC7 (Δ*angA*$_{ch}$ mutant strain) and the Δ*angA*$_{ch}$ (AC7) mutant strain complemented with the *angA*$_{ch}$ gene (AC8), complemented with the *angA*$_p$ gene (AC9), and harboring the empty vector pMMB208 (AC10). For the complementation experiments the plates were supplemented with 10 μg of chloramphenicol/ml and 1 mM IPTG to induce the genes cloned under the control of the inducible *Ptac* promoter.

Table 2). It is thus clear that both the chromosome and plasmid *angE* genes encode functional AngE proteins. This result was confirmed by complementing the double *angE* mutant with each one of the *angE* genes (Fig. 2B and Table 2).

The same functional redundancy was also found with the chromosome and plasmid homologues of *angC* by using the single *angC*$_p$ and *angC*$_{ch}$ mutants (Fig. 2C and Table 2) and confirmed by complementation of the double *angC* mutant AC13 (Fig. 2D and Table 2). However, this double mutant could produce small amounts of anguibactin (Fig. 2C and Table 2). Two plausible hypotheses can explain these results: (i) existence of a second chromosome *angC* homologue and (ii) presence of an isoenzyme capable of complementing the isochorismate synthase activity. It was previously demonstrated that MenF, which is involved in the biosynthesis of menaquinones, synthesizes isochorismate that results in the production of low amounts of enterobactin in an *E. coli entC* mutant (8). By performing protein sequence alignment of the MenF homologues from *E. coli* (P38051) and several *Vibrio* species, we designed degenerated primers that allowed us to clone the putative *V. anguillarum menF* gene. The predicted translated sequence of this gene shows similarity with MenF from *E. coli* (52% similarity and 38% identity). We then generated a deletion in this putative *menF* gene in the double *angC* mutant AC13, resulting in the complete suppression of anguibactin production (Fig. 2C). The fact that the AC13 strain can produce small amounts of anguibactin indicates that there is cross

talk between the two pathways at the level of the isochorismic acid produced by the *V. anguillarum* MenF protein as previously described for *E. coli* (8).

The chromosomal *angA* gene is essential for anguibactin production. To determine whether both $angA_p$ and $angA_{ch}$ can intervene in anguibactin biosynthesis, we constructed an $angA_{ch}$ mutant (AC7). This mutation resulted in the abolishment of the siderophore synthesis (Fig. 2E and Table 2), underscoring that the frame shift present in the $angA_p$ gene has impaired its functionality. The complementation experiments confirm that only the wild-type $angA_{ch}$ gene, not $angA_p$, could restore anguibactin biosynthesis in the AC7 strain (Fig. 2E and Table 2). Bioassays using *S. enterica* serovar Typhimurium strains (26) and chemical determinations (7) indicated that DHBA synthesis was abolished in this mutant. The ability of DHBA to restore the growth of this mutant under iron-limiting conditions corroborated that $AngA_{ch}$ is indeed involved in the biosynthesis of this anguibactin precursor (data not shown).

Transcriptional analysis of the plasmid and chromosomal *angC*, *angE*, and *angA* genes. We determined, using reverse transcription-PCR, that in each of the chromosomal and plasmid gene clusters the *angCE* genes are transcribed as an operon (data not shown). Figure 1B shows the transcriptional start points of these two operons as well as the two different transcription start points for the $angA_{ch}$ gene. This figure also shows a schematic diagram depicting the overlap between the divergent transcripts of $angA_{ch}$ and $angCE_{ch}$ as determined in the primer extension analysis (26). The in silico analysis of the $angCE_{ch}$, $angCE_p$ and $angA_{ch}$ promoters showed sequences with high identity to those described for the canonic Fur box (16) (Fig. 1B). In the case of $angCE_{ch}$ and $angA_{ch}$, this analysis suggests that Fur could control the expression of these genes by binding at a unique site.

We also determined that the transcription of both *angCE* operons was regulated in an iron- and Fur protein-dependent manner using RNase protection assay (RPA) (Fig. 3). We found that cultures grown in CM9 and CM9 plus EDDA expressed the chromosomal and plasmid *angCE* operons, while the presence of iron in CM9 leads to a complete repression of these genes (Fig. 3). It is important to note that the iron concentration in CM9 is sufficiently low to induce the expression of the anguibactin iron uptake system (3). In contrast with our results, Liu et al. (21) have recently shown that the *angE* gene harbored in a pJM1-like plasmid was not repressed in iron-rich conditions. The differences between our results and those observed by Liu et al. could be due to the different strains used or their specific experimental conditions. Therefore, it is tempting to speculate that in *V. anguillarum* 775 other environmental signals or growth conditions could preferentially induce the plasmid or chromosome *angE* genes.

The transcriptional analysis of $angA_{ch}$ indicated that this gene is also regulated by the iron concentration in a Fur-dependent manner (data not shown).

Conclusion. From our results it is reasonable to group the anguibactin biosynthesis genes in *V. anguillarum* 775 into those that are harbored only by the pJM1 plasmid (e.g., *angM*, *angR*, *angT*, *angU*, *angN*, and *angH)*, those harbored by both the plasmid and the chromosome (e.g., *angE* and *angC*), and those harbored only by the chromosome (e.g., *angA)*. The chromosomal gene cluster described here could have been the remains

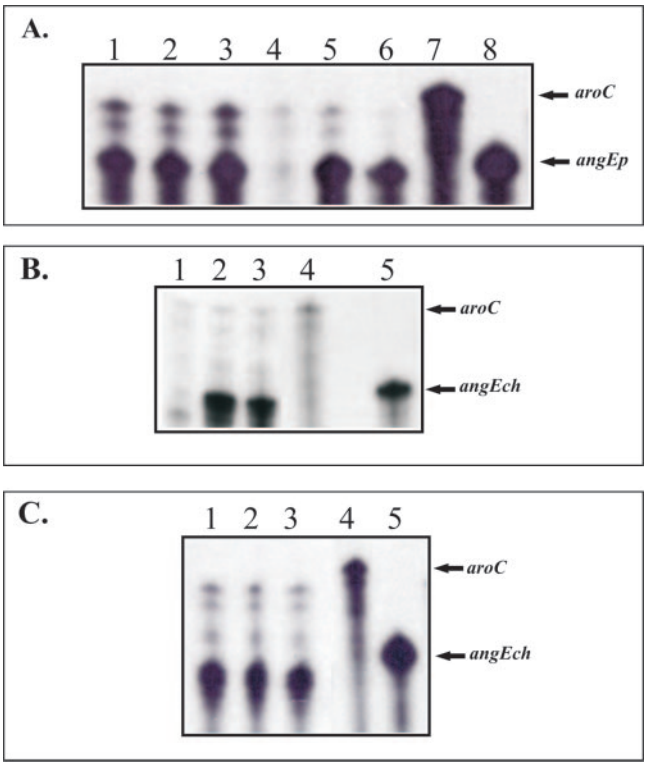

FIG. 3. Transcriptional regulation analysis by RPA of the $angE_p$ and $angE_{ch}$ genes. Total RNA was harvested from each strain grown under the conditions indicated for each lane. The riboprobes were synthesized using the Maxiscript T7/T3 kit from Ambion. In these experiments we used the *aroC* gene as an internal control because it was previously established that this gene is not regulated by iron (9). For *aroC* we used the plasmid pQSH6 (linearized with RsaI) (10, 15) and p32 for $angE_{ch}$ gene. For the $angE_p$ homologue we designed the primers EPPU (5′ CCGATAGATATCATCACGAAA 3′) and EPPRT7 (5′ TAATACGACTCACTATAGGGCGCGTAAAATCCG TTTTTATC 3′). The RPA assay was performed using the RPA III kit (Ambion) according to the manufacturer's specifications. Specific transcripts for *aroC*, $angE_{ch}$, and $angE_p$ were detected using the riboprobes synthesized as described above. (A) Analysis of the $angE_p$ gene. Lanes: 1, 2, and 3, RNA extracted from the *V. anguillarum* 775 MET 11 *fur* strain grown in CM9 supplemented with 4 μg of FAC, CM9, and CM9 supplemented with 2.5 μM EDDA/ml, respectively; 4, 5, and 6, RNA extracted from *V. anguillarum* strain 775 grown under the same conditions as described previously; 7, *aroC* riboprobe; 8, $angE_p$ riboprobe. (B) Analysis of the $angE_{ch}$ gene. Lanes 1, 2, and 3: RNA extracted from *V. anguillarum* strain 775 grown in CM9 supplemented with 4 μg of FAC, CM9, and CM9 supplemented with 2.5 μM EDDA/ml, respectively. (C) Lanes 1, 2, and 3: same as described for panel B but using RNA from the *V. anguillarum fur* mutant strain. Lanes 4 and 5 of both panels show the *aroC* and $angE_{ch}$ riboprobes.

of an earlier cluster involved in the biosynthesis of an ancestral DHBA-based siderophore. In this regard, it has been previously demonstrated that several plasmidless serotype O1 strains and all the plasmidless serotype O2 strains of *V. anguillarum* synthesize a chromosomally encoded catechol-type siderophore unrelated to anguibactin (11, 20). Since this chromosomally encoded siderophore can be utilized by the 775 strain as an iron source (20), it is possible that at some point in the evolution of this pathogen, an ancestor of this strain had the capability to synthesize this siderophore and that one or more chromosomal genes involved in its biosynthesis were silenced.

Chance and necessity (23) may have selected those organisms that by horizontal transfer acquired the new plasmid-mediated anguibactin biosynthesis and uptake genes. The lack of a functional $angA_p$ gene resulted in dependence of this incomplete plasmid system on the host $angA_{ch}$ gene. The important role of the anguibactin system in virulence strongly suggests that the newly acquired plasmid-mediated system, although partially duplicated in the chromosome, provided an evolutionary advantage to *V. anguillarum* 775 in its natural environment. Thus, our study generates new questions on the intersection of plasmid biology and the evolution of bacterial virulence.

Nucleotide sequence accession numbers. The nucleotide sequences described above were deposited in GenBank under the accession numbers AY738106 and AY738107.

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