Regulation of the iron uptake system in *Vibrio anguillarum*: Evidence for a cooperative effect between two transcriptional activators

(*lacZ* fusion genes/siderophore)

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ABSTRACT We have identified a 110-kDa polypeptide that has a trans-acting regulatory activity on the expression of the pJM1 plasmid iron-uptake genes in *Vibrio anguillarum*. This protein is encoded by the *angR* gene and maps in a 3.6-kilobase-pair pJM1 DNA region located downstream of the iron transport genes. Full expression of this gene occurs under iron-limiting conditions and requires a 2.9-kilobase-pair upstream region in cis that maps within the coding region of the OM2 outer membrane protein, essential for the transport of iron into the cell cytosol. Determination of the siderophore anguibactin levels as well as analysis of specific transcripts for anguibactin biosynthetic genes demonstrated that AngR and another transcriptional activator, Taf, regulate in a synergistic fashion the level of anguibactin production by activation of transcription of the anguibactin biosynthetic genes under iron-limiting conditions.

Vibrio anguillarum 775, a fish pathogen, carries the pJM1 plasmid that is essential for its virulence (1–4). This plasmid encodes an efficient iron-uptake system (1) that consists of the siderophore anguibactin (5) and an iron-transport system (6). Transposition mutagenesis analysis identified six genetic units (7). Five of these units were essential for the biosynthesis of the siderophore anguibactin. Cells carrying mutations in genetic unit II showed an iron-transport-deficient phenotype and had reduced levels of anguibactin production (7). Two components have been identified in the transport system, the outer membrane protein OM2 (6, 7) and a 40-kDa protein (8). We also reported that a DNA fragment noncontiguous to the iron-uptake region carries the genetic determinants for Taf, a transcriptional activator of anguibactin biosynthetic genes (7, 9). *V. anguillarum* strains producing higher levels of the siderophore anguibactin, as compared to the 775 strain, have been characterized (10). The increased siderophore production (Isp) phenotype was encoded by pJM1-like plasmids, such as pJHC1 in strain 531A (10). We report in this work the cloning of the region responsible for the Isp phenotype, the *angR* gene from pJHC1. This region encodes a 110-kDa polypeptide that is also found in cells harboring the cloned region from pJM1. The AngR protein together with the Taf product regulate the level of anguibactin production by activation of transcription of the anguibactin biosynthetic genes under iron-limiting conditions.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Transposition Mutagenesis. The *V. anguillarum* strains 775 (pJM1) (3), 531A (pJHC1) (10), H775-3 (plasmidless) (2), 775-Tn*1*–5 (pJHC91) (11), and

775-Tn*1*–6 (pJHC9-8) (11) and *Escherichia coli* strains HB101, LE392, JM107, C2110, and BN660 were described (12–14). Plasmids pHoHo1, carrying the transposon Tn*3*::HoHo1 and pSShe were used in transposition experiments (15). Transposition mutants of the pJHC1 and pJM1 cloned iron-uptake regions were carried out by insertion of Tn*3*::HoHo1 onto plasmids pJHC-T5.12 and pJHC-T2612, respectively (7). Plasmid pRK2073 (16) was used as a helper in conjugations of plasmids into *V. anguillarum*. Plasmids pUC4K (17, 18) and pHP45Ω (19) were used, respectively, as sources of the kanamycin gene and the Ω fragment carrying transcriptional terminators.

Molecular cloning of the *angR* locus from pJM1 (or pJHC1) was carried out by digestion of the plasmids with *Eco*RI and cloning into the *Eco*RI site of pJHC-S100, a plasmid containing the kanamycin (Km) fragment from pUC4K inserted into the *Pst* I site of pBR325. Plasmids pJHC-S200 and pJHC-S253, obtained in these experiments, contained the 9.6-kilobase-pair *Eco*RI fragment of pJM1 and pJHC1, respectively. Smaller subclones of pJHC-S200 and pJHC-S253 are shown in Figs. 1 and 2. Insertions into the recombinant clone pJHC-T5.12 were mobilized into *V. anguillarum* 775-Tn*1*–6 carrying *taf* determinants (7, 9).

Labeling of Proteins. Plasmid-encoded proteins labeled with [^{35}S]methionine were synthesized in a maxicell system using *E. coli* BN660 according to the procedure of Sancar *et al.* (20) or in a coupled-cell-free protein synthesizing system (Amersham). Polypeptides were analyzed by NaDodSO$_4$/12.5% polyacrylamide gel electrophoresis (PAGE).

Northern Blot Analysis. RNAs from *V. anguillarum*, isolated by a hot phenol procedure (21), were analyzed in formaldehyde/agarose gels and transferred to Nytran membranes. Equal loading and transfer of RNA was assessed by acridine orange staining. An *Xho* I fragment corresponding to genetic unit I was cloned into the Bluescript plasmid KS (Stratagene) and the riboprobe was prepared using [^{32}P]UTP and T3 RNA polymerase according to instructions of the manufacturer (Stratagene). Hybridizations were performed in 50% (vol/vol) formamide at 65°C (22).

RESULTS

Characterization of Transposition Mutants of pJHC-T5.12. Transposition mutants obtained by insertion mutagenesis of plasmid pJHC-T5.12, carrying the iron-uptake region from pJHC1 (strain 531A), with transposon Tn*3*:HoHo1 were analyzed for the minimal inhibitory concentration of the iron chelator ethylenediamine di(*o*-hydroxyphenyl)acetic acid, anguibactin production, and the synthesis of the OM2 protein, essential for the transport of iron into the cell cytosol (8, 11).

Abbreviations: Isp, increased siderophore production; Km, kanamycin.
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Strains carrying mutations 50, 11, 70, 75, and 62 (Fig. 1) were iron-uptake deficient since they were unable to grow in minimal medium containing more than 2 μM ethylene di(*o*-hydroxyphenyl)acetic acid and produced very low levels of anguibactin. However, mutation 160 was iron-uptake proficient but had a level of iron-regulated siderophore production of the order of that found with strains carrying the pJM1 plasmid or its cloned derivative pJHC-T2612 carrying the iron-uptake region (this level of siderophore production will be identified hereafter as normal). Cells carrying mutation 39, which mapped very close to mutation 160 but in reverse orientation, were iron-uptake deficient producing very low levels of anguibactin, comparable to the levels found in cells carrying mutations 50 and 11.

Cells harboring mutations 160 and 39 produced OM2, an iron-regulated outer membrane protein. Cells carrying the other mutations (mutants 50, 70, 75, and 62) failed to synthesize OM2 or synthesized low levels of iron-regulated OM2 (mutant 11).

Complementation Experiments. Since insertion 160, which maps in genetic unit III, affects only the level of anguibactin production and confers a similar phenotype as the wild-type strain 775, we initiated complementation studies using clones that overlapped this region. It could be expected that the Isp phenotype would only be restored by complementation with clones derived from pJHC-T5.12, rather than with clones derived from pJHC-T2612, which should give the phenotype conferred by this clone (normal anguibactin production). Fig.

1 shows the results of complementation tests carried out with mutations 11, 75, 160, and 39. Results with mutation 50 as well as with mutations 70 and 62 were identical to those obtained with mutations 11 and 75, respectively. The results confirmed our prediction: the Isp phenotype in each of the mutants could only be restored by clones derived from pJHC-T5.12 (such as pJHC-S253) (Fig. 1, line 3). However, it was still unclear how insertions 50, 11, 70, 75, and 62 affected the Isp phenotype, unless we assumed that the Tn*3*::HoHo1 insertion caused a polar effect downstream from its site of insertion. Also pJHC-S2557 (Fig. 1, line 5) and pJHC-A107 (Fig. 1, line 6) restored the iron-uptake proficient phenotype to mutants 11 and 75 albeit not the Isp phenotype. Similar results were obtained with mutants 50, 70, and 62. Therefore, our results are consistent with the hypothesis that insertions 50, 11, 70, 75, and 62 polarly affected the region responsible for the Isp phenotype. Plasmids pJHC-S2539 and pJHC-S2739 (Fig. 1, lines 7 and 8) were unable to change the phenotype of mutants 160 and 39. However, pJHC-S2571 (Fig. 1, line 9) was able to restore this phenotype in contrast to the clone carrying the identical fragment derived from pJM1 (Fig. 1, line 10). Since this cloned fragment starts downstream of mutations 50 and 11, we investigated whether these results were actually due to transcription initiation occurring at promoter(s) located upstream of the *Sal* I site in the vector DNA.

We inserted the Ω fragment carrying terminators for transcription and translation (19) into the *Sal* I site of
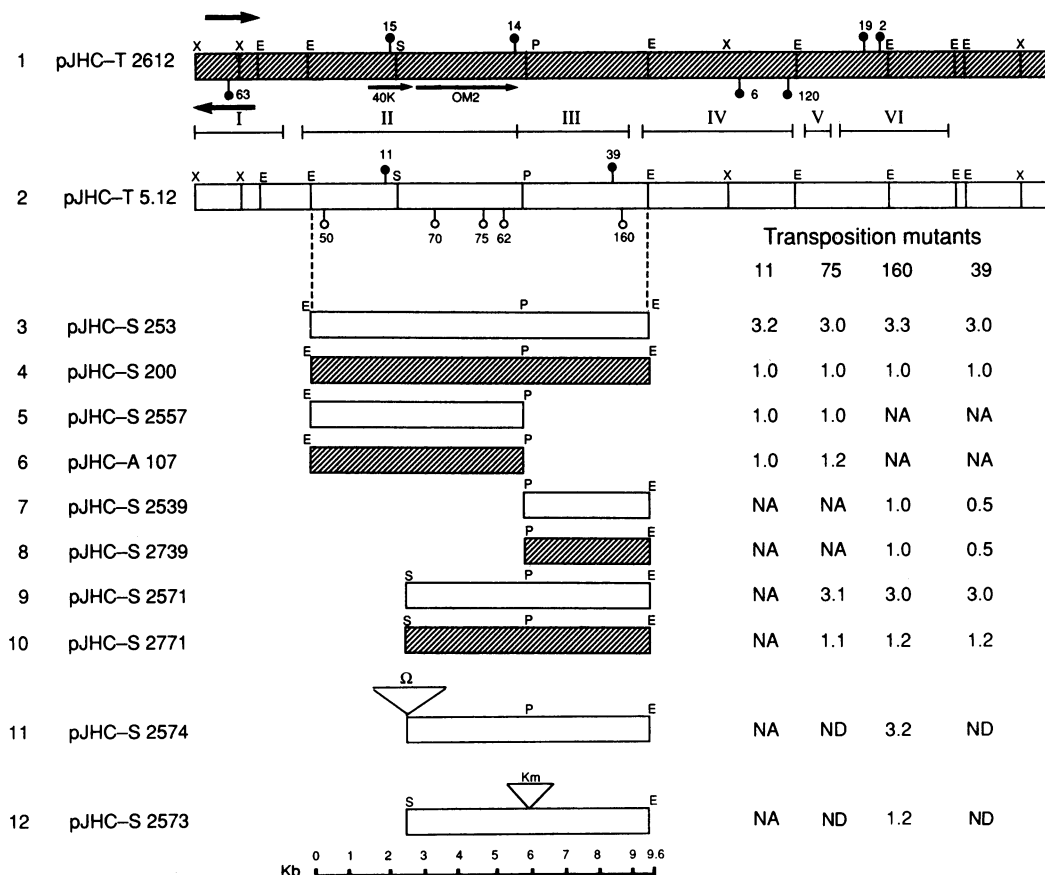

FIG. 1.   Complementation analysis of the transposition mutants located in genetic units II and III. The location of Tn*3*::HoHo1 mutants in the pJHC-T2612 and pJHC-T5.12 plasmids is shown on lines 1 and 2. Solid circles, mutations that express β-galactosidase; open circles, mutations that do not express β-galactosidase. Insertions in which the *lacZ* gene is oriented from left to right are above the bar and those with the *lacZ* gene oriented from right to left are shown below the bar. I–VI represent the six genetic units described by Tolmasky *et al.* (7). X; *Xho* I; E, *Eco*RI; S, *Sal* I; P, *Pst* I. The vector used in the construction of these clones is not represented in this figure. Open bars represent DNA fragments from pJHC1 and hatched bars represent DNA fragments from pJM1 plasmid. The values in each lane correspond to relative anguibactin production by the transposition mutants when complemented with the corresponding subclone. NA, not applicable; ND, not done; Ω, Ω fragment carrying transcription and translational terminators; Km, Km fragment from pUC4K. Arrows indicate the direction of transcription.
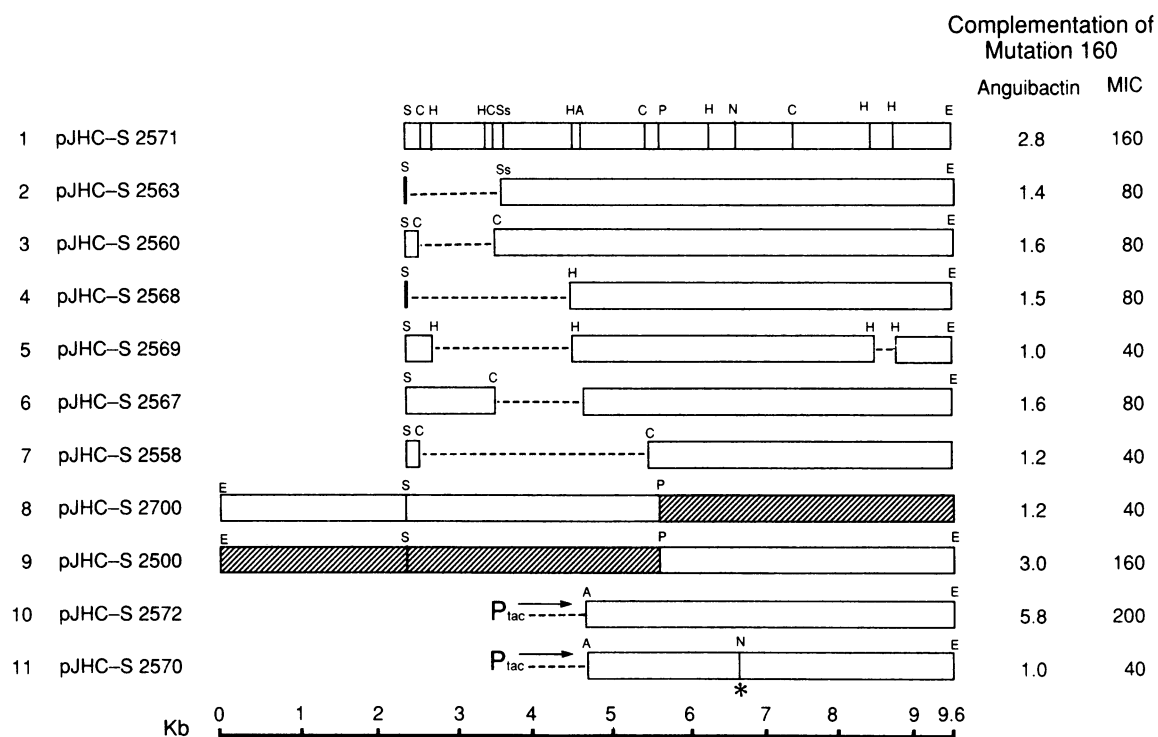
Biochemistry: Salinas *et al.*

Proc. Natl. Acad. Sci. USA 86 (1989)   3531

Complementation of
Mutation 160

		Anguibactin	MIC
1	pJHC–S 2571	2.8	160
2	pJHC–S 2563	1.4	80
3	pJHC–S 2560	1.6	80
4	pJHC–S 2568	1.5	80
5	pJHC–S 2569	1.0	40
6	pJHC–S 2567	1.6	80
7	pJHC–S 2558	1.2	40
8	pJHC–S 2700	1.2	40
9	pJHC–S 2500	3.0	160
10	pJHC–S 2572	5.8	200
11	pJHC–S 2570	1.0	40

Kb 0 1 2 3 4 5 6 7 8 9 9.6

FIG. 2. Complementation analysis of mutation 160 with pJHC-S2571 and its derivatives. Anguibactin production was measured by bioassays. E, *Eco*RI; S, *Sal* I; H, *Hin*dIII; C, *Cla* I; Ss, *Sst* I; A, *Ava* I; N, *Nco* I. All fragments, except those from pJHC-S2572 and pJHC-S2570, were cloned into pJHC-S100, a pBR325 derivative carrying the Km fragment from pUC4K cloned into the *Pst* I site. pJHC-S2572 contains the *Ava* I–*Eco*RI fragment cloned into pKK223-3 containing the *P*tac promoter. pJHC-S2570 is a derivative of pJHC-S2572 in which the *Nco* I site was modified by filling the cohesive ends by using the Klenow fragment of DNA polymerase I. The column labeled "complementation of mutation 160" corresponds to results of complementations obtained with each of these clones. Arrows indicate the direction of transcription initiated at the *P*tac promoter. Bars symbolize the regions from pJM1 (hatched bars) and pJHC1 (open bars).

pJHC-S2571. The new plasmid, pJHC-S2574, was then introduced into a *V. anguillarum* strain carrying mutation 160. The results (Fig. 1, line 11) indicated that pJHC-S2574 was able to restore the Isp phenotype, demonstrating that vector sequences were not responsible for these results. Plasmids pJHC-S2557 and pJHC-S2539 were unable to restore the Isp phenotype (Fig. 1, lines 5 and 7), but pJHC-S2571 (line 9) did restore the Isp phenotype; thus, it was clear that the unique *Pst* I site was important for the expression of this phenotype. This was confirmed by the fact that complementations carried out with pJHC-S2573, a *Sal* I–*Eco*RI clone from pJHC1 that has the Km-resistance gene inserted in this *Pst* I site, did not restore the Isp phenotype (Fig. 1, line 12).

To identify the minimum stretch of DNA necessary for expression of this phenotype, we generated several subclones derived from pJHC-S2571. Further complementation studies were carried out using only mutation 160 (Fig. 2). The complementation studies also indicated that subclones conferring intermediate levels of anguibactin production had deletions within the stretch of DNA limited by the *Sal* I and *Cla* I sites (coordinates 2.5–3.7 kilobase pairs). Since this DNA region is intact in the plasmid carrying mutation 160, the deleted DNA sequences in the subclones must contain an essential cis-acting element. The contribution of this cis-acting region to the Isp phenotype was assessed by constructing two hybrid clones: pJHC-S2700, carrying the *Eco*RI-*Pst* I fragment from pJHC1 and the *Pst* I–*Eco*RI fragment from pJM1, and pJHC-S2500, carrying the *Eco*RI-*Pst* I fragment from pJM1 and the *Pst* I–*Eco*RI fragment from pJHC1. Complementation experiments with mutant 160 (Fig. 2, lines 8 and 9) indicated that, although the upstream region was essential for this phenotype, it was inconsequential whether it was derived from pJHC1 or pJM1 DNA. However, the downstream *Pst* I–*Eco*RI fragment had to be derived from pJHC1 to restore the Isp phenotype. To further corroborate

this finding, we generated plasmid pJHC-S2572, which is an *Ava* I–*Eco*RI fragment cloned in the expression vector pKK223-3 under the control of the *P*tac promoter (23). The strain carrying this plasmid produced more anguibactin (which was still iron regulated) and had a higher minimal inhibitory concentration of ethylenediamine di(*o*-hydroxyphenyl)acetic acid than the strain carrying pJHC-S2571 (Fig. 2, lines 1 and 10). Thus, *P*tac appears to be used even more efficiently than the wild-type promoter. Moreover, the pJHC-S2570 clone, derived from pJHC-S2572, in which the unique *Nco* I site was modified (Fig. 2, line 11), was unable to increase the level of anguibactin production of mutant 160. Since this modification could have led to a frameshift mutation (see below), our results suggested that a putative truncated product encoded by this clone was unable to confer the Isp phenotype.

Analysis of the Polypeptides Encoded by Subclones of the *angR* Region. Polypeptides synthesized by the various plasmids in an *in vitro* transcription–translation system were analyzed by NaDodSO4/PAGE. Fig. 3A shows that the OM2 as well as the 110-kDa proteins were synthesized by pJHC-S200 (lane 1; the 110-kDa protein is barely visible in this case), pJHC-S253 (lane 2), and pJHC-S2571 (lane 3). Neither OM2 nor the 110-kDa protein were synthesized in extracts in which the template was pJHC-S2539 (lane 4) whereas OM2, but not the 110-kDa protein, was synthesized in extracts using as a template pJHC-S2573, a derivative of pJHC-S2571 that carries the Km fragment in the *Pst* I site (lane 5). The vector control is shown in lane 6.

To determine whether these proteins were also expressed *in vivo*, we analyzed the polypeptides made in a maxicell system. Plasmids pJHC-S2571 and pJHC-S2771 encoded OM2 and the 110-kDa proteins (data not shown). It was of interest that maxicells harboring pJHC-S2572 (*Ava* I–*Eco*RI fragment cloned under the control of the *P*tac promoter)
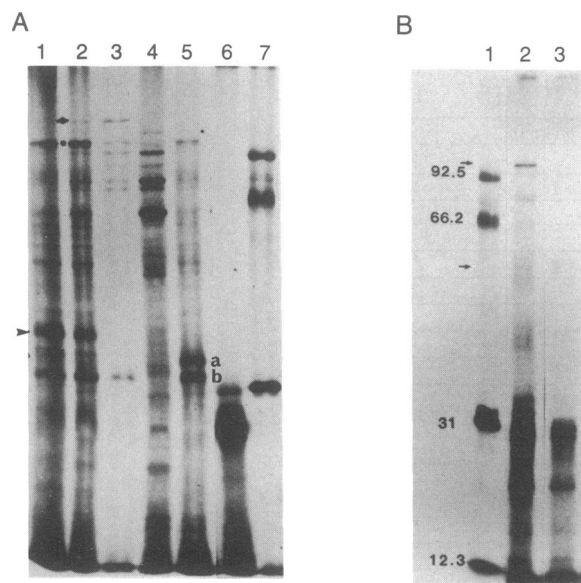
A B

FIG. 3. NaDodSO₄/PAGE of plasmid-encoded proteins. (*A*) *In vitro* transcription–translation system. Lanes: 1, pJHC-S200; 2, pJHC-S253; 3, pJHC-S2571; 4, pJHC-S2539; 5, pJHC-S2573; 6, pJHC-S100, vector control. ←, 110-kDa protein; *, OM2 protein; ▶, 40-kDa protein; a and b, precursor of β lactamase and β lactamase, respectively. (*B*) Plasmid-encoded proteins in the *E. coli* BN660 maxicell strain containing pJHC-S2572 (lane 2) and pJHC-S2570 (lane 3). Arrows indicate the position of the 110-kDa protein and the 57-kDa protein. Lanes 7 in *A* and 1 in *B* contain molecular mass markers whose sizes in kDa are to the left in *B*.

Table 1. Regulation of anguibactin production by AngR and Taf

Resident plasmid	Anguibactin, normalized value			
	None	pJHC-S2572 (AngR)	pJHC9-8 (Taf)	pJHC-S2572 + pJHC9-8 (AngR + Taf)
pJHC-T7::Tn3–1	1	2.5	4.2	23.4
pJHC-T7	1.6	2.7	9.4	23.6

Plasmids carrying the pJM1 iron-uptake region were pJHC-T7::Tn3–1, an *angR* mutant, and pJHC-T7, a wild-type gene. Complementing plasmids were pJHC-S2572 and pJHC9-8. Anguibactin levels, as determined by bioassays, were normalized to the anguibactin level of *V. anguillarum* harboring pJHC-T7::Tn3–1.

expressed the 110-kDa protein at higher levels than its parental clone pJHC-S2571 (Fig. 3*B*, lane 2). This result was correlated with the higher level of anguibactin production when this clone was used to complement mutation 160 in *V. anguillarum* (see above). Maxicells harboring pJHC-S2570 (with a modified *Nco* I site) did not express the 110-kDa protein but synthesized a new protein of 57 kDa, which is possibly a truncated product of the 110-kDa protein (Fig. 3*B*, lane 3). This clone did not increase the level of anguibactin production of mutant 160 (see above). These findings suggest that the 110-kDa protein gene may start just before the *Pst* I site and may extend through the *Nco* I site up to the two *Hin*dIII sites that mapped close to mutation 160 (Fig. 2). Therefore, this polypeptide must be the product of the *angR* gene.

Mode of Action of *angR*. To determine the possible mechanism of action of the *angR* gene, we introduced pJHC-S2571 as a source of the AngR protein into *V. anguillarum* strains containing the Tn3::HoHo1 *lacZ* gene fused to various genes of the anguibactin iron-uptake system and used those mutants in which the *lacZ* gene was expressed. The mutations analyzed were mutations 63 and 16, located in genetic unit I and affecting anguibactin biosynthesis (7); mutations 6 and 120, located in genetic unit IV; and mutations 2 and 19, located in genetic unit VI. All four mutations led to the shut off of anguibactin biosynthesis (7) (Fig. 1). The results indicated that the presence of pJHC-S2571 in mutant 63 resulted in a 2-fold increase of β-galactosidase activity, measured as described by Miller (24) under iron-limiting conditions. However, pJHC-S2571 was unable to modify the expression of the *lacZ* gene in mutations 16, 6, 120, 19, and 2 (data not shown). Therefore, the 110-kDa protein appears to regulate positively the expression of anguibactin biosynthetic genes in genetic unit I. Since insertion of Tn3::HoHo1 results in transcriptional fusions (15), the AngR protein must be controlling the expression of these genes at the transcriptional level.

Since Taf is another transcriptional activator of this iron-uptake system (7), we analyzed the contribution of AngR and Taf to regulation of the level of anguibactin production by

using *angR* mutant 1 and plasmids pJHC-S2572 and pJHC9-8 in complementation experiments. We also used pJHC-T7, which carries an intact iron-uptake system, as control for this experiment. Bioassays were performed to determine the level of anguibactin production on these *V. anguillarum* strains. Results in Table 1 indicated that the presence of AngR alone (provided by pJHC-S2572) resulted in a 2.5-fold increase whereas the presence of Taf alone (contributed by pJHC9-8) produced a 4.2-fold increase. However, the level of anguibactin production was 23.4-fold higher in the presence of both factors. Therefore, each factor could act independently. However, both factors together had a synergistic effect on the regulation of anguibactin biosynthesis. As expected, results with the wild-type plasmid pJHC-T7 were essentially similar to those obtained with the *angR* mutant, although the additional presence of pJHC-S2572 increased the level of anguibactin production due to the high expression of the *angR* gene under the control of the P_{tac} promoter.

To further characterize the mechanism of action of AngR, we analyzed the RNA levels from genes involved in anguibactin biosynthesis located in genetic unit I in which insertion of the *lacZ* gene (as in mutation 63) resulted not only in an impairment in anguibactin biosynthesis but also in a β-galactosidase activity that was regulated by the *angR* gene. The probe used was [^{32}P]UTP-labeled RNA made *in vitro* by using as a template an *Xho* I fragment from this region cloned in pKS (Bluescript).

Northern blot analysis showed that three transcripts of 3.1, 2.8, and 1.6 kilobases were recognized by the riboprobe in RNA obtained from strains carrying the iron-uptake region in the presence or absence of AngR and/or Taf. The three transcripts were iron regulated in all cases and were not present in the plasmidless *V. anguillarum* H775-3 nor in a strain carrying only pJHC9-8, a plasmid encoding Taf. Densitometry tracing indicated that, in the presence of Taf and AngR and under iron-limiting conditions, there was an 8-fold increase in the RNA levels of the 3.1- and 2.8-kb transcripts and a 4- to 6-fold increase in the 1.6-kb transcript, as compared to RNA from strains carrying only the *angR* gene or the *taf* gene (Fig. 4, lanes D, H, and J). These results suggest that Taf and AngR act synergistically on the level of these transcripts.

DISCUSSION

Iron, one of the most abundant metals on earth, is not readily available for bacterial growth in host vertebrates (26, 27). However, bacteria have evolved mechanisms to overcome this phenomenon by producing siderophores with a high affinity for iron (28). *E. coli* and enteric bacteria produce siderophores, such as enterobactin and aerobactin, in response to low iron conditions (4). This process is negatively regulated by the repressor molecule Fur (29–31). In contrast in this work, we have isolated and characterized *angR*, a regulatory gene encoding a 110-kDa protein, that in concert
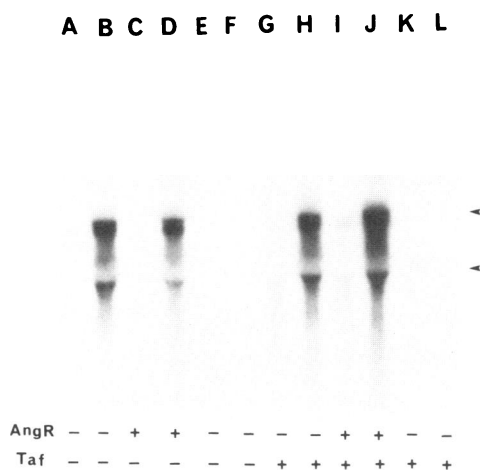
Biochemistry: Salinas *et al.*

Proc. Natl. Acad. Sci. USA 86 (1989)   3533

A B C D E F G H I J K L

AngR − − + + − − − − + + − −
Taf   − − − − − − + + + + + +

FIG. 4. Northern blot analysis. Total RNAs (20 μg) were fractionated in 1% formaldehyde gel. Lanes: A, C, E, G, I, and K, RNA extracted from *V. anguillarum* strains grown in 10 μM FeCl₃; B, D, F, H, J, and L, RNA from strains grown in ethylene di(*o*-hydroxyphenyl)acetic acid. Lanes: A and B, pJHC-T7::Tn*3–1* in strain H775-3; C and D, pJHC-T2612 in strain H775-3; E and F, strain H775-3 alone; G and H, pJHC-T7::Tn*3–1* and pJHC9-8; I and J, pJHC-T2612 and pJHC9-8; K and L, pJHC9-8 alone. Blot was hybridized with labeled RNA corresponding to genetic unit I involved in anguibactin biosynthesis. The blot was stripped and hybridized with a 720-base-pair DNA probe internal to the tetracycline gene of pVK102 (25) to assess equal loading (data not shown). Arrows indicate the position of the 23S and 16S *E. coli* rRNAs. +, Presence of indicated material; −, absence of indicated material.

with another regulatory gene, *taf* (7, 9), positively regulates the expression of the iron-uptake system in *V. anguillarum* under iron-limiting conditions.

Our results using hybrid clones strongly suggest that the 5′ upstream region and the amino-terminal portion of the 110-kDa protein in pJHC1 (up to the *Pst* I site) are not responsible for the Isp phenotype. Since the *angR* gene is functional in both strains 531A and 775 under iron-limiting conditions and encodes the 110-kDa protein, the differences observed between these strains might be due to mutation(s) within the coding region of the *angR* gene that could not be detected by restriction endonuclease analysis. An intriguing finding was that even when this gene was under the control of an external promoter, such as P_{tac}, anguibactin production was still iron-regulated.

Another important result was that Taf, a transcriptional regulator (7, 9), and AngR concertedly regulate the expression of iron transport as well as anguibactin biosynthetic genes under iron-limiting conditions. Both factors can act independently from each other; however, both together activate anguibactin biosynthesis in a synergistic manner. The increase in the levels of mRNA in the presence of Taf and AngR for one of the biosynthetic units was consistent with the increase in anguibactin levels, demonstrating that AngR and Taf also have a synergistic effect on the level of mRNA for anguibactin biosynthetic genes. The enhanced level of specific transcripts in the presence of AngR and Taf could be due to either an increase in transcriptional initiation or an increased mRNA stability. The fact that there is a measurable difference when we assess the levels of the fused β-galactosidase product suggests that at least part of this enhancement effect must be at the initiation level, since the 3′ terminus of the fused genes is now β-galactosidase sequences and in those instances examined so far mRNA stability depends on the action of ribonucleases with 3′-end exonuclease activity (32).

This type of positive regulation appears to be unique for the anguibactin iron-uptake system since the expression of other iron-uptake systems, such as enterobactin and aerobactin, has only been demonstrated to be negatively controlled by the *fur* gene product (29, 30). Moreover, the cooperative effect of two transcriptional activators is unusual in prokaryotic systems and the levels of activation by a single regulator and the two regulators in concert are comparable to those described in eukaryotic systems (33). It is possible that the AngR protein from strain 531A has a mutation in a putative activating domain that results in increasing activation functions as occur with point mutations in the *GAL4* gene (33).

Sequencing analysis of the *angR* gene and purification of the AngR protein will undoubtedly shed light on the nature of the mechanism by which these factors regulate the expression of the *V. anguillarum* iron-transport genes.

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