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Signal Transduction and Transcriptional and Posttranscriptional Control of Iron-Regulated Genes in Bacteria

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INTRODUCTION

"Some circumstantial evidence is very strong, as when you find a trout in the milk."
H. D. Thoreau, *Journal*, November 1850

Iron is the fourth most abundant metal on Earth; however, it is found in the environment as a component of insoluble hydroxides and it is present in biological systems chelated by high-affinity iron binding proteins or as a component of erythrocytes. Iron is essential for most organisms, with the exception of lactobacilli, and is found in all cells examined (172). It is essential because it is a component of key molecules such as cytochromes, ribotide reductase, and other metabolically linked compounds. However, iron can also be deleterious: hydroxyl free radicals generated through Haber-Weiss reactions catalyzed by iron can accumulate, leading ultimately to cell death (16, 19, 28, 29, 75, 79, 108, 121). It is not surprising, then, that production of the cellular components responsible for utilizing this "precious" metal is controlled by various parameters that act under different physiological and environmental conditions in either a negative (under iron-rich conditions) or a positive (under iron-limiting conditions) fashion. One important control comes directly from iron itself. High concentration of this metal leads to a shut-off of the expression of many genes involved in iron uptake; this occurs in conjunction with the Fur protein, which acts as a repressor together with iron (30). The other control mechanism acts once the bacterium encounters iron limitation conditions: this environmental status is transduced into the cell cytosol, and positive regulatory mechanisms are then turned on. The intention of this review is to concentrate on the mechanisms of regulation that occur under iron limitation conditions; however, since all the iron transport systems that are described are repressed by iron in conjunction with the Fur protein, I will devote the next few paragraphs to a brief report on this negative controlling mechanism.

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TURNOFF OF IRON UPTAKE GENE EXPRESSION BY IRON-Fur COMPLEXES UNDER IRON-RICH CONDITIONS

Escherichia coli mutants constitutive in the expression of iron-regulated genes showed a mutation at a single locus, designated *fur*, which mapped close to the *glnS* gene (46, 69, 70). The gene encodes a 148-amino-acid protein with a high content of histidine (9, 39–41, 70). By using pure Fur protein, it was possible to determine that it would bind to a DNA region upstream of the aerobactin biosynthesis genes, which is adjacent to the promoter and thus is probably an operator for the regulation of transcription of the aerobactin operon (40, 41). In combination with Mn^{2+} (because Fe^{2+} is more prone to oxidation than Mn^{2+} and the latter still forms a good complex with Fur), Fur protein protected a fragment of about 31 bp from cleavage with DNase I. In addition, it protected a second region of 19 bp when higher concentrations of the Fur protein-Mn^{2+} complex were used. The presence of Mn^{2+} enhanced binding to the 31-bp sequence by a factor of about 1,000. The site of protection extends from 7 bases upstream of the −35 sequence to the bp 1 of the −10 region. Binding of the Fur repressor in the presence of Mn^{2+} was also shown for regions upstream of the *cir* gene (60). From all these studies, a consensus sequence for the Fur-binding site was obtained: GAT AATGATAATCATTATC. This consensus was confirmed by introducing a synthetic nucleotide with the consensus sequence

upstream of an *ompF-lacZ* fusion. Iron-regulated *lacZ* expression was obtained upon transfer of the construct to *E. coli* (21). These results clearly showed that the complex of Fur and Fe^{2+} is a transcriptional repressor of iron-regulated genes. Later, sequences showing homology to the Fur-binding sites described for the aerobactin operon, the Shiga-like toxin gene, the *cir* gene, and upstream of the *fur* gene itself, were also demonstrated upstream of a large number of other iron-regulated genes (9, 39–41, 70). Fur-like proteins in *Yersinia pestis* (139, 140), *Vibrio cholerae* (95), *V. vulnificus* (96), *Pseudomonas aeruginosa* (124), *P. putida* (163), *Vibrio anguillarum* (159, 169), *Bordetella pertussis* (17), *Campylobacter jejuni* (176), *Legionella pneumophila* (78), *Neisseria meningitidis* (86, 151), *Neisseria gonorrheae* (11), and cyanobacteria (105) have been described. By different approaches, such as marker exchange for *V. cholerae* (95), *V. vulnificus* (96) and *Y. pestis* (119, 120) and selection with Mn^{2+} (since *fur* mutants are resistant to high concentrations of Mn^{2+} [71]) for *P. aeruginosa* (124) and *V. anguillarum* (174), it was possible to demonstrate that mutations in the *fur*-like gene in these bacteria lead to an impairment of iron regulation for specific iron-regulated systems.

By using an ordered *E. coli* cosmid library (146), several new Fur box-containing genes were identified, cloned, and mapped in the *E. coli* genome. The method was also very efficient in the identification of Fur box-containing genes from other gram-negative and some gram-positive bacteria and in the identification of genes whose products are involved in iron storage and/or binding. Recently, another excellent method, in vitro cycle selection of iron-regulated genes, was used to successfully identify 21 *Pseudomonas* iron-regulated genes (PIGs) (112). Novel targets of the Fur protein were isolated in a powerful in vitro cycle selection consisting of in vitro DNA-Fur interaction, binding to anti-Fur antibody, purification on protein G, and PCR amplification. By this method, DNA fragments obtained after three exponential enrichment cycles were cloned and subjected to DNA mobility shift assays, DNase I footprint analysis, and RNase protection to verify the interaction with the Fur protein and their iron-dependent expression. While 4 of the PIGs isolated by this method were identical to already known genes (*pfeR*, *pvdS*, *tonB*, and *fumC*), 17 PIGs were novel. Homology studies of the proteins encoded by these PIGs resulted in the identification of two novel siderophore receptors and three novel alternative sigma factors of the extracytoplasmic function (ECF) subfamily (97).

There is also enough evidence that Fur may play a variety of roles in controlling the expression of other genes and bacterial metabolism. It is very clear that Fur is a pleiotropic protein that regulates the expression of many iron-regulated and some non-iron-regulated genes (33). There are countless examples of the former, and in those cases ferrous iron is always found as a cofactor. For the latter, the example of the *sodB* gene regulation comes immediately to mind. *sodB* expression is regulated by Fur in a positive fashion, possibly by a direct DNA-protein interaction that does not require the ferrous iron (109). In another example of Fur pleiotropism, it is very well known that *E. coli fur* mutants cannot grow with glycerol, succinate, or fumarate as the carbon source, and it was shown that although the expression of certain genes in *Salmonella typhimurium* was repressed by high iron concentrations, a series of nine proteins were expressed more strongly in the presence of Fur and iron (48). Furthermore, Fur^- mutants were more sensitive to acid, a feature that may be important in the ability of this intestinal pathogen to travel through the stomach and also in its intracellular survival (48, 65).

A motif different from that present in gram-negative bacteria might be responsible for the binding of iron-dependent repressor in coryneform and other gram-positive bacteria. The first example of a gram-positive iron-regulated promoter/operator examined was that of the diphtheria toxin gene, *toxA*, of *Corynebacterium diphtheriae* (14, 147). Evidence was presented for direct regulation of diphtheria toxin gene transcription by an Fe^{2+}-dependent DNA-binding repressor, DtxR (14, 49). The 226-amino-acid DtxR protein shows little, if any, amino acid homology to the *E. coli* Fur protein. The specific binding of the Fe^{2+}-dependent DtxR protein to the *toxA* operator occurred on a 9-bp interrupted palindromic sequence (14) that showed only 25% homology to the Fur operator. Gunter et al. (63) identified in another gram-positive bacterium, *Streptomyces pilosus*, a region in the promoter for the deferrioxamine operon which is essential for iron regulation and is highly related to that of the *C. diphtheriae* DtxR-binding site. It is now clear that DtxR functions as a global iron-sensitive regulatory element in the control of gene expression in *C. diphtheriae*. Recent physical analysis by X-ray crystallography at 3.0-Å resolution and site-directed mutagenesis has led to the identification of the two potential metal ion-binding sites which may play a role in the activation of DNA binding by the repressor. The primary site functions directly in the activation of DNA binding, while the ancillary site contributes weakly to activation (42, 149).

TURN-ON OF IRON UPTAKE GENE EXPRESSION UNDER IRON LIMITATION

Once the bacterium reaches conditions of iron limitation, the negative control mediated by Fur cannot occur. In many systems, this is all that is required; derepression of the iron-regulated genes leads to biosynthesis of the transport and siderophore biosynthesis proteins. However, in other systems, when the bacterium meets conditions of iron deprivation, positive gene regulation is required for the synthesis of the iron transport proteins and/or siderophore biosynthetic enzymes; i.e., when the cell senses the lack of iron in the immediate environment, the need for expression of the iron transport system is, in some way, transduced inside the cell to make operational the actions of transcription enhancers for the particular iron transport system.

In this review, I concentrate on positive regulatory mechanisms in the most extensively studied systems: the ferric dicitrate system of *E. coli*, the siderophore-mediated induction in pseudomonads, the regulation of a virulence factor in *Vibrio cholerae*, and our own work on the regulation of the plasmid pJM1-mediated iron uptake system in *Vibrio anguillarum*. Finally, although I concentrate almost exclusively on positive regulatory mechanisms, I will also discuss our work on a unique repression mechanism that operates posttranscriptionally under iron-rich conditions to control iron transport gene expression in *Vibrio anguillarum.*

I do not pretend that this will be an exhaustive analysis of all regulatory systems related to iron transport, but it will, I believe, give the reader an account of some of the most recent information on the mechanisms of signal transduction and the subsequent regulation at the transcriptional and/or posttranscriptional level. Evidence for all the stages, from signal transduction from the environment to the actual regulatory process, is not available for all the systems. Therefore, I emphasize the stages that have been elucidated in each of the systems discussed.

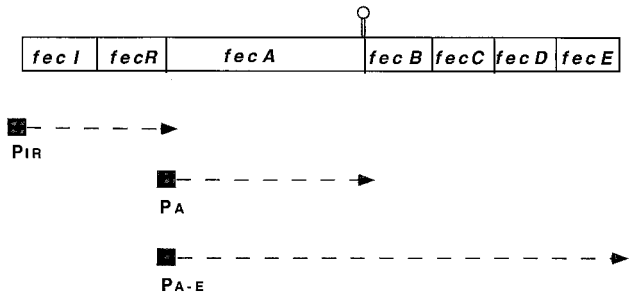

FIG. 1. Ferric dicitrate iron transport system of *E. coli*. Three transcripts have been identified. One corresponds to the *fecI-fecR* region (promoter PIR), another corresponds to the *fecA* region (promoter PA), and the third corresponds to the *fecA-fecE* region (promoter PA-E). Transcripts are symbolized by the dashed arrows and the respective promoters by the boxes. ⚲, hairpin at the end of the *fecA* gene.

FERRIC DICITRATE SYSTEM OF *E. COLI*

Transport of iron into the cell cytosol of *E. coli* can be induced by citrate at low iron concentrations (50, 67, 167). The ferric dicitrate system, shown in Fig. 1, is also repressed at high iron concentrations by the Fur protein (123). The evidence indicates that the outer membrane protein receptor FecA, as well as TonB, ExbB, and ExbD, is required for induction, since cells carrying mutations in any one of the genes encoding these proteins no longer expressed ferric dicitrate transport proteins whereas cells with mutations in genes required for transport through the cytoplasmic membrane were still fully inducible (177). Further evidence suggests that citrate does not need to enter the cytoplasm to induce the citrate system (81). Although the two regulatory genes, *fecI* and *fecR*, found upstream of the transport genes (123, 141) intervene in the transmembrane signalling, they do not show any homology to the two-component regulatory systems which are controlled by a phosphoryl transfer mechanism (6, 61, 143). In these systems, such as the EnvZ-OmpR system for porin gene regulation, the Ntr regulon, which senses nitrogen availability, or the chemotaxis system (80), a transmembrane protein serves as the sensor of the inducer and transmits the signal to the receiver, which in turn activates transcription. Signal transduction involves autophosphorylation of the sensor followed by phosphorylation of the receiver. The histidine phosphorylation site of the sensor and the aspartate phosphorylation site of the receiver are both contained in conserved sequences among the various two-component systems but are not present in FecR and FecI. Apparently, the ferric dicitrate transmembrane regulation follows other rules. The FecI sequence revealed a DNA-binding motif consisting of a helix-turn-helix motif. It is important to state that FecR was required for ferric dicitrate regulation. It is likely that FecR transduces the signal from the periplasm into the cytoplasm. To understand the mechanism of regulation in this system, Braun's laboratory carried out a very thorough genetic analysis of the transcription of the ferric dicitrate transport by using fusions to the β-galactosidase gene carried on a derivative of phage Mu (123, 141, 161).

Recently, this study was complemented by an analysis of the transcripts synthesized from the ferric dicitrate transport system in *E. coli* (38). The transcription of the regulatory and structural genes under various environmental conditions was studied, and the locations of the transcriptional start points and promoter regions were determined (Fig. 1). The main species observed in Northern hybridization analyses were a 2.5-kb mRNA, encoded by the outer membrane protein receptor gene *fecA*, and a 1.5-kb mRNA encoded by a region including the *fecIR* genes. The synthesis of the 2.5-kb *fecA* mRNA is regulated by citrate in a positive fashion and is repressed by iron. Furthermore, it was also determined that transcription of the *fecA* gene is dependent on the presence of FecI. The promoter region for the *fecA* mRNA, a likely site of action for FecI, is not related to the consensus promoter region for σ^{70} RNA polymerase in *E. coli* K-12. However, it shows the greatest similarity to promoters of genes regulated by a new subfamily of σ factors, i.e., the ECF σ factors, which are associated with the expression of genes involved in extracytoplasmic functions (97), suggesting that FecI may act as a specialized σ factor. It was also shown (Fig. 1) that the *fecBCDE* transport genes are linked in operon fashion to the *fecA* gene. Since the levels of the *fecBCDE* RNAs are extremely low compared to the level of *fecA* mRNA, it is likely that processing from the 3′ end must occur and stop near the end of the *fecA* gene, where a hairpin structure is located (Fig. 1).

To assess the contribution of FecR and FecI to the regulation of expression of the *fec* transport genes, several genetic and molecular approaches were followed by Braun's group. Clones containing portions of the *fecR* gene were prepared, and the proteins were examined functionally. Cells expressing truncated FecR derivatives no longer responded to ferric dicitrate, and they expressed the *fec* transport genes constitutively. The smallest active derivative was 59 amino acids long, compared to the wild type, which is 317 amino acids. The level of *fec* transport gene expression occurring in the constitutive derivative was lower than that obtained when the FecR wild-type strain was induced by ferric dicitrate. The model currently proposed by Braun and colleagues is that the ferric dicitrate effect is actually mediated by FecA, so that the N terminus of FecA, like the N terminus of PupB, is involved in the signal transduction process (88). There is a conformational change in FecA which promotes an interaction with FecR involving an N-terminal and periplasmic tail. FecR then activates FecI to transcribe the *fec* transport genes. Deletion analysis of *fecR* had also revealed that the response to ferric citrate is ascribed to the C-terminal portion of FecR while the interaction of FecR with FecI is localized to the N-terminal end of FecR (110).

At high ferric dicitrate concentrations, there is diffusion into the cell cytosol of a *fecA* mutant strain, with no evidence of induction of transcription of the ferric dicitrate system. However, there is a proposed involvement of FecA in induction, independent of its transport activity, in strains harboring missense point mutations within the *fecA* gene. In this case, transcription of the ferric dicitrate system was induced in the absence of ferric dicitrate. It appears that a conformational change is transmitted from FecA to FecR and that TonB, ExbB, and ExbD are part of the signal transduction mechanism in addition to the function of ferric dicitrate transport across the membrane (15, 64, 72).

Recently, Braun's laboratory (7) examined the mechanism of transcription enhancement by FecI by attempting to assess the role played by FecI in the process. To pursue this aim, the investigators cloned the *fecI* gene under the control of an ideal ribosome-binding site to obtain overexpression of the protein, since the chromosomal gene produces only low levels of FecI. The overexpressed FecI formed inclusion bodies. However, solubilization in *N*-dodecyl-*N*,*N*,-dimethyl-3-ammonio-1-propanesulfonate resulted in a product that could be purified by gel filtration in the active form. The purified FecI protein, in conjunction with the RNA polymerase core enzyme, was able to specifically transcribe the *fecA* promoter in vitro. Moreover, DNA fragments upstream of *fecA* were specifically retarded in mobility shift experiments in which core RNA polymerase was

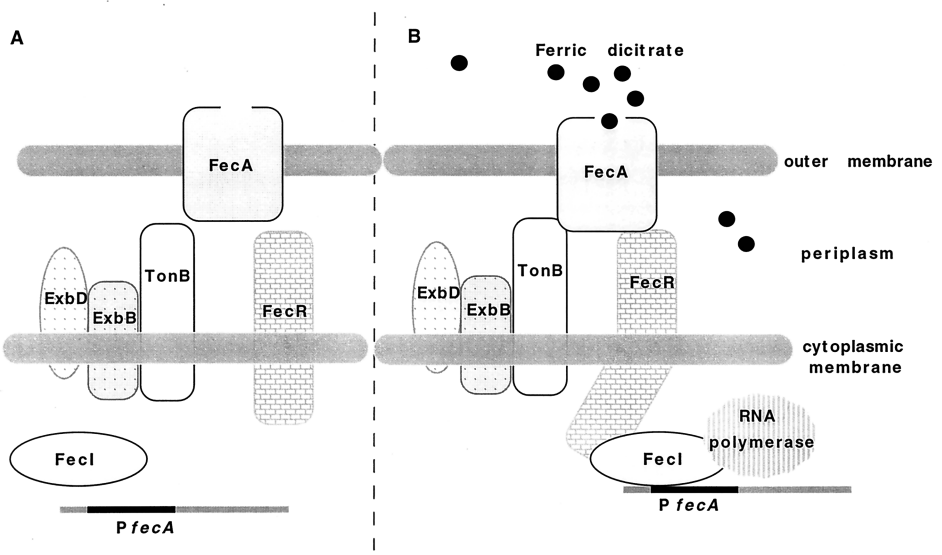

FIG. 2. Model of the regulation of the ferric dicitrate system. (A) Absence of ferric dicitrate; (B) presence of ferric dicitrate.

added to the fragments in conjunction with FecI. An additional finding was that ferric dicitrate, FecA, and FecR increased the amounts of promoter DNA bands that were shifted. Further in vivo genetic experiments with high-copy-number plasmid constructs containing the *fecA* promoter demonstrated that FecI is removed by the high-copy-number wild-type *fecA* promoter DNA from interaction with the chromosomal region but not by mutated *fecA* promoter DNA. The results indicated that FecI interacts most strongly with the +13 region, at nucleotide 2750 (position 13 relative to the transcription initiation site for *fecA* [7] as determined by primer extension [45]), which is an unusual site for a σ^{70}-like factor interaction. These results, however, strongly suggest that this DNA sequence must play an essential role in the FecI-mediated activation of RNA polymerase for transcription initiation. Previous transcriptional studies identified a region upstream of *fecA* that showed homology to ECF promoter regions for σ^{70}-regulated genes (45), such as those belonging to the ECF subfamily, i.e., *algD* of *P. aeruginosa* regulated by AlgU (87). A common feature of these ECF factors is that they sense a signal generated at or outside the cytoplasmic membrane. Induction of the ferric dicitrate transport system would fit into this category, since ferric dicitrate bound to FecA in the outer membrane induces synthesis of the ferric dicitrate transport proteins.

Braun and colleagues also studied whether the binding of FecI-RNA polymerase to the *fecA* promoter DNA depends on a signal. To assess this hypothesis, they performed binding experiments (7, 111) with lysates from an *E. coli* strain harboring the *fecIRA*, *fecIA*, and *fecIR* mutations. It was of interest that binding to the 267-bp promoter fragment occurred only in cells expressing the FecI, FecR, and FecA proteins if the cells were grown in the presence of ferric dicitrate. The fragment was shifted to the same position as when purified FecI together with RNA polymerase was used. Since no mobility shifts occurred in the absence of FecA and FecR, the investigators proposed that active FecI must be formed during growth on ferric citrate. It is therefore possible that ferric dicitrate binds to FecA and, through the activity of TonB, ExbB, and ExbD (64, 77, 107), triggers a conformational change in FecR that results in the activation of FecI (Fig. 2). It is obvious that the active conformation of FecI is maintained in the cell lysates.

POSITIVE REGULATION BY SIDEROPHORES OF OUTER MEMBRANE RECEPTOR EXPRESSION IN PSEUDOMONADS

P. aeruginosa, an important opportunistic pathogen of humans, possesses many systems devoted to the transport of iron into the cell cytosol, a finding consistent with the wide range of environmental conditions under which this microorganism is found (117, 135). The siderophores produced by this bacterium are pyochelin and pyoverdin (also called pseudobactin), although it can also use pyoverdins produced by other pseudomonads, as well as enterobactin, ferrioxamine B, and aerobactin (12, 13, 28, 29, 36, 92, 100, 104, 150, 173). The outer membrane cognate protein receptors for ferric pyochelin, ferric pyoverdin, and ferric enterobactin have now been identified (12, 74, 90, 100, 103, 136).

In addition to the control exerted by iron (115), expression of the receptors for ferric pyoverdin and ferric pyochelin in *P. aeruginosa* is regulated by the corresponding siderophore (52). The ferric enterobactin receptor, PfeA, which has a molecular mass of 78 kDa, shows homology to the corresponding enterobactin receptor, FepA, of *E. coli* (39). Induction of the PfeA receptor and of ferric enterobactin uptake is switched on not only by iron limitation but also by the presence of enterobactin. Likewise, induction of the ferrioxamine receptor requires the presence of ferrioxamine B. In the following sections, I discuss each of the siderophore uptake systems and their regulation in pseudomonads. The regulatory circuitry in *P. aeruginosa* iron transport gene expression is complex; therefore I have included in Table 1 a summary of the regulatory molecules and a comparison of their mechanisms with those of the other bacteria discussed. I have also included Fig. 3 and 4 to help in following the arguments presented.

Pseudobactin Uptake Systems

In addition to PupA, the pseudobactin 358 receptor, *P. putida* WCS358 possesses several other outer membrane receptors that recognize various pseudobactins. The transcriptional regulation of *pupB*, one of the receptor genes for the heterologous pseudobactins BN7 and BN8, occurs via two reg-

TABLE 1. Transcriptional regulators

Bacterium	Regulator	Action	Target
E. coli	FecI/FecR	Positive	*fec* system
	Fur	Negative	All iron uptake systems
P. putida	PupI/PupR	Positive	*pupB*
	PfrA	Positive	Pseudobactin 358 biosynthesis genes
P. fluorescens M114	PbrA	Positive	Pseudobactin 358, M114 biosynthesis, and cognate receptors
P. aeruginosa	PchR	Positive?	Pyochelin system
	RegA	Positive	*toxA*
	LasR	Positive	*las*
	PvdS	Positive	*pvdA* (pyoverdin system)
	Fur	Negative	Many genes
V. cholerae	IrgB	Positive	*irgA*
	Fur	Negative	Various systems
V. anguillarum	AngR/TAF	Positive	*fat* and *ang* systems
	Fur	Negative	*fat* and *ang* systems
	Fur	Positive	RNAα expression
	RNAα	Negative	*fat* iron transport system

ulatory genes, *pupI* and *pupR* (89, 90). The 19-kDa PupI protein is essential for the transcription of the *pupB* gene. There is a helix-turn-helix motif in the C terminus of PupI. The other component, PupR, appears to modify the activity of PupI in response to the availability of the specific heterologous pseudobactin-iron complex. As can be seen from these introductory statements, the PupI-PupR system is very similar to the FecI-FecR system; PupI shows homology to FecI, and PupR shows homology to FecR. However, the mechanism of action appears to be different because it was described that in a *pupR* mutant, the *pupB* gene is transcribed independently of pseudobactin BN8, suggesting that PupR must inhibit the activity of PupI in the absence of the siderophore. Therefore, pseudobactin must actually remove PupR from that inhibitory activity. However, in the same vein as that for the FecI-FecR system for expression of the *fecA* gene, the activity of the *pupB* promoter is considerably reduced in this *pupR* mutant, indicating that PupR must be required for maximal expression of *pupB*. In this system, the receptor PupB itself, as with the ferric dicitrate system for FecA, appears to be required for the pseudobactin enhancement of *pupB* transcription. Koster et al. (89, 90) constructed a chimera consisting of a *pupB-pupA* hybrid. This hybrid receptor, which had the first 86 amino acids of PupB and the rest of PupA, could still transport a complex of ferric pseudobactin 358 but had, via a mechanism mediated by the PupI-PupR system, now become able to induce the *pupB* promoter in response to pseudobactin 358, which suggests that the signal for expression of this system is not the siderophore itself but, rather, a transduction mechanism via the receptor upon which transport is occurring. The model proposed by these investigators is that PupB transduces a signal to the PupR protein during the transport of the siderophore. This signal results in a conformational change of PupR, and as a consequence it can no longer repress PupI but actually activates it, leading to transcriptional activation of *pupB*. In this case, as well as in the ferric dicitrate system, it is possible that the signal transduction processes occur by a cascade of conformational changes on the intervening proteins. It is known that ferric siderophore receptors can act as gated channels. These channels open in response to the action of a TonB energy-coupling system, and then the ferric-siderophore complex can travel across the outer membrane barrier (15, 68, 84, 85, 121, 122, 128). It is therefore possible that in the FecI-FecR and PupI-PupR systems, opening of the channel and a change in conformation of the siderophore receptor act as the signal recognized by the FecR or PupR components. As in the case of the FecI-FecR system, induction of *pupB* expression via PupI-PupR requires the TonB protein, supporting this model. The PupI-PupR system, as was the case for FecI-FecR, does not show homology to the two-component systems.

In *P. putida*, expression of biosynthetic genes for pseudobactin (structurally related to pyoverdin) also depends on the presence of pseudobactin (164). Biosynthetic genes for pseudobactin 358 and its cognate outer membrane protein receptor PupA are transcribed in the plant growth-promoting *P. putida* WCS358, under conditions of iron limitation, only if the product of the *pfrA* gene is present. PfrA is an 18-kDa polypeptide that has 58% identity to AlgQ, a positive regulator for alginate biosynthesis in *P. aeruginosa* (98, 99, 162). AlgQ possesses kinase activity, although it does not belong to the class of typical bacterial two-component signal transducing systems (143). PfrA is also able to complement an *algQ* mutant of *P. aeruginosa* (162) for restoration of mucoidy (alginate production). Conversely, although poorly, *algQ* could also complement a *pfrA* mutant in *P. putida*. Venturi et al. (163) described, in addition to PfrA, another positive transcriptional activator, PfrI, which shares homology with PupI and FecI. The 19.5-kDa PfrI contains a helix-turn-helix motif. PfrI does not up regulate the synthesis of the cognate receptor PupA; however, only pseudobactin 358 biosynthetic genes are affected by this transcriptional activator. The Fur protein can bind to the promoter region of *pfrI* and thus shut off siderophore synthesis. The *pupIR* promoter also has a sequence with homology to the Fur box, and evidence indicates that repression of the expression of siderophore biosynthetic genes occurs by shutting off the expression of *pfrI* and *pupIR* by means of the binding of a Fur-iron complex. The question is whether there is another component, PfrR, in the model in Fig. 3, which could be a homolog of PupR. In addition, these authors demonstrated that pseudobactin 358 itself plays a role in the biosynthesis of this siderophore. The experiments were carried out by using a mutant that can no longer synthesize pseudobactin 358. The results demonstrated that promoter activity of siderophore biosynthetic genes, as assessed with *lacZ*-promoter fusions, is significantly lower in the mutant than in the parent strain under iron-limiting conditions. The original promoter activity could be restored almost to full capacity by the addition of pseudobactin 358. Addition of other pseudobactins or of pyoverdin did not restore the activity of this mutant. A possible involvement of PupA in the regulatory circuitry induced by pseudobactin 358 was also proposed, since this protein possesses an uncommon N-terminal domain with 60 residues located in the periplasmic space. This is an array very similar to that described for the pseudobactin BN7 receptor PupB, which has been shown to be involved in signal transduction to the regulator PupR in response to the presence of the specific ferric siderophore.

As a consequence of iron limitation, *P. fluorescens* M114 shows an enhanced expression of a number of genes, including those involved in the biosynthesis of the siderophore pseudobactin M114 and its cognate receptor PbuA. By using transposition mutagenesis with a Tn*5-lacZ* derivative, a mutant was isolated that had lost the ability to express these iron-regulated genes and that showed a pleiotropic phenotype: a cosmid clone

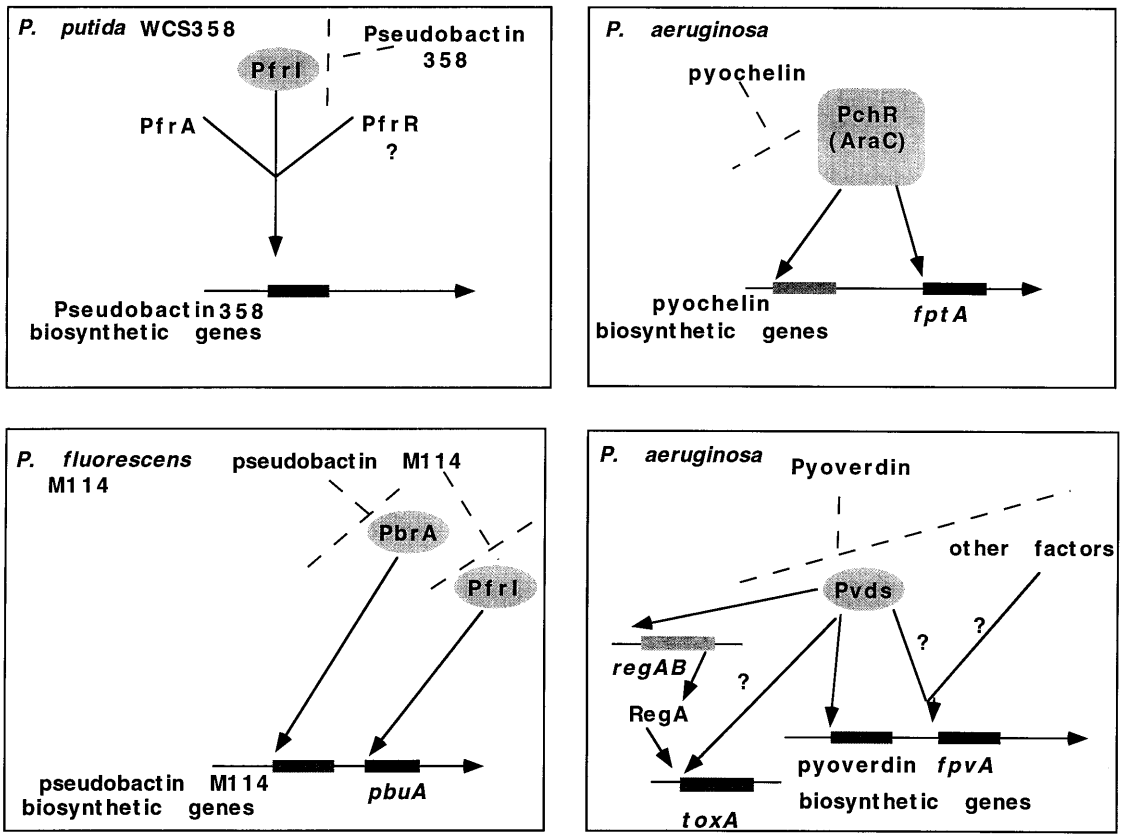

FIG. 3. Models of the regulation by ferric pseudobactin in *P. putida* WCS358 and *P. fluorescens* M114 and by ferric pyochelin and pyoverdin in *P. aeruginosa.*

containing the wild-type genes not only complemented this mutation but also restored the activation of a number of iron-regulated promoter fusions from *P. fluorescens* M114 and *P. putida* WCS358 (133). This clone carried *pbrA*, which could also promote the expression of these fusions in *E. coli*. The deduced amino acid sequence of the *pbrA* product, a polypeptide of 184 amino acids, showed significant domain homology to a number of ECF transcriptional regulators of the σ^{70} sigma factor family. These ECF factors included FecI, *P. aeruginosa* AlgU (which is involved in the positive regulation of extracellular alginate biosynthesis), and *P. syringae* HrpL (which controls transcription of a plant virulence factor). Members of the ECF group are regulated by environmental stimuli, such as iron and citrate for *fecI*, plant extracts for *hrpL*, and iron and osmolarity for *algU*. Furthermore, PbrA also showed homology to SpoIIIC, the C terminus of the *Bacillus* sporulation sigma factor, sigma-K, and to the *Rhizobium meliloti fixJ* gene (133). PbrA is essential for the transcriptional activation of all iron-regulated promoters of *P. fluorescens* tested, and, in contrast to PfrA, PbrA is required for the coordinate control of the transcription of both pseudobactin M114 and heterologous pseudobactin 358 biosynthetic and receptor promoter fusions, under iron-limiting conditions. The iron-mediated repression of the expression of all these genes might occur by a Fur-iron mediated repression of *pbrA* expression (133).

Pyoverdin Uptake System

The genetic loci involved in pyoverdin synthesis and uptake in *P. aeruginosa* are located within a 103-kb region at about 47 min of the PAO1 chromosome map. Pyoverdin biosynthetic genes have also been localized to the 23-min region of the revised PAO map and, more recently, to the 66- to 70-min region (104, 142). The *pvdA* gene encodes the enzyme L-ornithine N5-oxygenase, which catalyzes a key step of the pyoverdin biosynthetic pathway in *P. aeruginosa*: the formation of the hydroxamate ligands (L-N5-OH-ornithine residues) of pyoverdin (166). The product of the *pvdD* gene has been proposed to be a synthetase involved in the assembly of the peptidic moiety of the siderophore by a nonribosomal mechanism (102), while the gene *fpvA* encodes the outer membrane protein receptor for ferric pyoverdin complexes. It was originally thought that the products of the *envCD* gene cluster, now called *mexAB* (94, 119), could be involved in the secretion of pyoverdin (118), but there now seems to be less certainty that this cluster is strictly involved in siderophore export. More recent speculation indicates that it may be involved in the export of secondary metabolites (53). No recognition sequences for the iron regulator Fur were identified on the control regions of genes involved in pyoverdin biosynthesis and uptake (54, 100, 118) while such regions were found on the controlling regions of genes involved in pyochelin- and enterobactin-mediated iron transport in *P. aeruginosa* (8, 36, 76, 120).

The promoter controlling the expression of *pvdA* was found within a 154-bp sequence upstream of the *pvdA* translation start site (93). The minimum DNA region required for iron-regulated promoter activity was mapped from bp −41 to −154 relative to the ATG translation start for the *pvdA* gene (126). Two 5′ termini were mapped on *pvdA*-specific transcripts, one at 68 bp (T1) and the other at 43 bp (T2). The −10 region of the T1 promoter had a fair degree of similarity to −10 regions of AlgU-dependent promoters controlled by the σ^{E}-like factor

PvdS. The other promoter, T2, was located 43 bp upstream of the *pvdA* translational start and accounted for about 10% of the total *pvdA* mRNA. Both transcripts were about 1.6 kb and were apparently monocistronic. A 57-bp potential stem-loop structure was identified as being centered 255 bp downstream of the *pvdA* promoter (p*pvdA*) coding sequence. It is hypothesized that this sequence may serve as a generator of the 3′ terminus of the *pvdA* mRNA either by protection from 3′ exonuclease activity or by 3′ processing. It was of interest that a region between nucleotides −33 and −25 relative to the start site for the T1 transcript was homologous (6 of 9 nucleotides) to the corresponding region of the *regAB* p2 promoter (Reg is a regulator of various exo products of *P. aeruginosa*) and that the −35 region of this transcript was highly conserved compared with the consensus sequence (G/C)CTAAATCCC found in the promoter regions of *pvdA*, *pvdD*, *pvdE*, and *toxA*. It is noteworthy that within this region, mutations in the CTAAAT motif resulted in a strong reduction of promoter activity. PvdS and PbrA, which activate the transcription of pyoverdin genes in *P. aeruginosa* PAO1 and pseudobactin genes in *P. fluorescens* M114, respectively, show extensive domain homology to other ECF members in domains 2.4 and 4, which are responsible for site recognition of −10 and −35 sequences (93). PvdS was first described by Miyazaki et al. (106) and Cunliffe et al. (35).

With respect to the T2 transcription start site, it is remarkable that the region between T1 and T2 is essential for the *pvdA* promoter activity, since a deletion that extends upstream of the T2 start point resulted in complete cessation of promoter activity (93). There is some similarity between the DNA region upstream of T2 and the consensus for σ^{54}-dependent promoters. However, T2 may not be transcribed by a σ^{54}-like RNA polymerase, because the transcription initiation and the activity of the *pvdA* promoter are similar in *rpoN*$^+$ and *rpoN P. aeruginosa* strains. p*pvdA* was silent in *E. coli* unless a construct containing the *pvdS* gene was also present. It is noteworthy that once this *pvdS* gene was introduced, the expression of the *pvdA* gene became regulated by iron, underscoring the previous results that Fur may act on *pvdS* rather than directly on *pvdA* (93). In these experiments, excess iron did not completely repress transcription from p*pvdA*, which may be due to the large number of Fur-binding sequences on the *pvdS* promoter sequence, which titrate out the *E. coli* Fur repressor pool. A Fur-PvdS connection was also reported for the *pvdD* and *pvdE* promoters (35). Overexpression of *pvdS* by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) increased the β-galactosidase levels of the fusions even in the presence of high levels of iron. It is obvious that PvdS must confer to the *E. coli* RNA polymerase specificity for the pyoverdin promoters. However, there may be other activators in this bacterium, since the activity was significantly lower in *E. coli* carrying a multicopy *pvdS* gene than in *P. aeruginosa*. The *pvdA* promoter and the SP1 promoter of *P. fluorescens* thus are activated by iron-regulated σ^{E}-type factors, but they differ in that *pvdA* lacks a Fur-binding motif and is not directly controlled by Fur. Figure 3 shows a model of regulation in this system.

As presented before in the citrate system, in *P. putida* there is an interaction of the iron-siderophore complex with the surface receptor which leads to a signal transduction cascade that ultimately results in activation of the receptor gene expression. It is possible that the requirement for FpvA in *P. aeruginosa* up regulation by pyoverdin is due to a similar type of mechanism.

A recent finding has related PvdS to expression of the exotoxin A gene (113). Exotoxin A is produced by *P. aeruginosa* under iron-limiting conditions, and expression of the *toxA* gene is regulated at the transcriptional level by the products of the *regAB* operon. Expression of *toxA* and *regAB* is repressed under iron-rich conditions. The Fur protein does not interact with either the *toxA* or *regAB* promoters. Ochsner et al. (113) recently presented evidence that the repression by iron of the expression of these promoters is mediated by Fur repression of the expression of PvdS, which shares domain homology with alternative sigma factors. In a Δ*pvdS* deletion mutant, exotoxin A is produced at low levels (less than 5% of those in the wild-type strain). Introduction of a cloned *pvdS* gene restores the production of toxin A. Furthermore, neither *toxA* nor *regAB* mRNA could be detected in the Δ*pvdS* mutant, and overexpression of PvdS by using a construct in which the *pvdS* gene is under the control of the *tac* promoter leads to high-level production of exotoxin A, which becomes constitutive with respect to the iron concentration in the cell. This is not true in a Δ*regA* mutant, suggesting that PvdS must be required for activation of the *regAB* promoter. Kinetic experiments carried out during growth of the *Pseudomonas* strains carrying the p*tac pvdS* construct indicated that both *regAB* and *toxA* transcripts were highly expressed under iron-rich conditions during all growth phases if microaerobic conditions were used (10). However, under aerobic conditions, only *regAB* transcripts could be detected during all growth phases, while *toxA* transcripts were detected only during the exponential phase but not the early stationary phase of growth. The authors interpreted these results to be suggestive of the presence of a second regulatory mechanism of iron control of toxin A production beyond that controlled by PvdS and Fur. Recently, Hamood et al. described a second regulator, PtxR, which up regulates exotoxin expression by apparently activating *regA* expression. This protein also up regulates siderophore expression (66).

Pyochelin Uptake System

The product of the *pchR* gene is a 31-kDa AraC-like regulatory protein, which is required for production of the FptA ferric pyochelin receptor in response to iron limitation and to the presence of pyochelin and which appears to mediate the pyochelin-dependent expression of FptA (74–76). Heinrichs and Poole (75) used the transcriptional fusions *fptA-lacZ* and *pchR-lacZ* to study the regulation of gene expression for *fptA* and *pchR*. Their findings can be summarized as follows. Inactivation of *pchR* by insertion of an omega cartridge, with termination signals for transcription and translation in both orientations, led to a dramatic decrease in the expression of *fptA*. However, this effect was not as pronounced as that found when pyochelin was not present. It is noteworthy that insertional inactivation of *pchR* in a pyochelin-deficient background restored *fptA* expression to the levels found in the pyochelin-proficient PchR-deficient strain. Therefore, it is apparent that PchR must repress *fptA* expression in the absence of pyochelin. This was further proved by noticing that the cloned *pchR* gene caused a fivefold decrease in the expression of the *fptA-lacZ* fusion in *E. coli*. As with *pvdS*, *pchR* expression was repressed by iron. The data from this work indicated that PchR functions as both an activator and repressor in controlling the expression of *fptA* and *pchR* (74–76). The involvement of FptA in this control is unclear, although it may be important in mediating the effect of pyochelin on *fptA* expression, possibly by modulating PchR activity. The activities of PchR as a repressor and inducer are uncommon, although there are some regulators such as AraC that play such dual roles. AraC regulates the expression of genes involved in the metabolism (*araBAD*) and transport (*araFGH*) of arabinose. Expression of *araBAD* is repressed in the absence of arabinose and activated in its

presence. Repression occurs by the binding of an AraC dimer to two half-sites, *araO2* and *araI1*, which are 210 bp apart. This binding causes a loop formation of DNA upstream of *araBAD*. Arabinose may cause induction of the system by interacting directly with the amino-terminal portion of AraC, causing a conformational change which not only affects loop formation but also leads to an enhanced binding through its carboxy terminus to adjacent half-sites, *araI1* and *araI2*, which in turn leads to *ara* gene expression. Whether this model could be applied to the PchR-mediated regulation of *fptA-pchR* expression remains unclear. It is noteworthy that two partially conserved heptameric repeat sequences, CGAGGAA and CGT GGAT, were found upstream of the *fptA* −35 region. These sequences are also found upstream of the autoregulated *pchR* gene, suggesting that these sequences could function in PchR binding. Furthermore, XylS, an AraC-family regulator of the plasmid-mediated TOL genes encoding enzymes involved in the degradation of benzoate and *m*-toluate in *Pseudomonas*, should be mentioned. XylS also binds to direct repeats that are located immediately upstream of the −35 region of the TOL operon controlling the Pm promoter through weak DNA-protein interactions. However, the lack of available sequences further upstream of *fptA* has hampered the identification of the additional binding sites that would be necessary if a looping model were considered for regulation.

Another point is related to the inducibility of *pchR* expression by pyochelin, as assessed by using *pchR-lacZ* functions (66). The results strongly suggest that *pchR* expression, like that of *fptA*, responds to pyochelin. However, unlike that of *fptA*, there is no PchR-dependent activation of *pchR* gene expression by pyochelin. Therefore, PchR acts only as a repressor with regard to *pchR* regulation. It is possible that an interaction between ferric pyochelin and FptA initiates a signal transduction cascade which can lead to the release of an effector that interacts with PchR to cause either activation or repression. Alternatively, there could be a direct interaction between PchR and the terminal element in the cascade. The attractive feature in this model is that the siderophore does not need to be transported inside the cell for transduction of the signal of successful iron chelation by pyochelin and the concomitant need to enhance the expression of the *fptA* gene (Fig. 3).

The activation of *fptA* by PchR requires both FptA and PchR. The presence of only one of these two elements leads to just basal levels of expression (66). The repression of *fptA* (and *pchR*) present in a pyochelin-deficient strain requires both FptA and PchR. The lack of either of these two factors results in a basal level of *fptA* expression, indicating that *fptA* expression depends on the presence of these two proteins to be able to respond to the presence or absence of pyochelin. It is thus likely that FptA must sense the level of pyochelin in the culture and communicate it to PchR directly. Alternatively, the signal could be transduced by influencing *pchR* expression directly (Fig. 3).

It was of interest that the receptors for ferripyochelin and ferric pyoverdin were present in decreased amounts in strains grown in the presence of enterobactin. Furthermore, production of the ferripyochelin receptor is similarly depressed in strains that are actively producing pyoverdin (36). From these results, it appears that at least in *P. aeruginosa*, enterobactin is utilized preferentially over pyoverdin and pyochelin whereas pyoverdin is utilized preferentially over pyochelin. This hierarchy of ferric siderophore uptake system follows the relative affinity of these siderophores for ferric iron: enterobactin > pyoverdin > pyochelin. Therefore, the most successful siderophore in chelating the available iron will be in charge of delivering it to the cell, and thus up regulation of the particular uptake system for that ferric siderophore complex becomes essential. The results obtained by these investigators also indicate that siderophore production must be similarly regulated, since pyochelin levels are greatly reduced in cultures of pyoverdin-producing strains compared to cultures of pyoverdin-deficient strains.

Ferric Enterobactin Uptake System

P. aeruginosa can also utilize ferric enterobactin. This uptake system is inducible by growth in the presence of enterobactin. The ferric enterobactin receptor, PfeA, is an 80-kDa outer membrane protein which is iron repressible and enterobactin inducible (36, 116). This receptor is highly homologous to FepA (60% identity), the enterobactin receptor in *E. coli* (18, 43). It is noteworthy that PfeA can replace FepA in *fepA* mutant strains of *E. coli*. Differently from FpvA and FptA (see above on pyochelin- and pyoverdin-enhanced induction), PfeA shares all four homology regions with the TonB-dependent family of receptors. Regulation of PfeA expression by iron may take place by repression mediated by the Fur product (124), since *pfeA* possesses a consensus Fur-binding region, consistent with this regulation. In *P. aeruginosa*, a region upstream of *pfeA* is involved in the enterobactin-responsive regulation of PfeA expression (37). Two genes, *pfeR* and *pfeS*, were identified. The predicted translation product of PfeR showed significant homology to a number of response regulators such as the OmpR protein of *E. coli*, while the amino acid sequence of PfeS showed homology, albeit lower, to a number of histidine kinase sensors including the EnvZ protein of *E. coli* (37). Conserved regions of homology found in the N-terminal portion of the regulator and the C-terminal portion of the sensor proteins were also found in PfeR and PfeS, respectively (37).

Thus, sequences with a high degree of homology to all four of the conserved regions found in response regulators of two-component systems were also found appropriately spaced and ordered in the N-terminal region of PfeR. Furthermore, the invariant aspartate residues present in regions 1 and 2 of response regulators were also present in PfeR (37). In the C-terminal end of PfeS, five regions, also appropriately spaced and ordered, showed homology to the conserved regions of the sensor class of proteins (37). One of these regions, region 3, showed less homology to the corresponding region in other sensor proteins. However, the three invariant amino acids present in other sensor proteins are also found in PfeS, and, more important, the invariant histidine residue of region 1, which is apparently involved in autophosphorylation in sensor kinases, is also conserved in PfeS. Analysis of hydropathy profiles for these regions suggested a cytoplasmic location for PfeR and a cytoplasmic membrane location for PfeS (37). Further support for the conclusion that these regions play an important role in the enterobactin-mediated expression of PfeA was provided by the isolation of a mutant with a mutation in the *pfeR* gene, which became unresponsive to the presence of enterobactin in the medium, and by the fact that a *P. aeruginosa* strain transformed with a multicopy plasmid carrying *pfeR* and *pfeS* expressed higher levels of PfeA in the presence of enterobactin than did the vector-transformed strain (37).

Expression of the *pfeRS* operon is iron regulated, and there is evidence for Fur binding at a region upstream of *pfeR*, where there is a Fur box overlapping the start site of the *pfeR* coding region (112).

Figure 4 shows the mechanisms by which the signal is transduced from the environment to activate the expression of the enterobactin receptor gene. PfeS is a predicted cytoplasmic

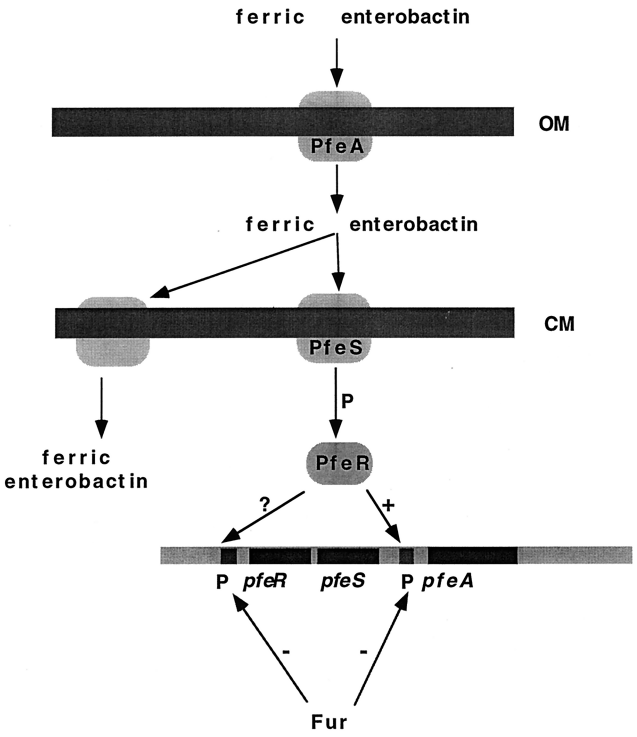

FIG. 4. Model of the regulation by ferric enterobactin of the expression of the ferric enterobactin receptor gene *pfeA* of *P. aeruginosa*. OM, outer membrane; CM, cytoplasmic membrane.

membrane-associated histidine kinase sensor, which may function by activating PfeR by phosphorylating an aspartate residue in response to a stimulus possibly provided by enterobactin. The activated PfeR can then activate the expression of *pfeA* by first binding upstream of this gene. The authors have confirmed this binding activity by gel shift experiments (38). Introduction of a clone harboring *pfeR* and *pfeS* into *P. aeruginosa* in a high-copy-number vector resulted in an enhancement of the enterobactin-dependent expression of *pfeA*, while a *pfeR* mutant did not show enhancement of *pfeA* synthesis in the presence of enterobactin. It is of interest that *pfeA* knockout mutants were still capable of acquiring enterobactin and still showed expression of a *pfeA-lacZ* fusion in the presence of enterobactin (38). These combined results would argue for the existence of another means by which enterobactin crosses the outer membrane to enhance the expression of *pfeA*.

REGULATION OF AN IRON-REGULATED OUTER MEMBRANE PROTEIN AND VIRULENCE DETERMINANT IN *V. CHOLERAE*

The causative agent of cholera, *V. cholerae*, acquires iron via the vibriobactin-mediated iron transport system (20, 145). This bacterium can also use iron contained in heme or hemoglobin, and it produces an iron-regulated hemolysin which may intervene in iron acquisition in vivo (144).

Goldberg et al. (59) used transposition mutagenesis to identify in *V. cholerae* a gene, *irgA*, that when mutated led to a decrease in the virulence of this bacterium in the suckling-mouse animal model. A concomitant loss of a major iron-regulated 77-kDa outer membrane protein was also detected. However, this mutation had no apparent effect on growth, suggesting that the protein must play a role in other stages of the infection process. This protein was not involved in iron transport of ferric vibriobactin, although it is a member of the family of TonB-dependent proteins (58). Later, an iron-regulated outer membrane protein of 74 kDa, ViuA, was identified as the actual receptor for ferric vibriobactin (20, 145).

Recently, Goldberg et al. (56) demonstrated that regulation of *irgA* by iron occurs at the transcriptional level. They also showed that there is an interrupted sequence of dyad symmetry in the vicinity of the promoter with homology to the Fur-binding site or Fur box. They also demonstrated that a region located about 900 bp upstream of the *irgA* transcription start site is required for transcription of *irgA*. An open reading frame of 894 bp, *irgB*, which is in inverse orientation to *irgA*, is included within this region (Fig. 5). IrgB is a positive transcriptional activator for *irgA* expression (57). Expression of *irgB* is also negatively regulated by iron. The promoters of *irgB* and *irgA* are divergent but overlap each other and the previously defined Fur-binding site (56, 57). In the presence of high iron

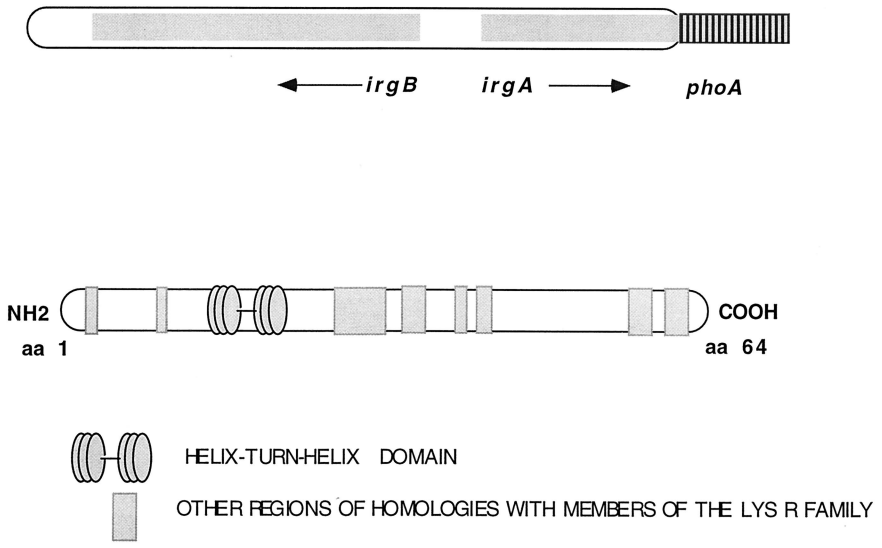

FIG. 5. Location of *irgB* and *irgA* of *V. cholerae* and schematic diagram of the positive transcriptional activator IrgB.

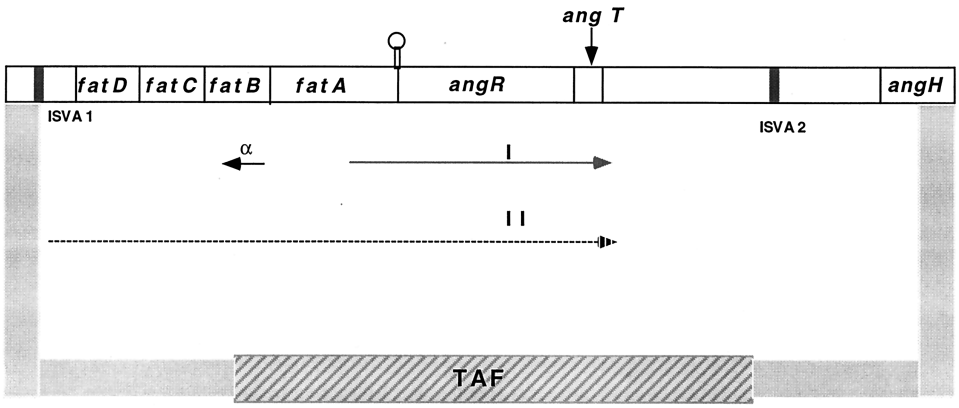

FIG. 6. Map of the iron uptake region of the *V. anguillarum* virulence plasmid pJM1 and location of transcripts. α, antisense RNA; I, *angR* transcript originating in a promoter internal to the *fatA* gene; II, polycistronic mRNA of the iron transport region and *angR*. ISV A1 and ISV A2 are insertion sequences. *angT*, which encodes a thioesterase, and *angH*, which encodes a histidine decarboxylase, are biosynthetic genes.

concentrations, transcription of both *irgA* and *irgB* is repressed by a Fur-like protein. The amino-terminal portion of IrgA is homologous to FepA, the *E. coli* enterobactin receptor, and is also similar to FepA in that its transcription is negatively regulated by iron with a Fur-binding sequence in its promoter region (56, 58, 59). It is of interest that both of these genes, *irgA* and *irgB*, are iron-regulated genes, divergently transcribed from promoters overlapping the same Fur-binding site. Under low-iron conditions, this negative regulation of transcription is removed and production of *irgB* leads to positive transcriptional activation of *irgA*. Analysis of the *irgB* sequence shows that the IrgB protein is homologous to the LysR family of positive transcriptional activators. Members of this family have been identified from several species of the family *Enterobacteriaceae*, such as *E. coli*, *S. typhimurium*, and *Enterobacter cloacae*. The analysis of IrgB secondary structure predicts a helix-turn-helix DNA-binding motif (Fig. 5). Like IrgB, all members of the LysR family possess a helix-turn-helix motif near the amino terminus. Like *irgB*, in several cases the encoding gene for the LysR family member is transcribed divergently from an operon or gene regulated by the LysR-like protein (57).

Several questions still remain with respect to the function of IrgA in virulence, as well as the evolutionary reasons why this protein shows all the characteristics of an iron transport receptor without being essential to iron transport.

REGULATION OF IRON UPTAKE IN *V. ANGUILLARUM*

As is clear from the preceding section, bacteria that belong to the genus *Vibrio* are common etiologic agents of disease in humans and animals. An important member of this group is the marine vibrio *V. anguillarum*, which causes the devastating and highly fatal salmonid disease vibriosis, consisting of a hemorrhagic septicemia that leads to death by hypoxia and dysfunction of various organs (33).

We have used the *V. anguillarum*-host fish system as a model to study the molecular mechanisms of host-pathogen interactions leading to human disease (32, 33). Several characteristics of this system were taken into account in deciding on its usefulness as a model. The disease caused by *V. anguillarum* has remarkable similarities to invasive septicemic disease in humans, and the sequence of events immediately after infection is very similar to that in mammalian inflammation except for obvious species-specific responses (33). Another important feature that makes the system unique is that this bacterium is an actual pathogen for the vertebrate host (31–33, 175). Therefore, inferences made from a study of the mechanism of interaction between the microorganism and the host will result in a valid assessment of the host-parasite relationship and will lead to an increased understanding of invasive diseases.

Regulation by AngR and TAF

For the past few years, we have been concerned with elucidating the genetics and molecular biology of *V. anguillarum*. We identified a 65-kb plasmid, pJM1, that is responsible for the high virulence of this bacterium and that encodes an iron uptake system consisting of the 348-Da siderophore anguibactin and the iron transport proteins FatABCD (1, 2, 4, 31–34, 91, 153, 154, 157, 171). Full expression of the system leads to the high-virulence phenotype and requires the presence of products from the TAF region, located noncontiguously with the other iron uptake sequences, as well as the AngR protein, which acts synergistically in the expression of anguibactin biosynthetic and also iron transport genes. Either AngR or the TAF products resulted in higher gene expression, perhaps 2- to 3-fold, whereas when they acted together, expression was more than 20-fold higher (47, 129, 130, 155, 158). As discussed in the previous sections and as is the case for TAF products and AngR, cooperativity between two factors plays a role in gene expression for some of the other environmentally controlled systems. We have also demonstrated that iron negatively regulates the expression of the pJM1 iron uptake system through a novel mechanism that involves a Fur protein and antisense RNA, RNAα (see the following section). A model of the pJM1 iron uptake system and its regulators is shown in Fig. 6.

The *V. anguillarum* 775 *angR* gene ($angR_{775}$) encodes a regulatory protein, AngR, of 1,048 amino acids (26, 47, 130, 158), which has two helix-turn-helix motifs typical of prokaryotic DNA-binding proteins (16, 27). One of them shows homology to the DNA-binding domain of the P22 phage protein Cro. The other was recently identified by using parameters set at lower stringency (27). Leucine zippers just upstream of each of these two helix-turn-helix motifs were also identified (125, 155). Figure 7 is a diagram of the AngR protein in which these and other important regions are depicted. In eukaryotic regulators, a leucine zipper is often followed by a basic DNA-binding region to form the so-called bZIP (22, 101, 114, 148). The

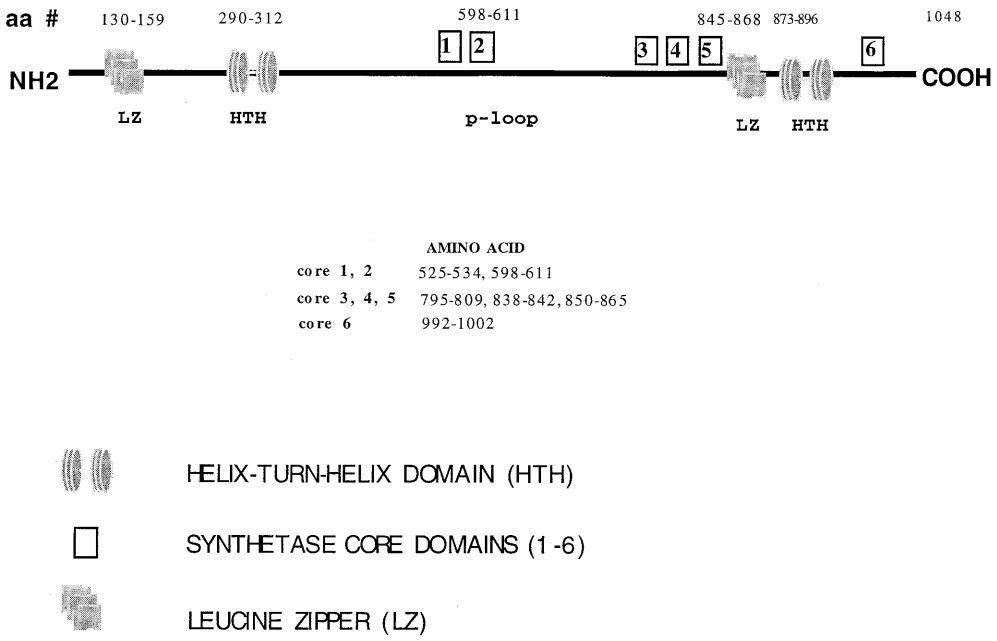

FIG. 7. Diagram of the AngR protein showing putative synthetase and regulatory domains.

presence of leucine zippers in prokaryotic proteins other than AngR was also recently reported (55). In eukaryotic systems, these regions interact with another regulatory protein molecule and bind DNA (5, 16, 55, 101, 114, 125, 160, 165, 170). Therefore, one or both of the leucine zipper helix-turn-helix domains found in AngR might play a role in protein and DNA recognition, and, specifically, one of them may be involved in the synergistic action found between AngR and the TAF products (130, 158).

Other important features identified from the analysis of the sequence are that AngR possesses the ATP-binding P-loop IIYTSGSTGLPKG, the covalent substrate amino acid-binding motif SDFFLDGGDAYNAIEV, and other domains that are conserved among the enzymes of the firefly luciferase family, which includes proteins from gram-positive as well as gram-negative bacteria that are involved in stages of nonribosomal peptide synthesis (51, 62, 73, 132, 152). Interestingly, high-molecular-weight protein 2 (HMWP 2) of *Yersinia enterocolitica* shows homology in various domains to AngR of *V. anguillarum* and belongs to the family of proteins involved in nonribosomal peptide synthesis (62).

The members of the nonribosomal peptide synthetases family catalyze two types of reactions, adenylation of their substrates and thioester formation (62, 152). These proteins are acid-thiol ligases and are responsible for the activation reactions in the biosynthesis of enterobactin and for the synthetases for tyrocidin, gramicidin S, and penicillin, also known as the firefly luciferase family. The enzymes belonging to this group are the gramicidin S and tyrocidine synthetase from *Bacillus brevis*; the 57-kDa polypeptide of the 4-chlorobenzoate dehalogenase from *Pseudomonas* spp.; the coumarate coenzyme A:ligase from *Petrosinum crispum*; the luciferase from *Photinus pyralis*; the D-alanine-activating enzyme from *Lactobacillus casei*; EntF, an enzyme of the enterobactin pathway that activates L-serine via an L-seryl-AMP intermediate, which, together with other Ent proteins, catalyzes a series of enzymatic reactions with enzyme-bound intermediates (127); and the 2,3-dihydroxybenzoate (2,3-DHBA)-AMP ligase (EntE) from *E. coli* (113). Based on the crystallographic structure and chemical analysis of anguibactin (1, 83), we predict that anguibactin is synthesized from the enzymatic modification of 2,3-DHBA, cysteine, and histamine. Recent investigations support this hypothesis, since both 2,3-DHBA and histamine are required for the biosynthesis of anguibactin (24, 156). The presence of both hydroxy acids and amino acids in the anguibactin molecule led us to hypothesize that anguibactin is synthesized, at least in part, by a nonribosomal peptide synthetase mechanism. The domain homology of AngR to EntE was especially interesting since EntE catalyzes the activation of 2,3-DHBA, an essential step in the biosynthetic pathway of the diphenolic siderophore enterobactin. Transformation of a clone expressing AngR into an *entE* mutant of *E. coli* demonstrated that AngR can indeed replace EntE, leading to the complementation of the *entE* mutation in *E. coli* (155). The anguibactin molecule also possesses a diphenolic ring, and anguibactin is synthesized from the chromosomally encoded 2,3-DHBA by pJM1-encoded enzymes (24). Therefore, it is possible that in addition to its regulatory functions, AngR plays a role as an activating enzyme in the biosynthesis of anguibactin.

Nonribosomal peptide synthesis occurs by a multistep process of adenylation, thioesterification, and sometimes racemization or N methylation of each amino acid or hydroxy acid, creating various peptide structures. This process involves distinct domains of the biosynthetic enzyme(s) that catalyzes the activation of constituent amino acids as acyladenylates and thioesterifies the activated amino acids through a covalent interaction with specific thiol groups (27). Recent biochemical data suggest that elongation of the peptide (transpeptidation) occurs via multiple cofactors of 4′-phosphopantetheine, which are covalently bound to the carboxy-terminal region of each amino acid-activating domain. It is currently believed that these cofactors covalently attach to a conserved serine residue within the thioester formation module, core 6. During this elongation, reaction intermediate peptides remain covalently

attached to their specific sites. Termination of nonribosomal peptide synthesis includes the release of the thioester-bound peptide from the enzyme complex either by cyclization, by the action of a thioesterase, or by transferring the peptide chain to a functional group such as a phospholipid (138). AngR possesses motifs 1 to 5, involved in adenylation, and motif 6, involved in cofactor binding and thioesterification, as determined for gramicidin synthetase (137; also, unpublished observations). AngR shares homology with all six motifs, as does EntF and the HMWP 2 of *Y. enterocolitica*. EntF is a nonribosomal peptide synthetase involved in serine activation during the synthesis of enterobactin in *E. coli*. HMWP 2 of *Y. enterocolitica* shares remarkable homology with AngR, although its function is unknown. Unlike EntE, AngR does possess a core 6 (implicated in thioesterification), and thus in addition to playing a role as an adenylating enzyme similar to EntE from *E. coli* by activating a DHBA molecule in anguibactin biosynthesis (28), it may be involved in incorporation of the activated hydroxy acid into the growing anguibactin molecule. However, the highly conserved serine residue, which is the actual binding site for the phosphopantetheine (137), is not present in AngR core 6, although it has a serine 4 amino acids prior to that site (unpublished observations). It is thus possible that anguibactin is produced by nonribosomal peptide synthesis via adenylation of 2,3-DHBA and incorporation of the activated molecules of cysteine and histamine. Subsequent cleavage of the newly synthesized peptide complex would occur via a thioesterase.

It is of interest that the *angR* gene is followed by *angT*, which encodes a thioesterase which may also be essential for anguibactin biosynthesis. Thioesterase genes are also essential in other nonribosomal peptide synthetase systems and are generally contiguous with the synthetase genes.

In addition to the biosynthetic motifs, AngR possesses predicted regulatory domains, i.e., secondary structures of helix-turn-helix motifs common to DNA-binding proteins. We also recently demonstrated that *fatB* and *fatA* expression under iron-limiting conditions is dramatically reduced in an AngR-deficient strain, suggesting a regulatory function for AngR (3). Therefore, AngR may play a critical role in both the biosynthesis of anguibactin and the regulation of iron transport gene expression. Thus, the iron transport gene expression and siderophore biosynthesis systems in *V. anguillarum* are intimately connected through the AngR protein. Therefore, it is possible that AngR possesses distinct domains that are related to either biosynthetic or regulatory functions. Our recent results indeed demonstrate that AngR is not merely a biosynthetic enzyme. We have demonstrated that AngR, together with the TAF product(s), has a positive regulatory effect on the expression of iron transport protein genes (3, 26). Furthermore, we have also shown that under iron-limiting conditions, expression of the *angR* gene is positively regulated by the product(s) of the TAF region, suggesting a cascade type of regulatory events in the control of the expression of iron uptake genes in *V. anguillarum*. However, an interaction between AngR and TAF products may also be essential for the synergistic regulatory activity of these two activators (129, 130).

By using site-directed mutagenesis, it was possible to generate mutations in the leucine zipper and helix-turn-helix domains of AngR. We included in this analysis $angR_{531A}$, a gene cloned from a pJM1-like plasmid, pJHC-1, found in *V. anguillarum* 531A, which produced high levels of anguibactin. Mobilization of this clone into the 775-type strain demonstrated that $AngR_{531A}$ was responsible for the increased siderophore production phenotype and higher MIC of ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDA) (155). All the mutant derivatives, as well as the $angR_{531A}$ gene, were sequenced, and this showed that the only difference between $angR_{531A}$ and $angR_{775}$ was a substitution of His (in $AngR_{775}$) for Asn (in $AngR_{531A}$) at amino acid 267 (Fig. 7). This mutation was therefore solely responsible for the increased siderophore production phenotype and higher MIC conferred by $AngR_{531A}$. It was of interest that the substituted amino acid is located between the first leucine zipper and helix-turn-helix motifs. Other amino acid changes were engineered at this position 267 of the protein by site-directed mutagenesis. Different EDDA MICs were obtained for the different mutations. Substitution of the His by Leu or Gln generated AngR derivatives that conferred EDDA MICs lower than that for the wild type to the *V. anguillarum* strain carrying them, demonstrating that this is an important location for AngR activity. The location of the mutation between a leucine zipper and a helix-turn-helix motif could indicate that its effect is on its regulatory role through modification of the protein-protein or DNA-protein interactions involving AngR. Some of the mutations in the helix-turn-helix domains consisted of substitutions of the original amino acid by Pro, which is known to disrupt helix structures. Some of the other mutations resulted in substitutions of one or more Leu or Val residues, producing a disruption of the leucine zipper. All of these mutations affected the function of AngR, resulting in a reduced or total shutoff of anguibactin production. However, one of these mutations also resulted in the loss of regulation of the expression of iron transport genes (unpublished observations).

We have recently shown that the transcript encoding the iron transport genes is polycistronic and that it is also associated with the *angR* gene (unpublished observations). The expression of this polycistronic transcript (Fig. 6) was positively regulated by AngR and TAF and repressed by iron and the Fur protein. Our previous results demonstrated that there are transcripts initiated within the *fatA* gene that encode *angR* and that these may be autoregulated by AngR (129). It is therefore possible that AngR represses these shorter transcripts while enhancing the synthesis of the polycistronic transcript.

Another important point is that the presence of anguibactin itself enhanced the transcription of the iron transport genes independently of AngR and the TAF products (26). The presence of either AngR (together with the TAF products) or anguibactin alone led to a partial iron transport gene expression, while full expression was achieved when AngR, the TAF products, and anguibactin were all present.

Mechanism of Posttranscriptional Control of Iron-Regulated Genes

The negative regulation of the expression of the iron transport genes in *V. anguillarum* is mediated by a chromosome-encoded Fur protein and a plasmid pJM1-derived antisense RNA (RNAα) (131, 159, 168, 169). With respect to Fur action, we know that there is constitutive synthesis of the iron transport gene products in a *fur* mutant of *V. anguillarum*; however, we do not know yet whether Fur action occurs by directly repressing the transcription of the iron transport genes or by repressing the transcription of the *angR* gene, resulting in a lack of availability of AngR to affect the expression of the polycistronic mRNA which contains the transport genes. RNAα is encoded within the *fatB* gene (Fig. 6) and is preferentially expressed under iron-rich conditions (131, 168, 169). By using a construct in which RNAα synthesis was under the control of a strong external promoter, we showed that a high level of RNAα synthesis led to a reduced expression of the iron transport genes *fatA* and *fatB* (131, 168). Direct evidence of the

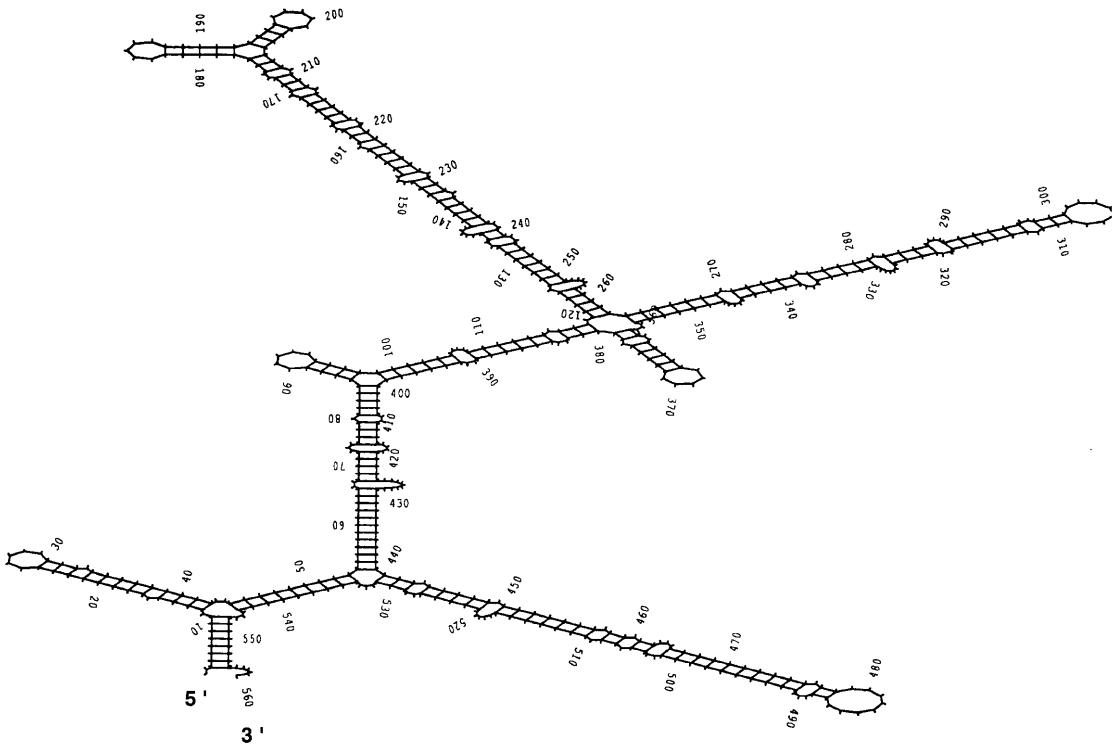

FIG. 8. Predicted secondary structure of ΔRNAα, an active truncated derivative of RNAα.

physiological role of RNAα transcribed from its own promoter in the control of the expression of these genes was recently provided and is discussed in this section (25, 131, 168, 169).

Antisense RNA plays an important role in regulatory events; including the control of transcription, translation, plasmid copy number, and plasmid killing; in the control of plasmid DNA replication; and in the bacteriophage lysis/lysogenic cycle (82, 134). RNAα is the first antisense RNA demonstrated to play a role in the control of expression of iron transport genes. Three possible transcription start sites, Ra, Rb, and Rc, have been mapped (131). Recently, by subcloning a fragment containing the promoters for each of these transcriptional start points, it was demonstrated that the major promoter activity is located in a region containing the pb and pc promoters (25). It was of interest that neither of these promoters, as assessed by fusion to a *cat* gene, was regulated by iron. However, RNAα can be found preferentially under high-iron conditions (131, 168). Further experiments proved that the stability of RNAα actually depends on the presence of iron (25). The mechanism of stabilization is not clear. One possible mechanism of iron stabilization of RNAα is the existence of a factor that is able to bind RNAα only in the presence of iron. This binding could protect RNAα from RNase attack either by a secondary-structure change of the RNA molecule or by a steric hindrance effect. Sequencing analysis demonstrated that indeed in RNAα there are several stem-loop structures at the 3′ end as well as in other regions of the molecule. It is of interest that the secondary structures found at the 3′ end are also found on a truncated RNAα molecule that lacks the first 100 nucleotides from the 5′ end synthesized under the control of an external promoter (21a). This truncated molecule (Fig. 8) is also stabilized by iron. It is possible that these secondary structures play a role in the stabilization mechanism. We recently demonstrated, by using a gene fusion containing the RNAα promoter pb/c fused to the *cat* gene, that the Fur protein was essential for RNAα expression at the transcription level independently of the iron status of the cell (25). How does Fur regulate the initiation of transcription of RNAα? Fur is a pleiotropic protein that regulates the expression of many iron-regulated and some non-iron-regulated genes (33). There are countless examples of the former, and in those cases ferrous iron is always found as a cofactor. For the latter, the example of *sodB* gene regulation comes immediately to mind. *sodB* expression is regulated by Fur in a positive fashion, possibly by a direct DNA-protein interaction that does not require the ferrous iron. Furthermore, neither *sodB* nor RNAα possesses a good *fur* box upstream of the gene (109).

There are various possibilities that could explain the mechanism by which Fur could regulate the synthesis of RNAα. For instance, it could serve as an activator via a direct interaction with the RNAα promoter. It could also be a repressor for another, still unidentified gene that encodes a repressor of RNAα synthesis, or, in turn, it could be an activator of other genes that could positively regulate RNAα expression. Recently, we used hydroxylamine mutagenesis of the RNAα promoter to generate 50 RNAα promoter mutants that are active in the *V. anguillarum* Fur-deficient strain. In all 10 mutants examined, the mutation mapped at a single nucleotide within the −10 region of the promoter pb, causing a change from TAGGCT to TAGaCT. This mutation resulted in an increased expression of RNAα in the absence of Fur (25). It is possible that this change in the −10 region of the RNAα promoter leads to a favorable structure for the function of RNA polymerase. This is true in another system, the *lac* operon. It is known that the catabolite activator protein (CAP) is essential for transcription initiation of the lac promoter. In the presence of cyclic AMP (cAMP), CAP binds to a region located at the −35 sequence of the *lac* promoter. This binding stimulates the

productive interaction of RNA polymerase with the promoter by leading to a protein-protein interaction with RNA polymerase or by leading to a distortion of the DNA sequence, which facilitates transcription initiation. A certain class of mutations, mapped to the −10 region of the *lac* promoter, facilitate expression in the absence of the CAP-cAMP complex. These mutations, called class III mutations, are thought to affect open-complex formation of RNA polymerase, the rate-determining step for the wild-type promoter and also the step influenced by the CAP-cAMP complex. The Fur-independent mutations of RNAα may provide a clue to the function of Fur. This protein may have a function similar to that of CAP for the *lac* system in the activation of RNAα expression. However, activation of RNAα transcription initiation in *E. coli* appears to be independent of Fur, possibly because of the existence of a different transcription machinery in this bacterium. At any rate, the absence of a Fur box at the RNAα promoter region and the fact that the positive regulation of RNAα by Fur is independent of iron strongly suggest that a unique mechanism must be involved in this regulation in which Fur interaction with DNA may occur with sequences other than the canonical Fur box. Alternatively, Fur may enhance RNAα synthesis by acting on another gene that is the actual regulator of RNAα synthesis. Further experiments are being carried out to assess the role of Fur on RNAα synthesis and on the control of expression of the pJM1 iron transport genes. An important consequence of these studies is that we were able to demonstrate that there was a correlation between the lack of RNAα and a dramatic enhancement in the levels of the *fatAB* polycistronic transcript in the Fur-deficient strain, strongly suggesting that under physiological conditions (i.e., at high iron concentrations), RNAα may indeed play an important role in the negative regulation of the expression of these iron transport genes.

CONCLUDING REMARKS

Siderophore-mediated iron uptake systems in microorganisms are highly regulated primarily by the iron status of the cell. In *E. coli* and most other bacteria, this regulation is mediated by a single regulator Fur, which acts as a transcriptional repressor in the presence of Fe^{2+}. In other bacteria, such as *V. anguillarum*, in addition to the Fur-mediated mechanism of control there is another hierarchy of control mediated by an antisense RNA that is stabilized by iron and that inhibits the expression of iron transport genes. When the concentration of iron is decreased, the Fur-repressed genes become derepressed and initiation of transcription can take place. Of course, with decreasing concentrations of iron, the *V. anguillarum* antisense RNA becomes more unstable and its inhibitory posttranscriptional action becomes less evident. However, for some systems, there is more to the whole mechanistic problem of iron transport gene expression, because concomitant with derepression there is a need for an activation of these genes under iron-limiting conditions. It is clear from this review that activation occurs by a plethora of mechanisms, including two-component systems, AraC-like regulators, and others such as FecI-FecR, PupI-PupR, or AngR-TAF, that do not fit into any of the artificial classifications into which we are tempted to place phenomena when we do not understand them. There appears to be a moral, which is especially clear in the examples given for the complex *Pseudomonas* systems and a feature that is repeated in both the ferric dicitrate system of *E. coli* and the anguibactin-mediated iron transport system of *V. anguillarum*: the siderophore itself will generate a signal that, when transduced inside the cell, induces the expression of genes leading to uptake systems for the ferric siderophore complex at hand or, if the cell finds itself under conditions in which various ferric siderophore complexes are present, will utilize the ferric siderophore complex with the highest affinity for iron. The fact that some other virulence factors such as ToxA in *Pseudomonas* can be controlled by regulatory proteins that also regulate the expression of iron transport genes is also a logical Darwinistic decision that may lead to coordinate regulation of various virulence factors. Thus, there appears to be a redeeming similarity in all these mechanisms of control that is somewhat lost when one is presented with the different pathways chosen to perform the regulation.

The question arises whether there are more points in common among all these bacteria. For instance, it would be expected that *Pseudomonas* and the *Vibrio* species, being somewhat environmental in their tastes for ecological niches, could have opportunities to interact and possibly share some of the regulatory wisdom fortuitously (or otherwise) evolved in the control of expression of their iron transport systems. Searches for these possible homologies have led to some interesting findings. We have already described in the text the domain homologies found between the AngR protein and the *Y. enterocolitica* HMWP 2, as well as members of the luciferase group. Furthermore, in addition to the receptors for pyoverdin, pyochelin, and enterobactin, a *Pseudomonas* gene was identified that showed good homology (36% identity, 15% similarity) to the gene encoding the *V. anguillarum* FatA receptor for anguibactin (99). The product of this gene, the putative receptor protein UfrA, was 83 kDa in size and showed homologies throughout the length of the proteins, including the conserved regions typical for TonB-dependent receptors. However, additional UfrA sequences were found at the N-terminal end of UfrA which were absent from FatA. These N-terminal extensions were similar to those found in FpvA, PupA, and PupB. It is tempting to speculate that the UfrA N-terminal extensions, like those in the other proteins, are somewhat involved in a signal transduction cascade that results in the expression of *ufrA*. Despite the homology of UfrA to FatA and the fact that pyochelin and anguibactin show some structural similarities, the investigators did not find any evidence for the role of UfrA in ferric pyochelin uptake, and *ufrA* knockout mutants behaved like the wild type with respect to growth, iron uptake, and expression of iron-regulated outer membrane proteins. This puzzle of *V. anguillarum* genes in other bacteria has been compounded recently by the finding that anguibactin can cross-feed strains of the opportunistic human pathogen *Acinetobacter baumannii* and that acinetobactin can cross-feed anguibactin-deficient mutants of *V. anguillarum* (unpublished data). Furthermore, acinetobactin was found to be structurally related to anguibactin, the only difference being that acinetobactin possessed an oxazoline ring instead of a thiazoline ring (178). The remarkable similarities between the components of iron transport in these pathogens and others (44) underscore the importance of possession of regulatory genes that, although using different mechanisms, act to enhance the expression of bacterial iron transport genes under conditions of iron limitation.

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