Molecular Characterization of Fur and AngR, Key Proteins Involved in the Regulation of Iron Acquisition in *Vibrio anguillarum*

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CERTIFICATE OF APPROVAL

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To Paul

Thank you to my parents, my family, my friends, my many professors and teachers -- all of whom gave me encouragement to do whatever my heart desired

Thank you to Jorge and Lidia for providing such a creative and spirited environment for research and intellectual discussions!

ABSTRACT

Vibrio anguillarum causes acute hemorrhagic septicemia in salmonids. Virulence of *V. anguillarum* is directly correlated to the presence of the plasmid pJM1. This virulence plasmid encodes an iron uptake system which consists of the siderophore anguibactin, and an energy dependent transport system whereby the anguibactin-Fe(III) complex is internalized into the bacterial cell. The pJM1 virulence plasmid also encodes at least two positive regulatory elements, AngR and TAF, which are necessary for full expression of the biosynthetic and iron transport gene transcripts under iron limiting conditions. Expression of the plasmid encoded iron transport genes is negatively regulated by the plasmid encoded anti-sense RNAα molecule. There is also a chromosomally encoded Fur protein which acts as a negative regulator, at the transcription initiation level.

The goals of my thesis are to a) elucidate the mechanism for Fur function at the molecular level and b) elucidate the role played by the AngR protein in iron acquisition. The Fur protein is a chromosomally encoded negative regulator which specifically interacts with Fe(II). Once complexed with Fe(II), Fur binds the operator sequence of the iron regulated genes. In Chapter 1, I present experiments in which I relate the subcloning and sequencing of the *V. anguillarum fur*, and analysis of recombinant *V. anguillarum* mutant Fur proteins in *E.coli*. In Chapter 2, I present a further analysis of Fur, using mutant Fur strains of *V. anguillarum*. This work revealed several key features, primarily that the Fur protein is essential and that without selective pressure, mutant strains revert to the wild type. In addition, I found that mutations within the third predicted helix of Fur resulted in reduced regulatory function and aberrant migration of the Fur protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In Chapter 3, I demonstrate that the iron transport genes are transcribed as a polycistronic mRNA, possibly from a strong iron-regulated promoter found within a 2.1 kb region upstream of *fatD*. The activity of this

promoter region is reduced by 90% when bacteria are cultured in the presence of 4 μM ferric ammonium citrate. My fourth chapter details the characterization of the positive regulator AngR. AngR has several domains found in regulatory proteins such as helix-turn-helix and leucine zipper motifs. In addition AngR contains a large region just beyond amino acid 500, which contains all 6 core regions found in non-ribosomal peptide synthetases. Some enzymes involved in biosynthesis of siderophores have recently been identified as non-ribosomal peptide synthetases. In *Escherichia coli*, for example, the EntE and EntF proteins have been found to be involved in non-ribosomal peptide synthesis of the *E.coli* siderophore enterobactin; EntE serves as an adenylating enzyme while EntF is a non-ribosomal peptide synthetase. Therefore, we generated a panel of site-directed mutants throughout *angR* to assess the structural-functional relationship of these predicted structural regions and their phenotypes. One mutation, designed to introduce a helix breaking proline into the first helix-turn-helix, led to the loss of both the regulatory and biosynthetic functions. All of the other mutants were capable of regulation and most had diminished biosynthetic function as assayed by anguibactin bioassay. Thus, AngR may also be a non-ribosomal peptide synthetase, most likely involved in activation of 2,3-dihydroxybenzoic acid (2,3-DHBA) and perhaps in its thioesterification with other precursors in anguibactin biosynthesis such as cysteine and histamine. These findings are consistent with AngR serving two functions, one as a regulatory protein essential for positive regulation of the iron transport genes, and the other as a biosynthetic enzyme.

INTRODUCTION

Part I: IRON: AN ESSENTIAL ELEMENT FOR LIFE

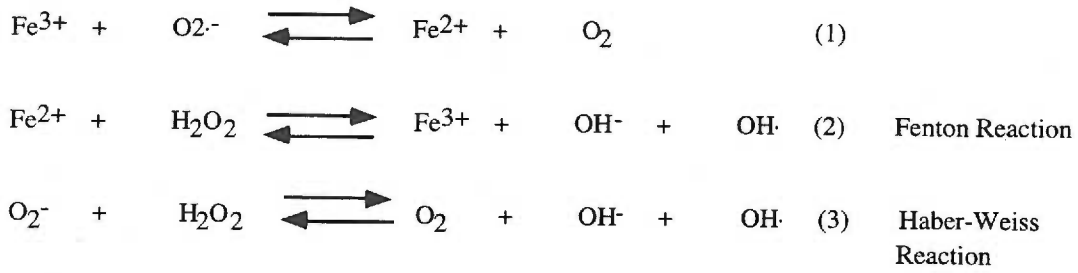
Iron, the second most abundant metal in the earth's crust (just behind aluminum) is remarkably insoluble at neutral pH. In nature, iron is primarily found as a constituent of insoluble oxyhydroxide polymers (e.g. goethite, hematite). Ironically, almost all living organisms require iron for critical cellular functions such as respiration and ribonucleotide synthesis (lactobacilli are the exception, as they utilize manganese and cobalt as biocatalysts in place of iron) (43).

Iron is remarkably versatile and serves many critical roles as a bio-essential element. An important role of iron is its ability to change valence. A variety of organic ligands subtly modify the ability of iron to change valence, thus providing electron transport proteins a wide range of redox potential (47). The oxidation capacity of iron as an electron transfer catalyst from ferredoxin to cytochrome C oxidase spans 1000 mV (74). Iron is also required for such processes as fixation of dinitrogen, photosynthesis, dioxyribotide synthetase and oxygen metabolism.

Iron may have been established as a bio-essential element during the anaerobic phase of early Earth. Under anaerobic conditions Fe(II) (ferrous) is stable and quite soluble at physiologic pH. Thus, in the early environment it is thought that specific ligands were not required and iron was assimilated by simple pathways analogous to those followed by other divalent metal ions (97). It was not until the anaerobic atmosphere was slowly converted to aerobic, an event which was probably achieved by the O_2 generating blue-green algae, that surface iron began to be oxidized and precipitated as insoluble oxyhydroxide polymers (74). It is this challenge of solubilizing the otherwise unavailable iron, which most living things must overcome, that makes the study of iron assimilation fundamentally important.

Iron's dark side: iron can be toxic.

Iron is essential. However, if not complexed, it may react with superoxides and hydrogen peroxide to generate reactive hydroxyl radicals, as summarized by the following three reactions (44):



These hydroxyl radicals are extremely reactive causing lipid peroxidation, DNA strand breaks, and degradation of various biomolecules (45, 47). Vertebrates deal with the toxic effects of iron by synthesizing extracellular transferrin and intracellular ferritins to retain iron in the unreactive Fe(III) (ferric) form. At physiologically relavent pH, ferritin is able to solubilize iron, capturing the highly toxic Fe(II), oxidizing it and finally sequestering it as Fe(III), thus limiting its toxicity (47, 115). Therefore, the major role of ferritin for vertebrates is to provide a means of iron storage. Ferritin is uniquely designed to keep polynuclear iron in a soluble and available form. Prokaryotes also synthesize ferritin, known as bacterioferritin. Bacterioferritin also captures the toxic Fe(II) and sequesters it as Fe(III), limiting iron toxicity within the bacterial cell. Ferritin-iron incorporation appears to follow conserved pathways from humans to bacteria: iron is taken up through pores in the ferritin protein shell, is oxidized at specific sites (at least initially) and then resides in a preformed cavity (47).

Iron absorption in eukaryotes:

Humans maintain, on average, about 3-5 g of iron (47). About two-thirds of this iron is found in circulating red blood cells as hemoglobin and 15-25% in storage as ferritin and hemeosiderin (16). The remaining iron is in myoglobin (about 8%) and in cytochromes

and iron containing enzymes. Plasma transferrin accounts for only 3 mg of iron. The main role of transferrin is iron distribution. On a daily basis there is about 1 mg of iron taken up from foodstuffs and 1 mg excreted, primarily from the gut and kidney (47).

Iron is in a constant state of flux in the host. The role of transferrin is to sequester iron as it is released from one area, and to transport it to another area that can either utilize or store it. On a daily basis, 22 mg of iron is released by destruction of red blood cells, 7 mg from parenchymal tissue, and about 1 mg from mucosal cells of the intestine. The iron from all these sources is bound by transferrin and deposited in the erythroid marrow and other tissues for synthesis of various iron proteins and for storage. The transfer of iron between transferrin and its various recipients and donors involves an interaction with specific receptors followed by endocytosis and recycling of apotransferrin and the transferrin receptor (33, 47, 106).

In humans, the major sites for iron storage include the liver, where about 1/3 of the body's iron is stored, followed by the spleen and bone marrow. Muscle is also an important storage site because of its large mass, although the actual concentration of iron in muscle tissue is quite low at 40 mg per kg (108).

In mammalian cells, ferritin is primarily localized in the cytoplasm (58). In the gastric mucosa, ferritin plays a role in the regulation of iron entry into the body by sequestering unneeded iron which is lost when the cells are desquamated (55, 98). In conditions of elevated body iron, ferritin levels reach a maximum and then hemeosiderin becomes prominent (56).

General strategies for iron acquisition:

Bacteria and fungi acquire iron primarily by producing small organic molecules with high affinity for Fe(III) (ferric) iron. These iron carriers (siderophores) may contain phenolate and/or hydroxamate moieties. A common feature of siderophores is that they

contain six coordinate sites for Fe^{3+} (73). Siderophores scavenge iron from various high affinity iron binding proteins such as ferritin, lactoferrin and transferrin (detailed in Part II).

Humans and other omnivores acquire the majority of their iron by consuming meat. Meat contains iron-rich heme which is more readily absorbed as compared to other iron sources. Plants tend to be poor sources of iron because of the presence of phosphates, phylates, and polyphenols which inhibit iron absorption (43).

Plants themselves have adopted several strategies for iron acquisition including, translocating iron into their root systems, releasing protons or organic acids leading to localized lowering of soil pH, or synthesizing reductants which convert Fe (III) to the more soluble Fe (II) (47). Some plants release chelating agents and others actually exploit their siderophore-producing symbiotic microbes and utilize the ferric-siderophore complexes.

PART II: IRON ACQUISITION IN BACTERIA

This section specifically addresses how different microbes acquire iron, emphasizing bacterial pathogens. I will begin by presenting the non-siderophore and siderophore mediated systems of iron acquisition. Then I will discuss the specific siderophore mediated systems in *E. coli* and *Pseudomonas spp.* I will also briefly discuss mechanisms used by the *Yersineae*. I will conclude this section with a discussion of the general mechanisms employed to regulate iron acquisition systems. Once a human pathogen enters the hostile environment of the host, the pathogen finds that iron is complexed with high affinity iron binding proteins such as transferrin, lactoferrin, and heme. Interestingly, even in the terrestrial and aquatic environments, iron is not readily accessible, as it is insoluble. As mentioned earlier, the actual assimilation of iron poses two very critical problems for microbes. First, the microbe must overcome the insolubility of iron in the particular environment, and second it must regulate iron uptake because iron has the potential for wreaking cellular havoc by way of hydroxyl radicals - therein lies a paradox. Not only is iron essential and found in various unavailable forms, it is also potentially toxic - even for microbes! In response to this paradox microbes have developed a variety of iron uptake systems and extensive mechanisms of regulation.

Microbes in anaerobic environments have it easy, as iron is far more accessible under physiologic conditions in the absence of oxygen. The iron acquisition mechanisms used by anaerobes resemble simple diffusion systems similar to other essential ions (97). The aerobes and facultative anaerobes are the creative ones as they have been forced to develop active mechanisms to acquire the nearly insoluble iron in their aerobic environment. Table 1 illustrates some examples of the strategies used by various microbes (120).

Table 1. Mechanisms of iron uptake by pathogenic bacteria.

(Modified from Wooldridge, 1993 (120))

Species	SIDEROPHORE MEDIATED		NON-SIDEROPHORE MEDIATED	
	Endogenous Siderophores	Exogenous Siderophores	Host Iron	Other
Bordetella pertussis			Transferrin, Ovotransferrin, Lactoferrin	
Campylobacter jejuni		Enterobactin	Heme, hemoglobin	
Corynebacterium diptheriae	Uncharacterized	Aerobactin		
Escherichia coli	Enterobactin Dihydroxybenzoic acid Dihydroxybensoyl-serine Aerobactin	Ferrichrome Ferricrysin, Ferricrosin Coprogen, Rhodotorulic acid Citrate FerrioxamineB	Heme	Alpha-hemolysin Ferrous Iron
Haemophilus influenzae		Enterobactin	Heme	
Neisseria meningitides			Heme, Hemoglobin Transferrin Lactoferrin	
Neisseria gonorrhoeae		Aerobactin	Heme, Hemoglobin Transferrin Lactoferrin	
Pseudomonas aeruginosa	Pyoverdin Pyochelin Enterobactin	Enterobactin	Pyocyanin	
Staphylococcus-aureus	Uncharacterized			
Vibrio anguillarum	Anguibactin Enterobactin-like	Enterobactin Ferrichrome	Transferrin	
Vibrio cholera	Vibriobactin		Heme Hemoglobin	Hemolysin
Vibrio vulnificus	Catechol-type, Hydroxamate-type		Heme	
Yersinia spp.	Uncharacterized	Aerobactin	Heme Hemoglobin	

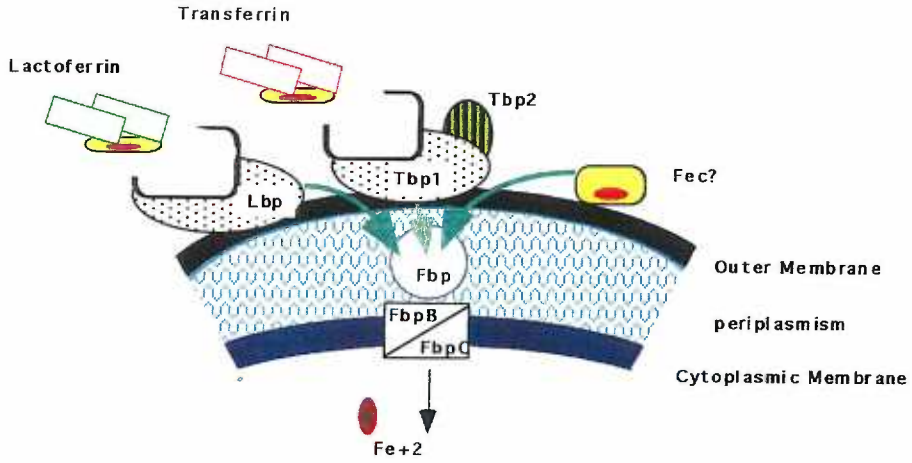
Figure 1. Comparison of the gene products that facilitate transport of iron for pathogenic *Neisseria spp.* (Modification of Mietzner, 1994(69)). Note that the general classes of proteins for gram-negative pathogenic *Neisseria spp.* involve an outer membrane receptor (Lbp1, or Tbp1), a periplasmic binding protein (Fbp), a cytoplasmic permease* (Fbp C), and a nucleotide binding protein* (Fbp B). In the pathogenic *Neisseria spp.* cartoon, Tbp 1 stands for the transferrin binding protein 1 , Tbp 2 is transferrin binding protein 2. Lbp 1 stands for the lactoferrin binding protein 1 and Fbp is the ferric binding protein. Fec stands for ferric dicitrate. *Fbp C and Fbp B are hypothetical nucleotide-binding protein and permease respectively.

IRON UPTAKE MECHANISMS

As illustrated in Table 1, a bacterium may possess multiple mechanisms for iron uptake. Although not discussed in this report, it should be noted that bacteria also have passive iron uptake mechanisms. The active mechanisms can be divided into two categories, non-siderophore binding mechanisms and siderophore synthesis mechanisms.

Non-Siderophore Binding Systems

There are two basic mechanisms of iron acquisition which are not mediated by a siderophore: 1) the direct reduction of ferric iron, 2) the direct utilization of host iron compounds (transferrin, heme, or hemoglobin as well as simply lysis of red blood cells) (63). Figure 1 illustrates some examples of binding mechanisms utilized by the pathogenic *Neisseriae*.



Iron acquisition by pathogenic *Neisseria*

Transferrin and lactoferrin uptake Studies of *Neisseria meningitidis* and *N. gonorrhea* in the early 1980's revealed that siderophores were possibly not the mechanism for iron utilization in *Neisseria*. An experiment was carried out using dialysis bags containing ferri-transferrin, where the bag was immersed in an iron limiting medium, and either *E.coli* or *N. meningitidis* were inoculated into the medium outside the bag. The researchers discovered that *E.coli* grew whereas *N. meningitidis* did not. Since *E. coli* secretes a siderophore which can transverse the dialysis bag, the siderophore will complex the iron and then be taken up by the *E.coli* for growth. Obviously, *N. meningitidis* required direct contact with the iron source ferric-transferrin (9, 69).

The next question was how is transferrin incorporated into the meningococcal cell. Isogenic mutants in the *tbpA* and *tbpB* genes were generated (57). These isogenic mutants were deficient in tranferrin binding protein 1 (Tbp1) and transferrin binding protein 2 (Tbp2), respectively. The isogenic mutants exhibited reduced binding of transferrin by whole cells and total membrane preparations and could not utilize transferrin for growth. This indicated an involvement of both proteins in transferrin binding at the meningococcal surface, as both proteins in wild type cells are surface exposed (69). Sequence searches revealed that the Tbp1 protein has a striking similarity with the TonB-dependent outer membrane receptors FepA, FecA, IutA, FhuA and FhuE (25) implying that a TonB analog must exist in pathogenic *Neisseria* species. Recently, a *tonB* homologue has been cloned and characterized from *N. meningiditis* (105). TonB is thought to be involved in the transmission of the electrochemical potential across the cytoplasmic membrane. The TonB protein is anchored in the cytoplasmic membrane and spans the periplasmic space to interact with the outer membrane bound receptor proteins to which the substrates to be transported are bound (18, 86).

In addition to binding transferrin, *Neisseriae* also bind lactoferrin. Some parameters of lactoferrin uptake are common to transferrin uptake but there are also distinctive differences. One important aspect of lactoferrin binding is that *Neisseriae* only bind human

lactoferrin whereas they can bind transferrin from many other species. Interestingly, production of receptors for both lactoferrin binding and transferrin binding appears to be linked through a non-receptor gene product essential for obtaining iron from both sources (15).

Once bound to its appropriate receptor, iron transferrin is transported onto a 37 kDa major iron-regulated protein, ferric iron-binding protein (Fbp) (32). All pathogenic *Neisseriae* strains examined to date possess Fbp as reported in a recent review (69). Fbp was recently localized to the periplasm and found to accept iron transiently as a basic periplasmic protein (see Figure 1).

In mammalian cells transferrin is internalized via receptor mediated endocytosis (121). The entire receptor protein-iron complex is internalized; subsequently the iron is removed from transferrin by cellular endosomes, generating apo-transferrin. The apo-transferrin is then released by the cell. However endocytosis does not occur in the case of bacterial uptake of transferrin. Bacterial transport of transferrin is distinct from the mammalian process and is not well understood (69).

Heme uptake Several microbes e.g., *Haemophilus influenzae* type B, *Vibrio vulnificus* (51), *Campylobacter jejuni* (82) and some *Neisseria* species rely on heme (37). The mechanism of uptake via heme requires a means of accessing the intracellular pool of hemoproteins (i.e., hemolysins, proteases, or cytolysins etc.). It also requires the ability to extract heme bound to serum carrier proteins. Additionally, specific outer membrane proteins as well as periplasmic binding proteins are required to transport intact heme.

Recently it was described that the meningococcal lactoferrin, transferrin and hemoglobin receptors share significant amino acid homology (104), suggesting at least some common structure. In addition, transport of heme, transferrin or lactoferrin all appear to be TonB dependent. An interesting feature of the heme uptake system involves the fact that there is no correlation between the ability to transport heme and virulence, although

heme binding has long been recognized as a virulence factor (80). It appears that the ability to aggregate heme around the exterior of the cell may involve proteins not engaged in transport. This accumulation of heme might act as an external storage reservoir for iron (81). In addition it may be serving as a type of armor with rudimentary antioxidant defense. As both heme and hemoproteins readily bind nitric oxide (63, 71), this shell may insulate the bacterium against the oxidative ravage of nitric oxide (63).

Siderophore systems

Siderophores are low molecular weight high affinity iron binding molecules (73). There are two basic classes of siderophore, phenolates (catechols) and hydroxamates. The prototypical phenolate is enterobactin (also called enterochelin) and aerobactin is the prototypical hydroxamate (74). Figures 2 and 3 illustrate the prototypical siderophores enterochelin and aerobactin, respectively, as well as their putative biosynthetic pathways.

***E.coli* uptake mechanisms** *E.coli* uses several iron uptake mechanisms such as: endogenous siderophore uptake, which include enterobactin and aerobactin; exogenous siderophore uptake i.e., uptake of siderophores made by other bacteria or fungi which include enterobactin, ferrichrome and citrate; and direct binding of host iron compounds such as heme. *E.coli* also possesses an alpha hemolysin, and can directly reduce ferrous iron (Table 1). Figure 4 illustrates the many different iron transport systems utilized by *E. coli*. The best described mechanisms of siderophore uptake are those involving enterobactin and aerobactin so I will briefly detail what is currently understood regarding the synthesis and transport of these two compounds. I will also briefly detail the ferric citrate uptake system in which some rather novel regulation mechanisms have been characterized recently (6). Siderophore transport (Figure 4) is an energy dependent mechanism involving several membrane bound proteins. The *E.coli*: TonB-ExbB-ExbD

Figure 2. Biosynthesis of enterobactin from chorismic acid (Silver 1992 (97)). Chorismic acid is converted to 2,3-dihydroxybenzoic acid sequentially by the action of three enzymes EntC, EntB, and EntA. 2,3-Dihydroxybenzoic acid and serine are coupled in a Mg^{2+}-ATP dependent reaction to form DHBS monomer, which is attached to the four-polypeptide biosynthetic protein complex (consisting of EntD, EntE, EntF and EntG). Two DHBS monomers are coupled to form the DHBS dimer, and a third DHBS monomer is added to form the trimer, which is still attached to the protein complex. The final ester linkage in trimeric DHBS (i.e. enterobactin) is formed with the release of the siderophore from the protein complex.

Figure 2. Enterobactin structure and biosynthesis pathway.

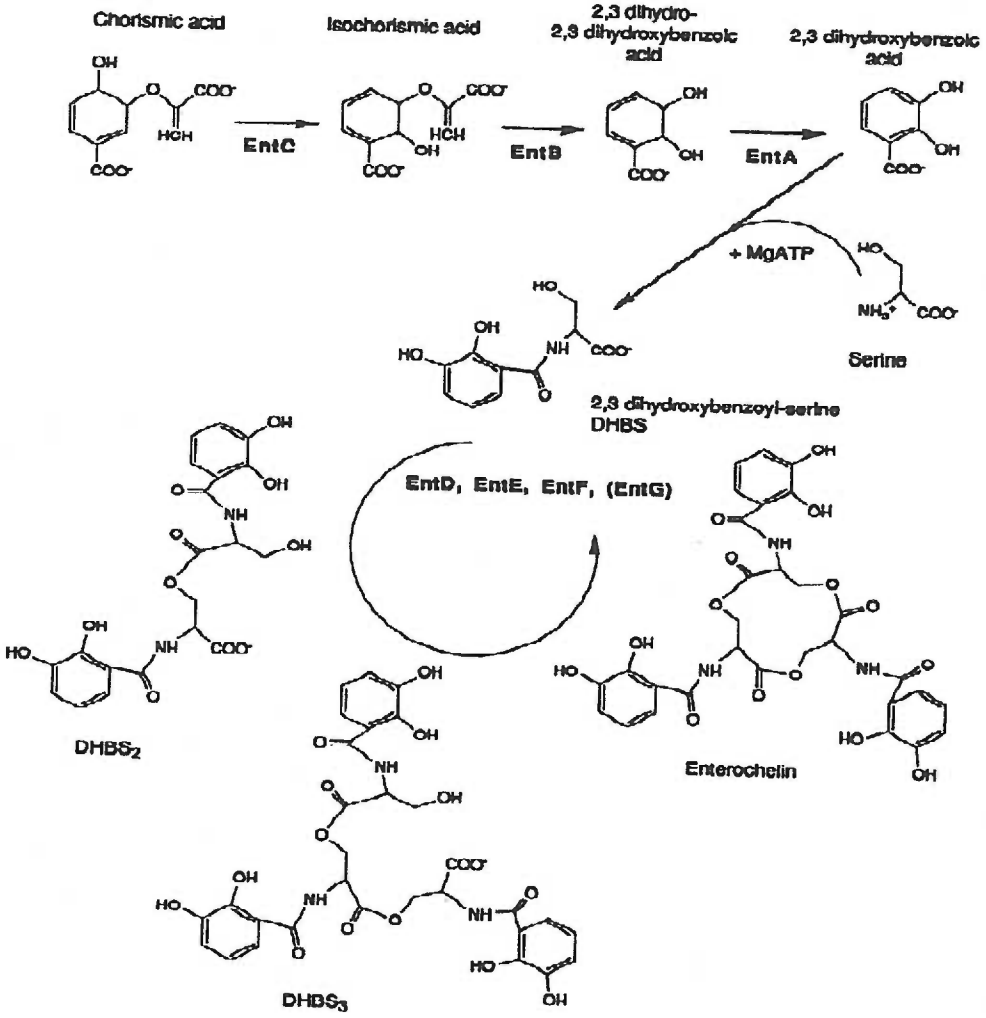


Figure 3. Biosynthesis of aerobactin (Silver, 1992 (97)). The ε-amino group of lysine is hydroxylated with molecular oxygen by the IucD polypeptide. An acetyl gorup from acetyl coenzyme A is added to the same ε-amino group by the IucB protein. Citrate is added to the other (α-) amino group of lysine by the IucA polypeptide, and then the second ε-*N*-hydroxylysine is added by the IucC protein, to complete the synthesis of aerobactin.

Figure 3. Aerobactin structure and biosynthesis pathway.

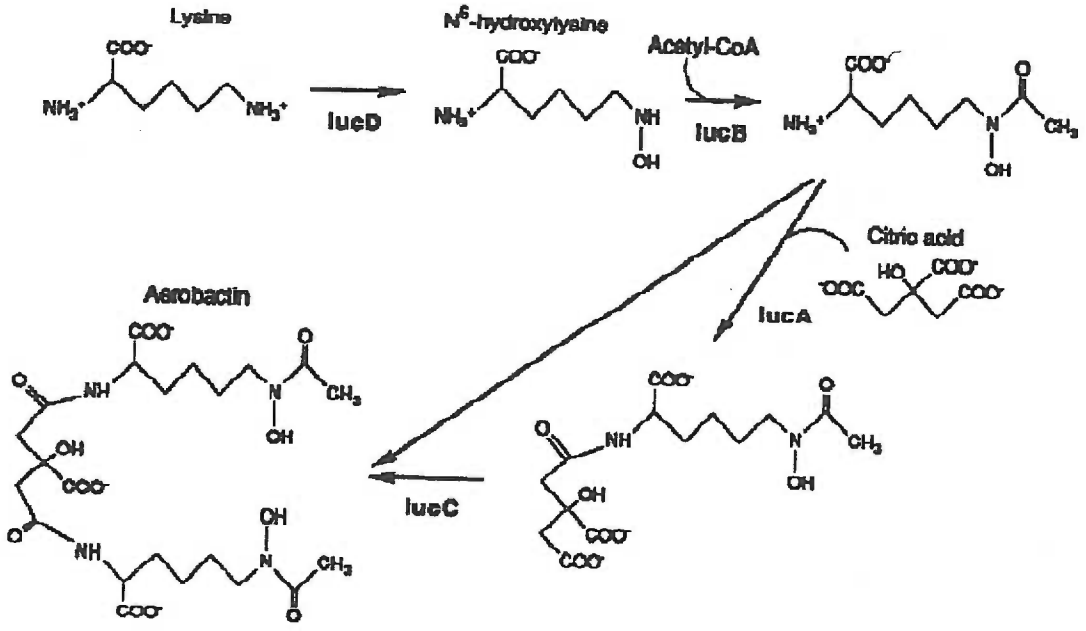
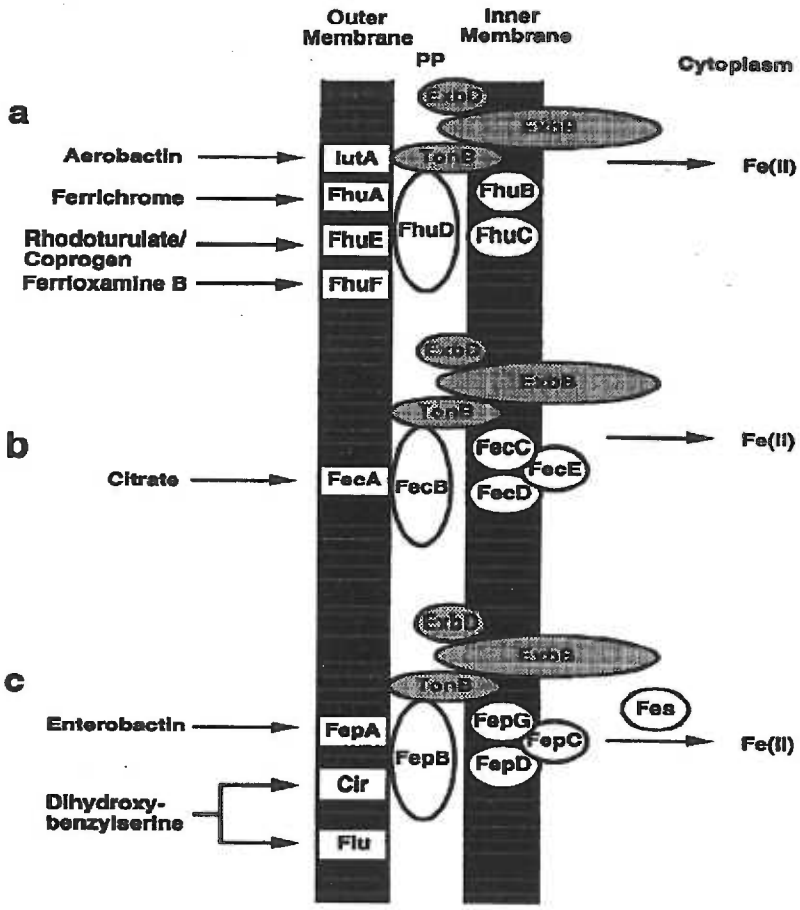


Figure 4. Scheme of iron-uptake systems in *Esherichia coli*. (Guerinot, 1994 (43)). Inner and outer membranes are shaded. Light shading indicates the three proteins known to be common to all three hydroxamate(a), citrate (b), and catechol (c) transport systems. PP=periplasm. Proteins are not drawn to scale.

Figure 4. Mechanisms of iron transport in *E.coli*



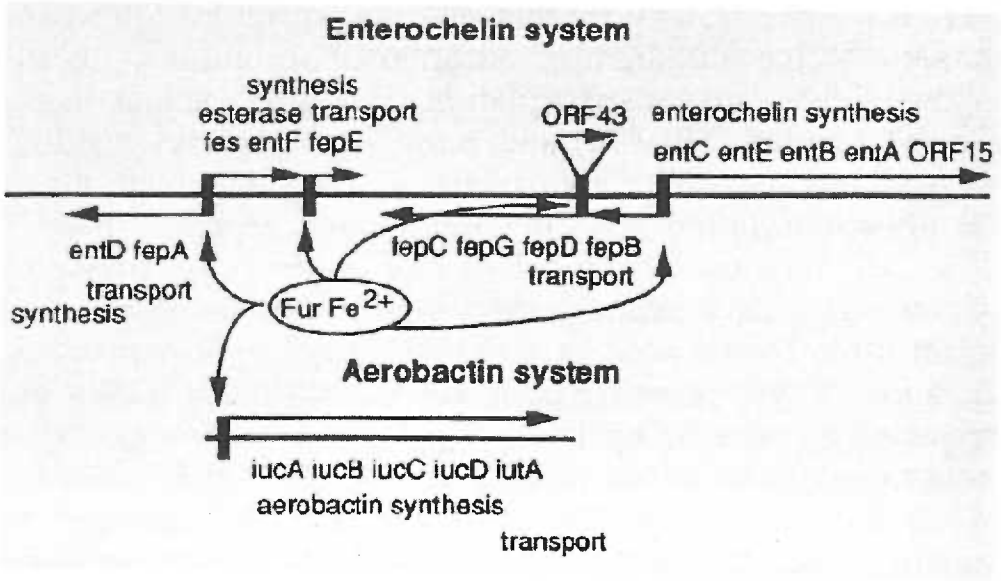
dependent systems include all specific ferri-siderophore binding outer membrane proteins: enterobactin (FepA), aerobactin (IutA), citrate (FecA), ferrichrome (FhuA) and Rhodoturulate (FhuE) (11).

Enterobactin: Enterobactin (Figure 2) is a cyclic trimer of 2,3-dihydroxybenzyl serine (DHBS) in which peptide bonds connect the serine to catechol and the serine residues are cyclized by ester linkages. Synthesis involves the *ent* genes and transport involves the *fep* genes, a total of 14 genes in 7 transcriptional units in both orientations (Figure 5). All of these fall within a 22 kb segment of the chromosome. Synthesis involves the interaction of some 10 proteins. EntE and EntF are of specific interest as they have been characterized quite extensively. These two proteins are directly involved in the active synthesis of enterobactin (34, 60, 89-91). EntE serves as the 2,3- dihydroxybenzoic acid (2,3-DHBA) AMP-ligase enzyme. EntF is the serine adenylating enzyme. These enzymes belong to the family of adenylating enzymes and non-ribosomal peptide synthetase enzymes (89-91, 99).

Transport of the ferric-siderophore complex occurs in an energy coupled manner and involves the outer membrane protein receptor FepA (72). FepA is proposed to contact Gln-165 (14, 72) of TonB in such a way that the potential of the cytoplasmic membrane is transduced providing the metabolic energy for transport of the ferri-siderophore complex through FepA. ExbB and ExbD function within the inner membrane to stabilize and activate TonB function. Fes is the enterochelin esterase which cleaves the ester backbone of enterobactin reducing the dissociation constant of the ferri-siderophore complex from 10^{-52}M to about 10^{-8}M (97). This is required to make the iron available for intracellular metabolism.

Figure 5. Fur protein binding to iron boxes (solid boxes) upstream of the seven enterobactin (*ent* and *fep* genes)operons on the *E.coli* chromosome and the *iuc* aerobactin operon on ColV plasmids (Silver, 1992 (97)). Individual genes and the directions of transcription are shown as arrows. The intact enterobactin system comprises 22kb.

Figure 5. Map of enterobactin and aerobactin synthesis and transport genes



Aerobactin: This siderophore is a hydroxamic acid consisting of two residues of ε-N-acetyl-ε-N-hydroxylysine connected via peptide bonds to the terminal carboxyl groups of citric acid (Figure 3). Synthesis involves the ε-amino group of lysine which is hydroxylated with molecular oxygen by the IucD polypeptide followed by acylation by the IucB protein. Citrate is added to the other alpha amino group of lysine by the IucA polypeptide and then the second ε-N-acetyl-ε-N-hydroxylysine is added by the IucC protein. A single transport protein, IutA , is produced from the final gene in the operon. This protein shows homology with FepA. Once iron is complexed with aerobactin it interacts with IutA and is internalized in a mechanism involving the ferric hydroxamate uptake proteins FhuB, FhuC, and FhuD in a process that is TonB, ExbB and ExbD dependent (11).

Ferric dicitrate: The uptake of ferric dicitrate requires the FecA, FecI and FecR proteins which are involved in signaling the presence of ferric citrate in the environment. FecA is the outer membrane protein receptor, FecR is a periplasmic iron dicitrate sensor which transmits a signal to FecI. FecR straddles the membrane so as to interact with FecA in the periplasm. FecR also interacts with FecI with in turn binds the Fec operator region on the DNA resulting in transcription of the *fec* transport genes. FecI/R is not related in sequence to the two component regulator family that involves phosphorylation for signal transfer. Activating the *fec* system requires both positive action by FecI and the absence of Fur (54). Then the cells respond to extracellular citrate. FecI has recently been shown to be an extracytoplasmic function (ECF) sigma factor (6).

Pseudomonads Depending on which species, Pseudomonads secrete several siderophores including pseudobactins (also called pyoverdins), pyochelins, and enterobactin. Synthesis of these siderophores involves several different operons (68). The psuedobactins are composed of a closely related chromophore, derived from 2,3-diamino-

6,7-dihydroxyquinoline, bound to a peptide moiety which has considerable substrate variation between pseudobactins produced by different strains and contain catechol, hydroxamate and α-hydroxy acids (73). The peptide backbone maybe linear, partially circular or completely circular and can consist of 5 to 12 amino acid residues with different chiral configurations (1). Some pyoverdins are synthesized via non-ribosomal peptide synthetase enzymes (68). The pyochelins are much simpler molecules consisting of phenolate and thiazoline moieties (73). Each siderophore has its own cognate receptor which is up regulated by the presence of its siderophore. In Pseudomonads, the transport of iron-siderophore complex requires not only iron limitation, but also the presence of the proper receptor (41, 83, 85).

***Yersineae* uptake mechanisms** The *Yersiniae* appear to synthesize neither classical siderophores nor siderophilin (host iron-transport protein) receptors but can obtain iron from a variety of host iron binding proteins including: ferritin, hemin and hemin complexes as discussed in the binding section. The mechanism for iron acquisition is still unclear but appears to involve expression of high molecular weight iron regulated proteins (101). However, siderophore activity has recently been observed and is currently being investigated (69). In addition, during infection *Yersiniae* often remain in endocytic vesicles which have been shown to be quite iron sufficient. It is believed that *Yersinia* can survive within these intracellular environments (67).

REGULATION:

Even in bacteria, iron is potentially toxic. Thus, it is essential not only to possess iron binding proteins such as ferritins, to keep iron stable and within bacterial cells, but it is also critical to regulate expression of the genes encoding components of the iron acquisition systems. Initially regulation relating to the iron transport and acquisition systems was tied directly to the ferric uptake regulator, Fur. In *E.coli* , fur was reported to serve as the master switch for signaling iron limitation vs. iron surplus (46). *fur* gene homologs have now been found in *Vibrio anguillarum*, *Klebsiella pneumonia*, *Pseudomanas aeruginosa*, *Salmonella typhimurium*, *Yersinia pestis*, *Bordetella pertusis*, *Campylobacter upsaliensis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio vulnificus*, *Staphylococcus epidermidis*, *Legionella pneumoniae*, and putative Fur box motifs , i.e. Fur DNA binding sites, have been found in *Bacillis subtillus* and *Serratia marcescens* suggesting that Fur homologs may exist in these species as well. (2, 7, 8, 12-13, 17, 22, 40, 42, 50, 53, 66, 102, 107, 112, 119). In iron rich conditions the Fur protein binds Fe(II) which allows the Fur-Fe(II) complex to bind DNA. Once Fur is bound to DNA at its specific recognition site, usually overlapping the -10/-35 promoter region, transcription via RNA polymerase is successfully halted (10, 38, 52, 93, 117). Indirect evidence suggests that when the concentration of iron begins to drop, the Fur-Fe(II) molecule dissociates and conformation changes, thus Fur no longer can effectively bind to its operator sequence (26). The originally described *E. coli* "Fur box" sequence is a 19 base pair dyad 5'-GAT(A/T)ATGAT(A/T)AT(C/T)ATTTTC, based on the operator sequences found upstream of the enterobactin and aerobactin synthesis and transport genes (Figure 5) (87, 114). However, as more Fur boxes became known via footprinting, a palindromic sequence 5'- GATAATGATAATCATTATC was chosen to represent the general Fur box (21, 36).

Researchers have uncovered novel systems involving sigma factors where Fur regulation is indirect (76, 77, 88) An entire class of alternative sigma factors has now

begun to be characterized. The extracytoplasmic function (ECF) subgroup of the sigma 70 family of eubacterial RNA polymerase sigma factors are environmentally responsive (96). Each ECF responds to specific extracellular signals. In *E.coli* there are at least two such ECF factors with different functions, one is a temperature-responsive heat shock sigma factor (39) and the other is FecI which is required for ferric iron dicitrate transport (54, 122). There are also ECF factors in *Pseudomonads.*

As mentioned, *Pseudomonads* are found in nearly every ecological niche and accordingly they possess numerous receptors for a variety of siderophores. In addition they have non-siderophore mediated iron uptake mechanisms. Thus, their mechanisms of regulation become quite complex; consider just three of these uptake mechanisms which involve pyoverdin, ferric enterobactin, and pseudobactin 358. In the case of pyoverdin uptake in *P. aeriginosa*, the *pvd* genes are required (70). Expression of *pvdS*, has recently been found to be directly controlled by Fur (64, 76). PvdS in turn appears to acts as a sigma factor (31) and is required for expression of *pvdA* (64) and other genes (78). *pvdA* codes for the L-ornithine N^5 oxygenase which catalyzes a key step of pyoverdin biosynthesis (113). Ferric enterobactin uptake requires PfeA, an 80 kDa outer membrane protein which is highly homologous to FepA, the enterobactin receptor. Expression of *pfeA* is regulated by a two component regulatory pair PfeR (the regulator) and PfeS (the sensor) (61). The latter pair's expression may be regulated by Fur although there are currently no direct binding experiments published (34). Uptake of Pseudobactin 358 requires products of the *pupI* and *pupR* genes which share homology with *fecI* and *fecR* of *E.coli* (62) Neither system shows homology with the two component regulator systems.

Fur has been found to regulate several of these components either directly or through multiple regulatory complexes (35). Specifically, *fur* mutations result in a species specific inability to use certain carbon sources. For instance in *V. cholerae* these include glycerol, acetate, succinate, lactate, and fumarate when the bacteria are grown in minimal medium (65). In *Yersinia pestis*, *fur* mutants show a loss of temperature control of hemin storage

and under iron rich conditions present increased sensitivity to pesticin (100). In addition, Fur has been found to be required for expression of some of the superoxide dismutase genes both in *E. coli* (75) and *P. aeruginosa* (49) as well as for the expression of the regulatory RNAα molecule in *V. anguillarum* , which also serves as a negative regulator of the iron transport genes (23). Therefore Fur appears to be involved in a multitude of cellular functions in addition to those directly related to iron uptake, including: metabolism, fimbriae production, production of hemolysins (84, 103), generation of toxins (21, 79) and Fur has recently been found to have multiple effects on the expression of superoxide dismutase genes (49,75).

PART III: *VIBRIO ANGUILLARUM* IRON ACQUISITION

The bacterial fish pathogen *V. anguillarum* is a gram negative rod possessing a polar flagellum. *V. anguillarum* causes vibriosis, a highly fatal hemorrhagic septicemic disease in salmonids. We use the *V. anguillarum*-host fish system as a model to study the molecular mechanisms of host-pathogen interactions leading to human disease. The disease caused by *V. anguillarum* has remarkable similarities to invasive septicemic disease in humans and the sequence of events immediately after infection are very similar to mammalian inflammation, except for obvious species-specific responses (27, 30). Another important feature of our system is the fact that this bacterium is an actual pathogen for the vertebrate host (30, 110, 118). Thus, inferences made from the study of the mechanisms 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.

Our laboratory has elucidated a key feature which enable pathogenic strains of *V. anguillarum* to survive within the salmonids: primarily the possession of a virulence plasmid which provides the bacteria with an iron-uptake system (Figure 6) (28, 30).

The plasmid encoded iron-sequestering system is comprised of three regulators: the positive regulators TAF (trans acting factor(s)), and AngR (anguibactin biosynthesis regulator), and a negative regulator, antisense RNA_{α}. The cast is completed by genes involved in iron transport such as *fatDCBA*, and several genes involved directly in siderophore production (Figure 6) (27-30). This plasmid mediated system centers upon the production and uptake of the siderophore anguibactin (Figure 7 and 8). Anguibactin has a molecular weight of 348 and its molecular composition is $C_{15}H_{16}N_4O_4S$ (3, 59). It has a unique structure containing both hydroxamate and catecholate structures. Single crystal structure determination of the Ga(III) complex of racemized anguibactin revealed a 1:1

metal to ligand stoichiometry in which the O-hydroxy group, the nitrogen of the thiazolin ring, the hydroxamate (N-O group), and the deprotonated nitrogen of the imidazole ring coordinate the metal ion (Figure 7) (59). The ferric-siderophore complex specifically binds to the outer membrane protein FatA (3, 4, 28, 30). The iron is transported into the cell via the interaction of at least 3 other proteins, FatB, a membrane-anchored periplasmic spanning lipoprotein (5), and the integral membrane proteins Fat C and FatD (Figure 8).

Figure 6. Plasmid pJM 1

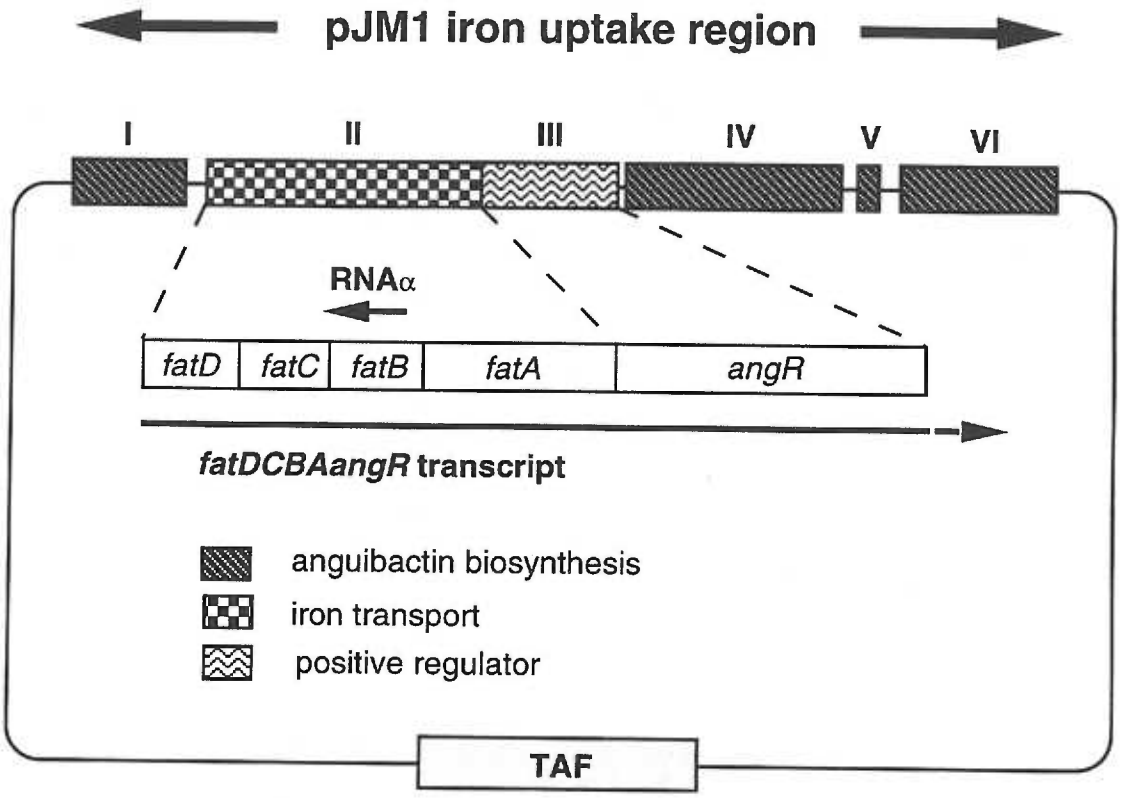


Figure 7: Symbols: empty spheres, carbons; red spheres, oxygen (O); blue spheres, nitrogen (N); dark blue spheres, sulfur (S); and yellow spheres, methoxy groups or water molecules; grey spheres, gallium (Ga). The structure was elucidated by Jalal et. al. (59).

Figure 7. Anguibactin structure complexed with gallium (59)

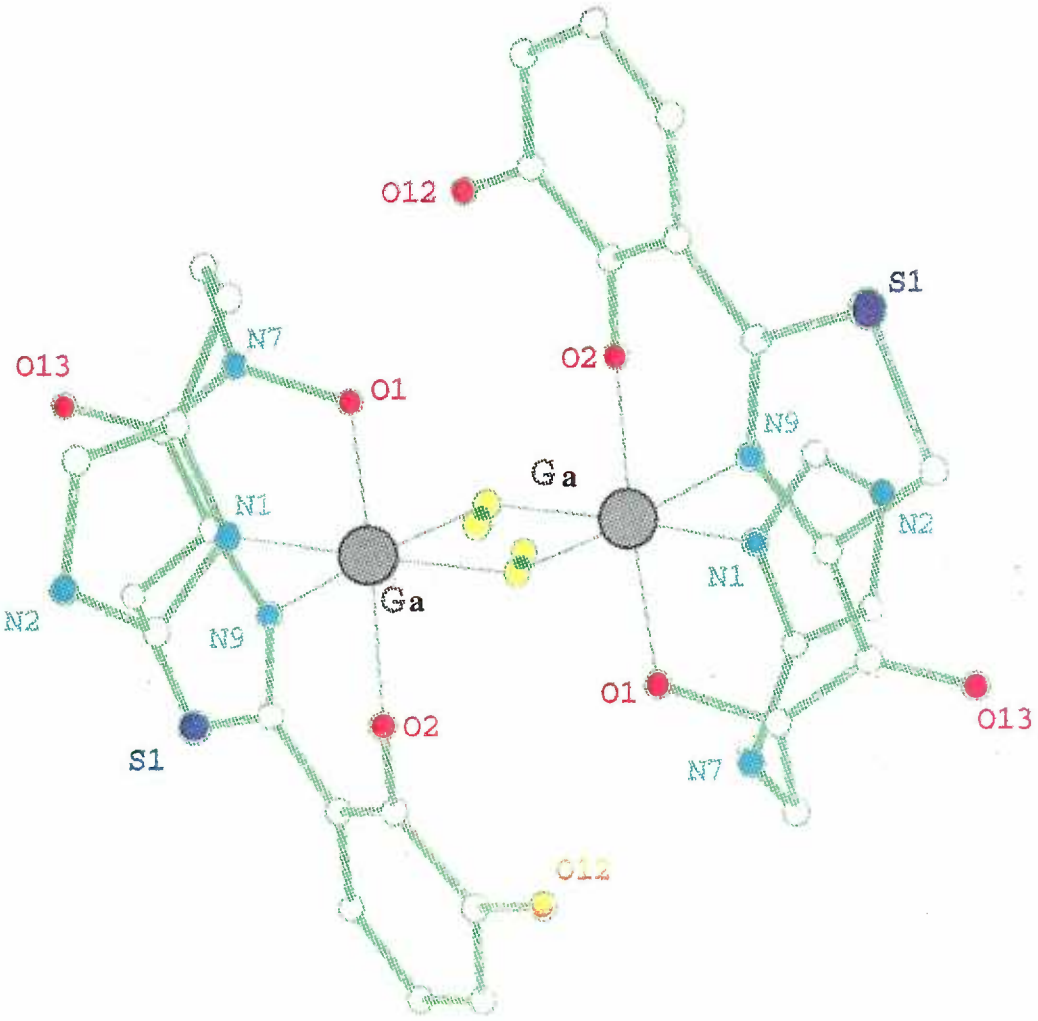
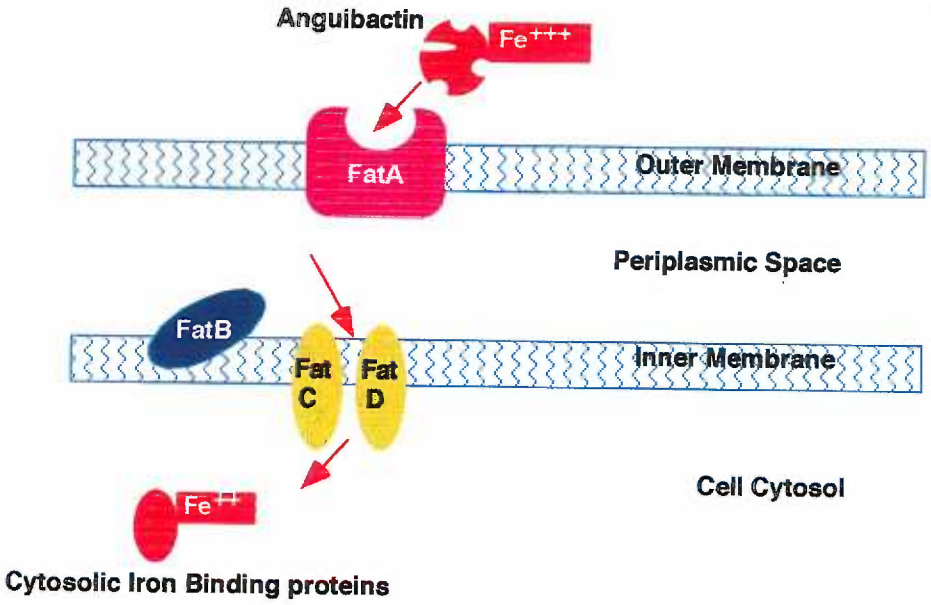


Figure 8. Ferric-anguibactin transport into *V. anguillarum*



In addition to the components encoded by the virulence plasmid, we have recently identified chromosomally encoded genes which code for proteins that are required for siderophore production such as Aro C, and regulation such as Fur (Ferric uptake regulator).

My thesis research focused on the mechanism of regulation of the plasmid mediated iron uptake system of *V. anguillarum.* Specifically, I analyzed the chromosomally-encoded negative regulator Fur, the mechanism of expression of the iron transport genes *fatDCBA*, and the plasmid encoded positive regulator AngR.

In *V. anguillarum*, at least four regulators, two positive (TAF and AngR) and two negative (Fur and antisense RNA α) are involved in regulating the expression of the iron transport genes *fatDCBA* (Figure 6). The *V. anguillarum* Fur protein is one member of the complex circuit, unlike in *E. coli* where regulation of much of the iron uptake gene expression depends primarily on the Fur regulator (46). The Fur protein was first described in *E. coli* (46). Although indirect, there is evidence implicating the C-terminal portion of the *E. coli* Fur protein in Fe(II) binding, which may induce a conformational change in the N-terminal region of the protein that allows the binding, possibly as a dimer, to the operator of Fur-regulated genes (26) However, the characteristic helix-turn-helix motif (19, 20) present in many DNA-binding proteins is not found. Despite initial NMR (nuclear magnetic resonance) and CD (circular dichroism) studies on *E. coli* Fur, its ligand-free or corepressor Fe(II)-bound atomic structures have not been described (92-94). Therefore, the study of the *V. anguillarum* Fur protein is of great importance not only to understand the regulation of the iron uptake system within *V. anguillarum* but also as a general model of regulation and DNA-protein interaction. In order to address these issues my thesis involved identification and isolation of the *V. anguillarum fur* gene (Chapter 1) (112), characterization of both wild type and mutant Fur proteins (Chapter 2) (116), and determination of the nature of transcription of the iron transport genes (Chapter 3).

AngR, a 110-kDa protein encoded by pJM1-like plasmids (110, 111), acts as a trans-activator of other genes of the iron uptake system (95, 111). AngR possesses two putative helix-turn-helix motifs each preceded by putative leucine zipper motifs (109) which are consistent with regulatory function. In addition to TAF and AngR, we recently discovered that the siderophore anguibactin itself was able to stimulate the expression of the iron transport genes *fatA* and *fatB,* although not to the same extent as when all three regulators are present (24).

Interestingly, it has also been demonstrated that AngR shares homology with several biosynthetic enzymes from the firefly luciferase family which all utilize ATP. As AngR possesses adenylation motifs and a thioester motif, we conducted experiments and demonstrated that AngR can complement the biosynthetic enzyme EntE from *E. coli.* EntE functions as the 2,3-dihydroxybenzoic acid AMP-ligase required for biosynthesis of the siderophore enterobactin (Figure 2) (89). The fact that AngR can complement EntE, further strengthens the concept that AngR is directly involved in anguibactin biosynthesis (109). Thus, AngR may well serve two functions, one as a DNA-binding protein and transcriptional activator, and the other as a biosynthetic enzyme. Although we have yet to elucidate the specific DNA region recognized by AngR we have begun to dissect the regions involved in activation and biosynthesis, my research has addressed these questions (Chapter 4).

LITERATURE CITED

1. **Abdallah, M. A.** 1991. Pyoverdins and pseudobactins, p. 139-153. *In* G. Winkelmann (ed.), Handbook of Microbial Iron Chelates. CRC press, Boca Raton.
2. **Achenbach, L. A., and W. Yang.** 1997. The *fur* gene from *Klebsiella Pneumoniae* - characterization, genomic organization and phylogenetic analysis. Gene. **185:**201-207.
3. **Actis, L. A., W. Fish, J. H. Crosa, K. Kellerman, S. R. Ellenberger, F. M. Jauser, and J. Sanders-Loehr.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775 (pJM1). Journal of Bacteriology. **167:**57-65.
4. **Actis, L. A., S. Potter, and J. H. Crosa.** 1985. Iron regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. Journal of Bacteriology. **161:**736-742.
5. **Actis, L. A., M. E. Tolmasky, L. M. Crosa, and J. H. Crosa.** 1995. Characterization and regulation of the expression of FatB, an iron transport protein encoded by the pJM1 virulence plasmid. Molecular Microbiology. **17:**197-204.
6. **Angerer, A., S. Enz, M. Ochs, and V. Braun.** 1995. Transcriptional regulation of ferric citrate transport in *Escherichia coli* K-12. FecI belongs to a new subfamily of sigma 70-type factors that respond to extracytoplasmic stimuli. Molecular Microbiology. **18:**163-74.
7. **Angerer, A., B. Klupp, and V. Braun.** 1992. Iron transport systems of *Serratia marcescens*. Journal of Bacteriology. **174:**1378-87.
8. **Ankenbauer, R. G., and H. N. Quan.** 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. Journal of Bacteriology. **176:**307-19.

9. **Archibald, F. S., and I. W. DeVoe.** 1979. Removal of iron from human transferrin by *Neisseria meningitidis*. FEMS Microbiology Letters. **6:**159-162.

10. **Bagg, A., and J. B. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry. **26:**5471-7.

11. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. [Review] [63 refs]. Microbiological Reviews. **51:**509-18.

12. **Baik, H. S., S. Bearson, S. Dunbar, and J. W. Foster.** 1996. The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids. Microbiology. **142:**3195-3200.

13. **Beall, B.W., and G. N. Sanden.** 1995. Cloning and initial characterization of the *Bordetella pertussis fur* gene. Current Microbiology. **30:**223-6.

14. **Bell, P. E., C. D. Nau, J. T. Brown, J. Konisky, and R. J. Kadner.** 1990. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. Journal of Bacteriology. **172:**3826-3829.

15. **Blanton, K. J., G. D. Biswias, J. Tsai, J. Adams, D. W. Deyer, et. al.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. Journal of Bacteriology. **172:**5225-5235.

16. **Bothwell, T. H., R. W. Charlton, J. D. Cook, and C. A. Finch.** 1979. Iron Metabolism in Man. pp1-576. Blacwell, Oxford.

17. **Bourke, B. B., S. T. Alrashid, H. L. Bingham, and V. L. Chan.** 1996. Characterization of *Campylobacter upsaliensis* Fur and its localization in a highly conserved region of the *Campylobacter* genome. Gene. **183:**219-224.

18. **Braun, V., S. Gaisser, C. Herrmann, K. Kampfenkel, H. Killmann, and I. Traub.** 1996. Energy-coupled transport across the outer membrane of

Escherichia coli: ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. Journal of Bacteriology. **178:**2836-45.

19. **Brennan, R.** 1991. Interactions of the helix-turn-helix binding domain. Curr. Opin. Struct. Biol. **1:**80-88.
20. **Brennan, R.** 1993. The winged-helix DNA-binding motif: another helix-turn-helix takeoff. Cell. **74:**1015-1017.
21. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Eschericia coli* is mediated by the *fur* locus. Journal of Bacteriology. **169:**4759-4764.
22. **Chen, L., L. P. James, and J. D. Helmann.** 1993. Metalloregulation in *Bacillus subtilis*: isolation and characterization of two genes differentially repressed by metal ions. Journal of Bacteriology. **175:**5428-37.
23. **Chen, Q., and J.H. Crosa.** 1996. Antisense RNA, Fur, iron, and the regulation of iron transport genes in *Vibrio anguillarum*. Journal of Biological Chemistry. **271:**1885-91.
24. **Chen, Q., A. M. Wertheimer, M. E. Tolmasky, and J. H. Crosa.** 1996. The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum*. Molecular Microbiology. **22:**127-134.
25. **Cornelissen, C. N., G. D. Biswis, and P. F. Sparling.** 1993. Expression of gonococcal transferrin binding protein 1 causes *Escherichia coli* to bind human transferrin. Journal of Bacteriology. **175**:2448-50.
26. **Coy, M., and J. Neilands.** 1991. Structural dynamics and functional domains of the *fur* protein. Biochemistry. **30:**8201-8210.
27. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Reviews. **53:**517-530.

28. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature. **284:**566-568.

29. **Crosa, J. H.** 1984. The relationship of plasmid mediated iron transport and bacterial virulence. Annual Review of Micriobiology. **38:**69-89.

30. **Crosa, J. H., L. Hodges, and M. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infection and Immunity. **27:**897-902.

31. **Cunliff, H. E., T. R. Merriman, .and I. L. Lamont**. 1995. Cloning and characterization of *pvdS* a gene required for pyoverdine synthesis in *Pseudomonas aeriginosa*: PvdS is probably an alternative sigma factor. Journal of Bacteriology **177**:2744-50.

32. **Daskaleros, P. A., and S. M. Payne.** 1987. The congo red/hemin binding pheotype also associated with increased infectivity of *Shigella flexneri* in a HeLa cell model. Infection and Immunity. **55:**1393-1399.

33. **Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish.** 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proceedings of the National Acadamy of Science U.S.A.. **80:**2258-2262.

34. **Dean, C. R., and K. Poole.** 1993. Cloning and characterization of the ferric enterobactin receptor gene (*pfeA*) of *Pseudomonas aeruginosa*. Journal of Bacteriology. **175:**317-24.

35. **de Lorenzo, V. L., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur (ferric uptake regulator) protein and CAP(catabolite activator protein) modulate transcription of *fur* gene in *Escherichia coli*. European Journal of Biochemistry. **173:**537-546.

36. **de Lorenzo, V. L., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid pColV-K30 binding the ferric uptake regulation (*fur*) repressor. Journal of Bacteriology. **169:**2624-2630.

37. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infection and Immunity. **55:**2171-2175.

38. **Elkins M. F., and C.F. Earhart.** 1989. Nucleotide sequence and regulation of the *Escherichia coli* gene for ferrienterobactin transport protein FepB. Journal of Bacteriology. **171:**5443-51.

39. **Erickson, J. W., and C. A. Gross.** 1989. Identification of the subunit of *Escherichia coli* RNA polymerase: a second alternative factor involved in High-temperature gene expression. Genes and Development. **3:**1462-1471.

40. **Expert, D., C. Sauvage, and J. B. Neilands.** 1992. Negative transcriptional control of iron transport in *Erwinia chrysanthemi* involves an iron-responsive two-factor system. Molecular Microbiology. **6:**2009-17.

41. **Gensberg, K., P. A. Lambert, and A. W. Smith.** 1992. Iron uptake in *Pseudomonas aeruginosa*: induction by growth in the presence of siderophores. Abstracts of the Third International Symposium on Iron Transsport, Storage and Metabolism. France 87.

42. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1990. Transcriptional regulation by iron of a *Vibrio cholerae* virulence gene and homology of the gene to the *Escherichia coli* fur system. Journal of Bacteriology. **172:**6863-70.

43. **Guerinot, M. L.** 1994. Microbial Iron Transport, p. 743-772. *In* Ornston and Balows and Greenberg (ed.), Annual Review of Microbiology, vol. 48. Annual Reviews Inc., Palo Alto, CA.

44. **Haber, F., and W. Weiss.** 1934. Catalytic Decomosition of Hydrogen Peroxide by Iron Salts. Prec. Roy. Soc. Ser. A. **147:**332-333.

45. **Halliwell, B., and J. M. C. Gutteridge.** 1984. Oxygen toxicity , oxygen radicals, transition metals and disease. Biochemical Journal. **219:**1-14.

46. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Molecular and General Genetics. **197:**337-341.

47. **Harrison P. M., and P. Arosio.** 1996. The ferritins: molecular properties, iron storage function and cellular regulation. [Review] [477 refs]. Biochimica et Biophysica Acta. **1275:**161-203.

49. **Hassett, D. J., P. A. Sokol, M. L. Howell, J. F. Ma, H. T. Schweizer, U. Ochner, and M. L Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomanas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. Journal of Bacteriology. **178**:3996-4003.

50. **Heidrich, C., K. Hantke, G. Bierbaum, and H. G. Sahl.** 1996. Identification and analysis of a gene encoding a Fur-like protein of *Staphylococcus epidermidis*. FEMS Microbiology Letters. **140:**253-9.

51. **Helms, S. D., J. D. Oliver, and J. C. Travis.** 1984. Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. Infection and Immunity. **45:**345-349.

52. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Molecular Microbioly **4:**1621-1628.

53. **Hickey, E. K., and N. P. Cianciotto.** 1997. An iron- and Fur-repressed *Legionella pneumophila* gene that promotes intracellular infection and encodes a protein with similarity to the *Escherichia coli* aerobactin synthetases. Infection & Immunity. **65:**133-143.

54. **Hove, B. V., H. Staudenmaier, and V. Braun.** 1990. Novel two component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* -K12. Journal of Bacteriology. **172:**6749-6758.

55. **Huebers, H., E. Huebers, W. Rummel, and R. R. Chrichton.** 1976. Isolation and characterization of iron-binding proteins from rat intestinal mucosa. European Journal of Biochcemistry. **66:**447-455.

56. **Iancu, T. C., and H. B. Neustein.** 1977. Ferritin in human liver cells of homozygous beta-thalassaemia: ultrastructural observations. British Journal of Haemotolgy. **37:**527-535.

57. **Irwin, S. W., N. Averil, C. Y. Cheng, and A. B. Schryvers.** 1993. Preparations and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB* from *Neisseria meningitidis*. Molecular Microbiology. **8:**1125-1134.

58. **Jacobs, A., and M. Worwood.** 1975. Ferritin in serum. Clinical and biochemical implications. New England Journal of Medicine. **292:**951-956.

59. **Jalal, M., D. Hossain, J. van der Helm, J. Sanders-Loerh, L.A. Actis, and J.H. Crosa.** 1989. Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. Journal of the American Chemimal Society **111**:292-296.

60. **Klug, C. S., W. Su, J. Liu, P. E. Klebba, and J. B. Feix.** 1995. Denaturant unfolding of the ferric enterobactin receptor and ligand-induced stabilization studied by site-directed spin labeling. Biochemistry. **34:**14230-6.

61. **Koster M., J. van de Vossenberg, J. Leong, and P. J. Weisbeek.** 1993 Identification and characterization of the *pupB* gene encoding an inducible ferric-pseudobactin receptor of *Pseudomonas putida* WCS358. Molecular Microbiology. **8**:591-601.

62. **Koster. M., W. van Klompenburg, W. Bitter, J. Leong, and P. J. Weisbeek.** 1994. Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. EMBO Journal. **13**:2805-13.

63. **Lee, B. C.** 1995. Quelling the red menace: heme capture by bacteria. Molecular Microbiology. **18:**383-390.

64. **Leoni, L., A. Ciervo, N. Orsi, and P. Visca.** 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. Journal of Bacteriology. **178:**2299-313.

65. **Litwin, C. M., and S. B. Calderwood.** 1994. Analysis of the complexity of gene regulation by *fur* in *Vibrio cholerae*. Journal of Bacteriology. **176:**240-8.

66. **Litwin, C. M., and S. B. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of *fur* mutant by *in vivo* marker exchange. Journal of Bacteriology:706-715.

67. **Lucier, T. S., F. J. D. Fetherston, R. R. Brubaker and R. D. Perry.** 1996. Iron uptake and iron-repressible polypeptides in *Yersinia pestis.* Infection & Immunity. **64:**3023-31.

68. **Merriman, T. R., M. E. Merriman, and I. L. Lamont.** 1995. Nucleotide sequence of *pvdD*, a pyoverdine biosynthesis gene from *Pseudomas aeruginosa*: *pvdD* has similarity to peptide synthetases. Journal of Bacteriology. **177:**252-258.

66. **Mietzner T. A., and S. A. Morse.** 1994. The role of iron-binding proteins in the survival of pathogenic bacteria. Annual Review of Nutrition. **14:**471-93.

70. **Miyazaki. H., H. Kato, T. Nakazawa, and M. Tsuda.** 1995. A positive regulatory gene, *pvdS*, for expression of pyoverdin biosynthetic genes in *Pseudomonas aeruginosa* PAO. Molecular & General Genetics. 248:17-24.

71. **Nathan, C.** 1992. Nitric oxide as a secretory product of mammalian cells. J. FASEB. **6:**3051-3064.

72. **Nau, C. D., and J. Knonisky.** 1989. Evolutionary relationships between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequiences of the *Escherichia coli* colicin I receptor gene. Journal of Bacteriology. **171:**1041-1047.

73. **Neilands, J. B.** 1995. Siderophores: structure and function of microbial iron transport compounds. [Review] [43 refs]. Journal of Biological Chemistry. **270:**26723-6.

74. **Neilands, J. B., R. C. Hider, A. Chimiak, A. Liu, Y. Sugiura, and N. Nomoto.** 1984. Structure and Bonding, vol. 58. Springer-Verlag, Berlin Heilelber, New York, Tokyo.

75. **Niederhoffer, E. C., C. M. Naranjo , K. L. Bradley, and J. A. Fee.** 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. Journal of Bacteriology. **172:**1930-8.

76. **Ochsner U. A., A. I. Vasil , and M. L.Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. Journal of Bacteriology. **177:**7194-201.

77. **Ochsner U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proceedings of the National Academy of Sciences of the United States of America. **93:**4409-14.

78. **Ochsner U. A., Z. Johnson, I. L. Lamont, H. E. Cunliffe, and M. L. Vasil.** 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron regulated *pvdS* gene encoding an alternative sigma factor. Molecular Microbiology. **21:**1019-1028.

79. **Payne, S. M.** 1988. Iron and viulence in *Shigella*. Molecular Microbiology. **3:**1301-1306.

80. **Perry, R. D., and R. R. Bruebaker.** 1979. Iron accumulation by *Yersiniae*. Journal of Bacteriology. **137:**1290-1298.

81. **Perry, R. D., T. S. Lucier, D. J. Sikkema, and R. R. Brubaker.** Storage reservoirs of hemin and inorganic iron in *Yersinia pestis*. 1993. Infection and Immunity. **61:**32-39.

82. **Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. Jusef.** 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infection and Imunity. **60:**3872-3877.

83. **Poole, K., Q. Zhao, S. Neshat, D. E. Heinrichs, and C. R. Dean.** 1996. The *Pseudomonas aeruginosa tonB* gene encodes a novel TonB protein. Microbiology. **142:**1449-58.

84. **Poole, K., and V. Braun.** 1988. Iron regulation of *Seratia marcescens* hemolysin gene expression. Infection and Immunity. **56:**2967-2971.

85. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernandez, S. Kim, M. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. Journal of Bacteriology. **178:**5487-92.

86. **Postle, K.** 1993. Ton B protein and energy transduction between membranes. Journal of Bioenergetics and Biomembranes. **25:**5591-5610.

87. **Pressler, U., H. Staudenmaier, L. Zimmermann, and V. Braun.** 1988. Genetics of the iron dicitrate stransport system of *Escherchia coli* K-12. Journal of Bacteriology. **170:**2716-2724.

88. **Rombel, I. T., B. J. McMorran, and I. l. Lamont.**1995. Identification of a DNA sequence motif required for expression of iron-regulated genes in Pseudomonads. Molecular & General Genetics. **246:**519-28.

89. **Rusnak, F., W. S. Faraci, and C. Walsh.** 1989. Subcloning, expression, and purification of the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP

ligase: demonstration of enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. Biochemistry. **28:**6827-35.

90. **Rusnak, F., J. Liu, N. Quinn, G. Berchtold, and C. Walsh.** 1990. Subcloning of the enterobactin biosynthetic gene *entB*: expression, purification, characterization, and substrate specificity of isochorismatase. Biochemistry. **29:**1425-35.

91. **Rusnak, F., M. Sakaitani, D. Drueckhammer, J. Reichert, and C T.Walsh** 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry. **30:**2916-27.

92. **Saito, T., D. Duly, and R. P. J. Williams.** 1991. The histidines of the iron-uptake regulation protein, Fur. European Journal of Biochemistry. **197:**39-42.

93. **Saito, T., and R. P. J. Williams.** 1991. The binding of the ferric uptake regulation protein to a DNA fragment. European Journal of Biochemistry. **197**:43-47.

94. **Saito, T., M. R. Wormald, and R. P. J. Williams.** 1991. Some structural features of the iron uptake regulation protein. European Journal of Biochemistry. **197:**29-38.

95. **Salinas, P., L. Waldbeser, and J. H. Crosa.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as repressor. Gene. **123:**33-38.

96. **Sexton, R., P. R Gill, M. J. Callanan D. J. O'Sullivan, D. N. Dowling, and F. O'Gara.** 1995. Iron-responsive gene expression in *Pseudomonas fluorescens* M114: cloning and characterization of a transcription-activating factor, PbrA. Molecular Microbiology. **15:**297-306.

97. **Silver, S., and M. Walderhaug.** 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. [Review]. Microbiological Reviews. **56:**195-228.

98. **Skikne, B. S., P. Whittaker, A. Cooke, and J. D. Cook.** 1995. Ferritin excretion and iron balance in humans. British Journal of Haemotology. **90:**681-687.

99. **Stachelhaus, T., and M. Marahiel.** 1995. Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide sythesis. FEMS Microbiology Letters. **125:**3-14.

100. **Staggs T. M., J. D. Fetherston, and R. D. Perry.** 1994. Pleiotropic effects of a *Yersinia pestis* fur mutation. Journal of Bacteriology. **176:**7614-24.

101. **Staggs T. M., and R. D. Perry.** 1992. Fur regulation in *Yersinia* species. Molecular Microbiology. **6**:2507-16.

102. **Staggs, T. M., and R. D. Perry.** 1991. Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. Journal of Bacteriology. **173:**417-425.

103. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infection and Immunity. **56:**2891-2895.

104. **Stojiljkovic, I., V. L. Hwa, D. S. Martin, P.O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Molecular Microbiology. **15:**531-541.

105. **Stojiljkovic, I., and N. Srinivason.** 1997. *Neisseria meningitidis tonB*, *exbB*, and *exbD* genes: Ton-dependent utilization of protien bound iron in *Neisseriae*. Journal of Bacteriology. **179**:805-812.

106. **Theil, E. C., and P. Aisen.** 1987. , p. 491-520. *In* Iron Transport in Microbes Plants and Animals. G. Winklelmann and D. V. d. Helm and J. B. Neilands (ed.), VCH, Weinheim.

107. **Thomas, C. E., and P. F. Sparling.** 1996. Isolation and analysis of a *fur* mutant of *Neisseria gonorrhoeae*. Journal of Bacteriology. **178:**4224-32.

108. **Tipton, I. H., and M. J. Cook.** 1963. Health Phys. **9:**103-143.

109. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyperproduction of anguibactin. Infection and Immunity. **61:**3228-3233.

110. **Tolmasky, M. E., L. A. Actis, A. Toranzo, J. Barja, and J. H. Crosa.** 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. Journal of General Microbiology. **131:**1989-1997.

111. **Tolmasky, M. E., and J. H. Crosa.** 1991. Regulation of plasmid-mediated iron transport and virulence in *Vibrio anguillarum*. Biol. Metals. **4:**33-35.

112. **Tolmasky, M. E., A. M. Wertheimer, L. A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* gene: role in regulation of expression of the FatA outer membrane protein and catechols. Journal of Bacteriology. **176:**213-220.

113. **Visca, P., A. Ciervo, and N. Orsi.** 1994 Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthesis enzyme L-ornitine N^5-oxygenase in *Pseudomonas aeruginosa*. Journal of Bactreiology. **176**:1128-1140.

114. **Wee, S., J. B. Neilands, M. L. Bittner, B. C. Hemming, B. L. Haymore, and R. Seetharam.** 1988. Expression, isolation, and properties of Fur (ferric uptake regulation) protein of *Escherichia coli* K-12. Biol. Metals. **1:**62-68.

115. **Welch, S. (ed.).** 1992. The chemistry and biology of iron. CRC, Boca Raton, FL.

116. **Wertheimer, A. M., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1994. Structural and Functional Analyses of Mutant Fur Proteins with Impaired Regulatory Function. Journal of Bacteriology. **176:**5116-5122

117. **Williams, R. G., M. R. Wormald, R. C. Hider, and J. Neilands.** 1989. Spectroscopic properties of the FUR (Ferric Uptake Regulation) protein of *Escherichia coli* K-12., p. 189-196. *In* D. Hamer and D. Winge (ed.), Metal ion homeostasis: molecular biology and chemistry. Alan Liss Inc., New York.

118. **Wolf, M. K., and J. H. Crosa.** 1986. Evidence for the role of a siderophore in promoting *Vibrio anguillarum* infections. Journal of General Microbiology. **132:**2949-52.

119. **Wooldridge, K. G., P. H. Williams, and J. M. Ketley.** 1994. Iron-responsive genetic regulation in *Campylobacter jejuni*: cloning and characterization of a Fur homolog. Journal of Bacteriology. **176:**5852-6.

120. **Wooldridge, K. G., and P. H. Williams.** 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiology Reviews. **12:** 325-348.

121. **Zenke, M., P. Steinlein, E. Wagner, M. Cotten, H. Beug, and M. L. Birnstiel.** Receptor mediated endocytosis of transferrin polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. Proceedings of the National Acadamy of Science U.S.A. **87:**3655-3659.

122. **Zimmermann, L., K. Hantke, and V. Braun.** 1984. Exogenous induction of the iron dicitrate transport system of *Eschericia coli* K-12. Journal of Bacteriology. **159:**271-277.

CHAPTER 1

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Characterization of the *Vibrio anguillarum fur* Gene: Role in Regulation of Expression of the FatA Outer Membrane Protein and Catechols.

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ABSTRACT

The chromosomally-encoded *Vibrio anguillarum fur* gene was characterized. The amino acid sequence of the Fur protein showed a very high degree of homology with those of *V. cholerae* and *V. vulnificus.* The degree of homology was lower, although still high, with the *Escherichia coli* and *Yersinia pestis* Fur amino acid sequences, while the lowest degree of homology was found with the *Pseudomonas aeruginosa* Fur protein. The C-terminal portion of Fur is the least conserved region among these Fur proteins. Within this portion, two regions, spanning amino acids 105 to 121, and 132 to the end, are the least conserved. A certain degree of variation is also present in the N-termini spanning amino acids 28 to 46. Regulation of expression of the *V. anguillarum fur* gene by iron was not detected by immunoblot analysis. Mutations in the cloned *fur* gene were generated either by site-directed mutagenesis (the Lys_{77} was changed to a Gly to generate the derivative FurG77) or by insertion of a DNA fragment harboring the *aph* gene in the same position. FurG77 was impaired in its ability to regulate a reporter gene with the Fur-box in its promoter, while the insertion mutant was completely inactive. *V. anguillarum fur* mutants were obtained by isolating manganese-resistant derivatives. In one of these mutants, which encoded a Fur protein with an apparent lower molecular weight, the regulation of the production of catechols and synthesis of the outer membrane protein FatA were partially lost. In the case of another mutant, no protein was detected by anti-Fur serum. This derivative showed a total lack of regulation of biosynthesis of catechols and FatA protein by iron.

INTRODUCTION

The ability of a bacterial pathogen to scavenge iron from its host's fluids is an important factor in virulence (34, 60). Most bacteria possess efficient iron uptake systems that are expressed when the bacterial cell enters a mammalian host, which is a low-iron environment, allowing it to capture iron from the high affinity host's iron binding proteins. In *Escherichia coli*, the iron regulation of the iron-uptake genes expression depends on a single regulatory gene, *fur* (*f*erric *u*ptake *r*egulator), which acts as a classical repressor, blocking transcription in the presence of high concentrations of iron (5, 28, 44). The C-terminal portion of the *E. coli* Fur protein binds Fe^{2+}, inducing a conformational change in the N-terminal region of the protein which allows the binding to the operator of Fur-regulated genes (13). This operator consists of a 21-bp dyad symmetric consensus sequence (10, 11, 20-22). Fur was subsequently found in other bacterial species: *Salmonella typhimurium* (25), *Yersinia pestis* (45), *Vibrio cholerae* (33), *V. vulnificus* (35), and *Pseudomonas aeruginosa* (37). It was demonstrated that Fur also controls the expression of toxins and other virulence factors apparently unrelated to iron metabolism, e.g., hemolysin in *V. cholerae* (46), Shiga-like toxin of *E. coli* (10), and pH-regulated proteins in *S. typhimurium* (25).

V. anguillarum is the causative agent of the fish disease vibriosis (40). Some virulent strains carry a virulence plasmid, such as pJM1 in strain 775 (14-16, 53), which encodes a very efficient iron-uptake system composed of anguibactin, a siderophore that has hydroxamate and catechol moieties in its molecule (1, 30), and membrane components that play a role in internalization of iron(III)-anguibactin complexes inside the cell (3, 32). This is one of the first systems in which a perfect correlation of virulence and iron uptake was demonstrated (14, 19, 52, 61). The expression of many genes of this system was shown to be regulated by the concentration of iron in the medium (2, 17, 49). Studies of the regulation of expression of genes encoding different components of this iron uptake system

demonstrated that, in the *V. anguillarum* pJM1-encoded system, plasmid-encoded factors such as the AngR protein, the *trans*-acting factor(s) (TAF) and an antisense RNA, RNAα, play roles in regulation (41, 42, 49, 52, 55). However, we recently demonstrated the presence of a chromosomally-encoded Fur-like activity in *V. anguillarum* (58) and identified the presence of a putative Fur binding site within the promoter region of the gene encoding the bifunctional protein AngR (24), a protein that plays a role as a regulator and can also complement an *entE* mutant of *E.coli* (50). These findings suggested a role for Fur in the pJM1-mediated iron uptake system. Hence, we conducted this study on the *V. anguillarum fur* gene and present in this report its characterization and nucleotide sequence, as well as evidence of Fur-regulation of expression of the outer membrane protein FatA and synthesis of catechols, which are presumed to be intermediates in the biosynthesis of anguibactin. In addition, the isolation and characterization of *V. anguillarum fur* mutants by the manganese resistance selection method (28) are described.

(Part of this research was presented at the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga. 16-20 May 1993.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The genotype and source of strains and plasmids used in this study are shown in Table 1. *E. coli* HB101 or JM107 was used as the bacterial hosts. Plasmid pBCSK+ (Stratagene, La Jolla, CA) was used as vector for DNA sequencing and site-directed mutagenesis. Plasmid pT7-5 was used for overexpression of Fur with *E. coli* BL21(DE3)(pLysE) as the host. The uses of other plasmids and strains are described below. *V. anguillarum* was grown in either trypticase soy broth or agar supplemented with 1% NaCl or M9 minimal medium containing either 50 μM $FeCl_3$ (iron-rich) or 2.5 μM ethylenediamine-di-(*o*-hydroxyphenyl) acetic acid (EDDHA [iron-limiting]).

Chemical and enzymatic determinations. Levels of 2,3-dihydroxybenzoic acid (DHBA) in culture supernatants were determined with the Arnow phenolic assay (4). β-galactosidase levels were measured by the method described by Putnam and Koch (38). Units were determined as described by Miller (36). All experiments to determine 2,3-DHBA and β-galactosidase levels we performed at least four times. The ratios between the values among the different strains were constant. Protein concentrations were determined as described by Bradford (9).

General DNA procedures. Plasmid DNA was purified according to the method of Birnboim and Doly (7) with the modifications described by Weickert and Chambliss (59). Transformations were performed by the method of Cohen et al. (12). Sequencing of double stranded DNA was performed by the dideoxy chain-termination method (43) with the Sequenase kit (US Biochemical, Cleveland, OH) with the T7 and T3, and, in some cases, with specific synthetic primers. Site directed mutagenesis of pTAW1.8 was carried out with the Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio Rad Laboratories,

Richmond, CA) and the synthetic mutagenic oligonucleotide AAGGTGGAGGATCCGTTT, following the recommendations of the manufacturer with modifications previously described (26), to generate plasmid pMETAW3.12. This mutation was confirmed by the fast DNA sequencing method (51) using the appropriate primers. Basically, the method consisted of electrophoresis of the sequencing reaction mixtures obtained as described above (43) in a small gel 18 cm long run at 1,000 V for 20 minutes. Plasmid pMETAW4.1 was generated by insertion mutagenesis performed by ligating the kanamycin resistance (Km^r) fragment of pUC4K into the *Bam*HI site generated after site-directed mutagenesis. Hybridization experiments were carried out at 37°C under low-stringency conditions (0.75 M sodium chloride, 0.075 M sodium citrate, Denhardt's solution, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 25% formamide). After hybridization, the filters were washed at 50°C in a solution containing 0.3 M sodium chloride, 0.03 M sodium citrate, and 0.1% SDS.

Immunoblot Analysis. Antiserum against Fur was raised in 6-month old rabbits as previously described for FatA (2) by using purified *E. coli* Fur protein (a gift from J. Neilands, University of California, Berkeley). Antiserum against FatA was prepared as described before (2). Proteins from cytosol, total cell extracts or outer membrane preparations were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Crosa and Hodges (17), with prestained high-range protein molecular weight standards, (Bio-Rad, Richmond, CA). After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper (0.2 µm pore size, BAS 83, reinforced NC, [Schleicher & Schuell]) essentially as described by Towbin et al. (56) using the Genie electrophoretic blotter (Idea Scientific Company, Minneapolis, MN) under the conditions recommended by the supplier. The blots were incubated in the presence of the anti-Fur or anti-FatA serum and developed by reaction with peroxidase and staining with H_2O_2 and horseradish peroxidase color development reagent (2).

Isolation of Fur mutants by manganese selection. Isolation of Fur mutants in the presence of manganese was carried out essentially as described by Hantke (28) for *E. coli*, *Serratia* spp., and *Klebsiella* spp. with the modifications of Prince et al. (37) for *Pseudomonas aeruginosa*. *V. anguillarum* 775 was cultured in Trypticase soy broth supplemented with 1% NaCl at 24°C overnight. Aliquots (100 µl) of this culture were spread onto Trypticase soy agar plates supplemented with 1% NaCl and 10 mM $MnCl_2$. After 5 days incubation at 24°C, the Fur proteins in growing colonies (about 5 per plate) were analyzed by immunoblotting.

Overexpression of Fur in *E. coli* BL21(DE3)(pLysE). Both intact and interrupted *fur* genes from recombinant clones pTAW1.8 and pTAW4.1, respectively were cloned under the control of the ϕ10 promoter using the vector pT7-5 (48). Ligation of *Xba* I- and *Eco*RI- digested pT7-5 with the *Xba* I-*Eco*RI fragment containing the *fur* gene in pTAW1.8 generated pTAW2.1, and ligation with the *Xba* I-*Eco*RI fragment containing the truncated *fur* gene in pTAW4.1 generated pMET31. Recombinant clones pTAW2.1 and pMET31 were transformed into *E. coli* BL21(DE3)(pLysE). Cells were grown in Luria broth containing ampicillin at 37°C until the optical density at 600 nm reached 0.7. Protein expression was then induced by addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were incubated for an additional three hours and collected by centrifugation. After washing with one volume of 10 mM magnesium sulfate the cells were lysed with SDS-PAGE sample buffer at 100°C for 10 min. and analyzed by SDS-PAGE.

Nucleotide sequence accession number. The nucleotide and predicted amino acid sequences of the *V.anguillarum fur* gene will appear in the EMBL and Gen Bank sequence libraries under accession no. L19717.

RESULTS

Cloning of the *V. anguillarum fur* gene. A recombinant clone was isolated from the plasmidless *V. anguillarum* H775-3 library (54) by screening pools of recombinant plasmid DNA by Southern blot hybridization under low stringency conditions with a *Hin*dIII-*Bgl*I DNA fragment encompassing most of the *fur E. coli* gene (27) used as a probe. Recombinant plasmids in a hybridizing pool were then screened individually. Recombinant clone pMET67 was isolated in this manner and its biological activity was tested by introduction of this plasmid and the Fur reporter plasmid pSC27.1 (11) into the *fur* mutant *E. coli* BN4020. The activity of β-galactosidase was inhibited in the presence of high concentrations of iron indicating that pMET67 produced a Fur-like activity (Figure 1). The Fur activity elicited by pMET67 was comparable to that of the *E. coli* Fur protein coded for by pMH15 (Figure 1).

The *V. anguillarum fur* gene was subcloned by performing a *Sau*3A I partial restriction endonuclease treatment of pMET67 followed by ligation to *Bam*HI digested pBCSK+. Transformation of the Fur reporter *E. coli* RRJC1 produced a few white colonies (which represented about 1% of the total transformants) when plated on McConkey agar supplemented with 100 μM ferric chloride. One of these white colonies was further analyzed and was shown to carry a recombinant plasmid, pTAW1.8, which had a 1.6 kbp insert.

Nucleotide sequence and expression of the *V. anguillarum fur* gene. The *V. anguillarum fur* gene included in pTAW1.8 was sequenced. The nucleotide sequence (Figure 2) showed homology to the nucleotide sequence of *fur* genes from other bacterial species. Homologies to the *fur* coding sequence of *V. cholerae* (33), *V.*

vulnificus (35), *Y. pestis* (45), *E. coli* (44) and *P. aeruginosa* (37) were 81.6, 80.4, 70.1, 70.0, and 60.9% respectively. An open reading frame of 149 amino acids was present which had high degree of homology with the amino acid sequence of Fur proteins of the other bacteria (Figures 2 and 3), especially*V. cholerae* and *V. vulnificus*. To identify the *V. anguillarum* Fur protein, the pTAW1.8 insert was recloned into the vector pT7-5 to generate pTAW2.1 which was transformed into *E. coli* BL21(DE3)(pLysE). Upon induction with IPTG, a protein with a mass of about 20 kDa was detected which is the approximate mass of the predicted *V. anguillarum* Fur protein (Figure 4). The identity of this band was confirmed by analysis of the protein extract of *E. coli* BL21(DE3)(pLysE)(pLyseE) harboring a derivative consisting of the insertionally mutated *fur* (see below) cloned in pT7-5 (plasmid pMET31). Figure 4 (lane F) shows that the 20 kDa band is indeed Fur since pMET31 did not express this protein. Immunoblot analysis of a gel identical to that in Figure 4 with anti-Fur serum further confirmed that the 20 kDa protein overexpressed by *E. coli* BL21(DE3)(pLysE) (pLysE, pTAW2.1) corresponds to Fur (data not shown).

Regulation of expression of the *V. anguillarum fur* gene.

To determine whether the biosynthesis of the *V. anguillarum* Fur protein was iron-regulated, cells were grown under iron-rich and iron-limiting conditions and the cytosolic extracts were subjected to immunoblotting. Immunoblot analysis (Figure 5) demonstrates that the same amount of Fur protein was expressed in both conditions, indicating that its expression is constitutive in *V. anguillarum*. The production of 2,3-DHBA was used as a control for the expression of iron-regulated products. The optical densities at 510 nm obtained when the Arnow reaction was carried out, as described in Materials and Methods for the cultures under iron-rich and iron-limiting conditions, were 0.030 and 0.353, respectively, showing that 2,3-DHBA production is iron-regulated.

Mutagenesis of the *V. anguillarum fur* gene. The *V. anguillarum fur* gene in pTAW1.8 was mutated by site-directed mutagenesis; nucleotides 229 and 230 were changed from AA to GG (Figure 2) to generate plasmid pTAW3.12. As a result of this mutation, a *Bam*HI site was generated and the amino acid at position 77 was changed from Lys to Gly (this mutant derivative was called $FurG_{77}$) (Figures 2 and 3). Another mutant was generated by inserting the Km^r fragment from pUC4K in the newly generated *Bam*HI site to originate plasmid pTAW4.1. The mutated recombinant clones were transferred to the *E. coli fur* mutant RRJC1 and plated on McConkey agar plates supplemented with 100 μM $FeCl_3$. *E. coli* RRJC1 with no plasmid or with pTAW4.1 produced red colonies indicating that β-galactosidase was being produced. Colonies of *E. coli* RRJC1(pTAW1.8) were white as a result of the inhibition of production of β-galactosidase in the presence of Fur and iron. *E. coli* RRJC1(pTAW3.12) generated pink colonies, indicating that the $FurG_{77}$ encoded by this mutant is leaky. Next, β-galactosidase levels were determined for these strains in iron-rich and iron-limiting conditions (Table 2). The results indicate that the insertion mutant pTAW4.1 is a null mutant while pTAW3.12 encodes a protein, $FurG_{77}$, which has some Fur activity (Table 2).

The plasmid pTAW4.1 was used to attempt to construct a *V. anguillarum fur* mutant by marker exchange. However, as was already described for *P. aeruginosa* (37), we were unable to isolate a *V. anguillarum fur* mutant by this technique. Therefore, we attempted to select *V. anguillarum fur* mutants using the positive selection method first described by Hantke (28). *V. anguillarum* cells were spread onto plates containing 10 mM manganese chloride and the plates were incubated for 5 days at 24°C. The colonies that grew in these conditions were analyzed by immunoblotting to identify proteins that run differently than the wild type Fur in SDS-PAGE. Two types of mutants were obtained, one that showed the presence of a Fur related protein but migrates with an apparently lower molecular mass (*V. anguillarum* 775MET9) and another which showed no reaction against the anti-Fur serum (*V. anguillarum* 775MET11) (Figure 6).

These mutants were used to analyze whether components of *V. anguillarum* iron-uptake system are under the control of Fur. For this purpose, the strains were cultured in iron-rich or iron-limiting minimal medium and the biosynthesis of catechols and the FatA outer membrane protein was analyzed. As shown in Table 3, although in the wild type strain 775 the production of catechols is iron-regulated, in *V. anguillarum* 775MET11 all regulation by the iron concentration in the milieu was lost. A degree of regulation can still be seen in the case of *V. anguillarum* 775MET9. This could indicate that *V. anguillarum* 775MET11 is a null or nearly null mutant while strain 775MET9 is leaky.

The 86-kDa outer membrane protein FatA (2,53) is an important component of the receptor for the ferric anguibactin complexes (2). Expression of the *fatA* gene is highly regulated by the concentration of iron in the medium and at least two regulatory factors have already been identified, TAF and RNAα (42, 49, 58). The results from experiments analyzing regulation of expression of *fatA* using an *E. coli* Fur mutant and a recombinant clone harboring the *E. coli fur* gene were inconclusive (58). Therefore, the *V. anguillarum* manganese-resistant *fur* mutants were used to study regulation of expression of *fatA* by Fur. Wild type *V. anguillarum* and the mutants 775MET9 and 775MET11 were cultured under iron-rich and iron- limiting conditions, and outer membrane proteins were isolated. The presence of FatA was detected by immunoblotting with anti-FatA serum. Figure 7 shows that while no FatA was detected in the wild type strain grown under iron-rich conditions, this protein was present in the outer membrane preparation of both mutants. However, it is also clear from Figure 7 that in the case of mutant 775MET9 there is still a certain degree of regulation while the amounts of FatA detected in mutant 775MET11 grown under iron-rich and iron-limiting conditions are virtually the same. This is likely due to the fact that *V. anguillarum* 775MET9 behaves as a leaky mutant while the mutation in derivative 775MET11 is null or nearly null.

DISCUSSION

The pJM1-mediated iron uptake system of *V. anguillarum* is highly regulated by the concentration of iron in the surrounding medium. Recently, several plasmid-encoded regulators of this system have been identified: TAF (49); the bifunctional protein AngR which besides its function as a regulator can complement an *entE* mutant of *E. coli* (50, 55), and RNAα, an antisense RNA that regulates the expression of FatA and FatB (42, 58). Besides these plasmid-mediated regulators, a chromosomally-encoded protein with a Fur-like activity was also detected (58). Here we report the cloning and sequencing of the gene encoding that protein. The highest degree of homology of the *V. anguillarum* amino acid sequence of the Fur protein was found with the Fur amino acid sequences reported for *V. cholerae* and *V. vulnificus* (33, 35). The degree of homology was lower (although still high) with the *E. coli* and *Y. pestis* Fur amino acid sequences (44, 45). The lowest degree of homology was found with the *P. aeruginosa* Fur protein, which seems to be the most distant from the rest of the Fur amino acid sequences reported so far. The C-terminal portion of the protein seems to be the less conserved region of the Fur proteins. The regions spanning amino acids 105 to 121, and 132 to the end (shaded in Figure 3) are the least conserved within this C-terminal portion which has been described to play a role in the binding of the metal ions (13, 29, 31). A certain degree of heterogeneity is also found in the N-termini spanning amino acids 28 to 46 (shaded in Figure 3).

It has been suggested previously that expression of the *E. coli fur* gene is autoregulated by its product (22); however, no iron regulation of the *fur* genes was detected in *V. cholerae* or *V. vulnificus* (33, 35). Our results obtained by immunoblotting of extracts of *V. anguillarum* cultured under iron-rich or iron-limiting conditions indicate that, in this bacterium, regulation of expression of *fur* by iron either does not exist or is very low. We are presently performing transcription and operon fusion analysis to confirm these results.

Studies of metal ion activation of Fur proteolysis demonstrated that, in the presence of $MnCl_2$ the *E. coli* Fur protein is digested by trypsin between the Lys_{76} and the Ser_{77} suggesting that upon binding the metal ion, the protein undergoes a conformational change that makes this region available to trypsin (13). As a result of this conformational change, the N-terminus of the Fur protein binds the DNA. This region has been postulated as one probable boundary between the DNA-binding N-terminus and the metal ion-binding C-terminus (13) and, with the exception of the *P. aeruginosa* Fur, its amino acid sequence is conserved in all Fur proteins sequenced so far (see Figure 3). We generated a mutant by site-directed mutagenesis in this region (plasmid pTAW3.12). The Lys_{77} (equivalent to the Lys_{76} of the *E. coli* Fur) was changed to a Gly, generating the derivative FurG77. Analysis of this mutant showed that FurG77 was impaired in its ability to regulate a gene that has the Fur-box in its promoter.

Attempts to use pTAW4.1 to generate a *V. anguillarum fur* mutant by marker exchange were unsuccessful. A similar result has been recently reported in the case of the *P. aeruginosa fur* gene (37). A possibility is that a null mutant of Fur is lethal for some bacteria. However, it is puzzling that, by manganese selection, we generated a mutant that seems to be null or nearly null. Explanations could be that this mutant is not null and the activity left is enough to support viability; or that, since it is known that Fur regulates other traits, this mutant lost the ability to regulate some genes but is still partially or totally able to regulate others. It is of interest that a manganese-resistant Fur mutant of *P. aeruginosa* is affected in regulation of biosynthesis of siderophore but is not affected in regulation of production of exotoxin A (37).

Of the manganese-resistant Fur mutants isolated by these techniques, two were further analyzed in this work. One of them, *V. anguillarum* 775MET9, partially lost its ability to regulate the production of catechols as determined by the Arnow reaction. Precursors for biosynthesis of the siderophore anguibactin as well as anguibactin itself are detected by the Arnow reaction. Other factors have also been implicated in regulation of the biosynthesis

of anguibactin (41, 49, 55). Therefore, we cannot yet discriminate the step that is Fur regulated in the synthesis of anguibactin. This mutant,*V. anguillarum* 775MET9, produced a Fur protein with an apparently lower molecular weight, as determined by immunoblotting. In the case of the other mutant, *V. anguillarum* 775MET11, no protein was detected by anti-Fur serum. This could be due to a total loss of Fur production or to the production of a very small Fur derivative or a protein that lost all recognizable epitopes. Since the anti-Fur serum is polyclonal, this last possibility seems improbable. Iron-regulation of biosynthesis of catechols in this mutant was completely lost.

In the case of FatA, a protein that is part of the receptor that recognizes ferric-anguibactin complexes, regulation by iron was impaired in mutant 775MET9 and almost lost in mutant 775MET11, as determined by immunoblot analysis. The presence of higher levels of FatA in the mutants than in the wild type strain, when cultured under iron limiting conditions, may be due to the existence of background levels of Fur in the wild type that can exert some negative regulation even at the low iron concentrations present in those conditions. Regulation of *fatA* by Fur was studied before in an *E. coli* background using a clone of the *E. coli* Fur, with inconclusive results (58). We showed before that expression of *fatA* is under the control of other regulatory factors such as TAF (49) or RNAα (42, 49). The results in this manuscript prove that Fur plays an important role in regulation of *fatA*, adding a new factor to the regulatory circuit of this gene. Experiments are presently being carried out to assess the degree of contribution of TAF, RNAα, and Fur to the regulation of *fatA*.

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LITERATURE CITED

1. **Actis, L. A., W. Fish,J.H. Crosa,K. Kellerman,S. Ellenberger,F. Hauser, and J. Sanders-Loher.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). J. Bacteriol. **167:**57-65.
2. **Actis, L.A., S. Potter, J.H. and Crosa.** 1985. Iron-regulated outer membrane protein OM2 of*Vibrio anguillarum* is encoded by virulence plasmid pJM1. J. Bacteriol. **161:**736-742.
3. **Actis, L. A., M. E. Tolmasky, D. Farrell, and J. H.Crosa.** 1988. Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid-mediated iron-transport system. J. Biol. Chem. **263**:2853-2860.
4. **Arnow, L.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118:**531-537.
5. **Bagg, A., and J. Neilands.** 1985. Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. **161:**450-453..
6. **Bagg, A., and J. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli.* Biochemistry **26:**5471-5477.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7**:1513-1523.
8. **Boyer, H. W., and D. Roulland-Doussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* J. Mol. Biol. **41**:459-472.
9. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**:249-252.

10. **Calderwood, S.B., and J. Mekalanos.** 1987. Iron-regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759-4764.

11. **Calderwood, S.B., and J. Mekalanos.** 1988. Confirmation of the *fur* operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015-1017.

12. **Cohen, S. N., A.C.Y. Chang, and L. Hsu.** 1972. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69:**2110-2114.

13. **Coy, M., and J. Neilands.** 1991. Structural dynamicsand functional domains of the fur protein. Biochemistry **30:**8201-8210.

14. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **284**:566-568.

15. **Crosa, J. H.** 1984. The relationship of plasmid mediated iron transport and bacterial virulence. Annu. Rev. Microbiol. **38**:69-89.

16. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53**:517-530.

17. **Crosa, J. H., and L. Hodges.** 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum* 775. Infect. Immun. **31**:223-227.

18. **Crosa, J.H., L. Hodges, and M. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infect. Immun. **27**:897-902.

19. **Crosa, J.H., M. Schiewe, and S. Falkow.** 1977. Evidence for plasmid contribution to the virulence of the fish pathogen *Vibrio anguillarum*. Infect. Immun. **18**:509-513.

20. **De Grandis, S., J. Ginsberg, M. Toone, S. Climie, J. Friesen, and J. Brunton.** 1987. Nucleotide sequence and promoter mapping of the *Escherichia coli* Shig-like toxin operon of bactriophage H-19B. J. Bacteriol. **169:**4313-4319.

21. **de Lorenzo, V.L., M. Herrero, F. Giovannini, and J.B. Neilands.** 1988. Fur (ferric uptake regulator) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli.* Eur. J.Biochem. **173:**537-546.

22. **de Lorenzo, V.L., S. Wee, M. Herrero, and J.B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid pColV-K30 binding the ferric uptake regulation (*fur*) repressor J. Bacteriol. **169:**2624-2630.

23. **Echenique, J. R., H. Arienti, M. E. Tolmasky, R. R. Read, R. J. Staneloni, J.H. Crosa, and L. A. Actis.** 1992. Characterization of a high-affinity iron transport system in *Acinetobacter baumanii.* J. Bacteriol. **174:**7670-7679.

24. **Farrell, D., P. Mikesell, L. Actis, and J.H. Crosa.** 1990. A regulatory gene, *angR*, of the iron uptake system of *Vibrio anguillarum*: similarity with phage P22 *cro* and regulation by iron. Gene **86**:45-51.

25. **Foster, J.W., and H. Hall.** 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174:**4317-4323.

26. **Gammie, A., M. E. Tolmasky, and J. H. Crosa.** 1993. Functional characterization of a replication initiator protein. J. Bacteriol. **175:**3563-3569.

27. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol. Gen. Genet. **197:**337-341.

28. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12. Mol. Gen. Genet. **210:**135-139.

29. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol. Microbiol. **4:**1621-1628.

30. **Jalal, M., D. Hossain, J. van der Helm, J. Sanders-Loerh, L.A. Actis, and J.H. Crosa.** 1989. Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. J. Amer. Chem. Soc. **111**:292-296.

31. **Klug, A., and D. Rhodes.** 1987. Zinc fingers: a novel protein motif for nucleic acid recognition. Trends biochem. Sci. **12:**464-469.

32. **Koster, W. L., L.A. Actis, L. Waldbeser, L., M.E. Tolmasky, M. E., and J.H. Crosa.** 1991. Molecular characterization of the iron transport system mediated by the pJM1 plasmid in *Vibrio anguillarum* 775. J. Biol. Chem. **266**:23829-23833.

33. **Litwin, C., S., and S. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clinical Microbiol. Rev. **6**:137-149.

34. **Litwin, C., S., and S. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by *in vivo* marker exchange. J. Bacteriol. 175:706-715.

35. **Litwin, C., S. Boyko, and S. Calderwood.** 1992. Cloning, sequencing and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897-1903.

36. **Miller, J.** 1972. Assay of β-galactosidase. p. 352-355. *In* J.H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold spring Harbor, N.Y.

37. **Prince, R., C. Cox, and M. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589-2598.

38. **Putnam, S., and A. Koch.** 1975. Complications in the simplest cellular enzyme assay: lysis of *Escherichia coli* for the assay of β-galactosidase. Anal. Biochem. 63:350-360.

39. **Read, R., and J. H. Crosa.** Unpublished data.

40. **Rucker, R., B.J. Earp, and E.J. Ordal.** 1953. Infectious diseases of Pacific salmon. Trans. Am. Fish. Soc. **83**:297-312.

41. **Salinas, P., M.E. Tolmasky, and J.H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: evidence for a cooperative affect between two transcriptional activators. Proc. Natl. Acad. Sci. U. S. A. **86:**3529-3533.

42. **Salinas, P., L. Waldebesser, and J.H. Crosa.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as repressor. Gene **123:**33-38.

43. **Sanger, F., S. Nicklen, and A. Coulsen.** 1979. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463-5467.

44. **Schaffer, S., K. Hantke, and V. Braun.** 1985. Nucleotide sequence of the iron regulatory gene *fur*. Mol. Gen. Genet. **200**:110-113.

45. **Staggs, T., and R. Perry.** 1991. Identification and cloning of a *fur* regulatory gene in Yersinia pestis. J. Bacteriol. **173:**417-425.

46. **Stoebner, J.A., and S. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891-2895.

47. **Studier, F., A. Rosenberg, J. Dunn, and J., Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. **185:**60-89.

48. **Tabor, S., and C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. U.S.A. **82:**1074-1078.

49. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1988. Genetic analysis of the iron uptake region of the *Vibrio anguillarum* plasmid pJM1: molecular cloning of genetic determinants encoding a novel *trans* activator of siderophore biosynthesis. J. Bacteriol. **170**:1913-1919.

50. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids results in hyperproduction of anguibactin. Infect. Immun. **61:**3228-3233.

51. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. Virulence plasmids. *In* (Rickwood, D., and Hame, B., eds.), Plasmids: a practical approach. IRL Press, Oxford University Press, Oxford, in press.

52. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. J. Bacteriol. **160**:860-866.

53. **Tolmasky, M. E., and J. H. Crosa.** 1991. Regulation of plasmid-mediated iron transport and virulence in *Vibrio anguillarum.* Biol. Metals **4**:33-35.

54. **Tolmasky, M. E., A. Gammie, and J. H. Crosa.** 1992. Characterization of the *recA* gene of*Vibrio anguillarum.* Gene **110:**41-48.

55. **Tolmasky, P. Salinas, L. A. Actis, and J. H. Crosa.** 1988b. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum.* Infect. Immun. **56**:1608-1614.

56. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gelsm to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76:**4350-4354.

57. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259-268.

58. **Waldbeser, L., M.E., Tolmasky, L.A. Actis, and J.H. Crosa.** 1993. Mechanisms for negative regulation by iron of the*fatA* outer membrane protein gene expression in *Vibrio anguillarum* 775. J. Biol. Chem. **268:**10433-10439.

59. **Weickert, M., and G. Chambliss.** 1989. Acid-phenol minipreps make excellent sequencing templates. USB Editorial Comments **16:**5-6.

60. **Weinberg, C.** 1978. Iron and infection. Microbiol. Rev. **42:**45-66.

61. **Wolf, M., and J. H. Crosa.** 1986. Evidence for the role of a siderophore in promoting *Vibrio anguillarum* infections. J. Gen. Microbiol. 132:2949-2952.

62. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13phage cloning vectorsand host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype	Source or reference
Bacterial strains:		
E. coli HB101	*rec13ara-14 proA2, rpsL20, xyl-5, hsdS20, lacY1, galK2, mtl-l, supE44, (rB-,mB-))*	8
JM107	*thi*Δ*(lac-proAB), gyrA96, endA1, hsdR17, relA1, supE44,* F'[*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	62
BL21(DE3)(pLysE)	*hsdS gal* (λc Its 857 *ind*1 *Sam* 7 *nin*5 lac UV5-T7 gene1)(Studier and Moffatt 1986)	Novagen(47)
BN4020	*fur*::Tn5	5
RRJC1	derivative of *E. coli* BN4020 (*fur*$^-$)with a *lacZ* reporter under the control of the*huF* Fur box	39
V. anguillarum 775	natural isolate, prototype, (pJM1)	14
H775-3	plasmidless derivative of 775	18
775MET9	*fur* mutant isolated in the presence of 10 mM $MnCl_2$	This work
775MET11	*fur* mutant isolated in the presence of 10 mM $MnCl_2$	This work
Plasmids:		
pBCSK+	cloning vector	Stratagene, La Jolla, CA
pT7-5	expression vector	48
pUC4K	contains the Tn*903 aph* in a restriction site mobilizing element	57
pSC27.1	Fur reporter gene. β-galactosidase is under the control of Fur	11
pMH15	*E. coli fur* gene cloned in pACYC184	27
pTAW1.8	*fur V. anguillarum* gene cloned in	This work
pTAW3.12	Site-directed mutagenized *fur* mutant derivative of pTAW1.8. Nucleotides 229 and 230 were changed from AA to GG	This work
pTAW4.1	Km resistance fragment from pUC4K inserted in the *Bam*HI site in pTAW3.12	This work
pTAW2.1	*V. anguillarum fur* gene cloned in pT7-5	This work
pMET31	Insertionally mutated fur from pTAW4.1 cloned in pT7-5	This work

Table 2. β-galactosidase activities

Plasmid in *E. coli* RRJC1	β-galactosidase activity[a]		Ratio iron-limiting / iron-rich
	iron-rich	iron-limiting	
None	1,762	1,486	0.84
pTAW1.8	155	1,304	8.41
pTAW3.12	352	1,679	4.76
pTAW4.1	2,081	2,243	1.07

[a] In Miller units (36).

Table 3. Production of catechols by *V. anguillarum* strains.

V. anguillarum strain	Catechols (OD_{510})[a]	
	iron-rich	iron-limiting
775	0.019	0.260
775MET9	0.118	0.323
775MET11	0.458	0.471

[a]Catechols were determined by the method of Arnow (4) on overnight cultures as described before (23).

Figure 1: β-galactosidase activities for *E.coli* derivatives. β-galactosidase activities, expressed as Miller units (36), were determined for *E. coli* BN4020 harboring: pSC27.1 (empty square) ; pSC27.1 and pMH15 (solid diamond); pSC27.1 and pMET67 (solid square) grown in minimal medium with the addition of 1 μM EDDHA and the $FeCl_3$ concentrations indicated.

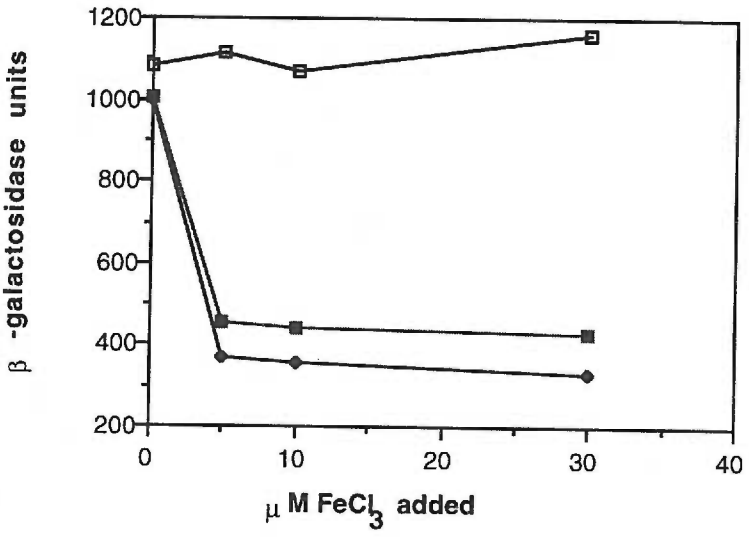

1200
1000
800
600
400
200
0
10
20
30
40
β -galactosidase units
μ M FeCl3 added

Figure 2: Nucleotide and predicted amino acid sequences of the *V. anguillarum fur*. This sequence will appear in the EMBL/GenBank nucleotide sequence libraries under the accession number L19717.

```
                10                  30                  50
                .                   .                   .
  1 ATGTCAGATAATAACCAAGCGCTCAAGGATGCAGGTCTTAAAGTTACCCTTCCTAGGCTA   60
  1 M  S  D  N  N  Q  A  L  K  D  A  G  L  K  V  T  L  P  R  L    20

                70                  90                  110
                .                   .                   .
 61 AAAATTTTAGAAGTGCTACAGCAGCCTGAATGCCAACATATCAGCGCTGAAGAACTGTAT  120
 21 K  I  L  E  V  L  Q  Q  P  E  C  Q  H  I  S  A  E  E  L  Y    40

                130                 150                 170
                .                   .                   .
121 AAGAAATTGATTGATCTTGGTGAAGAAATCGGTCTTGCGACTGTTTATCGAGTATTAAAC  180
 41 K  K  L  I  D  L  G  E  E  I  G  L  A  T  V  Y  R  V  L  N    60

                190                 210                 230
                .                   .                   .
181 CAATTTGATGATGCGGGTATTGTCACTCGTCACCATTTTGAAGGTGGAAAATCCGTTTTT  240
 61 Q  F  D  D  A  G  I  V  T  R  H  H  F  E  G  G  K  S  V  F    80

                250                 270                 290
                .                   .                   .
241 GAACTTTCAACACAACACCACCACGACCACTTAGTGTGCTTAGATTGTGGTGAAGTGATT  300
 81 E  L  S  T  Q  H  H  H  D  H  L  V  C  L  D  C  G  E  V  I   100

                310                 330                 350
                .                   .                   .
301 GAGTTTTCAGATGAGGTGATAGAACAACGCCAAAGAGAGATTGCCGAGCAATATAATGTA  360
101 E  F  S  D  E  V  I  E  Q  R  Q  R  E  I  A  E  Q  Y  N  V   120

                370                 390                 410
                .                   .                   .
361 CAGCTCACCAATCACAGCCTTTATCTATATGGTAAATGTGCCGACGGCAGTTGCAAGCAG  420
121 Q  L  T  N  H  S  L  Y  L  Y  G  K  C  A  D  G  S  C  K  Q   140

                430                 450
                .                   .
421 AACCCTAACGCGCACAAGTCAAAAAGATAG  450
141 N  P  N  A  H  K  S  K  R  *    149
```

Figure 3: Alignment of deduced amino acid sequences of Fur proteins from a variety of bacterial species. The *V. anguillarum* (V.a) Fur protein amino acid sequence (top) is compared with the Fur proteins from *V. cholerae* (V. c.), *V. vulnificus* (V. v.), *E. coli* (E. c.), *Y. pestis* (Y. P.), and *P. aeruginosa* (P.a.). Differences are typed and blank spaces represent identity with the *V. anguillarum* Fur sequence. Solid diamonds indicate spacing changes to maximize alignment. The percentages of identity and similarity, respectively, of the *V. anguillarum* Fur sequence with the other sequences are as follows: *V. cholerae*, 98% and 94%; *V. vulnificus*, 97% and 92%; *E. coli*, 88% and 76%; *Y. pestis*, 84% and 76%; *P. aeruginosa*, 76% and 56%, respectively. Dots show conserved amino acids substitutions; one or two dots indicate degree of similarity. Shaded areas indicate regions with the least amino acid sequence conservation. The **G** represents the position of the FurG77 site-specific mutation.

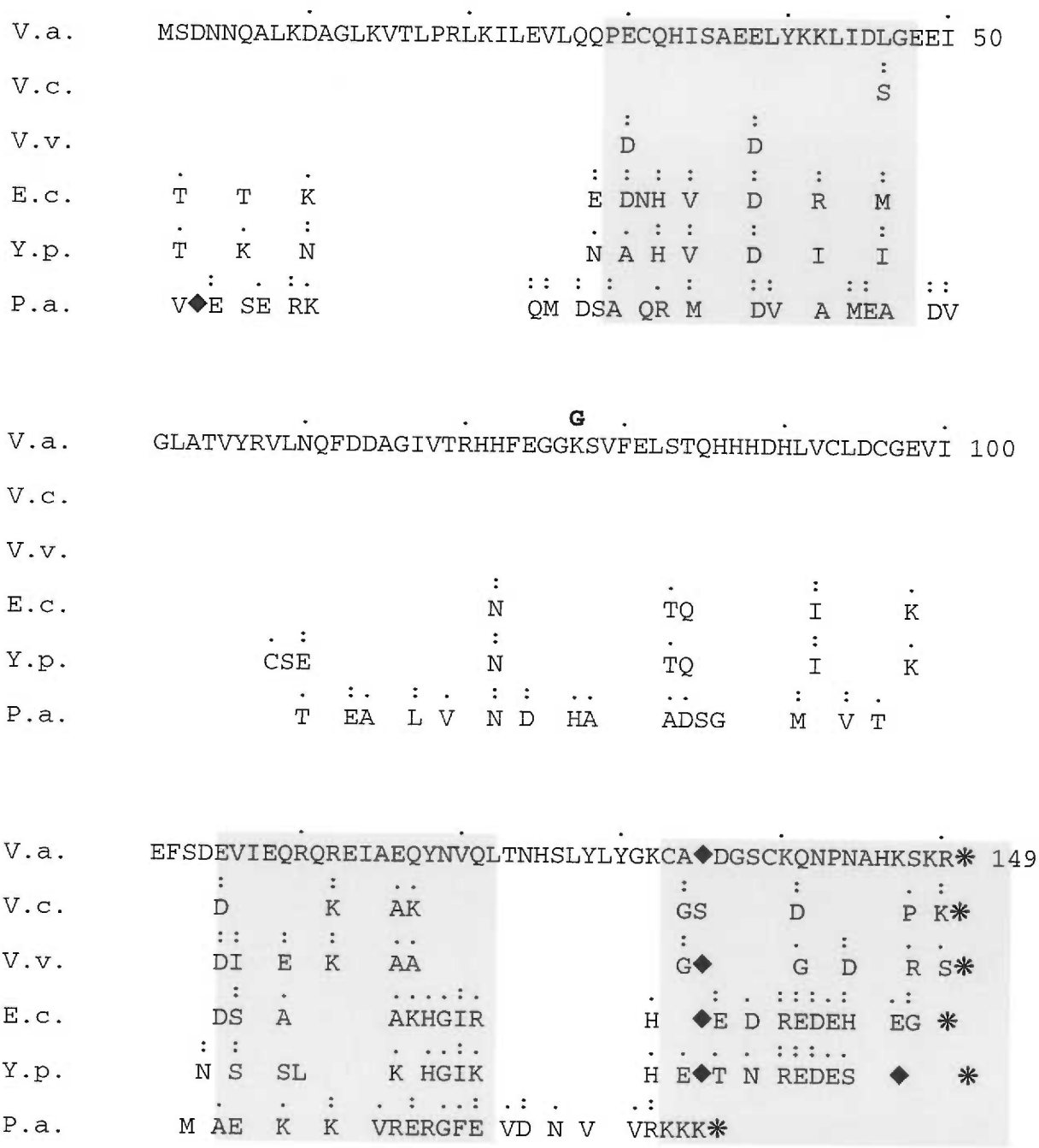

V.a. MSDNNQALKDAGLKVTLPRLKILEVLQQPECQHISAEELYKKLIDLGEEI 50
V.c.
V.v.
E.c.
Y.p.
P.a.
G
V.a. GLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVI 100
V.c.
V.v.
E.c.
Y.p.
P.a.
V.a. EFSDEVIEQRQREIAEQYNVQLTNHSLYLYGKCA◆DGSCKQNPNAHKSKR* 149
V.c.
V.v.
E.c.
Y.p.
P.a.

Figure 4: Expression of Fur in *E. coli* BL21(DE3)(pLysE). The wild type and mutated *fur* genes were subcloned in pT7-5 and transformed in *E. coli* BL21(DE3)(pLysE). Proteins were analyzed by SDS-PAGE and immunobloting as described in Materials and Methods. *E. coli* BL21(DE3)(pLysE) with: A and B, pT7-5; C and D, pTAW2.1 (wild type *fur*); E and F, pMET31 (*fur* mutated by insertion). A, C, and E non induced. B, D, and F, induced with 0.4 mM IPTG. The arrow indicates the band corresponding to Fur.

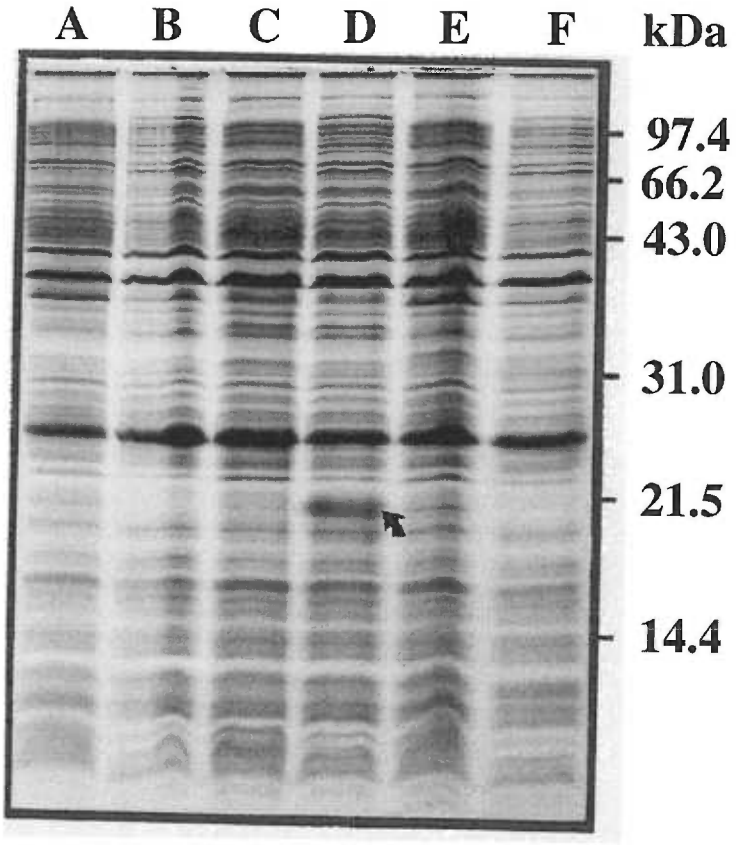

A
B
C
D
E
F
kDa
97.4
66.2
43.0
31.0
21.5
14.4

Figure 5: Regulation of expression of the *V. anguillarum fur* gene. Immunoblot analysis of the cytosolic extracts of wild type *V. anguillarum* grown in minimal medium under iron-rich (lane A) or iron-limiting conditions (lane B) as described in Materials and Methods, using anti-Fur serum. Total extracts (50 μg of protein) obtained by sonication, ultracentrifuged at 100,000 x g for 2 h and the cytosolic soluble protein were subjected to SDS-PAGE and immunoblotting, using anti-Fur serum, as described in Materials and Methods. Molecular masses (in kDa) are shown to the right.

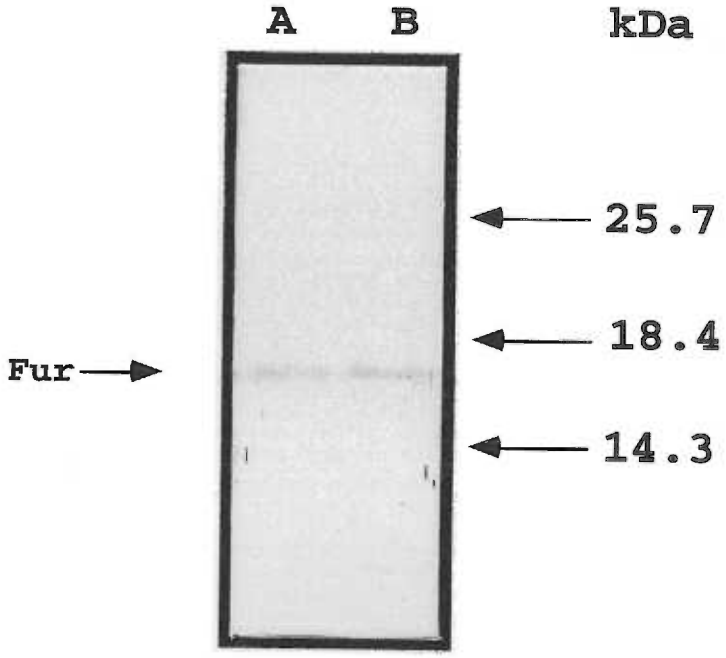

A
B
kDa
25.7
18.4
14.3
Fur

Figure 6: Immunoblot analysis of *V. anguillarum fur* mutants. The Fur proteins present in total protein extracts of the *V. anguillarum fur* mutants selected in the presence of 10 mM $MnCl_2$ were analyzed by SDS-PAGE and immunoblotting, using anti-Fur serum, as described in Materials and Methods. Strains analyzed were *V. anguillarum* 775 (lane A), *V. anguillarum* 775MET9 (lane B), *V. anguillarum* 775MET11 (lane C). Molecular masses (in kDa) are shown to the right.

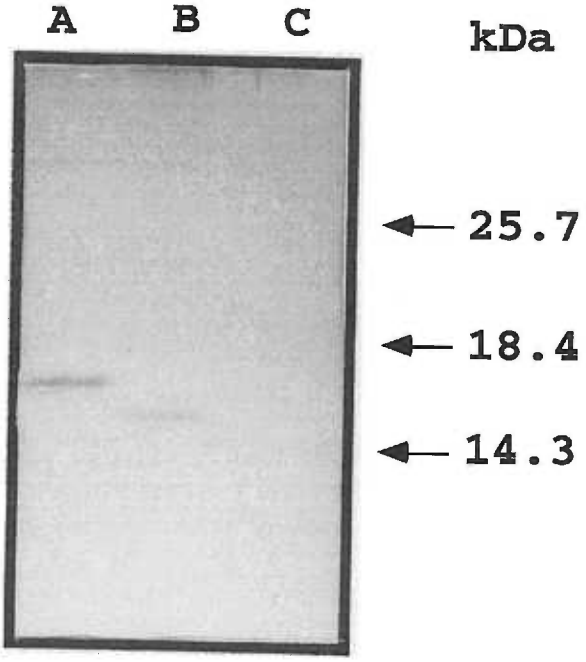
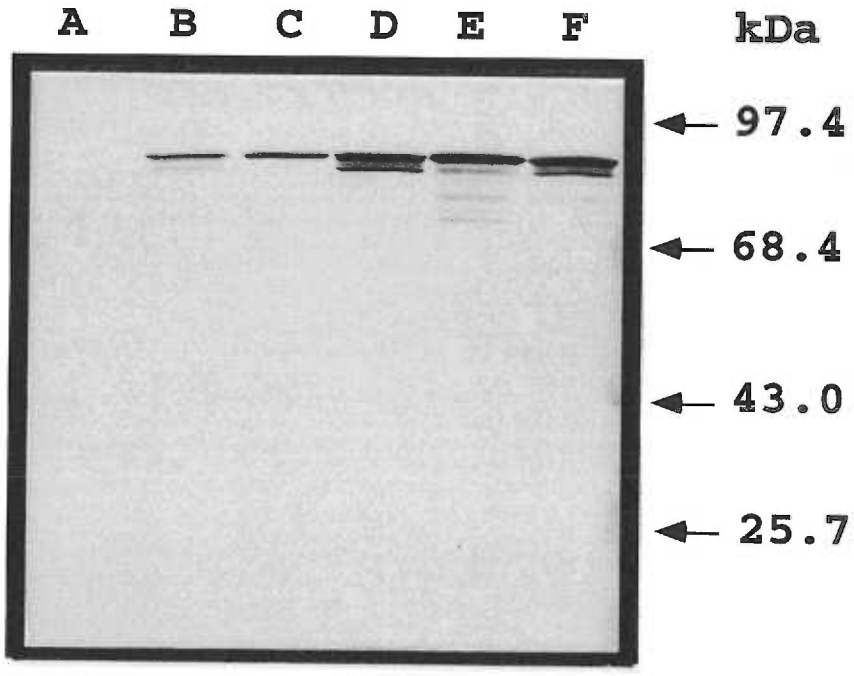

A
B
C
kDa
25.7
18.4
14.3

Figure 7: Regulation of expression of FatA in *V. anguillarum* mutants. Outer membrane proteins of *V. anguillarum* 775 (lanes A and B), 775MET9 (lanes C and D); 775MET11 (lanes E and F), cultured under iron-rich (lanes A, C, and E) or iron-limiting (lanes B, D, and F) conditions, as described in Materials and Methods, were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, incubated with anti-FatA serum, and developed as described in Materials and Methods. Molecular masses (in kDa) are shown to the right.


A
B
C
D
E
F
kDa
97.4
68.4
43.0
25.7

CHAPTER 2

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Structural and Functional Analysis of Mutant Fur Proteins with Impaired Regulatory Function

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ABSTRACT

Vibrio anguillarum Fur mutants, 775met9 and 775met11 were characterized. *V. anguillarum* 775met9 had a change D_{104}- G_{104} located in the carboxy terminus resulting in impaired Fur activity. Computer analysis predicts perturbation of an alpha-helix in the carboxy terminus which may interfere with Fur protein conformation. Strain 775met11 had a change in the start codon resulting in no protein synthesis. The mutants are unstable and reversion to wild type occurs frequently.

An important virulence factor of the fish pathogen *Vibrio anguillarum* is its plasmid-mediated iron uptake system, composed of the siderophore anguibactin and a receptor complex that recognizes ferric-anguibactin (1, 13, 14, 22). In some bacterial species such as *Escherichia coli*, regulation of the iron-uptake gene expression depends on a single gene, *fur* (*f*erric *u*ptake *r*egulator) (20), whose product acts as a classical repressor, blocking transcription in the presence of high concentrations of iron (4, 21, 36). We recently reported the cloning and characterization of the *V. anguillarum fur* gene and determined that Fur plays a role in regulation of the synthesis of anguibactin and the outer membrane protein FatA (45, 42). In addition, in *V. anguillarum* there are at least three plasmid-mediated regulators besides the chromosomally-mediated Fur (33, 34, 40, 41). The *V. anguillarum* Fur protein shares a high degree of homology with the other Fur protein sequences reported in the literature (5, 23, 24, 28, 37, 42).

Fur exerts its regulatory function by binding to the operator of Fur-regulated genes (4, 12, 16, 17). Although indirect, there is evidence implicating that the C-terminal portion of the *E. coli* Fur protein binds Fe^{2+} which may induce a conformational change in the N-terminal region of the protein allowing the binding, possibly as a dimer, to the operator of Fur-regulated genes (12). However, the characteristic helix-turn-helix motif (8,9) present in many DNA binding proteins is not present. Therefore, the study of the *V. anguillarum* Fur protein is of great importance not only to understand the regulation of the iron uptake system but also as a general model of regulation and DNA-protein interaction. As part of these studies we characterized two *V. anguillarum fur* mutants that we recently isolated (42) using the $MnCl_2$ resistance selection method (21). In this work we characterize the products of these mutations and investigate the implications of our findings to the structure function relationships of Fur.

(Part of this research was presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, NV., 23-27 May 1994).

Complementation of *fur* mutants by the *V. anguillarum* wild type Fur. We have recently isolated two *V. anguillarum fur* mutants, 775met9 and 775met11 (Table 1), by selection in the presence of $MnCl_2$ (42). To determine the nature of these two mutations we performed complementation studies with the wild type *fur* gene clone from *V. anguillarum*, pMET67 (45). We conjugated this plasmid with, either the reporter plasmid pSC27.1 or the control plasmid pRT240 (10), into the mutant strains 775met9 and 775met11. The transconjugants possessed wild type Fur activity as measured by the β-galactosidase activity of the reporter constructs (Table 2).

Cloning and sequencing of the mutant *fur* genes. Clones from *V. anguillarum* Fur mutant 775met9 as well as the parent strain *V. anguillarum* 775 Nal^r Rif^r, carrying the wild type *fur* gene, were obtained after PCR using the appropriate primers to amplify the *fur* open reading frame (18). To detect functional Fur proteins we used the reporter strain RRJC1(Table 1). This strain possesses a chromosomally encoded β-galactosidase under the control of a promoter carrying an *fhuF* Fur box, which in the presence of a functional Fur, is down regulated under high iron, resulting in white colonies on MacConkey plates with 100 μM $FeCl_3$. Therefore, after transformation of the PCR clones containing either wild type *fur* or mutant 9 *fur* inserts, into the reporter *E. coli* RRJC1, we obtained derivatives with mutant phenotypes. In the case of mutant strain 775met11, we amplified by PCR the upstream region as well as the open reading frame to analyze the sequence directly instead of cloning, as discussed in the following section.

Three of the clones containing mutation 9 were sequenced. In all three derivatives, we identified a single point mutation, a base change from T_{332} to C_{332} , which results in an amino acid change from D to G at position 104 . This mutation occurs in an amino acid conserved in most known Fur proteins.

In the case of mutant 11, sequence analysis revealed a single point mutation of nucleotide G3 to T3 resulting in a change of G to U in the translation initiation codon This mutation results in the infrequently recognized initiation codon AUU (46).

We also sequenced the upstream region of the mutant *fur* genes up to about 200 base pairs (bp) upstream from the Fur start site containing putative -10 and -35 sequences (44, 47). Features of this region are depicted in (Figure 1). Comparison of this sequence with those of *V. vulnificus* and *V. cholerae* shows that there is a ca. 100 bp deletion occurring in the *V. anguillarum* and *V. vulnificus* regions, while the remainder of the upstream region remains considerably conserved, including the anticipated ribosomal binding site, the predicted -35 and -10 promoter regions, and the predicted transcription start site. There also is a region resembling a Fur box which overlaps both the putative -10 and the +1 site of the *V. anguillarum fur* gene that is closer to the consensus sequence, as compared to the same region in the *fur* genes from the other two Vibrios. No differences were found in the upstream regions of mutant 9 nor mutant 11. These results were in agreement with the fact that approximately similar levels and sizes of *fur*- specific transcripts were detected in all samples from *V. anguillarum* 775, 775met9, and 775met11 under both iron-rich and iron-limiting conditions (data not shown).

Analysis of the predicted secondary structure of Fur proteins. The *E. coli* Fur protein has a high content of histidine (12). By different approaches, such as marker exchange for *V. cholerae* (24), *V. vulnificus* (23) and *Y. pestis* (37) and by using selection in the presence of $MnCl_2$ for *Pseudomonas aeruginosa* (28) and *V. anguillarum* (42), it was possible to demonstrate that mutations in the *fur*-like gene in these bacteria also led to constitutivity of specific iron regulated systems. Structural analysis of *E. coli* Fur using nuclear magnetic resonance and circular dichroism revealed that approximately 50% of the protein is alpha helical which is especially concentrated in four regions comprising residues 15 to 36, 80 to 96, 105 to 123, and 130 to 147 (48). These alpha helices may

interact with each other or with other regions of the protein upon the binding of metal ion and/or DNA (32). However, these helices which are 21 residues in length, do not conform to the classical helix-turn-helix motif which involves a two to three turn helix, a 4 residue turn , followed by a four turn helix (8,9). Although, extensive work has been carried out on the phenotype caused by *fur* mutations and some structural characterization has been worked out in *E. coli* Fur, very little is known about the structure and function relationships of the Fur proteins.

To gain information on the structure-function relationships of Fur we examined our model system, the *V. anguillarum* Fur protein. We analyzed the computer-predicted secondary structure, as well as the predicted structural changes resulting from a D_{104} to G_{104} mutation, in the amino acid sequence of the *V. anguillarum* Fur protein using both the Genetics Computer Group (1991), Program Manual for the GCG Package , Version 7. and Mac Vector software (International Biotechnology's, Inc., New Haven CT). Figure 2 shows the computer-predicted secondary structure, as well as the predicted effect of a simulated D_{104} to G_{104} mutation in the secondary structure of the Fur proteins from *V. cholerae* (panels E and F), *V. vulnificus,* (panels G and H) and *E. coli* (panels C and D).

The computer analysis confirms secondary structure for *E. coli* Fur similar to the experimental analyses cited above. We found that the general alpha helical pattern found in the *E. coli* Fur occurs to a similar extent in the wild type Fur from *V. anguillarum, V. cholerae* and *V. vulnificus*. The alpha helix patterns in the carboxy terminus of *V. anguillarum*, *V. cholerae* and *V. vulnificus* Fur proteins are quite similar, yet these patterns differ from the similar region in the *E. coli* Fur protein.

The D104 to G104 mutation results in a critical perturbation in the putative alpha helix region found in the carboxy terminus of this protein, causing an interruption of the alpha helix. (Figure 2, panels A and B) (27,31). As is the case for the *V. anguillarum* Fur protein, introduction of a D104 to G104 mutation in the *V. cholerae* and *V. vulnificus* Fur proteins, would also lead to a disruption in the alpha helix pattern. However, in these

cases, the result of the mutation is that the initial alpha helix region of about 10 amino acid residues, is no longer part of the alpha helix motif. It appears that this region in the wild type Fur proteins from *V. cholerae* and *V. vulnificus,* may be less constrained to alpha helicity as that of the wild type Fur from *V. anguillarum.* It is of interest that the *E. coli* Fur protein alpha helix motif does not appear to be significantly altered by the D104 to G104 mutation, a prediction which correlates with the fact that this alpha helix motif does not begin until residue 105 (48). Thus, based both on our computer model as well as the fact that the amino acid flanking this single mutation is a serine in the *Vibrio's* Fur protein sequence this greatly increases the probability that the D104 to G104 mutation causes termination of alpha helicity at this region of the carboxy terminus.

Analysis of the expression of the mutant Fur proteins The PCR insert from mutant 9 with the D104 to G104 mutation in the *V. anguillarum* Fur protein, was subcloned into the expression vector pT7-5 and then transformed into the *E. coli* strain BL21(DE3)(pLysE) (39). Using IPTG to induce gene expression, we demonstrated that this mutant *fur* gene could be expressed in *E. coli* (Figure 3, lane D). It can be seen in this figure that the mutant protein migrates more rapidly than the 17 kDa wild type protein (compare lanes C and D). To corroborate that our findings are not an artifact of the overexpression of the cloned mutant 9 gene in *E. coli*, we also analyzed the mobility of the chromosomally-encoded mutant 9 protein synthesized by the *V. anguillarum* strain 775met9 as compared to that synthesized by the cloned mutant 9 *fur* gene in *E. coli.* Figure 4, lanes C and E, shows that both proteins migrate similarly.

To further assess that this single point mutation was responsible for the faster migration, as well as for the diminished Fur function (42), we used pAWPCR9.1 which is the mutant 9 gene cloned using pBluescript SK+ as the vector. This recombinant plasmid was used to perform site-directed mutagenesis to revert the mutated *fur* gene to the wild type sequence (pAWPCR9.1M) as well as to assess Fur activity. Transformation of

pAWPCR9.1M DNA into *E. coli* RRJC1 resulted in white colonies on MacConkey agar containing 100 μM $FeCl_3$, indicating that the wild type form of Fur was recovered. As shown by immunoblot analysis (Figure 4, lanes E and F), concomitant with the reversion from G_{104} to D_{104}, the wild type mobility of the revertant Fur is also recovered.

Finally, we also performed site-directed mutagenesis of the cloned wild type Fur open reading frame reproducing the mutation 9. This mutated clone encoded a mutant Fur protein that migrated with the same mobility as the mutant 9 Fur protein isolated from 775met9 (data not shown). It is also apparent from Figure 4 that similar levels of Fur protein are produced from both wild type and mutant 9 *fur* clones in *E. coli*, as is the case for wild type and mutant 9 proteins in *V. anguillarum*.

The different migration and diminished activity of Fur mutant 9 occurs not only when the mutated chromosomal *fur* gene is expressed in the *V. anguillarum* cell environment but also as a cloned gene in an *E. coli* background, suggesting that the D104 to G104 mutation results in a change that is intrinsically related to the protein encoded by the mutated gene, either by changing the conformation or by making the protein more susceptible to degradation. The loss of such a large negatively charged amino acid has previously been determined to cause aberrant migration of a protein in SDS-PAGE (17b).

Our results showed that mutant 775met11 led to the constitutive expression of both, the FatA outer membrane protein receptor, as well as catechols under both iron-rich and iron-limiting conditions (2). Inspection of the total proteins with the Fur antiserum showed that this mutant did not synthesize any Fur protein (Figure 4, lane B, and ref. 42). However, if a five fold excess of the extract from 775met11 is loaded, a very faint band appears at the proper molecular weight for wild type Fur (compare lanes A and B in Figure 4). In this mutation, the initiation AUG codon was changed to AUU (46). Site directed mutagenesis changing the AUU to AUG restored production of Fur protein (data not shown).

Influence of the environment on the stability of *fur* mutations. We have experienced a loss of the *fur* mutant phenotype in several cultures of mutants 775met9 and 775met11. We proceeded to investigate this phenomenon with the 775met9 strain. Originally, we observed a decrease in the loss of the constitutive phenotype as determined by Arnow assay for catechols (2). When total cell extracts were made from these cultures of 775met9 (Figure 5, lane D), two protein bands appeared, one at the position of the wild type Fur and one migrating as mutant 9 Fur protein. We hypothesize that the presence of both species of proteins may be due to the fact that in the culture there is a mixture of mutant and revertant cells.

Reversion to wild type Fur protein mobility was also observed for 775met9 extracts after the strain had been passed from trypticase soy agar to trypticase soy broth. Such reversion also resulted from serial passage from trypticase soy agar to trypticase soy broth to complete minimal medium (Figure 5, lanes D and E), and a final passage to complete minimal medium with added iron (Figure 5, lane F) and complete minimal medium containing EDDHA (iron-limiting) (Figure 5 lane G). It was of interest that when the final passage was from complete minimal medium into iron-limited minimal medium (Figure 5, lane G) we detected less wild type Fur protein. Furthermore, when the inoculum was taken directly from the frozen stock into the iron-limited complete minimal medium (Figure 5, lane I) the migration of the mutant 775met9 Fur protein was maintained with no reversion detected, even after a further passage in this medium. When an inoculum was taken directly from the same trypticase soy agar plate as that used to initiate these experiments involving 775met9, and directly inoculated into iron-limited complete minimal medium (Figure 5, lane H) we detected more mutant 9 Fur protein as compared to the wild type form. Figure 5 lanes A through C also shows that similar culture manipulations for the 775 Nal^r Rif^r parent strain do not cause any changes in the wild type mobility of the Fur protein.

Having demonstrated the ability to revert, we attempted to define a medium which prevented reversion. When cultures were grown on trypticase soy agar with 1% NaCl medium with increasing $MnCl_2$ concentrations we discovered the disappearance of wild type Fur protein in total cell extracts run in SDS-PAGE (Figure 6). Cultures grown from frozen inoculum streaked onto trypticase soy agar with 1% NaCl with 4 mM $MnCl_2$, typically required 24-48 hours to grow, and only mutant 9 Fur protein was detected (Figure 6, lane E) yet at lower concentrations wild type Fur protein is present (Figure 6, lanes C and D). The wild type 775 strain, also streaked directly from frozen inoculum, consistently failed to grow within 48 hours on trypticase soy agar with 1% NaCl plates supplemented with 4mM $MnCl_2$. An interesting note is that these reversions to wild type mobility of the 775met9 protein in *V. anguillarum* were not seen in any of the clones containing the mutant 9 *fur* gene in *E. coli.*

We have consistently seen selection for bacteria carrying the wild type Fur proteins under nutrient rich and /or high iron conditions. Thus, we compared the growth of 775 and 775met9 in iron rich and iron limiting minimal medium (Table 3) and found that the mutant Fur strain, 775met9 consistently grows poorly as compared with the wild type strain. We also demonstrate that while the 775 wild type strain thrives in iron rich as compared to iron limiting conditions, the mutant strain 775met9 grows considerable less voraciously in non-iron limiting conditions yet it grows similarly to wild type in iron-limiting medium. This difference in growth leads us to hypothesize that in these mutant cultures once reversion to wild type Fur begins, the portion of the population containing wild type Fur will grow more rapidly and out compete the mutant cells in that population especially under iron rich conditions unless there is specific selection pressure in the form of $MnCl_2$ to maintain the mutant population. We are quite interested in why the mutant strain does not thrive in iron rich medium. As Fur has been shown in other bacteria to function pleotropically, many possibilities for poor growth exist. Perhaps, high intracellular iron concentration could be deleterious to the cell, for instance, by catalyzing

the production of free radicals as by a Haber-Weiss-type reaction (19). Functional Fur protein may successfully coordinate or bind the metal ion and prevent damage by the free iron or somehow maintain the intracellular iron balance.

Understanding the function-structure relationships leading to the interaction of the Fur protein with iron, DNA, and other parameters will likely be increased by analysis of these, and other mutants that we isolated. Thus, it has become clear that we have isolated two very different types of mutations which lead to loss of Fur function. Fur mutant 9 gives us the opportunity to examine questions involving structure-function of this DNA binding protein while mutant 11 draws our attention to a more general role played by Fur within the bacterium.

ACKNOWLEDGMENTS

Special thanks to Dick Brennan for his assistance in interpreting our secondary structure predictions for Fur. This work was supported by NIH grant AI19018.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains:		
E. coli		
XL1 Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A 46*thi rel*A *lac*- F'[*pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]	Stratagene
BL21(DE3)(pLysE)	*hsdS gal* (λc Its 857 *ind*1 *Sam* 7 *nin*5 lac UV5-T7 gene1)(Studier and Moffatt 1986)	Novagen(38) (38)
RRJC1	BN4020 derivative (*E. coli fur* mutant) with a β-galactosidase reporter gene under the control of the *fhuF* Fur box	(30)
V. anguillarum		
775 Nal^r Rif^r	parent strain from which *fur* mutants derived	(13)
775met9	fur mutant isolated in the presence of 10 mM $MnCl_2$	(42)
775met11	fur mutant isolated in the presence of 10 mM $MnCl_2$	(42)
775met9(pMET67)	pMET67 (wild type V. *anguillarum fur*) conjugated into 775met9	(42)
775met11(pMET67)	pMET67 (wild type V. *anguillarum fur*) conjugated into 775met11	(42)
Plasmids:		
pBluescript SK$^+$	Cloning vector	Stratagene
pT7-5	Expression vector	(39)
pT7-6	Expression vector	(39)
pMET67	*V. anguillarum fur* cloned in pVK102	(45)
pMETAW1.8	*fur V. anguillarum* cloned into pBCSK$^+$	(42)
pAWPCR9.1	mutant *fur* from 775met9 subcloned into pBluescript SK+	This work
pAWPCR9.1B	mutant *fur* from 775met9 subcloned into pT7-5	This work
pAWPCR9.1M	site-directed mutagenized pAWPCR9.1 restoring wild type D_{104} in *fur* subcloned into pBluescript SK$^+$	This work
pAWPCR11.3	site-directed mutagenized mutant *fur* from 775met11 restoring wild type AUG initiation codon subcloned into pBluescript SK$^+$	This work
pAWPCR11.3M	site-directed mutagenized pAWPCR11.3 creating a G_{104} mutation in *fur* , subcloned into pBluescript SK+	This work
pSC27.1	*fur* reporter gene. β-galactosidase gene cloned under the control of the *ompF* promoter with a Fur between the promoter and the open reading frame	(10)
pRT240	Control plasmid for *fur* reporter gene constructs with *ompF* promoter without Fur Box	(10)
pTAW2.1	*V. anguillarum fur* cloned into pT7-5	(42)

DNA procedures: Modifications of already described methods for genomic PCR (3) were used. Genomic DNA was isolated form cultures of *V. anguillarum* grown overnight at 27°C in trypticase soy broth supplemented with 1% NaCl using a modification of the method of Birnboim and Doly (6). All transformations were performed by the method of Cohen et al. (11).

Table 2. β-galactosidase activities of *V. anguillarum* derivatives

V. anguillarum strains[1]	775 (pSC27.1)	775met9 (pSC27.1)	775met9 (pSC27.1, pMET67)	775met11 (pSC27.1)	775met11 (pSC27.1, pMET67)
β-galactosidase[2]	1.8	5.9	0.5	11.7	0.4

[1] Cultures were grown under iron rich conditions 75-100 μM $FeCl_3$. Conjugations between *V. anguillarum* and *E. coli* were performed as described previously (40).

[2] Units values were determined as described by Miller (26, 29). The results reported are representative of three independent experiments.

Table 3. Comparison of growth of wild type *V. anguillarum* 775 and mutant strain 775MET9

STRAIN	OD_{600} Minimal Medium with 100 µM $FeCl_3$	OD_{600} Minimal Medium with 4 µM EDDHA	RATIO $FeCl_3$/EDDHA
775	2.5	0.94	2.7
775met9	1.2	0.75	1.5

Comparison of growth between wild type 775 *V. anguillarum* and mutant strain 775met9. An inoculum was taken directly from frozen cultures diluted into minimal medium then 100 µl was inoculated into minimal medium supplemented with 100 µM $FeCl_3$ and into minimal medium supplemented with 4 µM EDDHA then cultures were grown overnight at 27°C.

Figure 1: Analysis of the upstream region of *V. anguillarum fur* in comparison to *V. vulnificus (V. v.)* and *V. cholerae (V. c.)* illustrating a putative -35 and conserved -10 TATAAT sequences, transcriptional start site (down arrow), Shine-Dalgarno (SD) and protein start site (plus sign). Sequencing of double-, single-stranded DNA or gel purified PCR fragments, was performed by the dideoxy chain termination method (35) using Sequenase kits (US Biochemical, Cleveland, OH).

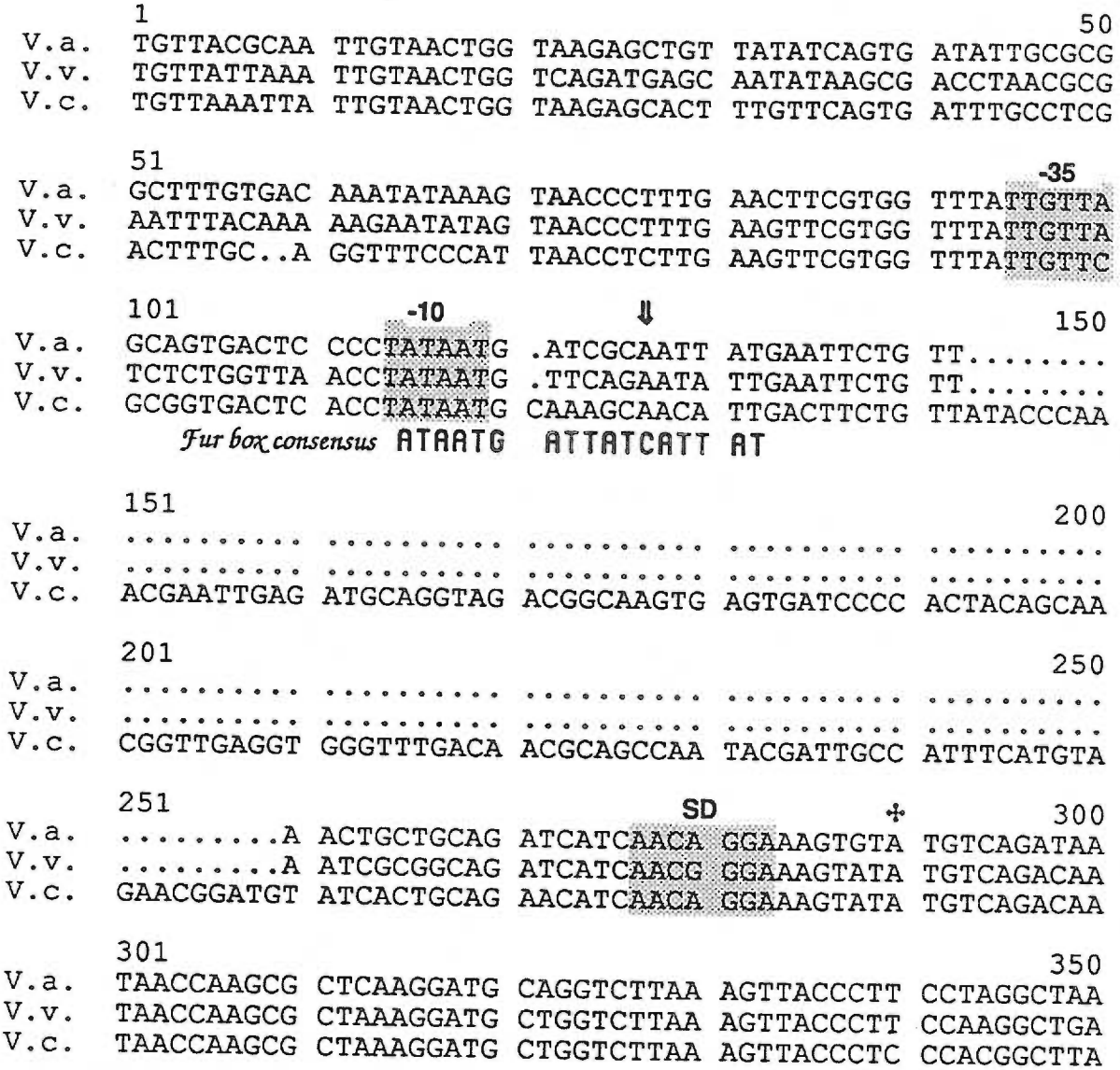
1
50
V.a. TGTTACGCAA TTGTAACTGG TAAGAGCTGT TATATCAGTG ATATTGCGCG
V.v. TGTTATTAAA TTGTAACTGG TCAGATGAGC AATATAAGCG ACCTAACGCG
V.c. TGTTAAATTA TTGTAACTGG TAAGAGCACT TTGTTCAGTG ATTTGCCTCG
51
-35
V.a. GCTTTGTGAC AAATATAAAG TAACCCTTTG AACTTCGTGG TTTATTGTTA
V.v. AATTTACAAA AAGAATATAG TAACCCTTTG AAGTTCGTGG TTTATTGTTA
V.c. ACTTTGC..A GGTTTCCCAT TAACCTCTTG AAGTTCGTGG TTTATTGTTC
101
-10
150
V.a. GCAGTGACTC CCCTATAATG .ATCGCAATT ATGAATTCTG TT.....
V.v. TCTCTGGTTA ACCTATAATG .TTCAGAATA TTGAATTCTG TT.....
V.c. GCGGTGACTC ACCTATAATG CAAAGCAACA TTGACTTCTG TTATACCCAA
Fur box consensus ATAATG ATTATCATT AT
151
200
V.a. .....
V.v. .....
V.c. ACGAATTGAG ATGCAGGTAG ACGGCAAGTG AGTGATCCCC ACTACAGCAA
201
250
V.a. .....
V.v. .....
V.c. CGGTTGAGGT GGGTTTGACA ACGCAGCCAA TACGATTGCC ATTTCATGTA
251
SD
300
V.a. .....A ACTGCTGCAG ATCATCAACA GGAAAGTGTA TGTCAGATAA
V.v. .....A ATCGCGGCAG ATCATCAACG GGAAAGTATA TGTCAGACAA
V.c. GAACGGATGT ATCACTGCAG AACATCAACA GGAAAGTATA TGTCAGACAA
301
350
V.a. TAACCAAGCG CTCAAGGATG CAGGTCTTAA AGTTACCCTT CCTAGGCTAA
V.v. TAACCAAGCG CTAAAGGATG CTGGTCTTAA AGTTACCCTT CCAAGGCTGA
V.c. TAACCAAGCG CTAAAGGATG CTGGTCTTAA AGTTACCCTC CCACGGCTTA

Figure 2: Predicted Fur protein motifs depicting the secondary structure as calculated using both Chou-Fasman and Robson-Garnier algorithms for Fur proteins from V. *anguillarum* 775 (panels A and B), *E. coli* (panels C and D), *V. cholerae* (panels E and F), and *V. vulnificus* (panels G and H). Predicted secondary structure for wild type Fur (panels A, C, E, and G), and from the resulting proteins after a D104 to G104 change is introduced (panels B, D, F, and H). The arrows indicate the characteristic perturbations introduced by the D104 to G104 mutations.

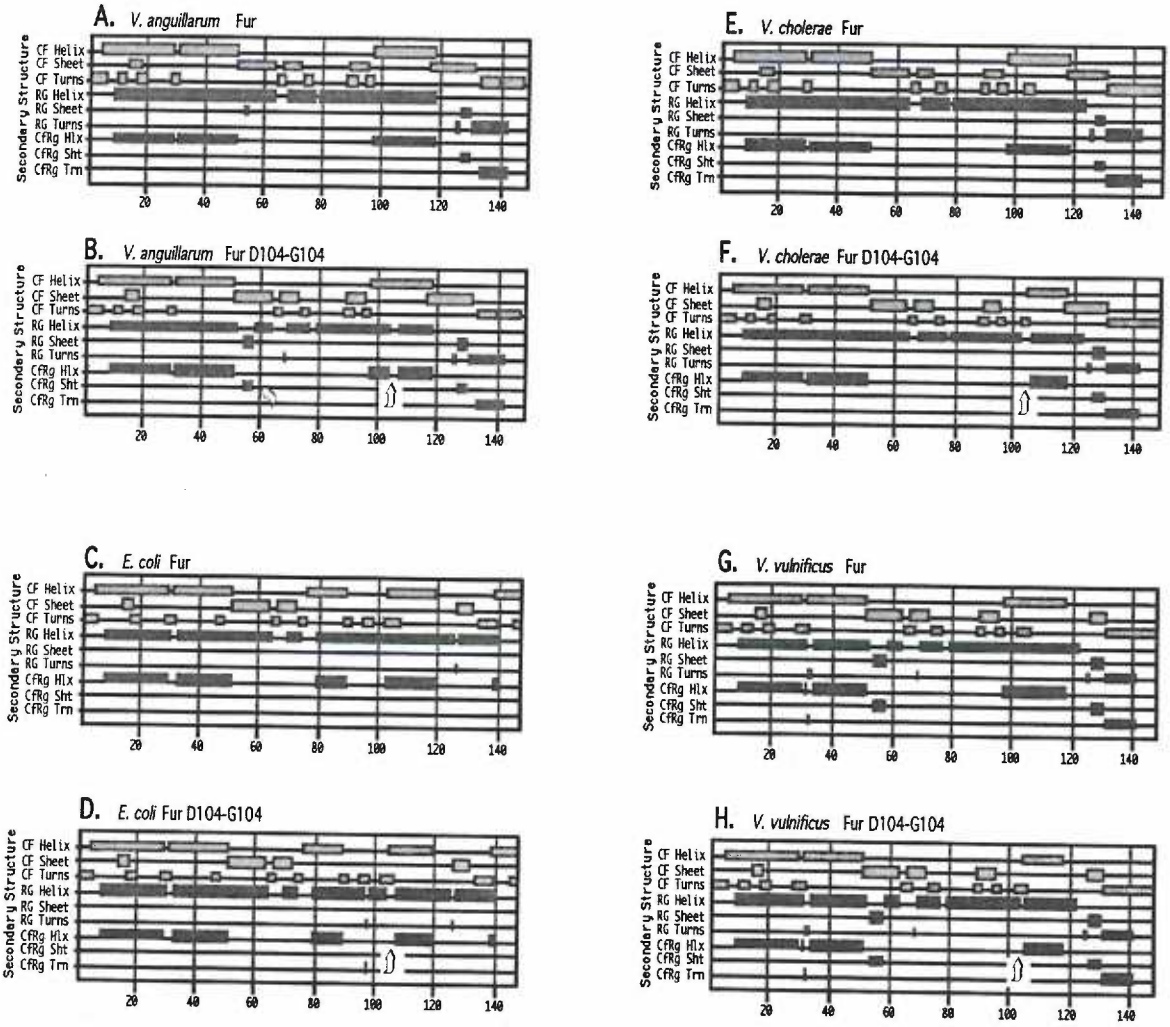
A. V. anguillarum Fur
B. V. anguillarum Fur D104-G104
C. E. coli Fur
D. E. coli Fur D104-G104
E. V. cholerae Fur
F. V. cholerae Fur D104-G104
G. V. vulnificus Fur
H. V. vulnificus Fur D104-G104
Secondary Structure
CF Helix
CF Sheet
CF Turns
RG Helix
RG Sheet
RG Turns
CfRg Hlx
CfRg Sht
CfRg Trn
20
40
60
80
100
120
140

Figure 3: Overexpression of wild type and mutant 9 *fur* genes in *E. coli* BL21(DE3)(pLysE) (pLysE)(1,15,25). Total extracts were electrophoresed on 15% SDS-PAGE and Coomassie blue stained. Extracts from cells harboring pTAW1.8 (wild type *V. anguillarum fur* gene) (lanes A and C) or pAWPCR9B (mutant 9 Fur) (lanes B and D) were obtained from cultures that were uninduced (lanes A and B) or induced in the presence of IPTG (lanes C and D). Lane E, broad range Bio Rad molecular weight markers, the molecular sizes are shown to the left.

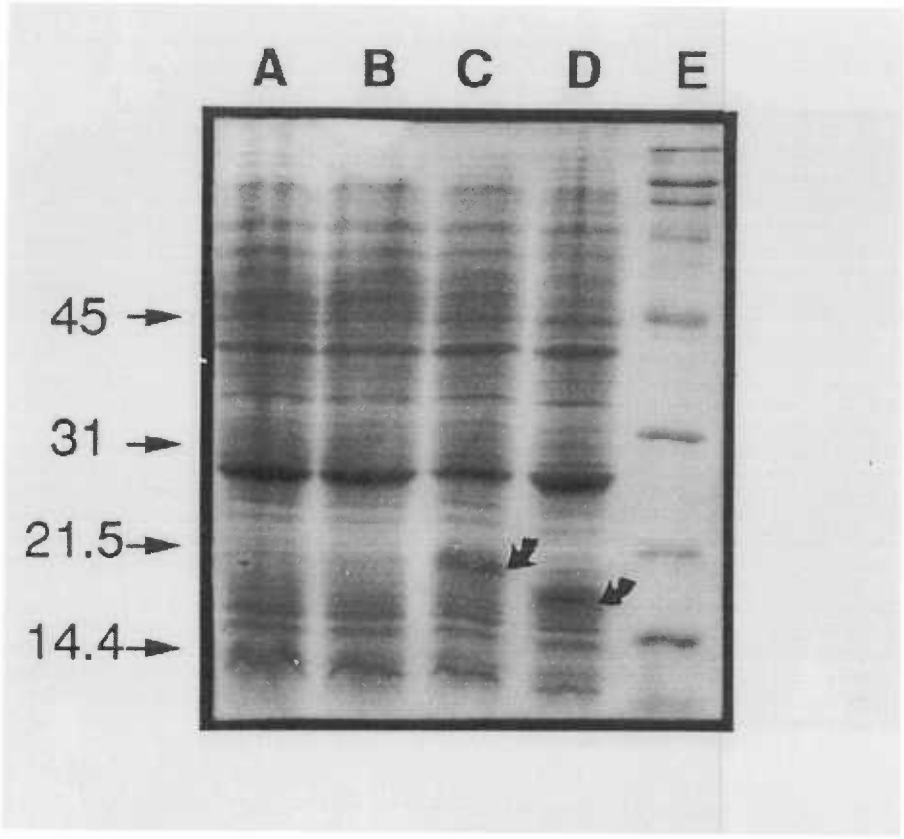
A
B
C
D
E
45
31
21.5
14.4

Figure 4: Detection of Fur proteins by immunoblot analysis from total cellular lysates(1,7,15,25). Proteins synthesized from: Lanes A and B: *V. anguillarum* 775met11 (lane A, 5 times more total protein loaded); Lane C, *V. anguillarum* 775met9; Lane D, *E. coli* RRJC1 harboring the wild type cloned *V. anguillarum fur* gene in plasmid pTAW1.8; Lane E, *E. coli* RRJC1 harboring the cloned *fur* mutant 9 in plasmid pAWPCR9.1; Lane F, *E. coli* RRJC1 harboring the G_{104}-D_{104} revertant obtained by site-directed mutagenesis of the cloned *fur* mutant 9 in plasmid pAWPCR9.1 generating pAWPCR9.1M; Lane G, *V. anguillarum* 775. Molecular weight standards are indicated to the left.

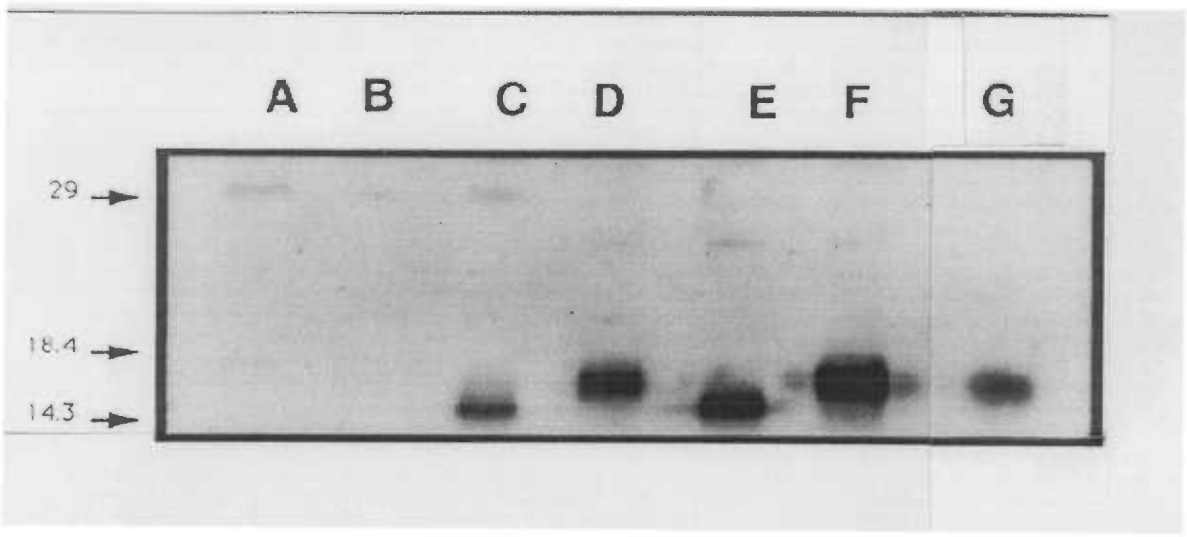
A
B
C
D
E
F
G
29
18.4
14.3

Figure 5: Detection of wild type and mutant Fur proteins from *V. anguillarum* cells grown in different media. Immunoblot of total cellular lysates probed with anti-Fur anti serum(1,7,15,25). Fur proteins from *V. anguillarum* 775 (lanes A-C) and mutant 775met9 (lanes D-I) obtained from respective trypticase soy broth agar plates and grown in: Lanes A and D, trypticase soy broth; Lanes B and E, complete minimal medium inoculated from the previous trypticase soy broth culture; Lanes C and F, iron-rich complete minimal medium inoculated from the previous complete minimal medium culture. Lanes F and G, iron-rich complete minimal medium (lane F) and iron-deficient complete minimal medium (lane G). In this case, cells were first obtained from the same complete minimal medium culture used to prepare the proteins shown in lane E and then inoculated into either the iron-rich or iron-deficient complete minimal medium. In the case of lane H, proteins were obtained from cells of mutant 775met9 grown in complete minimal medium that had been directly inoculated from the same trypticase soy broth agar plate used to generate the cultures described in lanes D-I; Lane I shows the proteins from 775met9 cells grown in complete minimal medium obtained directly from a frozen stock. Lane J, extract from the nearly null mutant 775met11 as a control for non-specific binding. Molecular weight standards are indicated to the left.

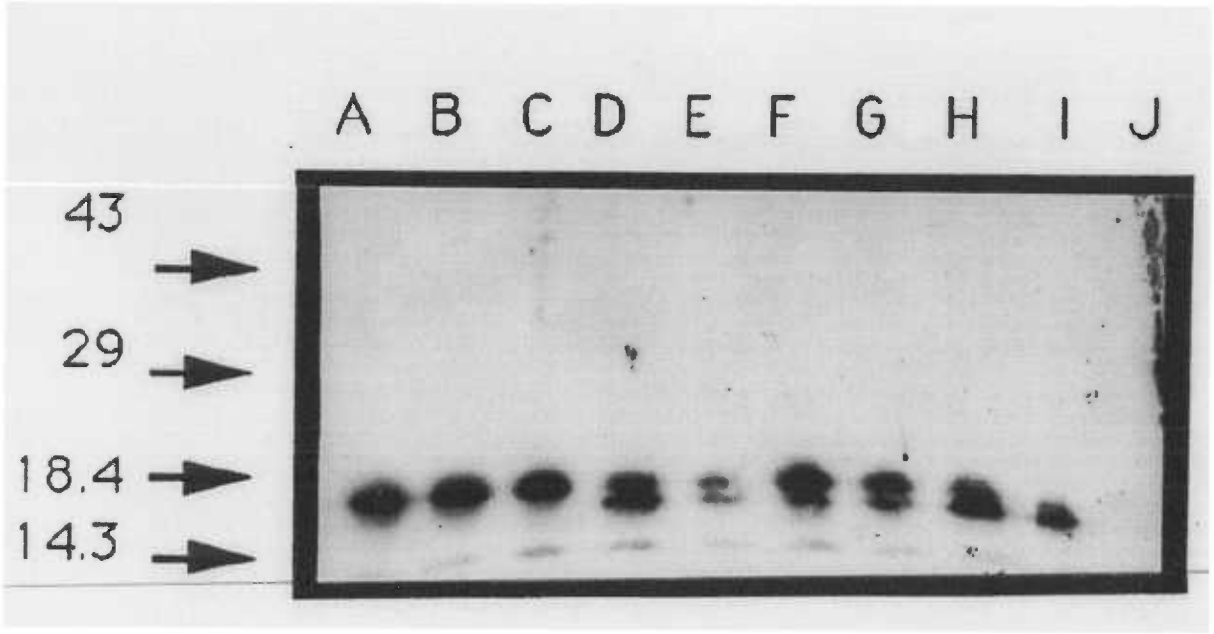
A B C D E F G H I J
43
29
18.4
14.3

Figure 6: Detection of wild type and mutant Fur 9 proteins grown in the presence of $MnCl_2$. Immunoblot of total cellular lysates probed with anti-Fur anti serum (1, 15, 25). Fur proteins from *V. anguillarum* 775 (lane A) and mutant 775met9 (lane B) obtained from cultures grown in trypticase soy broth with 1% NaCl and minimal medium respectively. Lanes C, D, and E show proteins from 775met9 after plating frozen inoculum directly onto trypticase soy agar with 1% NaCl supplemented with 1mM $MnCl_2$, 2mM $MnCl_2$, and 4mM $MnCl_2$. Molecular weight standards are to the left, and markers indicating wild type Fur and mutant Fur 9 are to the right.


A
B
C
D
E
29
18.4
14.3
FUR
FUR 9

LITERATURE CITED

1. **Actis, L.A., S. Potter, J.H. and Crosa.** 1985. Iron-regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. J. Bacteriol. **161:**736-742.
2. **Arnow, L.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118:**531-537.
3. **Ausubell, F.M.**, et. al., 1990. Current Protocols in Molecular Biology. John Wiley and Sons. NY.
4. **Bagg, A., and J. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli.* Biochemistry **26:**5471-5477.
5. **Berish, S., S. Subbarao, C. Chen, D. Trees, and S. Morse.** 1993. Identification and cloning of fur homolog form *Neisseria gonorrhea.* Infect. Immun. **61:**4599-4606.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acids Res. **7**:1513-1523.
7. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**:249-252.
8. **Brennan, R.** 1991. Interactions of the helix-turn-helix binding domain. Current Opinion in Structural Biology. **1**:80-88.
9. **Brennan, R.** 1993. The Winged-Helix DNA-Binding Motif: Another Helix-Turn-Helix Takeoff. Cell. **74**:773-776.
10. **Calderwood, S.B., and J. Mekalanos.** 1988. Confirmation of the *fur* operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015-1017.

11. **Cohen, S. N., A.C.Y. Chang, and L. Hsu.** 1972. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69:**2110-2114

12. **Coy, M., and J. Neilands.** 1991. Structural dynamic and functional domains of the Fur protein. Biochemistry **30:**8201-8210.

13. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **284**:566-568.

14. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53**:517-530.

15. **Crosa, J. H., and L. Hodges.** 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum* 775. Infect. Immun. **31**:223-227.

16. **de Lorenzo, V.L., M. Herrero, F. Giovannini, and J.B. Neilands.** 1988. Fur (ferric uptake regulator) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. Eur. J. Biochem. **173:**537-546.

17. **de Lorenzo, V.L., S. Wee, M. Herrero, and J.B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid pColV-K30 binding the ferric uptake regulation (*fur*) repressor J. Bacteriol. **169:**2624-2630.

18. **Gammie, A., M. E. Tolmasky, and J. H. Crosa.** 1993. Functional characterization of a replication initiator protein. J. Bacteriol. **175:**3563-3569.

19. **Haber, F., and J. Weiss.** 1934. Catalytic Decomosition of Hydrogen Peroxide by Iron Salts. Proc. R. Soc. London A **147:**332-351.

19b. **Hames, B. D** 1981.Introduction to PAGE p. 1-91. *In* B. D Hames, D. Rickwood (ed.), Gel Electrophoresis of Proteins: A practicle approach. IRL Press LMT, London, England.

20. **Hantke, K.** 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol. Gen. Genet. **197:**337-341.

21. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12. Mol. Gen. Genet. **210:**135-139.

22. **Jalal, M., D. Hossain, J. van der Helm, J. Sanders-Loehr, L.A. Actis, and J.H. Crosa.** 1989. Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. J. Amer. Chem. Soc. **111**:292-296.

23. **Litwin, C., S., and S. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by *in vivo* marker exchange. J. Bacteriol. 175:706-715.

24. **Litwin, C., S. Boyko, and S. Calderwood.** 1992. Cloning, sequencing and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897-1903.

25. **Maniatis, T., E.F. Fritsch, and J. Sambrook.** 1982. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

26. **Miller, J.** 1972. Assay of b-galactosidase. p. 352-355. *In* J.H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold spring Harbor, N.Y.

27. **Presta, L. G., and G.D. Rose.** 1988. Helix Signals in Proteins. Science **240**:1632-1641.

28. **Prince, R., C. Cox, and M. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589-2598.

29. **Putnam, S., and A. Koch.** 1975. Complications in the simplest cellular enzyme assay: lysis of *Escherichia coli* for the assay of b-galactosidase. Anal. Biochem. 63:350-360.

30. **Read, R., M. Tolmasky, A. Wertheimer, and J. Crosa.** Unpublished data.

31. **Richardson, J.S., and D. Richardson.** 1988. Amino acid preferences for specific locations at the ends of a helices. Science **240**:1648:1652.

32. **Saito, T., and J.P. Williams** 1990. The binding of the ferric uptake regulation protein to a DNA fragment. FEBS **197**:43-47.

33. **Salinas, P., M.E. Tolmasky, and J.H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: evidence for a cooperative affect between two transcriptional activators. Proc. Natl. Acad. Sci. U. S. A. **86:**3529-3533.

34. **Salinas, P., L. Waldebesser, and J.H. Crosa.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as repressor. Gene **123:**33-38.

35. **Sanger, F., S. Nicklen, and A. Coulsen.** 1979. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463-5467

36. **Schaffer, S., K. Hantke, and V. Braun.** 1985. Nucleotide sequence of the iron regulatory gene *fur*. Mol. Gen. Genet. **200**:110-113.

37. **Staggs, T., and R. Perry.** 1991. Identification and cloning of a *fur* regulatory gene in *Yersinia pestis.* J. Bacteriol. **173:**417-425.

38. **Studier, F., A. Rosenberg, J. Dunn, and J., Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. **185:**60-89.

39. **Tabor, S., and C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. U.S.A. **82:**1074-1078.

40. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. J. Bacteriol. **160**:860-866.

41. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1988. Genetic analysis of the iron uptake region of the *Vibrio anguillarum* plasmid pJM1: molecular cloning of genetic determinants encoding a novel *trans* activator of siderophore biosynthesis. J. Bacteriol. **170**:1913-1919.

42. **Tolmasky, M. E., A. Wertheimer, L. A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* gene. Role in regulation of expression of the FatA Outer Membrane Protein and Catechols. J. Bacteriol. **176:**213-220.

43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76:**4350-4354

44. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259-268.

45. **Waldbeser, L., Tolmasky, M.E., L.A. Actis, and J.H. Crosa.** 1993. Mechanisms for negative regulation by iron of the FatA outer membrane protein gene expression in *Vibrio anguillarum* 775. J. Biol. Chem. **268:**10433-10439.

46. **Watson, J.** 1987. Molecular Biology of the Gene, p. 443. Benjamin Cummings Publishing Company, Menlo Park, CA.

47. **Weickert, M., and G. Chambliss.** 1989. Acid-phenol minipreps make excellent sequencing templates. USB Editorial Comments **16:**5-6.

48. **Williams. R. G., M.R. Wormald, R.C. Hider, and J. Neilands.** 1989. spectroscopic properties of the FUR (Ferric Uptake Regulation) protein of *Escherichia coli* K-12. In, (D. Hamer, and D. Winge, eds.), Metal Ion Homeostasis: Molecular Biology and Chemistry, pp. 189-196. Alan Liss Inc, New York.

CHAPTER 3

The Iron Transport Genes Encoded by the Virulence Plasmid pJM1 in *Vibrio anguillarum* are Transcribed as a Polycistronic mRNA

Anne M. Wertheimer, Marcelo E. Tolmasky, Luis A. Actis, Qian Chen, and Jorge H. Crosa.

ABSTRACT

Vibrio anguillarum owes its virulence to a plasmid mediated iron uptake system which is composed of the siderophore anguibactin and the four transport proteins FatA, B, C, and D. Expression of the genes encoding these proteins is repressed under iron rich conditions by a chromosomal mediated Fur protein and a plasmid encoded antisense RNA, and is positively regulated by the plasmid mediated AngR protein and TAF products. We have performed reverse transcription coupled polymerase chain reaction (RT-PCR) to examine the transcript(s) carrying *fatDCBA*. We found not only individual linkages between *fatD* to *fatC*, *fatC* to *fatB*, and *fatB* to *fatA*, but we have also identified a link from *fatD* to the *angR* gene. Additionally, transposition insertions in the upstream regions, within *fatD*, resulted in the polar loss of expression as assessed at both the transcription and translation level. We also demonstrated that downstream mutations i.e. in *fatB*, result in the production of a *fat DCBA* truncated transcript containing *fatDC* and a portion of *fatB*. These data demonstrate the polycistronic nature of the transcribed iron transport genes *fatDCBA*. Using reporter gene fusions we have also identified a region containing an iron regulated promoter element upstream of *fatDCBA*.

INTRODUCTION

The bacterial fish pathogen *Vibrio anguillarum*, a gram negative pollarly flagellated rod, is responsible for both marine and fresh water fish epizootics throughout the world (13, 24). *V. anguillarum* causes vibriosis, a highly fatal hemorrhagic septicemic disease in salmonids and other fishes including eels (6, 21). We use the *V. anguillarum* system as a model to study the molecular mechanisms of host-pathogen interactions. The disease caused by *V. anguillarum* has remarkable similarities to invasive septicemic disease in humans and the sequence of events immediately after infection are very similar to mammalian inflammation except for obvious species-specific responses (12).

The key feature which enables the pathogenic strains of *V. anguillarum* to survive within the vertebrate host is the possession of a virulence plasmid, pJM1, which provides the bacteria with an iron-sequesering system crucial in overcoming the non-specific defense mechanisms of the host (9-11). This system centers upon both the synthesis and transport of the siderophore (iron scavenging compound) anguibactin. The siderophore is produced by the virulent strains in the host and in any other environment in which the bacteria's sole source of iron is chelated by high affinity iron binding compounds. The plasmid encoded iron-sequestering system is controlled via the concentration of available iron, through three plasmid-encoded regulators: the positive regulators TAF [trans acting factor(s)], and AngR (anguibactin system regulator) and a negative regulator, anti-sense RNAα); repression also requires the chromosomally encoded Fur protein (7, 9-11, 19, 25, 26, 29, 31). To assess the interaction of these regulators at the molecular level it was essential to first determine how the iron transport genes *fatDCBA* are transcribed. In this work we demonstrate that the pJM1 plasmid encoded iron transport genes are transcribed as a polycistronic mRNA. Furthermore, we have identified a region containing an iron regulated promoter element upstream of *fatDCBA*..

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *V. anguillarum* (pJHC-T2612, pJHC9-8) is a strain harboring the recombinant clone pJHC-T2612 which possesses all the genes required for synthesis of anguibactin and the iron transport complex and pJHC9-8 which encodes the regulator TAF which is essential for full expression of the iron uptake system (25). For maps see Table 1 and Figure 1. *V. anguillarum* strains harboring either (pJHC-T2612#20, pJHC9-8), (pJHC-T2612#17, pJHC9-8), or (pJHC-T2612#15, pJHC9-8) have a Tn3Ho-Ho1 insertion denoted with a #, within the *fatD, fatC, or fatB* genes respectively, carried on parental plasmid pJHC-T2612. The insertion mutants have been described previously (25) and transferred into *V. anguillarum* (pJHC9-8) by conjugation using the helper plasmid pRK2073 (25). Plasmid pBluescript SK(+) (Stratagene) was used for cloning of DNA fragments forsite-directed mutagenesis and sequencing. *V. anguillarum* was cultured in either trypticase soy broth (or agar) supplemented with 1% NaCl (TSBS and TSAS respectively). For experiments determining iron uptake characteristics, the strains were first grown on trypticase soy agar with 1% NaCl supplemented with the appropriate antibiotics and passed to M9 minimal agar with the appropriate antibiotics. The resulting cultures were tested either on plates for bioassay or inoculated into M9 minimal medium supplement with ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA) for iron limiting condition. Antibiotic concentrations used were ampicillin 500 μg/ml and tetracycline 5 to 10 μg/ml.

General DNA procedures. Plasmid DNA was prepared using the alkaline lysis method of Birnboim and Doly (3). Sequence quality plasmid DNA was generated using the appropriate Qiagen kits (Chatsworth , CA). Automated sequencing was performed by the Molecular Microbiology and Immunology (MMI) Core Facility on the PE/ABD377 DNA Sequencer using dye-terminator or dye-primer cycle sequencing chemistry or the

A.L.F Pharmacia's fluorescent sequencer and either dye-primer sequencing with Sequenase, or cycle sequencing with Taq polymerase. Manual sequencing was performed by the dideoxy chain-termination method using the Sequenase Kit (US Biochemical, Cleveland, OH) again using appropriate primers. Primers were all synthesized by the MMI Core Facility on a PE/ABD 394 automated synthesizer using standard phosphoramidite chemistry on polystyrene solid supports. Restriction endonuclease digestion of DNA was performed under the conditions recommended by the supplier (Life Technologies, Inc.). Transformations and other cloning strategies were carried out as described in Molecular Cloning (16.). DNA and protein sequence analysis were carried out at the NCBI using the BLAST network service, and also using the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (GCG). The GCG programs PILEUP and BESTFIT were used for comparisons of amino acid sequences.

Reverse transcription polymerase chain reaction (RT-PCR). The parental strain *V. anguillarum* (pJHC-T2612, pJHC9-8) or the mutant strains harboring either (pJHC-T2612#20, pJHC9-8), (pJHC-T2612#17, pJHC9-8), or (pJHC-T2612#15, pJHC9-8), grown in M9 minimal medium supplemented with 10μM EDDA for wild type and 1μM for the mutants. Total RNA from these strains was then harvested by a modified hot phenol method (28). Reverse transcription was carried out using 1 μg of RNA. Both single and two tube reactions were performed (30). In the single tube method the reaction was done in the presence of all the required materials for reverse transcription and subsequent PCR. The two tube method involved a separate reverse transcription reaction in the presence of a single primer for generation of the cDNA. Then an aliquot from this reverse transcription reaction was used in a PCR reaction using two primers to amplify the target region. The avian myeloblastosis virus (AMV) reverse transcriptase was used for synthesis of the complementary DNA, which served directly as the template for PCR. Reaction conditions were: 53°C for 30 minutes; 95°C for 5 minutes; 40 cycles of: 95°C for

10 sec, 53°C for 20 sec, 73°C for 1 min.; followed with a final extension at 73°C for 5 minutes. The negative controls involved using RNA which had not been reverse transcribed with AMV or, when the single tube method was used the reaction tube containing reaction buffer and enzymes (TAQ [Promega-Madison WI] and AMV]Gibco BRL-Gaithersburg, MD])was heated to 100°C for 10 minutes. The reaction tube was then cooled and the remainder of the reagents were added and cycling was performed. Cycling parameters varied with the length of product anticipated but generally the manufacturers parameters were as followed. In addition, we used the Titan one Tube RT-PCR kit from Boehringer Mannheim (Indianapolis-IN) to generate longer fragments. We followed the directions from the kits with minor modifications, again the negative control involved heating the enzymes in buffer prior to their addition. Products were electrophoresed on 0.7-1.0% agarose gels. In addition to being the predicted size, products were sequenced to verify they contained the iron transport genes. The primers used were Primer 1.fat: 5' CCG CCG CAT TAG GGT TGA TAC TCG, Primer 2.fat: 5' GGA CTC GTA ACC CAT AAT AGA TGG CG, Primer 3.fat: 5' GGA CCA ACT GCG TTC ATG GG, Primer 4.fat: 5' CCA AGG TCC GAA ATG TCA GGA GTG, Primer 5.fat: 5' GAT CGC ACA GCG GTC ATG GAA GGC Primer 6.fat: GAA TGT GTT TGT CTG TGT ACC C, and AngR.up: 5' CGT TAC TGC AGA ACG CGT ATC GGC C.

Measurement of the production of chloramphenicol acetyl transferase (CAT) from the reporter constructions. *V. anguillarum* strains were cultured in Trypticase soy agar with 1% NaCl (TSAS) with the appropriate antibiotics, then passed onto M9 agar with the appropriate antibiotics, into M9 minimal medium with the appropriate antibiotics, then inoculated at 1:50 into either iron limiting conditions (M9 minimal medium alone or with 5 μM EDDA) or into iron supplemented conditions (M9 minimal medium supplemented with 4 μM ferric ammonium citrate and grown to an optical density of 0.8 to 1.2 at 600 nm. A commercial CAT ELISA kit (Boehringer Mannheim)

was used and 1ml samples were prepared following the supplier's instructions. This particular enzyme linked immunosorbant assay (ELISA) detects the actual production of chloramphenicol acetyl transferase (CAT) synthesized. All samples were normalized for total protein levels prior to assay. All points represent samples assayed at least in duplicate per assay and at two different linear values. All assays were repeated at least twice.

Ribonuclease protection assays. A 1:50 inoculum from a saturated overnight culture grown in minimal medium plus appropriate antibiotics was used. When the culture reached an OD_{600} of 0.4-0.6, the total RNA was collected using the hot phenol method (28) and stored at -70°C until analyzed. Between 10-20μg of RNA was used for the RNase protections. The RNA was annealed 8 to 16 hrs at 43 to 45°C, with either *fatA*, or *fatB*, and *aroC* probes (as an internal control for RNA concentrations) (1-5 $x10^5$cpm $\alpha^{32}P$). Riboprobes were generated using in-vitro transcription using construct pMET13.1 (linearized with *Cla*I), pJHC-LW260 (linearized with *Eco*RI) and pQC3.5 (linearized with RsaI) phenol chloroform extracted after complete restriction digestion and ethanol precipitated, then transcribed with T3 RNA polymerase generating the *fatA*, *fatB* and *aroC* probe respectively. Each probe was gel purified, and resuspended in annealing buffer and kept at -70°C prior to use. After annealing, ribonuclease A and T1 were added (to the samples but not to the tubes containing the control probe) the samples were incubated 30 minutes at 30°C, proteinase K was then added and incubated an additional 30 minutes at 37°C. Samples were then treated with phenol chloroform, followed with an ethanol precipitation and electrophoresed in a standard sequencing gel. Gels were exposed to X-ray film for 1 to 4hrs with intensifying screens at -70°C.

Protein Analysis. The proteins encoded by the iron transport region were analyzed by immunoblot. Immunoblot analysis to detect FatA was carried out with anti-FatA serum as described previously (1). Immunoblot analysis to detect FatB was carried out with anti-FatB serum as described previously (2).

RESULTS

Transposition mutagenesis of the iron transport region. The diagram above Figure 2 illustrates that the iron transport genes *fatD, fatC, fatB,* and *fatA* are positioned in a head to tail fashion which suggested that these genes could be part of an operon (15). Transposition mutations within the same region all resulted in an iron transport deficient phenotype (25). However, we were not able to isolate any cA. 5 kb mRNA corresponding to *fatDCBA* using Northern blots possibly because of transcript instability (Unpublished observation-Waldbeser and Crosa). To demonstrate that the iron transport genes are transcribed as an operon we analyzed the expression of *fatA* and *fatB* at both the translation and transcription level in each one of the transposition mutants. Using polyclonal antiserum we were able to demonstrate in western blots as seen in Figure 2 panel A and panel B, that transposition events upstream of both *fatA* and *fat B* lead to a significant loss of protein synthesis from the genes downstream of the insertion. Specifically, insertion mutations within *fatD* (#20), *fatC* (#17) and *fatB* (#15) resulted in a significant loss of both FatA (panel A) and FatB (panel B) protein. This polar effect of insertion mutations suggests that the genes may be contained within the same mRNA transcript.

Having shown that at the level of protein these genes appeared to be linked, we next analyzed the effect of these mutations on transcription of *fatA* and *fatB.* Riboprobes transcribed from constructs pMET13.1, and pJHC-LW260 using T3 RNA polymerase, were used to detect specific *fatA* and *fatB* transcript, respectively. Figure 3 panel A and panel B illustrate that indeed transposition events lead to a significant loss of transcription from genes downstream of the insertion. Specifically insertion mutations within *fatD* (#20), *fatC* (#17) and *fatB* (#15) result in significant loss of both *fatA* (panel A) and *fatB* (panel B) mRNA. In the case of insertion 15 (panel B lane 15), a truncated version of *fatB* mRNA can be detected, an expected result since the riboprobe used hybridizes to both a

region of *fatB* upstream and to one overlapping the actual site of insertion. Thus, the *fatB* mRNA which is protected by the riboprobe is smaller than the entire riboprobe. This polar effect of insertion mutations suggests that the genes may be contained within the same mRNA transcript. Therefore by combining the results of protein and mRNA analysis it is clear that the *fatDCBA* must be transcribed as a polycistronic mRNA.

Linkage analysis of the iron transport genes by RT-PCR. In order to determine if genes *fatD, fatC, fatB, fatA* were actually contained in a polycistronic message we conducted RT-PCR experiments using both the wild type template as well as the constructs with insertion mutants upstream of *fatA* and *fatB*. Initially we had designed several primers (Figure1) in order to span each intergenic region. Thus, primer 1 is positioned such that in conjunction with primer 2, the segment between *fatD* and *fatC* will be amplified. The same concept was used for primer 3, and primer 4 (within *fatC* and *fatB* respectively) and primer 5 and primer 6 (within *fatB* and *fatA* respectively). Figure 4 panel A and B illustrates the results of our RT-PCR which detected a transcript spanning each of the genes described. We also successfully detected a link between a primer within *fatC* to *fatA* (see lane marked CA in panel B).

We also isolated RNA from each of the insertion mutants and used primers to detect the *fatDC* transcript. Figure 4 panel C shows the results of this analysis. Insertions within *fatD* or *fatC* lead to a loss of the *fatDC* transcript, however an insertion within *fatB* has no effect on the upstream transcript thus we were able to detect *fatDC* in lane 5. These results suggested that these genes were contained in a single transcript of about 4.9kb. In order to confirm that such a transcript contained all of the *fat* genes, we used the primer within *fatD* and designed a new primer in the 5' region of *angR*. Using Boehringer Mannheim's Titan RT-PCR kit we were able to amplify the predicted 4.9 kb fragment as seen in Figure 4 panel D lane 1).

Identification of an iron regulated promoter activity upstream of *fatD*. Our genetic and RT-PCR experiments indicate that there is a polycistronic message transcribed from the *fatDCBA* operon. Therefor, we constructed plasmids containing various size segments of the *fatD* upstream region, in order to identify promoter elements. Figure 5 panel A, illustrates the various constructions generated. The upstream regions to be characterized for promoter activity were inserted after a strong translational and transcriptional stop and just upstream of the chloramphenicol acetyl transferase (CAT) gene in the pKK232-8 vector (Pharmacia). The ELISA procedure used detects the actual concentration, pg/well of CAT produced which correlates with the promoter activity of the DNA segment cloned into the reporter vector. Each subclone containing various upstream regions of *fatDCBA* was first created in pBluescript, then subcloned into the pKK232-8 reporter vector and finally mobilized into *V. anguillarum* 775. The smallest clone, pAWEH.C containing the 200bp *Eco*RI-*Hind*III fragment, had no significant promoter activity. Plasmid pAWCH.C containing the *Cla*I-*Hind*III fragment also had extremely low level of activity, which may or may not be physiologically relevant although, it is significantly above background levels. Plasmid pMET23.10 containing the *Bste*II- *Cla*I fragment showed significant promoter activity. Plasmid pAWEEH.C encompassing the entire 2.1kb *Eco*RI-*Eco*RI-*Hind*III fragment also provides significant promoter activity. The region where we find this promoter activity is consistent with the location of the promoter for the polycistronic message as determined from our transposition mutagenesis and RT-PCR analysis. Since the iron transport genes are regulated by the iron status of the cell we next assayed whether this promoter region was regulated by iron.

We grew the strains carrying the constructs being assayed under either iron limiting conditions (M9 minimal medium without supplement of iron) or under iron limiting condition of M9 with 5 μM EDDA supplemented, or under iron rich conditions where M9 medium is supplemented with 4μM ferric ammonium citrate. Figure 5 panel B shows the bar graph comparing the growth conditions with the concentration of actual CAT

protein as measured in pg/well of CAT protein produced per 1mg/ml of total protein assayed. As shown in Figure 5, the promoter activity within the 2.1 kb construct is significantly iron regulated. The promoter activity within the 2.1kb fragment was most active when the strain was grown in 5μM EDDA, synthesizing between 5587 to 5929 pg/well CAT protein. When the cells were cultured in M9 minimal medium, the promoter activity was slightly less, between 4681 to 4826 pg/well of CAT protein. When the same cells were cultured in M9 supplemented with 4 mM ferric ammonium citrate the amount of CAT protein generated was quenched to 528 to 770 pg/well, approximately a 90% decrease in promoter activity.

DISCUSSION

Our initial characterization of the virulence plasmid pJM1 involved transposon mutagenesis in order to begin mapping functional regions of pJM1 (25). These studies revealed that a particular region spanning nearly 20 kb was required for iron acquisition. A discrete sub region spanning just under 10 kb specifically was required for iron transport. This region mapped to within a single *Eco*RI fragment (Figure 1). As this region was further analyzed, a considerable amount of circumstantial evidence (1, 2, 15, 25, 27, 29) suggested that the genes were transcribed as a single transcript. In addition, our discovery of a chromosomally encoded Fur protein heightened the need to characterize the nature of the mRNA transcribed from the iron transport region since Fur binds to the operator region of several iron transport systems (14, 17, 18, 22). Earlier Northern blot experiments were only able detect the existence of a 2.3 to 2.5 kb transcript (1, 29) rather than the anticipated c.a. 5 kb. It is possible that the c.a. 5 kb mRNA containing *fatDCBA* is quite unstable, thus found only at low concentrations, while processed products such as the 2.3 kb species accumulate (29). Therefore, physical manipulations during isolation, or simply the nature of the transcript was preventing us from physically isolating this large mRNA molecule. In this work we present genetic evidence showing that expression of both *fatB* and *fatA* genes decreases upon insertion of a transposon within any of the other upstream *fat* genes (Figure 2 and 3), suggesting a polar effect of this upstream insertion and thus the existence of a polycistronic mRNA. We decided to use reverse transcription coupled polymerase chain reaction (RT-PCR) in an attempt to physically detect this putative mRNA containing the *fat* genes. The advantage of using RT-PCR is that one is able to detect even a few molecules of an unstable long transcript and generate a cDNA molecule. Once the cDNA is synthesized, PCR is used to exponentially duplicate the molecule to a level which is detectable by electrophoresis in an agarose gel. If the genes are transcriptionally linked the transcript amplification of the cDNA will occur. Conversly, if there is a gap where the

transcript stops and a separate transcript starts, the cDNA will be only be as long as the transcript and thus no amplification will occur in the subsequent PCR, as long as the primers are positioned properly on the ends of each transcript. Thus we endeavored to show whether transcriptional linkage occurred between each *fat* gene and those genes located upstream and downstream. Reverse transcription was used to generate a cDNA from the total RNA then Taq polymerase was used to amplify the intergenic region. This method worked remarkably well as seen in Figure 4 (panel A and B). Using the extended PCR detailed in the results section, we were able to span the entire *fatDCBA* locus and generated the predicted 4.9 kb product, which we confirmed contained the *fat* genes by using restriction digestion and sequencing (Fig 4 panel D).

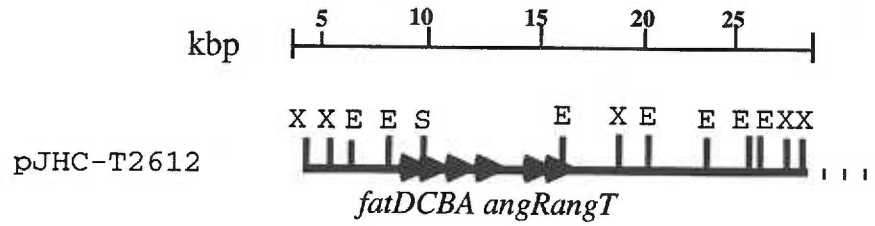
We also verified that when the total RNA was isolated from transposition mutants we are only able to detect the transcript linkage prior to the site of insertion (Figure 4, panel C). Thus, the DC linkage is detectable only for the strain with the transposition event occurring within *fatB*.

In order to identify potential promoter regions upstream of *fatD* we generated various constructs with the CAT reporter gene to dissect the region We found that the entire 2.1 kb region possesses significant promoter activity as analyzed by CAT ELISA (Figure 5). The majority of promoter activity was found upstream of the *Cla*I restriction site upstream of *orf7*, which as yet has no identifiable function (23). This promoter element is significantly affected by the iron concentration of the cell. It is of interest that the transposition mutations used in this study carry a promoterless *lacZ* gene, which we previously found to be iron regulated (25). This promoter element may indeed be involved in the control mechanism for at least a portion of the negative regulation by iron on the iron transport genes. Work is currently in progress to characterize this promoter as well as any cis elements involved in the iron regulation of iron transport gene expression.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype	Source or reference
Bacterial strains:		
Escherichia coli		
XL1 blue	*recA1, endA1, gyrA46, thi, hsdR17, supE44, relA, lac* F' [*proAB*$^{+}$, *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^{r})]	Stratagene La Jolla, CA
HB1O1	*supE44, hsdS20,*(r_B^-,m_B^-), *rec13, ara-14,proA2, lacyI, galK2, rpsL20 , xyl-5, mtl-1*	(5)
JM109	*recA1, endA1, gyrA96, thi*$^{-}$ Δ, *hsdR17, supE44, relA1, (lac-proAB),* [F' *traD36, proAB, lacI*^{q} *lacZ*ΔM15]	(20)
Vibrio anguillarum		
775	wild type, prototype, (pJM1).	(9)
H775-3	plasmidless derivative of 775.	(11)
Plasmids:		
pBluescript SK+	cloning vector.	Statagene, La Jolla CA
pVK102	cloning vector.	(4)
pKK232-8	CAT Reporter Vector.	Pharmacia
pJHC-T2612	recombinant clone carrying the iron uptake region of pJM1 cloned in pVK102.	(24)
pJHC-T2612#20	recombinant clone pJHC-T2612 with Tn3::-HoHo1 insertion in *fatD iron* uptake deficient, FatD mutated.	(24)
pJHC-T2612#17	recombinant clone pJHC-T2612 with Tn3::-HoHo1 insertion in *fatC iron* uptake deficient, FatC mutated.	(24)
pJHC-T2612#15	recombinant clone pJHC-T2612 with Tn3::-HoHo1 insertion in *fatB iron* uptake deficient, FatB mutated.	(24)
pJHC9-8	pJM1 derivative carrying only the TAF region.	(25)
pAW EEH.S	1.9 kb*Eco*13 fragment of pJM1 cloned into pAWEH.S.	This Work
pAW EEH.C	the *Sal*1-*Hind*III fragment of pAWEEH.S cloned into the pKK232-8 cat reporter construct.	This Work
pAWEH.S	The 300bp *Eco*R1-*Hind*III fragment of pJM1 just overlapping *fatD* cloned into pBluescript SK-II+.	This Work
pAWEH.C	The *Sal*1-*Hind*III fragment of pAWEH.S cloned into the pKK232-8 cat reporter construct.	This Work
pAW CH.S	The 600bp *Cla*I-*Hind*III region of pJM1 cloned into pBluescript SK-II.	This Work
pAWCH.C	The *Bam*HI-*Hind*III region of pAWCH.S cloned into the pKK232-8 reporter construct.	This Work
pMET23.10. S	The 600bp BstEII-*Cla*I region of pJM1 cloned into pBluescript SK-II.	(23)
pMET23.10.CAT	The 600bp *Bam*HI-*Cla*I region of pMET23.10 cloned into the pKK232-8 reporter construct.	(23)
pMET13.1	92bp *Cla*I-*Sal*I fragment of the *fatA* region cloned in pBluescript-IISK+.	(29)
pJHC-LW260	191 bp ThaI-RsaI fragment of the *fatB* region cloned in pBluescript-II SK+.	(29)
PQC3.5	420bp *Sal*I-*Cla*I fragment of the *aroC* gene cloned in pBluescript-II SK+.	(8)

Figure 1: Genetic and physical map of plasmids used in this work. Illustration of the location for the transposition insertions, in pJHC-T2612. The transposition insertion is approximately 12 kb and carries the ampicilin resistance marker in addition to a promoterless *lacZ* (25). Restriction sites are as follows: X, *Xho*I; E, *Eco*RI; S, *Sal*I;. The pVK102 vector DNA is represented as either hatched The insert DNA from pJM1 is represented by thick lines. The dark circle symbolizes the Tn3::HoHo 1 insertion mutants #20, #17, and #15. The arrows indicate the specific genes and their direction of transcription.


kbp
5
10
15
20
25
pJHC-T2612
X X E E S
E X E
E EEXX
fatDCBA angRangT

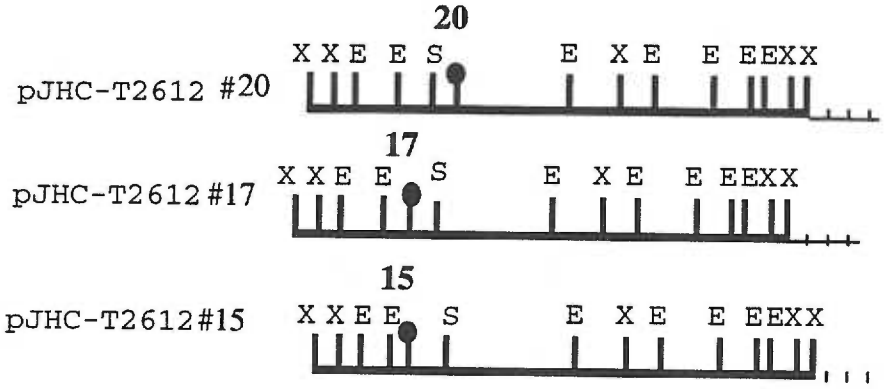

20
pJHC-T2612 #20
X X E E S
E X E
E EEXX
17
pJHC-T2612#17
X X E E S
E X E
E EEXX
15
pJHC-T2612#15
X X E E S
E X E
E EEXX

Figure 2: Immunoblot analysis revealing the polar effect of insertion mutations in various upstream positions on FatA and FatB synthesis. Total membrane proteins from *V. anguillarum* strains cultured under iron limiting conditions (CM9 minimal medium with the addition of 5μM EDDA) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose paper, incubated with anti-FatA (Panel A) or anti-FatB (panel B) serum and developed by reaction with peroxidase and staining with H_2O_2 and horseradish peroxidase luminescent development reagent. Panel A: Mutant strains are indicated above each lane, WT stands for wild type *V. anguillarum* iron transport region. Lane 1: *V. anguillarum* H775-3 (pJHC-T2612#15, pJHC9-8)), Lane 2:*V. anguillarum* H775-3 (pJHC-T2612#17, pJHC9-8) Lane 3: *V. anguillarum* H775-3 (pJHC-T2612#20, pJHC9-8), and Lane 4: *V. anguillarum* H775-3 (pJHC-T2612, pJHC9-8) or 775. Panel B: identical loading as Panel A.

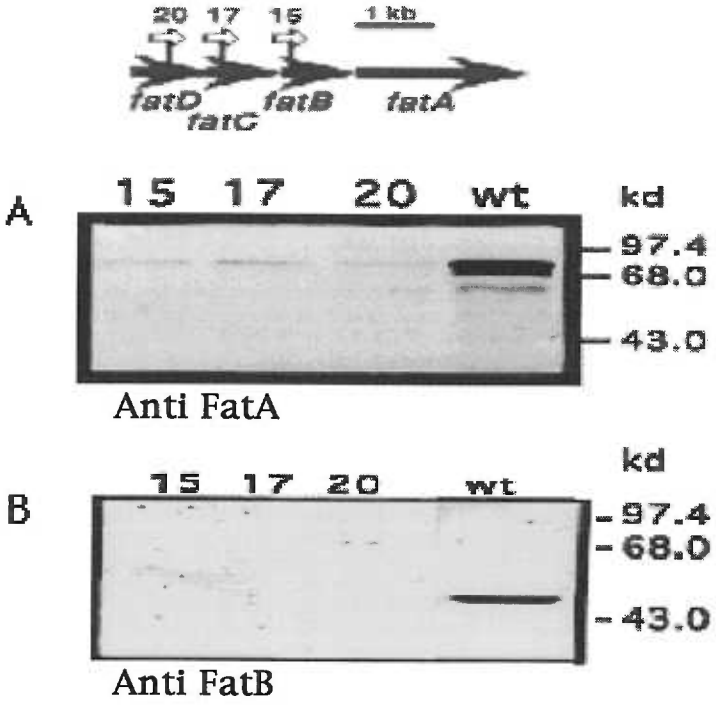

20 17 15
1 kb
fatD
fatC
fatB
fatA
A
15 17 20 wt
kd
97.4
68.0
43.0
Anti FatA
B
15 17 20 wt
kd
97.4
68.0
43.0
Anti FatB

Figure 3: The effect of insertion mutations on transcription of *fatA* and *fatB*. Total RNA was harvested from *V. anguillarum* strains harboring various recombinant clones grown under iron-limiting conditions. Riboprobes were generated using construct pMET13.1, pJHC-LW260 and pQC3.5 and transcribed with T3 RNA polymerase which generated the *fatA*, *fatB* and *aroC* probe, respectively. The *aroC*-specific riboprobe was included in the hybridization buffer as an internal control. Panel A: Mutant strains are indicated above each lane, WT stands for wild type *V. anguillarum* iron transport region probed with riboprobe to detect *fatA*. transcript. Lane 1: *V. anguillarum* H775-3 (pJHC-T2612#15, pJHC9-8)), Lane 2: *V. anguillarum* H775-3(pJHC-T2612#17, pJHC9-8) Lane 3: *V. anguillarum* H775-3 (pJHC-T2612#20, pJHC9-8), and Lane 4: *V.anguillarum* H775-3 (pJHC-T2612, pJHC9-8) or 775. Panel B: Riboprobe used to detect *fatB* RNA is identical loading as Panel A.

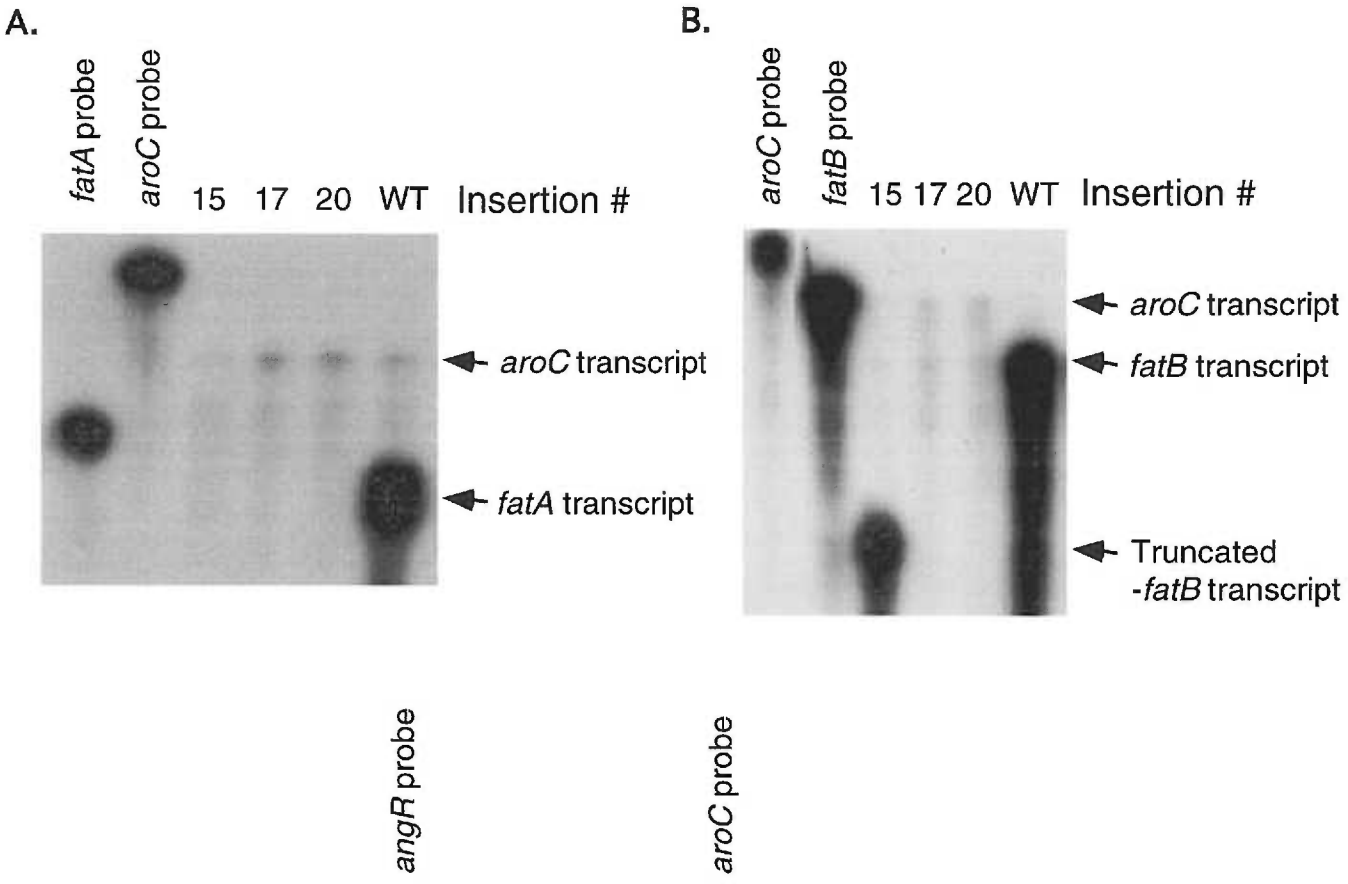
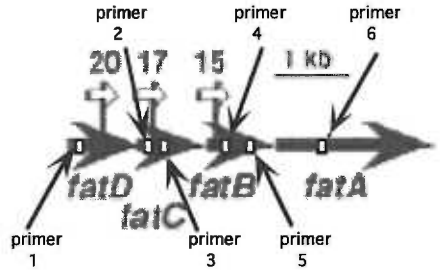
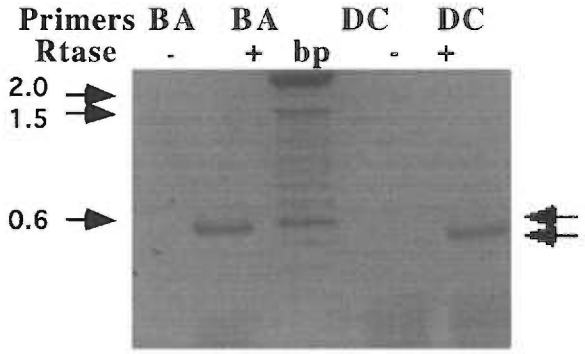

A.
fatA probe
aroC probe
15
17
20
WT
Insertion #
aroC transcript
fatA transcript
angR probe
B.
aroC probe
fatB probe
15 17 20 WT
Insertion #
aroC transcript
fatB transcript
Truncated
-fatB transcript
aroC probe

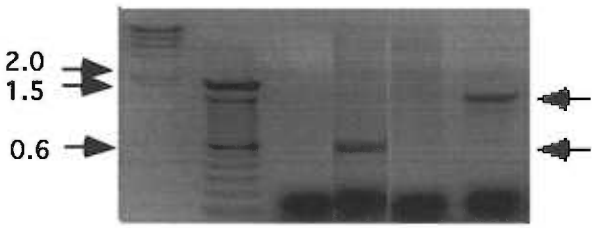
Figure 4: Detection of the *fatDCBA* polycistronic mRNA by RT-PCR using RNA from the wild type and mutant strains. **Panel A**: Detection of the mRNA spanning *fatB-fatA*, *fatD-fatC*. This panel shows the products spanning *fatB-fatA* (lane 1 and 2), and *fatD-fatC* (lane 4 and 5). Lanes 1 and 4 are controls where the identical cocktail (BA, and DC respectively) was used but no reverse transcriptase was added. bp stands for the molecular weight marker, the 100 bp ladder (lane 3). Arrows to the left indicate the kilobase pairs for this molecular weight marker. Arrows to the right indicate products. **Panel B:** Detection of mRNA spanning *fatC-fatB*, *fatC-fatA*. This panel shows the products spanning *fatC-fatB* (lanes 3 and 4), *fatC-fatA* (lanes 5 and 6). Lanes 3 and 5 are controls where the identical cocktail (CB and CA) was used but no reverse transcriptase was added. bp stands for the molecular weight markers, the lambda *Hind*III ladder and the 100 bp ladder (lane 1 and 2 respectively). Arrows to the left indicate the kilobase pairs for this molecular weight marker. Arrows to the right indicate products. **Panel C:** Detection of mRNA spanning *fatD-fatC* using RNA obtained from the mutant strains. This panel shows products spanning *fatD-fatC* (lanes 2-7). The RNA was isolated from the insertion mutants specified, #15, #17, and #20 respectively. Lanes 2-4 are controls where the identical cocktail (DC) was used but no reverse transcriptase was added. bp stands for the molecular weight marker, the 100bp ladder (lane 1). Arrows to the left indicate the kilobase pairs for this molecular weight marker. Arrows to the right indicate products.


primer 2
primer 4
primer 6
20
17
15
1 kb
fatD
fatC
fatB
fatA
primer 1
primer 3
primer 5

A


Primers BA BA DC DC
Rtase - + bp - +
2.0
1.5
0.6

B


Primer CB CB CA CA
Rtase bp bp - + - +
2.0
1.5
0.6

C

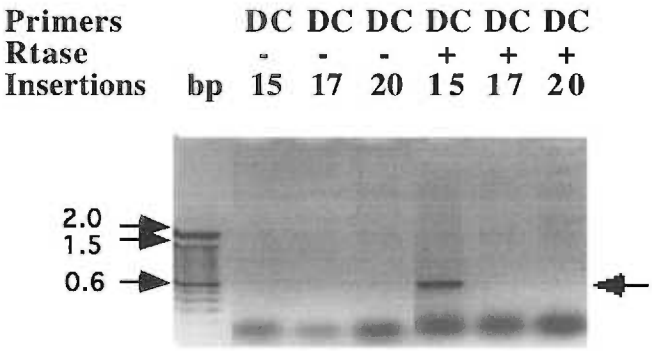

Primers DC DC DC DC DC DC
Rtase - - - + + +
Insertions bp 15 17 20 15 17 20
2.0
1.5
0.6

Figure 4 (continuation), Panel D: Detection of mRNA spanning *fatD-angR, fatC-angR, fatB-orf6* and *fatB-angR*. This panel shows the products spanning *fatD-angR*, (Lane 1), *fatC-angR* (Lane 2), *fatB-orf6* (Lane 3), and *fatB-angR* (lane 4). Lane 5 is the control where the reverse transcriptase was heat inactivated but the cocktail contained primers it amplify *fatD-fatC*. kbp stands for the molecular weight marker, the lambda *Hind*III ladder. Arrows to the right indicate the kilobase pairs for this molecular weight marker.

D

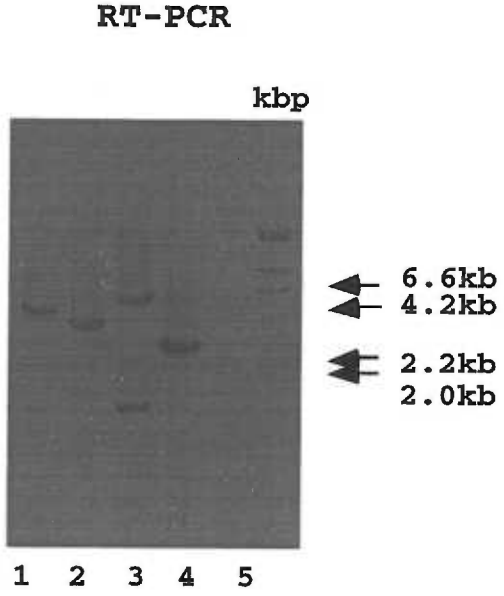
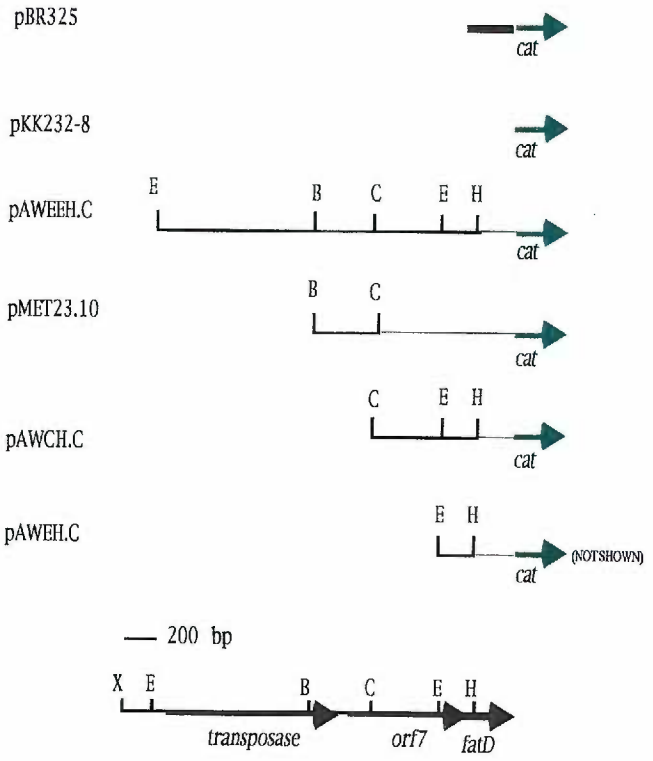
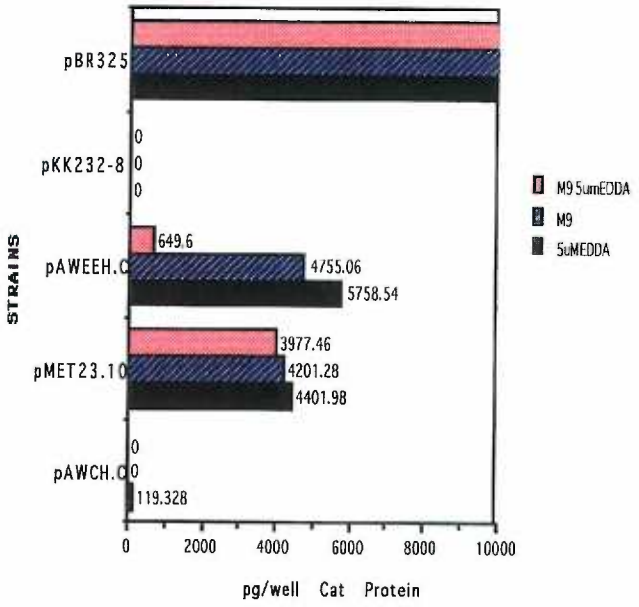


Figure 5: Promoter activity of pJM1 DNA constructs. **Panel A**: Genetic and physical map of pKK232-8 constructions. Restriction sites are as follows: X, *Xho*I; E, *Eco*RI; B, *Bste*II; C, *Cla*I; and H, *Hind*III. The vector DNA is represented as thin lines for pKK232-8, the promoterless *chloramphenical transacetylase* gene *(cat)* is shown as a green arrow. The posive control is pBR325 which has the entire *cat* gene with its natural promoter (shown as a thick line preceeding the arrow). The insert regions from pJM1 are also represented by thick lines and they have restriction sites marked. The dark arrows represent transcripts and their direction, the transcript name appears below them. **Panel B:** Bar graph illustrates pg/well of CAT protein. Each construct is tested in duplicate at two protein concentrations that are in the linear range of the standard curve. pBR325 is the positive control as mentioned above, pKK232-8 is the reporter construct without an insert in front of the reporter CAT. pAWEEH.C is the 2.1 kb fragment, pMET 23.10 is the 500 bp *Bste*II-*Cla*I construct, pAWCH.C is the 600 bp *Cla*I-*Hind*III construct. The strain tested appears on the X axis. The Y axis illustrate the pg/well of CAT. All constructs were tested in *V. anguillarum* 775.

PANEL A



PANEL B



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LITERATURE CITED

1. **Actis, L. A., S. Potter, and J. H. Crosa.** 1985. Iron regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. Journal of Bacteriology. **161:**736-742.
2. **Actis, L. A., M. E. Tolmasky, L. M. Crosa, and J. H. Crosa.** 1995. Characterization and regulation of the expression of FatB, an iron transport protein encoded by the pJM1 virulence plasmid. Molecular Microbiology. **17:**197-204.
3. **Birnboim H, C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. **7:**1513-1523.
4. **Bolivar F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene. **4:**121-126.
5. **Boyer, H., and D . Roulland-Doussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* Journal of Molecular Biology. **41:**459-472.
6. **Canestrini, G.** 1893. La malattia dominante delle anguile. Atti dell'Intituto veneto di scienze, lettere ed arti. **7:**809-814.
7. **Chen, Q., and J. H. Crosa.** 1996. Antisense RNA, Fur, iron, and the regulation of iron transport genes in *Vibrio anguillarum.* Journal of Biological Chemistry. **271:**1885-91.
8. **Chen , Q., A. M. Wertheimer, M. E. Tolmasky , and J. H. Crosa.** 1996. The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum.* Molecular Microbiology. **22:**127-134.

9. **Crosa J, H., M. Schiewe, H,, and S. Falcow.** 1977. Evidence for plasmid contribution to the virulence of fish pathogen *Vibrio anguillarum*. Infection and Immunity. **27:**509-513.

10. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio Anguillarum* specifies an iron-sequestering system. Nature. **284:**566-568.

11. **Crosa, J. H., L. Hodges, and M. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infection and Immunity. **27:**897-902.

12. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore medieated iron transport in bacteria. Microbiological Reviews. **53**:517-530.

13. **Harbell, S. O., H. O. Hodginds, and M. H. Schiewe.** 1979. Studies on the pathology of vibriosis in coho salmon. Journal of Fish Diseases. **2:**527-535.

14. **Hunt M.D, G. S. Pettis, and M. A. McIntosh.** 1994. Promoter and operator determinants for fur-mediated iron regulation in the bi-directional fepA-fes control region of the *Escherichia coli* enterobactin gene system. Journal of Bacteriology. **176:**3944-55.

15. **Koster W. L., L. A. Actis, L. S. Waldbeser, M. E. Tolmasky, and J. H. Crosa.** 1991. Molecular characterization of the iron transport system mediated by the pJM1 plasmid in *Vibrio anguillarum* 775. Journal of Biological Chemistry. **266:**23829-23833.

16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

17. **Martinez, J. I., M. Herrero, and V. de Lorenzo.** 1994. The organization of intercistronic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the *iucABCD iutA* genes. Journal of Molecular Biology. **238:**288-93.

18. **Neilands J.B.** 1992. Mechanism and regulation of synthesis of aerobactin in *Escherichia coli* K12 (pColV-K30). [Review]. Canadian Journal of Microbiology. **38:**728-33.

19. **Salinas, P. C., M. E. Tolmasky, and J. H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: Evidence for a cooperative effect between two transcriptional activators. Proceedings of the National Acadamy of Sciences U. S. A. **86:**3529-3533

20. **Sanger, F., S. A. Nicklen, and R.Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Acadamy of Sciences U.S.A.. **74:**5463-5467.

21. **Schiewe, M., J. H. Crosa, and E. J. Ordal.** 1977. Deoxyribonucleic acid relationships among marine Vibrios pathogenic to fish. Canadian Journal of Microbiology. **23:**954-958.

22. **Svinarich D . M, and S. Palchaudhuri** 1992. Regulation of the SLT-1A toxin operon by a ferric uptake regulatory protein in toxinogenic strains of *Shigella dysenteriae* type 1. Journal of Diarrhoeal Diseases Research. **10:**139-45.

23. **Tolmasky M. E, and J. H. Crosa.** 1995. Iron transport genes of the pJM1-mediated iron uptake system of *Vibrio anguillarum* are included in a transposonlike structure. Plasmid. **33:**180-90.

24. **Tolmasky, M. E., L. A. Actis, A. Toranzo, J. Barja, and J. H. Crosa.** 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. Joournal of General Microbiology. **131:**1989-1997.

25. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular Cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. Journal of Bacteriology. **160:**860-866.

26. **Tolmasky, M. E., P. C. Salinas, L. A. Actis, and J. H. Crosa.** 1988. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum.* Infection and Immunity. **56:**1608-1614.

27. **Tolmasky, M. E., A. M. Wertheimer, L. A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* Gene: Role in Regulation of Expression of the FatA Outer Membrane Protein and Catechols. Journal of Bacteriology. **176:**213-220.

28. **von Gabain A, J. Belasco , J. Schottel , A. Chang , and S. Cohen.** 1983. Proceedings of the National Acadamy of Sciences U.S.A. **80:**653-657.

29. **Waldbeser, L. S., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1993. Mechanisms for negative regulation by iron of the fatA outer membrane protein gene expression in *Vibrio anguillarum* 775. Journal of Biological Chemistry. **268:**10433-9.

30. **Wang R, F., W.W. Cao, and M.G Johnson.** 1992. Biotechniques. **12:**702-704.

31. **Wertheimer, A. M., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1994. Structural and Functional Analyses of Mutant Fur Proteins with Impaired Regulatory Function. Journal of Bacteriology. **176:**5116-5122.

CHAPTER 4

Characterization of AngR, a Protein Essential for Regulation of Iron Transport Gene Expression and Siderophore Biosynthesis in *Vibrio anguillarum*

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ABSTRACT

AngR, a 110kDa protein, is encoded on the pJM1 virulence plasmid in *Vibrio anguillarum*. Earlier investigations revealed that AngR may play a role in both regulation of the expression of the iron transport genes *fatDCBA* as well as in the biosynthesis of the siderophore, anguibactin. To determine the functions associated with various domains of AngR, we introduced a panel of site-specific mutants of *angR* into an AngR deficient strain, H-775-3 (pJHC-T2612#4, (pJHC9-8), of *V. anguillarum*. This strain is impaired in both regulation of the iron transport gene expression as well as in anguibactin biosynthesis. The site-specific mutations were in predicted helix-turn-helix motifs or leucine zippers, or in motifs implicated to be essential for anguibactin biosynthesis. In this report, we present evidence that anguibactin production was significantly affected by every mutation we introduced into the *angR* gene. Only one mutation, occurring in the region encoding the first putative helix-turn-helix motif, results in both loss of regulation of iron transport gene expression and reduction in anguibactin production. Our evidence also shows that regulation may not require the entire AngR molecule, since two different truncated AngR molecules retain the ability to regulate iron transport gene expression. In addition, we have demonstrated that *angT* a gene downstream of *angR* encoding a protein with domain homology with thioesterases, is essential for anguibactin biosynthesis, as is AngR.

INTRODUCTION

The pathogenic bacterium *Vibrio anguillarum* is the causative agent of vibriosis, a fulminant septicemic disease in salmon. *V. anguillarum* requires the plasmid, pJM1 which encodes an active iron uptake system consisting of the siderophore anguibactin and the iron transport proteins Fat A, B, C, and D for virulence (1, 9). Synthesis of anguibactin requires expression of genes from the chromosome and the virulence plasmid pJM1. Anguibactin production increases when the available iron is limited and diminishes in iron rich growth conditions. Expression of the genes encoding the transport proteins Fat A, B, C, and D is also repressed under iron rich conditions. We have recently demonstrated that expression of the iron transport genes is negativly regulated by the chromosomally encoded Fur protein and a plasmid encoded antisense RNA, and is positively regulated by the plasmid mediated AngR protein and TAF products as well as by the siderophore anguibactin (6, 10, 22, 32, 33, 35, 38).

Recent evidence, based primarily on homology with other proteins, suggested that AngR may also be a biosynthetic enzyme in anguibactin biosynthesis (14, 28). AngR possesses domains such as several putative adenylation motifs including an ATP binding P-loop, and a substrate binding motif nearly matching the consensus for the 5' phosphopantethiene attachment motif. These domains are conserved among the enzymes involved in non-ribosomal peptide synthesis (13-15, 24, 27). Another protein classified as a non-ribosomal peptide synthetase, the high molecular weight protein 2 of *Yersinia enterocolitica*, shows homology to various regions of AngR (14). Non-ribosomal peptide synthetases generally catalyze two types of reactions, adenylation of their substrate and thioester formation. These enzymes are acid thiol ligases and are responsible for the activation reactions in the biosynthesis of enterobactin and the synthesis of tyrocidine, gramicidine S and penicillin (17, 18, 25, 26). There are six distinctive cores found in these enzymes which have been associated with the functions of adenylation, transpeptidation

and thioesterification (25). The possibility that AngR may posses a biosynthetic function as predicted from the presence of all six of the cores found in non-ribosomal peptide synthetases, was strengthened by the result that an AngR clone was able to complement an *Escherichia coli* mutant defective in the 2,3-dihydroxy benzoic acid (DHBA) adenylating enzyme EntE required for biosynthesis of the *E. coli* siderophore enterobactin (19). Anguibactin, like enterobactin possesses a DHBA moiety in addition to a catechol moiety (16). These results suggest that AngR may function by adenylating the anguibactin precursor 2,3-DHBA during anguibactin biosynthesis in *V. anguillarum* (28).

In order to determine which regions of AngR are required for the regulation of iron transport gene expression and anguibactin biosynthetic function, we performed complementation analysis of an AngR deficient mutant with plasmids carrying site-specific mutant derivatives of AngR at various predicted structural regions. Our results demonstrate that biosynthesis of anguibactin requires a fully functional AngR. However, the regulatory ability of AngR appears to be limited to discrete regions of the AngR protein. In addition, we demonstrate that *angT,* a gene found downstream of *angR,* shows homology with thioesterases and is essential for anguibactin biosynthesis.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *V. anguillarum* H775-3 (pJHC-T2612, pJHC9-8) is a strain harboring two plasmids, the recombinant clone pJHC-T2612 which possesses all the genes required for synthesis of anguibactin and the iron transport complex and pJHC9-8 which encodes the regulatory product(s) TAF which is essential for full expression of the iron uptake system (31). For maps see Table 1 and Figure 1.*V. anguillarum* (pJHC-T2612#4, pJHC9-8) harbors the mutant derivative pJHC-T2612#4 which has a Tn3Ho-Ho1 insertion within the *angR* gene. Plasmid pBluescript SK(+) (Stratagene) was used for cloning of DNA fragments forsite-directed mutagenesis and sequencing. *V. anguillarum* was cultured in either trypticase soy broth (or agar) supplemented with 1% NaCl (TSBS or TSAS respectively). For experiments determining iron uptake characteristics, the strains were first grown on TSAS supplemented with the appropriate antibiotics then passed to M9 minimal agar with the appropriate antibiotics. Cultures were then tested either on plates in bioassays or inoculated into M9 minimal medium supplemented with ethylenediamine-di-(*o*-hydroxyphenylacetic) acid (EDDA) for iron limiting conditions.

General DNA procedures. Plasmid DNA preparations were performed using the alkaline lysis method of Birnboim and Doly (2). Sequence quality plasmid DNA was generated using the appropriate Qiagen kits (Chatsworth , CA). Automated sequencing was performed by the Molecular Microbiology and Immunology (MMI) Core Facility on the PE/ABD377 DNA Sequencer using dye-terminator or dye-primer cycle sequencing chemistry or the A.L.F Pharmacia's fluorescent sequencer and either dye-primer sequencing with Sequenase, or cycle sequencing with Taq polymerase. Manual sequencing was performed by the dideoxy chain-termination method using the Sequenase Kit (US Biochemical, Cleveland, OH) again using appropriate primers. Primers were all

synthesized by the MMI Core Facility on a PE/ABD 394 automated synthesizer using standard phosphoramidite chemistry on polystyrene solid supports. DNA and protein sequence analysis were carried out at the NCBI using the BLAST network service, and also using the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (GCG). The GCG programs PILEUP and BESTFIT were used for comparisons of amino acid sequences.

Site directed mutagenesis. The *SalI-Eco*RI fragment containing the *angR* and *angT* genes from pJM1 was cloned into pBluescript SK(+) to generate pJHC-S2771 then mutagenized using the Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio-Rad Laboratories, Richmond, CA) and synthetic mutagenic oligonucleotides (Table 2). Site-specific mutations were confirmed by DNA sequencing with appropriate primers. In addition, the entire *angR* gene was sequenced for mutants 16.1, 16.5, 16.7 and 16.11 to verify that no other region of *angR* was affected during mutagenesis. Once mutated, the *SalI-Eco*RI fragments from each derivative were subcloned into pJHC-S100 to generate the plasmids carrying the *angR* derivatives listed in Table 1 and Table 2 and transferred by conjugation into H775-3(pJHC-T2612#4, pJHC9-8) as described previosly (31). Each transconjugant was tested for its MIC for the iron chelator EDDA, tested for anguibactin biosynthesis with bioassays, and for regulation of gene expression by using a ribonuclease protection assay, with the iron transport gene specific riboprobes.

Reverse transcription polymerase chain reaction (RT-PCR).
Total RNA from the parental strain 775 grown in M9 minimal medium supplemented with 10μM EDDA was harvested by a modified hot phenol method (34). 1 μg of RNA was used per reaction. Both single and two tube RT-PCR reactions were performed (37). Avian myeloblastosis virus (AMV) reverse transcriptase was used for synthesis of the complementary DNA which served directly as the template for PCR. Reaction conditions

were: 53°C for 30 minutes; 95°C for 5 minutes; 40 cycles of: 95°C for 10 sec, 53°C for 20 sec, 73°C for 1 min.; followed with a final extension at 73°C for 5 minutes.

The negative controls involved using RNA which had not been reverse transcribed with AMV or, when the single tube method was used, the reaction tube containing reaction buffer and enzymes [(*Thermus aquaticus* (Taq) DNA polymerase (Promega-Madison WI) and AMV(Gibco BRL-Gaithersburg, MD)] were heated to 100°C for 10 minutes. The reaction was cooled then the remainder of the reagents was added and cycling was performed. Products were electrophoresed on 0.7-1.0% agarose gels. In addition to confirming their predicted size, products were also sequenced to verify they contained *angT* or *angR*.

Detection of anguibactin and receptor activity. Bioassays were performed to determine whether the mutant *angR* constructs would complement the siderophore deficient phenotype of *V. anguillarum* (p2612#4,pJHC9-8). Eachsite-directed mutant, once subcloned into pJHC-S100, was conjugated into the H775-3(pJHC-T2612#4, pJHC9-8) background. Bioassays were carried out as described previously by using either culture supernatant or by testing the strains grown on a minimal medium plate and then patched onto the bioassay lawn (32). The bioassay lawn is composed of an overnight culture of receptor proficient but anguibactin deficient *V. anguillarum* (pJHC9-16) (36). As a negative control the receptor deficient and anguibactin deficient *V. anguillarum* (pJHC9-8) was used (36). The following strains were each tested on the same bioassay plate: the positive control, wild type *V. anguillarum* 775; the strain H775-3 (pJHC9-8, pJHC-T2612#4,pJHC-S2771) which contains the entire *angR* and *angT* region cloned into JHC-S100, which serves as the positive control for complentation of the AngR deficient strain; the negative control, the AngR deficient strain JHC9-8(pJHC-T2612#4); and each mutant . The plate was allowed to dry and incubated at 27°C overnight. In addition, each

mutant was tested for its ability to take up ferric siderophore. This "reverse" bioassay uses the mutant strain as the lawn to assess if the mutant lawn can be cross fed with ferric anguibactin.

For each mutant we determined the MIC for EDDA using liquid cultures of each mutant as previously described (31)

Ribonuclease (RNase) protection assays. Cultures were grown with EDDA supplemented to just below the MIC to achieve maximal iron limiting stress for each strain tested. Total RNA was prepared when the culture reached an OD_{600} of 0.4 to 0.6. by using the hot phenol method (34) and stored at -70°C until analyzed. Between 10-20 μg of RNA was used for the RNase protections. The RNA was annealed 8 to 16hrs at 43-45 C, with the *fatB* and *aroC* riboprobes (1-5 x10^5cpm α^{32}P). The *fatB*-specific riboprobe was made from plasmid pJHC-LW260 linearized with *Eco*RI and transcribed with T3 RNA polymerase. The *aroC* specific riboprobe was made from plasmid pQC3.5, linearized with RSAI and transcribed with T3 RNA polymerase. The hybridized RNA was subsequently digested for 30 minutes at 30°C with RNase A and T1, then digested with protenase K 30 minutes at 37°C, and treated with phenol chloroform prior to ethanol precipitation, and elecrophoresed on a standard sequencing gel. Gels were exposed for 1to 4hrs with intensifying screens at -70°C.

***In vitro* transcription-translation.** The proteins encoded by the *angR* derivatives were analyzed by coupled cell-free transcription translation(Amersham Corp, Arlington Heights, IL). The [^{35}S] methionine-labeled proteins were elecrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), the gel was dried and exposed to X-ray film.

RESULTS

AngR possesses essential motifs for non-ribosomal peptide synthetases.

Figure 2 illustrates the exact location as well as the distribution of the core sequences within gramicidin S (GrsA), AngR, HMWP2, and EntF. AngR has homology with all six core sequences found in non-ribosomal peptide synthetases, although there is a discrepancy in the position of a serine in core 6, in the AngR motif, the serine is four amino acids earlier than in the other enzymes. Biochemical and genetic analyses of the gramicidin S synthetase (Figure 2, line 1,GrsA) revealed that cores 1 through 5 are required for adenylation , cores 2, 3, and 5 are involved in ATP binding and core 4 has an ATPase motif, and core 6 is the 4' phosphopantetheine binding site involved in thioester formation (25). Figure 2 illustrates the core motifs found in the high molecular weight protein 2 (HMWP 2) from *Yersinia entercolitica*, e.g., AngR is remarkably similar to HMWP 2 , as well as the EntF, which is involved in serine activation during biosynthesis of the *E. coli* siderophore enterobactin (20).

We have previously demonstrated that AngR can complement *E.coli* EntE which is the enzyme involved in adenylating 2,3-dihydroxybenzoic acid (DHBA) that is then incorporated into enterobactin (19). EntE is not shown in Figure 2 as EntE is strictly an AMP-ligase, only possessing the first 4 motifs. EntE is 536 amino acid residues and lacks cores 5 and 6. We hypothesize that AngR may serve a similar function as EntE but since AngR does have cores 5 and 6, we suspect that AngR may also be involved in thioesterification.

Site-specific mutations affect biosynthesis of anguibactin and MIC for EDDA. We performed bioassays on the strains containing each of the 13 site-specific AngR mutants to determine whether or not they could synthesize anguibactin. In designing the mutations we chose to introduce amino acids that are predicted to alter the

structure of a given region. Figure 3 shows a schematic diagram of AngR depicting potential regulatory and biosynthetic regions and identifies the location of each mutation. The non-ribosomal peptide synthetase motifs occur starting at amino acid 500 of AngR and continue toward the carboxy terminus.

Each strain was grown in minimal medium supplemented with 1-2 μM EDDA. Anguibactin production was measured by using a bioassay as described in the Materials and Methods section. Mutation of residue 267 of AngR, modulated anguibactin production in the following ways: when histidine (H) 267 was replaced with asparagine (N), anguibactin levels increased, when H_{267} was replaced with glutamine (Q), anguibactin levels decreased below that of the wild type derivative with H_{267}, although glutamine is quite similar to asparagine, simply one methylene group shorter. All the other mutants had no measurable anguibactin production as reported in Table 2. Table 2 also shows the minimal inhibitory concentration (MIC) for EDDA for each mutant strain. In order to assess the ability of the strain to survive in iron limiting conditions, we determined the MIC in the presence of EDDA. Survival in elevated levels of the chelator EDDA correlates with the strains ability to produce and/or uptake siderophore (9). Each mutant as well as wild type 775 and H775-3 (pJHC9-8) (pJHC-T2612#4) (pJHC-S2771), and H775-3 (pJHC9-8) (pJHC-T2612#4) was cultured in minimal medium to an OD_{600} of 2.0, sub-cultured at 1:50 into various concentrations of EDDA and grown overnight at 27°C. These results indicate (Table 2) that all mutants except those at H_{267} have very similar low MIC in EDDA, within 1μM of each other. As expected, mutation at H_{267} results in a very dramatic change in the MIC for EDDA which direclty correlates to the anguibactin production levels detected using the bioassay (Table 2).

In order to rule out a deficiency in the uptake of siderophore resulting in a low MIC in EDDA, we assayed the ability of each mutant to internalize the siderophore anguibactin. Each mutant was grown in minimal medium overnight at 27°C. The cultures were each

used to inoculate separate M9 plates supplemented with 10μM EDDA. All mutants were able to be cross fed when supplemented with a disc containing 5 μl of anguibactin.

In order to verify that plasmids containing the site-specific mutations in *angR* were indeed synthesizing the AngR protein, we performed *in vitro* coupled transcription-translation. Figure 4 illustrates that all ten of the mutants selected synthesized a protein of approximately 110 kDa, or slightly less in the case of mutant 16.3 which is a frame shift mutant. Furthermore mutants 16.1, 16.5, 16.7, and mutant 16.11 were also sequenced, verifying that no other nucleotide alterations had occurred during mutagenesis.

Effect of site-specific mutations and truncation derivatives of AngR on the regulation of the expression of iron transport genes. Our earlier research demonstrated that both *fatB* and *fatA* are up regulated in the presence of functional AngR in iron limiting conditions (7). RNase protection assays were used to assess the effect of each AngR mutation on the regulation of the iron transport genes. Each site-specific mutantation within *angR* was tested for its ability to complement the *angR* deficiient stratin. The *angR* deficient strains, carrying the recombinant clones encoding the site-specific *angR* mutants to be tested for complentation, were grown in minimal medium at the maximum iron limitation (based on the MIC of the mutant). The construct pJHC-LW260 served as a template for the *fatB* specific riboprobe synthesis using T3 RNA polymerase. The construct pQC3.5 served as a template for the *aroC*-specific riboprobe synthesis using T3 RNA polymerase. The *aroC* mRNA is constitutively expressed in *V. anguillarum* and is an internal control. Figure 5 illustrates the results from the ribonuclease protection assays detecting *fatB* specific transcripts using the *fatB* specific riboprobe. These results show that only mutant 16.5, Q_{304} to P_{304}, has a significant decrease in the level of the *fatB* -specific transcript. All other mutants contain high levels of *fatB* transcript. The negative control H775-3 (pJHC9-8) (pJHC-T2612#4) shows a minimal level of *fatB* transcript, while the positive control H775-3 (pJHC9-8 (pJHC-T2612)

shows wild type transcription. These results are consistant with the mutation of the Q_{304} to P_{304} (#16.5) introducing a putative helix breaking proline within the recognition helix of the first predicted helix-turn-helix of AngR leading to a decrease in the regulation of *fatB* expression, in addition to affecting anguibactin production. None of the other anguibactin synthesis-defective mutants caused a decrease in the *fatB*-specific transcript, suggesting that these regions are not required for the regulation of the iron transport genes. Even mutant 16.3, the frame shift mutant at position H_{888}, retained full regulatory activity, although it lost the ability to synthesize anguibactin. These findings, together with the observation that all the biosynthetic motifs occur after amino acid 500, led us to hypothesize that regulation might occur in a truncated AngR molecule that retained only the first predicted regulatory features localized in the N-terminal 500 amino acids. To test this hypothesis we used plasmid pJHC-S2570 which contains a truncated *angR* encoding for a 60 kDa AngR protein (22). This construct was generated by modifying the *Nco*I site within the *angR* cloned from the *V. anguillarum* strain 531A, with Klenow fragment of DNA polymerase I and then subcloning the modified *angR* behind the strong pTac promoter in pKK223-3. The wild type *angR* from 531A (which is identical to *angR* except that the gene encodes the AngR which has the H_{267} changed to N_{267}) was similarly subcloned (pJHC-S2572), this construct served as the wild-type control (Figure 1) (22). This truncation preserves the first helix-turn-helix and leucine zipper in the amino terminus of AngR, and no longer encodes the predicted biosynthetic motifs.

In order to examine these constructs in the same background as the other site-specific mutants, we conjugated each plasmid into the *V. anguillarum* 775-type AngR deficient strain, H775-3 (pJHC-T2612#4) (pJHC9-8), (Figure 1). Each strain was tested for its MIC for EDDA as well as its ability to regulate the *fatB* transcript. The strain containing pJHC-S2572 had the expected MIC of 10 μM, while the strain harboring the truncated 60 kDa AngR construct, pJHC-S2570 could not survive any addition of EDDA. These results are consistant with our earlier analysis of these constructs (22). The ribonuclease

protection assay revealed that the truncated AngR retained the ability to regulate *fatB* to similar levels (Figure 6) as the wildtype. These results, suggest that the truncated AngR protein which contains the DNA region encoding the predicted helix-turn-helix motif, is sufficient for regulation. However, its biosynthetic activity is lost as determined by the MIC for EDDA and anguibactin bioassay.

AngT is required for biosynthesis of the siderophore anguibactin.

Earlier work identified a open reading frame, ORF6, immediately downstream of the *angR* gene, which shows significant homology with two eukaryotic S-acyl fatty acid synthase thioesterases (12). The translated protein product of ORF6 is predicted to be 28kDa (12). We hypothesized at that time that ORF6, now designated *angT* may play a role in anguibactin biosynthesis (12). In order to determine if AngT is indeed involved in biosynthesis, we generated a deletion derivative of pJHC-S2771 called pMET26. This clone has the *angT* open reading frame deleted yet it preserves the stop codon in the *angR* transcript. This construct was conjugated into the siderophore deficient/regulation deficient recipient strain, *V. anguillarum* (pJHC9-8, pJHC-T2612#4). The transconjugant was assayed for its ability to synthesize anguibactin. The bioassay was negative but regulation was restored to the recipient strain (Table 2, and data not shown). RT-PCR was used to detect a transcript spanning *angR* and *angT*. We used a primer in *angT* and one in *angR* and we were able to amplify a 300bp fragment linking the downstream region of *angR* with the 5' region of *angT* (Figure 7).

DISCUSSION

The AngR protein has features consistent with roles in both anguibactin production as well as regulation of gene expression. In this work we used site-directed mutagenesis and truncation of the AngR protein in order to dissect these two distinct functions. It is likely that the regulatory functions of AngR depend on the characteristic structural domains such as helix-turn-helix motifs, while the biosynthetic functions may require not only the 6 core motifs but the overall structure of AngR.

Based on the crystallographic structure and chemical analysis of anguibactin (1, 16), we predict that anguibactin is synthesized from the enzymatic modification of 2,3-DHBA, cysteine and histamine. Recent investigations support this hypothesis as both 2,3-DHBA and histamine are required for biosynthesis of anguibactin (5, 29). The analysis of the structural formula of anguibactin shows imidizols consistant with a modified cystein moiety (16). 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 non-ribosomal peptide synthetase mechanism (25, 26). Both enterobactin, a siderophore and gramicidin S, an antibiotic, are compounds which are the result of condensation of hydroxy acids and amino acids via this type of synthetic mechanism (25). Non-ribosomal peptide synthesis occurs by a multi-step process of adenylation, thioesterification and sometimes racemization or N-methylation of each amino acid or hydroxy acid creating various peptide structures (17, 25, 26). This process involves distinct domains of the biosynthetic enzyme or enzymes that catalyze the activation of constituent amino acids as acyladenylates and thioesterify the activated amino acids through a covalent interaction with specific thiol groups (26). Recent biochemical data suggest that elongation of the peptide (transpeptidation) occurs via multiple co-factors of 4' phosphopantetheine which are covalently bound to the carboxy terminal region of each amino acid activating domain (Figure 8). It is currently believed that these cofactors covalently attach to a conserved

serine residue located within the thioester formation module, core 6 (Figure 2). During this elongation, reaction intermediate peptides remain covalently attached to their specific sites. Termination of non-ribosomal peptide synthesis includes release of the thioester bound peptide from the enzyme complex either by cyclization, the action of thioesterase, or by transferring the peptide chain to a functional group such as a phosopholipid (26). It is possible that anguibactin is produced by non-ribosomal 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 may occur via a thioesterase.

It has previously been suggested by sequence alignment that AngR possesses both the adenylation motifs as well as the motifs associated with thioester formation in non-ribosomal peptide synthetases (14). Figure 2 shows motifs 1-5, involved in adenylation, and motif 6, the motif involved in co-factor binding and thioesterification, as determined for gramicidin synthetase (25). AngR shares homology with all 6 motifs, as does EntF and the HMWP 2 of *Yersinia enterocolitica.* EntF has been demonstrated to be a non-ribosomal peptide synthetase involved in serine activation during synthesis of enterobactin in *E. coli.* The HMWP2 of *Yersinia* is also included as it shares remarkable homology with AngR, although its function is unknown.

Our genetic evidence indicates that AngR may serve a role as an adenylating enzyme similar to EntE from *E.coli* by activating a DHBA molecule in anguibactin biosynthesis (28). Furthermore, AngR, has both core 5 and core 6 (implicated in thioesterification) unlike EntE (25). AngR may be involved in incorporation of the activated hydroxy acid into the growing anguibactin molecule. However, as shown in Figure 2, the highly conserved serine residue which is the actual binding site for the phosphopantetheine (25), is not present in the AngR core 6. This core in AngR does however have a serine four amino acids prior to that site (Figure 2). It is of interest that the *angR* gene is followed by a required gene *angT*, which is also essential for anguibactin biosynthesis (Table 2). We

hypothesize that *angT* encodes a thioesterase based on sequence homology. Thioesterase genes are also essential in other non-ribosomal peptide synthetase systems, and are generally found contiguous with the synthetase genes. Our current work reveals that AngT may indeed serve as a thioesterase releasing anguibactin from the biosynthetic complex (25). *angR* and *angT* are also contiguous as reported in this work (Figure 7).

In addition to the biosynthetic motifs shown in Figure 2, AngR also 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 *V. anguillarum* (pJHC9-8, pJHC-T2612#4), suggesting a regulatory function for AngR (7). In this report, using a panel of site-specific mutants, we demonstrate that several regions are required for biosynthesis of anguibactin, yet only one specific region was found to affect regulation. One mutant, 16.5, containing a proline in place of a glutamine in the predicted helix-turn-helix, lost the ability to regulate *fatB*. The data suggest that the predicted helix-turn-helix motif is critical in the regulatory functions associated with AngR, although, this mutation also led to the loss of anguibactin biosynthesis. Eventhough only some of the mutations were specifically in predicted biosynthesis domains of *angR*, all (except 16.9) had diminished biosynthetic activity. Truncation of the AngR molecule by modifying the *Nco*I restriction site resulted in an approximately 60 kDa product (21). This derivative in which all the biosynthetic motifs were missing also lost biosynthetic function.

The 60 kDa truncated AngR molecule however, retained its ability to positively regulate the iron transport genes. Therefore, AngR plays a critical role in both the biosynthesis of anguibactin as well as 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 protein AngR.

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LITERATURE CITED

1. **Actis, L. A., W. Fish, J. H. Crosa, K. Kellerman, S. R. Ellenberger, F. M. Jauser, and J. Sanders-Loehr.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). Journal Bacteriology. **167:**57-65.
2. **Birnboim H, C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research. **7:**1513-1523.
3. **Bolivar F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene. **4:**121-126.
4. **Boyer, H., and D. Roulland-Doussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* Journal of Molecular Biology. **41:**459-472.
5. **Chen Q, L. A. Actis, M. E. Tolmasky, and J. H. Crosa.** 1994. Chromosome-mediated 2,3-dihydroxybenzoic acid is a precursor in the biosynthesis of the plasmid-mediated siderophore anguibactin in *Vibrio anguillarum.* Journal of Bacteriology. **176:**4226-34.
6. **Chen, Q., and J. H. Crosa.** 1996. Antisense RNA, Fur, iron, and the regulation of iron transport genes in *Vibrio anguillarum.* Journal of Biological Chemistry. **271:**1885-91.
7. **Chen, Q., A. M. Wertheimer, M. E. Tolmasky , and J. H. Crosa.** 1996. The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum.* Molecular Microbiology. **22:**127-134.

7b **Crecy-Lagard, V., V. Blanc, P. Gil, L. Naudin, S. Lornzon, A. Famechon, N. *Bam*as-Jacques, J. Crouzet, and D. Thibaut.** 1997 Pristinamycin I Biosynthesis in *Streptomyces pristinaespiralis*: Molecular Characterization of the First Two Structural Peptide Synthetase Genes. Journal of Bacteriology. **179**:705-713.

8. **Crosa, J. H., M. H. Schiewe, and S. Falkow.** 1977. Evidence for plasmid contribution to the virulence of fish pathogen *Vibrio anguillarum.* Infection and Immunity. **27:**509-513.

9. **Crosa, J. H.** 1984. The relationship of plasmid mediated iron transport and bacterial virulence. Annual Review of Microbiology. **38:**69-89.

10. **Crosa, J.H.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as a repressor. Gene. **123:**33-8.

11. **Crosa, J. H., L. Hodges, and M. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen Vibrio anguillarum. Infection and Immunity. **27:**897-902.

12. **Farrrell, D. H., P. Mikesell, L. A. Actis, and J. H. Crosa.** 1990. A regulatory gene, *angR*, of the iron uptake system of *Vibrio anguillarum*: similarity with phage P22 *cro* and regulation by iron. Gene. **86:**45-51.

13. **Fuma, S., Y. Fujishima, N. Corbell, C. D'Souza, M. Nakano, P. Zuber, and K. Yamane.** 1993. Nucleotide sequence of 5' portion of *sfrA* that contains the region for competence establishment in *Bacillus subtilis.* Nucleic Acids Research. **21:**93-97.

14. **Guilvout, I., O. Mercereau-Puijalon, S. Bonnefoy, A. Pugsley, and E. Carniel.** 1993. High molecular weight protein 2 of *Yersinia entercolitica* is homologous to AngR of *Vibrio anguillarum* and belongs to a family of proteins involved in nonribosomal peptide synthesis. Journal of Bacteriology. **175:**5488-5504.

15. **Heaton, M. P.** 1992. Biosynthesis of D-Alanyl-Lipoteichoic Acid: Cloning, Nucleotide Sequence, and Expression of the *Lactobacillus casei* Gene for the D-Alanine-Activating Enzyme. Journal of Bacteriology. **174:**4707-4717.

16. **Jalal, M., D. Hossain, J. van der Helm, J. Sanders-Loehr., L. A. Actis, and J. H. Crosa.** 1989. Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. Journal of the American Chemical Society **111:**292-296.

17. **Marahiel M.A.** 1992. [Molecular biology and regulatory mechanisms of antibiotic production in Bacillus]. [Review] [German] Molekularbiologie und Regulationsmechanismen der Antibiotikaproduktion in Bacillus. Naturwissenschaften. **79:**202-12.

18. **Merriman, T. R., M. E. Merriman, and I. L. Lamont.** 1995. Nucleotide sequence of *pvdD*, a pyoverdin biosynthesis gene from *Pseudomas aeruginosa*: *pvdD* has similarity to peptide synthetases. Journal of Bacteriology. **177:**252-258.

19. **Rusnak F, W. S. Faraci , and C.T. Walsh.** 1989. Subcloning, expression, and purification of the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP ligase: demonstration of enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. Biochemistry. **28:**6827-35.

20. **Rusnak F, M. Sakaitani , D. Drueckhammer , J. Reichert, and C. T. Walsh.** 1991. Biosynthesis of the *Escherichia coli* siderophore enterobactin: sequence of the *entF* gene, expression and purification of EntF, and analysis of covalent phosphopantetheine. Biochemistry. **30:**2916-27.

21. **Salinas, P., L. S. Waldbeser, and J. H. Crosa.** 1993. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as repressor. Gene. **123:**33-38.

22. **Salinas, P. C., M. E. Tolmasky, and J. H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: Evidence for a cooperative effect

between two transcriptional activators. Proceedings of the National Acadamy of Science U.S.A.Proceedings of the National Acadamy of Science U.S.A.. **86:**3529-3533.

23. **Sanger , F., S. Nicklen , and R. A. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Acadamy of Science U.S.A.. **74:**5463-5467.
24. **Scholten, J. D., K. Chang, P. Babbit, H. Charest, M. Sylvestre, and D. Dunaway-Maiano.** 1991. Novel enzymatic hydrolytic dehalogenation of a chlorinated aromatic. Science. **253:**182-185.
25. **Stachelhaus, T., and M. A. Marahiel.** 1995. Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. Journal of Biological Chemistry. **270:**6163-9.
26. **Stachelhaus, T., and M. A. Marahiel.** 1995. Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. FEMS Microbiology Letters. **125:**3-14.
27. **Toh, H.** 1991. Sequence analysis of firefly luciferase family reveals a conservative sequence motif. Protein Seq. Data Anal. **4:**111-117.
28. **Tolmasky, M. E., L. A. Actis , and J. H. Crosa** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyper production of anguibactin. Infection and Immunity. **61:**3228-3233.
29. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1994. A histidine decarboxylase gene encoded by the *Vibrio anguillarum* plasmid pJM1: histamine is a precursor in the biosynthesis of anguibactin. Molecular Microbiology.
30. **Tolmasky, M. E., L. A. Actis, A. Toranzo, J. Barja, and J. H. Crosa.** 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. Journal of General Microbiology. **131:**1989-1997.

31. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular Cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. Journal of Bacteriology. **160:**860-866.

32. **Tolmasky, M. E., P. C. Salinas, L. A. Actis, and J. H. Crosa.** 1988. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum.* Infection and Immunity. **56:**1608-1614.

33. **Tolmasky, M. E., A. M. Wertheimer, L. A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* Gene: Role in Regulation of Expression of the FatA Outer Membrane Protein and Catechols. Journal of Bacteriology. **176:**213-220.

34. **von Gabain, A., J. Belasco, J. Schottel, A. Chang, and S. Cohen.** 1983. Proceedings of the National Acadamy of Science U.S.A. **80:**653-657.

35. **Waldbeser, L. S., M. E Tolmasky, L. A. Actis, and J. H. Crosa.** 1993. Mechanisms for negative regulation by iron of the FatA outer membrane protein gene expression in *Vibrio anguillarum* 775. Journal of Biological Chemistry. **268:**10433-9.

36. **Walter, M., S. Potter, and J. H. Crosa.** 1983. Iron uptake system mediated by *Vibrio anguillarum* plasmid pJM1. Journal of Bacteriology. **156:**880-887.

37. **Wang R, F., W. W. Cao, and M. G. Johnson.** 1992. Biotechniques. **12:**702-704.

38. **Wertheimer, A. M., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1994. Structural and Functional Analyses of Mutant Fur Proteins with Impaired Regulatory Function. Journal of Bacteriology. **176:**5116-5122.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype	Source or reference
Bacterial strains:		
Escherichia coli		
XL1 blue	*recA1, endA1, gyrA46, thi, hsdR17, supE44, relA, lac* F' [*proAB*$^{+}$, *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^{r})]	Stratagene La Jolla, CA
HB1O1	*supE44, hsdS20,(r_B^-,m_B^-), rec13, ara-14,proA2, lacyI, galK2, rpsL20 , xyl-5, mtl-1*	(4)
JM109	*recA1, endA1, gyrA96, thi*$^{-}$Δ *hsdR17, supE44, relA1, (lac-proAB*$^{+}$*),* [F' *traD36, proAB, lacI*^{q} *lacZ*ΔM15]	(23)
Vibrio anguillarum		
531A(pJM1-like)	wild type, Atlantic prototype	(8)
775(pJM1)	wild type, prototype, (pJM1).	(8)
H775-3	plasmidless derivative of 775.	(11)
Plasmids:		
pJM1	natural isolate, endogenous plasmid in strain 775	(8)
pJHC-S100	cloning vector.	(22)
pBluescript SK+	cloning vector.	Statagene La Jolla CA
pVK102	cloning vector.	(3)
pJHC-T2612	recombinant clone carrying the iron uptake region of pJM1 cloned in pVK102.	(30)
pJHC-T2612#4	recombinant clone pJHC-T2612 with Tn3::-HoHo1 insertion in *angR* iron uptake deficient, deficient in AngR.	(30)
pJHC9-8	pJM1 derivative carrying only the TAF region.	(31)
pJHC9-16	pJM1 derivative carrying the iron transport region, deficient in siderophore production but iron uptake proficient.	(36)
pJHC-S2771	*angR*$_{775}$ cloned in pBR325km.	(22)
pJHC-S2572	*angR* 531A gene cloned at *Ava*I and *Eco*RI into pKK223-3.	(22)
pJHC-S2570	*angR* 531Agene with *Nco*I modification cloned at *Ava*I and *Eco*RI into pKK223-3.	(22)
pJHC-LW260	191 bp ThaI-RsaI fragment of the *fatB* region cloned in pBluescript-II SK+.	(35)
PQC3.5	420bp *Sal*I-*Cla*I fragment of the *aroC* gene cloned in pBluescript-II SK+.	(7)

Table 2. [1] *V. anguillarum* strains harbor pJHC9-8 and pJHC-T2612#4 in addition to the indicated recombinant clones. [2]Bioassay results reported measurable anguibactin production measured as detailed in the Material and Methods section as: Neg., no measurable anguibactin production, (Δ), extremely low levels, (+) low level , (++) wild type production, (+++++) hyper-production. [3]MIC for EDDA was determined by bioassay as described in Materials and Methods. [4]Iron uptake proficiency was determined by bioassay as described in Materials and Methods. A (~) following a constructs indicates the construct was created previously(18), or (~~) indicated the construct was created and partially analyzed (23). (n/d) indicates the particular assay was not done.

Table 2: Properties of the *angR* mutants

Recombinant	mutation Plasmid[1]	aa# residue	domain location	Bioassay[2]	MIC for (EDDAμM)[3]	IUP[4]
pMET16.1~~	H to L CAT to CTT	267		Δ	2.5	+
pMET16.2	T to P ACT to CCT	888	2nd HTH	Δ	2.5	+
pMET16.3~~	T to P CAT to ACCT	888	2nd HTH FRAMECHANGE	Δ	2.5	+
pMET16.4	L to P CTA to CCA	889	2nd HTH	Δ	2.5	+
pMET16.5	Q to P CAG to CCG	304	1st HTH	Neg	2.5	+
pMET16.6	V to D GTT to GAT	151	1st LZ	Neg	2.5	+
	L to R CTT to CGT	158				
pMET16.7	V to K GTC- to AAG	852	2ndLZ	Δ	2.5	+
pMET16.8	I to P ATT to CCT	859	2ndLZ	Δ	2.5	+
pMET16.9~~	H to N CAT to AAT	267		+++++	20	+
pMET16.10~~	H to Q CAT to CAA	267		+	5	+
pAW16.11	K to G AAG to GGA	609	p loop	Δ	2.5	+
pAW16.12	K to F AAG to GAC	609	p loop	Δ	2.5	+
pAW16.13	V to L GTT to CTT	151	1st LZ	Δ	n/d	n/d
pJHC-S2771~			*angR* from pJM1	++	10	+
pMET26			Δ*angT*	Δ	2.5	+
pJHC-S2572~			*angR* from pJHC1	n/d	20	n/d
pJHC-S2570~			*angR* from pJHC1*Nco*I*	n/d	0	n/d

Figure 1: Genetic and physical map of plasmids used in this work. Restriction sites are as follows: X, *Xho*I; E, *Eco*RI; S, *Sal*I; A, *Ava*I; N*, *Nco*I-site modified with the Klenow fragment of DNA polymerase I. The vector DNA is represented as either hatched marks for pVK102, slim lines for pBR325, and darker lines for pKK223-3. The insert DNA from pJM1 is represented by thick lines, the speckled lines represent insert DNA from pJHC1 DNA. The dark circle symbolizes the Tn3::HoHo 1 insertion mutant #4. The arrows indicate the specific genes and their direction of transcription.

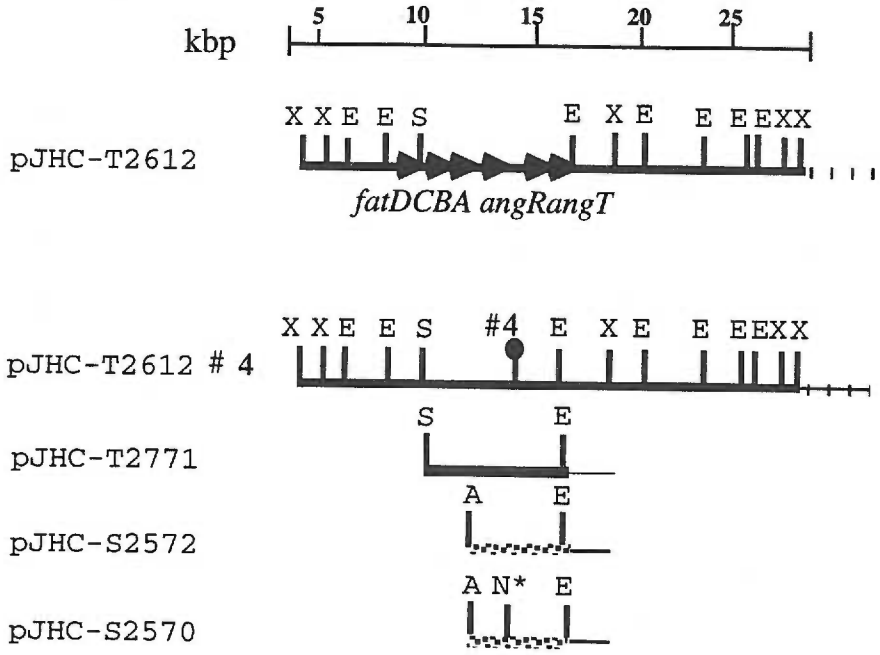

kbp
5
10
15
20
25
pJHC-T2612
X X E E S
E X E
E E E X X
fatDCBA angRangT
pJHC-T2612 # 4
X X E E S
#4
E X E
E E E X X
pJHC-T2771
S
E
pJHC-S2572
A
E
pJHC-S2570
A N*
E

Figure 2: Homology alignments for the non-ribosomal peptide synthetase core motifs. The top line shows the consensus sequence derived from alignments from gramicidin S (GrsA) from *Bacillus brevis* (25). The next lines align the motifs found within AngR from *V. anguillarum*, HMWP 2 from *Yersinia enterocolitica*, and EntF from *E. coli*. Biochemical and genetic analyses of the gramicidin S revealed that, cores 1-5 are required for adenylation: cores 2,3, and 5, ATP binding and core 4 an ATPase motif, and core 6 is involved in thioester formation (4' phosphopantetheine binding site). Identical amino acids, compared with GrsA , are in bold type.

Core Motif comparison.

	CORE 1	CORE 2	CORE 3	CORE 4	CORE 5
GrsA	**LKAGGAYVPID**	**YSGT TGXPKGV**	**GELCIGGXGXARGYL**	**YXTGD**	**VKIRGXRIELGEIE**
AngR	**LYAGAIYVPVS**	**YTSGSTGTPKGV**	**GELWIGGDGIALGYF**	**YRTGD**	**VKVGGYRIELGEIE**
HmwP	**LLAGAVYPVS**	**YTSGSTGTPKGV**	**GELWIGGIGVAEGYF**	**YRTGD**	**VKVGGYRIELGEIES**
EntF	**VEAGAAWLPLD**	**FTSGSTGRPKGV**	**GDLYLTGIQLAQGYL**	**YRTGD**	**LKIRGQRIELGEIDR**

Adenylation

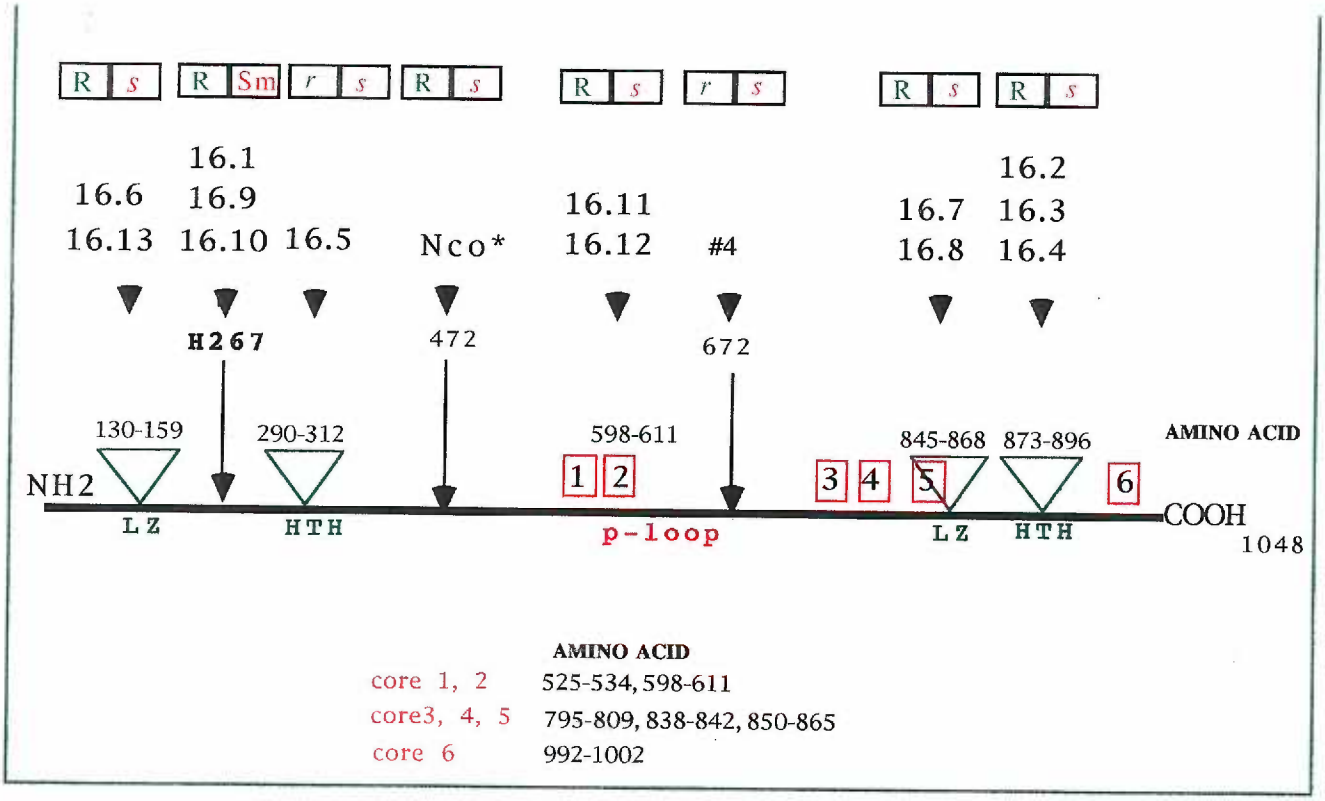
	CORE6
GrsA	**DNFYXLGGHSL**
AngR	**DFFLS GGDAYNA**
HmwP	**DFFQQ GGHSLLA**
EntF	**DFFAL GGHSLLA**

Thioester formation

AMINO ACID RESIDUES IN AngR

core 1, 2	525-534, 598-611
core 3, 4, 5	795-809, 828-842, 850-865
core 6	992-1002

Figure 3: An illustration showing the location and properties of *angR* mutants. A linear representation illustrating: regions of site-specific mutations, restriction site modification (*Nco** with arrow), and transposon insertion (#4 with arrow). Triangles indicate that the region has structures predicted to be involved in regulation. Cubes indicate regions where the core motifs found in biosynthetic enzymes are located. The boxes above each mutant indicate that the derivative is wild type for regulation (R) or siderophore biosynthesis (S) or that the mutations resulted in a loss of biosynthesis (s) or loss of regulation functions (r). (Sm) indicates that anguibactin synthesis was increased or decreased depending on the substitution at this site. The amino acids corresponding to the predicted domain are numberd above the triangle or arrow. LZ, represents predicted leucine zipper, HTH represents predicted helix-turn-helix, P-loop represents the predicted ATP binding site. The experimental designation of the mutant is listed above the black triangles.



AMINO ACID	
core 1, 2	525-534, 598-611
core3, 4, 5	795-809, 838-842, 850-865
core 6	992-1002

Figure 4: Analysis of the synthesis of the AngR protein in ten of the AngR mutants. SDS -PAGE of polypeptides synthesized using coupled cell-free transcription-translation assays of recombinant clones harboring the mutated *angR* gene derivatives. Numbers on top of lanes refer to pMET16.n recombinant clones. Numbers 25 and 27 stand for the controls pJHC-S2551 and pJHC-S2751 carrying the *angR531A* and *angR775* genes respectively. BR represents the vector pJHC-S100(22). The electrophoretic mobility (in kDa) of rabbit muscle phosphorylase b (97.4), bovine serum albumin (66.2) and hen egg white ovalbumin (45) used as markers are indicated on the right margin. Tr stands for truncated AngR, when the frame shift at amino acid 888 is introduced in mutant 16.3 a trucation is predicted.

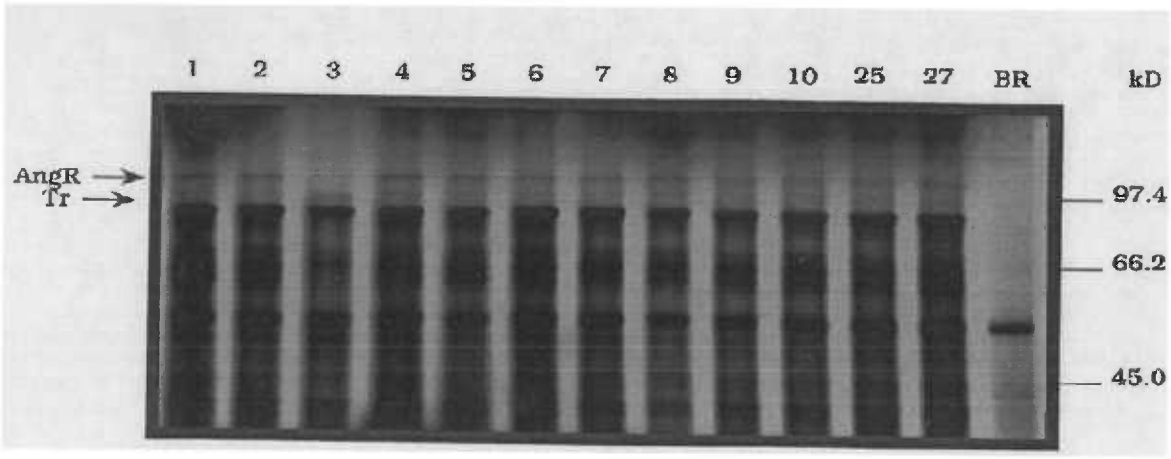
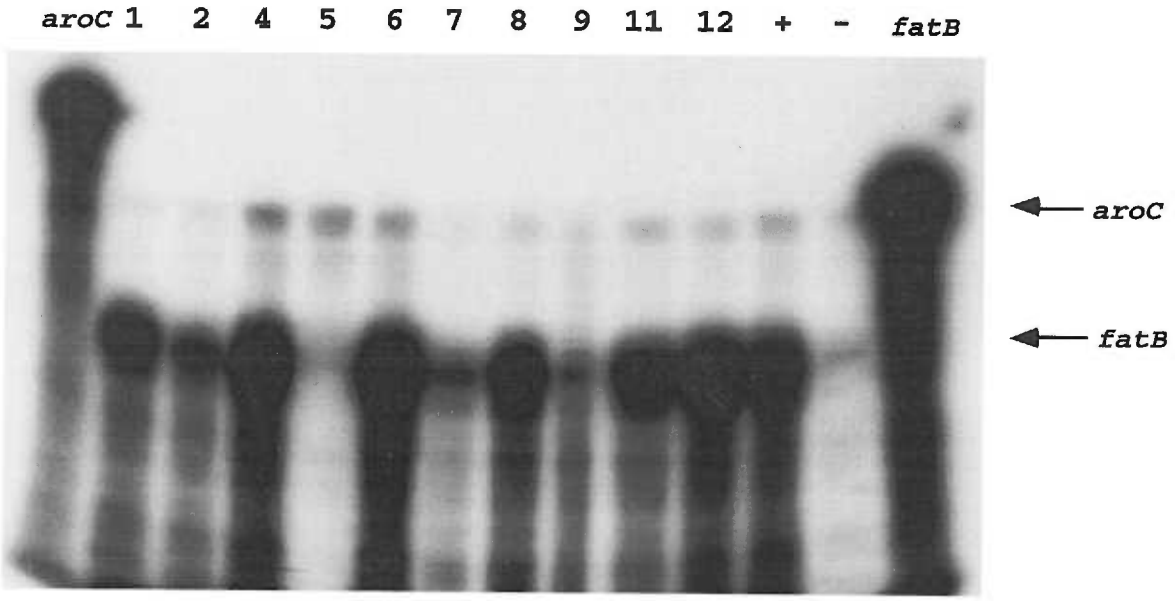

1
2
3
4
5
6
7
8
9
10
25
27
BR
kD
AngR
Tr
97.4
66.2
45.0

Figure 5: Regulation of iron transport gene expression by site-specific mutants of *angR*. Total RNA was harvested from *V. anguillarum* H775-3 strains harboring various recombinant clones grown under iron-limiting conditions. Strains were grown under their respective MIC for EDDA to achieve maximal iron stress for each strain.: bioassay deficient mutants, 2.5μM EDDA; bioassay proficient mutants, 5 and 10μM EDDA. Specific transcripts for *fatB* and *aroC* are detected by RNAse protection using riboprobes for specifc recognition of transcripts in the *fatB* or *aroC* region respectively. The *aroC* mRNA is constitutively expressed in *V. anguillarum* and is an internal control. For Panels A and Panels B: RNA obtained from the wild type (WT) harboring clones pJHC-T2612 and pJHC9-8 is indicated with a plus sign (+), while the negative control carrying clone pJHC-T2612#4 and pJHC9-8 is indicated with a minus sign (-) above the lane. Lanes marked *aroC* and *fatB* are the free riboprobe without RNase treatment. Regulation by site-directed mutants 16.1-16.12 (Panel A) and 16.3, 16.9, and 19.10 (Panel B). Lanes numbered 1-12 represent the mutant construct clone, all clones are in the *V. anguillarum* strain H775-3 with pJHC-T2612#4 and pJHC9-8. Lane marked +/ Fe is the positive control grown in iron supplemented condions to illustrate that *fatB* is not expressed. The *fatB* and *aroC* transcripts are indicated by arrows.

A


aroC 1 2 4 5 6 7 8 9 11 12 + - fatB
aroC
fatB

B

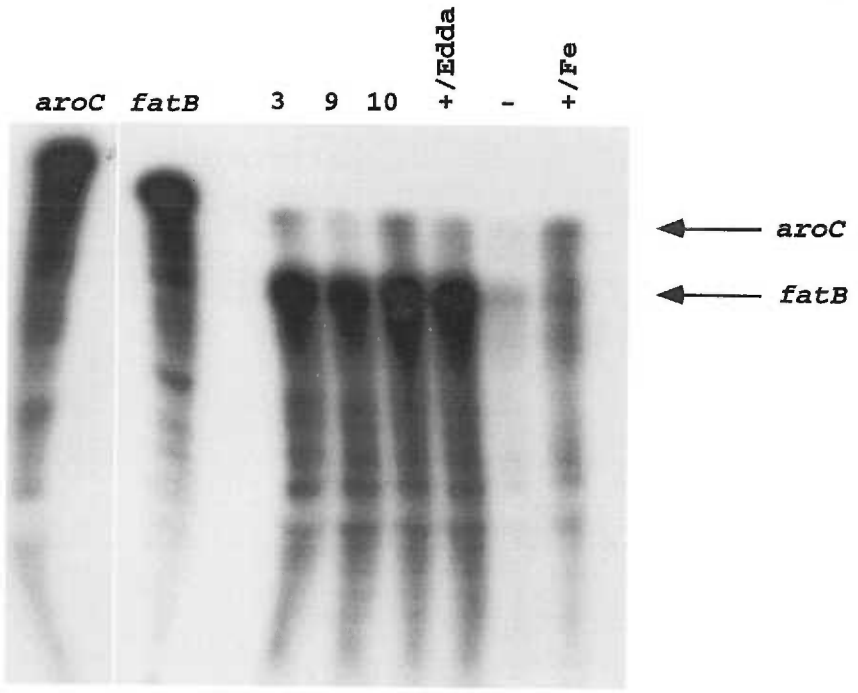

aroC fatB 3 9 10 +/Edda - +/Fe
aroC
fatB

Figure 6: Regulation of iron-transport gene expression by *Nco** modified AngR. Detection of *fatB* -specific and *aroC*-specific transcript levels. The presence or absence of AngR is indicated by a '+' or '-', respectively, above each lane. The presence of the truncated AngR is indicated by the '+*'. Total RNA was harvested from *V. anguillarum* strains harboring various recombinant clones grown under iron-limiting conditions. Clones: Lane 1, pJHC-T2612#4, pJHC9-8 and pJHC-S2572, Lane 2, pJHC-T2612#4, pJHC9-8 and p2570, Lane 3, pJHC-T2612#4, pJHC9-8 and pJHC-T2771, Lane 4, pJHC-T2612#4, and pJHC9-8, Lane 5, pJHC-T2612, pJHC9-8. Lanes marked *aroC* and *fatB* are the free riboprobe without RNase treatment. The *fatB* and *aroC* transcripts are indicated by arrows.

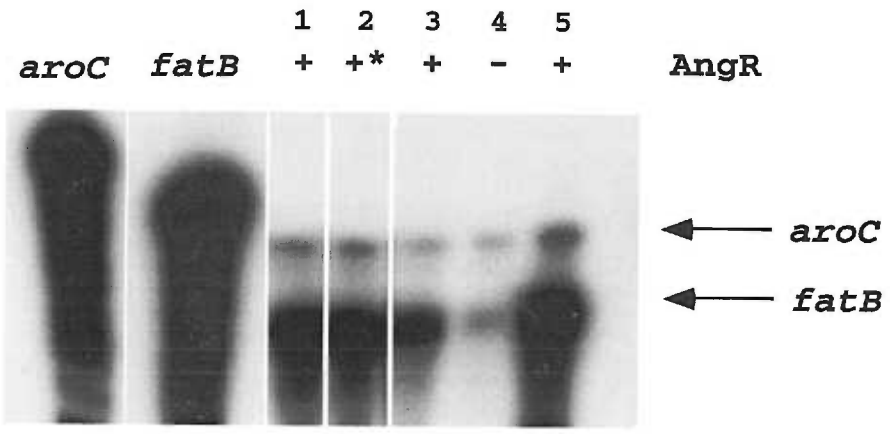

1 2 3 4 5
aroC fatB + +* + - + AngR
aroC
fatB

Figure 7: Reverse transcription polymerase chain reaction (RT-PCR) identifies a single transcript containing *angR* and *angT*. Ethidium bromide stained 0.7% agarose gel (negative photo) of 10µl the PCR reaction shows the 300 bp product generated from RT-PCR using one primer upstream inside of *angR* and one primer downstream in *angT*. Total RNA was harvested from the *V. anguillarum* strain harboring both the TAF containing plasmid pJHC9-8, and pJHC-T2612 containing the iron uptake genes. Lane 1 shows the product from the *angR* and *angT* primers yielding the predicted 300 bp fragment. Lane 2 shows the product from *fatD* and *fatC* yielding the predicted 350 bp fragment. Lane 3 shows the product from an internal control for *aroC* with two primers within the *aroC* gene yielding the predicted 700 bp fragment. Lanes 1', 2', and 3' are the reactions with the same RNA and primers as 1, 2, and 3 except no reverse transcription was performed prior to PCR.

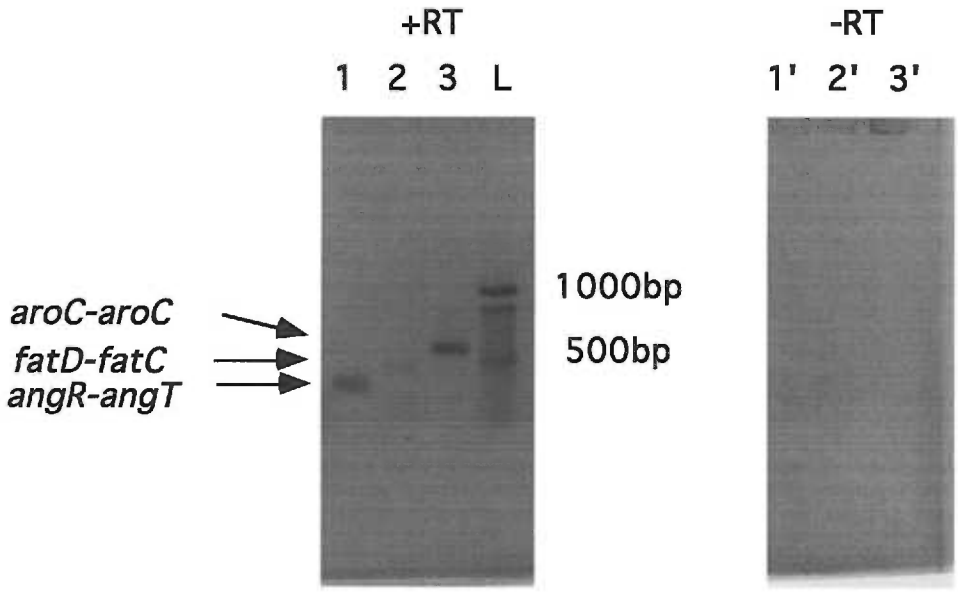
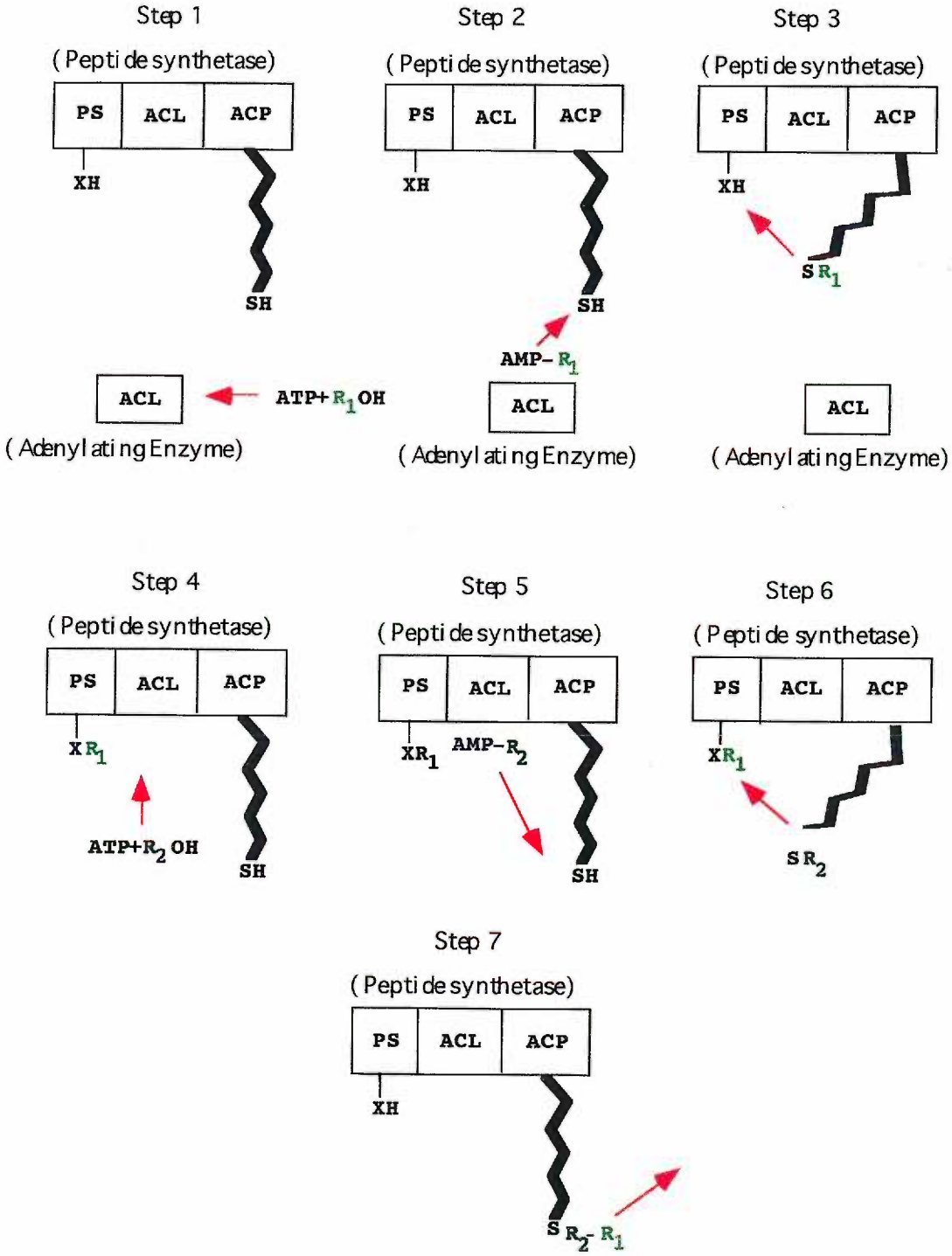

+RT
1 2 3 L
-RT
1' 2' 3'
aroC-aroC
fatD-fatC
angR-angT
1000bp
500bp

Figure 8. Putative nonribosomal peptide synthesis (Modified from Crecy-Lagard et.al (7b)). The model presents the interaction of a non-ribosomal peptide synthetase and an adenylating enzyme; such as EntF is a serine activing enzyme which may also be involved in thioesterification (non-ribosomal peptide synthetase), while EntE is proposed to be an AMP ligase (adenylating enzyme). The abbreviations are: PS, peptide synthase (elongation domain containing the HHxxxDG motif); ACL, acyl-CoA ligase (adenylate-forming domain (cores 1-5)); ACP, acyl carrier protein domain containing the phospho pantethenyl attachment site (core 6); X = serine or threoinine; S = cysteine; R= amino acid, or hydroxy acid; OH= hydroxyl group. Steps 1 to 4: step 1 initiation, transfer of the strarter chain to the loading site of the adenylating enzyme; step 2, transfer to the 4'-phosphopantetheinyl arm of the non-ribosomal peptide synthetase enzyme; step 3, transfer to the waiting/ elongation site. Steps 4 to 7, elongation, step 4, transfer of the elongation chain to the loading site of the nonribosomal peptide synthetase enzyme; step 5, transfer to the 4-phospopantetheinyl arm; step 6, condensation of the elongation chain on the 4-phosphopantetheinyl arm with the starter chain at the waiting/elongation site (PS), elongation domain containing the HHxxxDG motif; step 7, transfer of the elongated chain to a waiting/ elongation site, of another module within the same synthetase, or to yet another non-ribosomal peptide synthetase.


Step 1
(Peptide synthetase)
PS
ACL
ACP
XH
SH
ACL
ATP+R1OH
(Adenylating Enzyme)
Step 2
(Peptide synthetase)
PS
ACL
ACP
XH
SH
AMP-R1
ACL
(Adenylating Enzyme)
Step 3
(Peptide synthetase)
PS
ACL
ACP
XH
SR1
ACL
(Adenylating Enzyme)
Step 4
(Peptide synthetase)
PS
ACL
ACP
XR1
ATP+R2OH
SH
Step 5
(Peptide synthetase)
PS
ACL
ACP
XR1
AMP-R2
SH
Step 6
(Peptide synthetase)
PS
ACL
ACP
XR1
SR2
Step 7
(Peptide synthetase)
PS
ACL
ACP
XH
SR2-R1

CONCLUSIONS AND DISCUSSION

Bacteria have evolved various mechanisms to acquire iron. These mechanisms involve scavenging the metal from the various iron sources, often using high affinity iron binding molecules known as siderophores, and internalizing the scavenged iron. The bacterial system studied in our laboratory, *Vibrio anguillarum* , possesses the virulence plasmid pJM1 which encodes several essential molecules that enable the bacterium to survive within the iron limiting conditions of the host (11, 13). These molecules include biosynthetic enzymes required for siderophore synthesis, the iron transport proteins FatA, FatB, FatC, and FatD, and several regulatory elements, two positive regulators, AngR and TAF and the negative regulatory molecule, antisense RNAα (11, 13, 29, 30). The siderophore anguibactin is synthesized when the *V. anguillarum* cells experience iron limitation, for example, when the bacteria are within the host tissues. Anguibactin is released from the bacterial cell and once in the extracellular milieu, it interacts with the host iron binding proteins and scavenges the iron. The ferric anguibactin complex is then imported into the cell via the iron transport proteins, FatA, FatB, FatC, and FatD (12). The outer membrane receptor, FatA specifically recognizes the ferric-anguibactin complex (1, 2). Infection experiments demonstrated that *V. anguillarum* strains which cannot synthesize anguibactin but do possess receptors for transport, are still virulent when co-infected with siderophore producing strains; yet if anguibactin producing strains do not synthesize the iron transport proteins they do not survive (35). Clearly the ability to produce anguibactin and express receptors is crucial for virulence. However, excess intracellular iron is toxic for the cell, so the genes required for iron acquisition are strictly regulated. Thus, the bacterium senses the iron concentration and responds by either increasing or decreasing the expression of the iron transport and siderophore biosynthesis genes as well as other genes required for iron acquisition. The overall aim of this thesis was to understand how two key proteins, Fur and AngR regulate the expression of genes involved in iron acquisition in *V. anguillarum.*

Regulation of the expression of iron-regulated genes in *E. coli* and other bacteria involves the transcriptional regulator Fur (10,26). Fur binds the intracellular ferrous iron as a complex and then binds DNA at specific consensus sequences (5'-GATAATGATAATCATTATC) usually within the -10, and -35 promoter region of the genes it regulates (10). When the intracellular iron concentration drops, Fur and iron disassociate and Fur releases the DNA allowing transcription of the regulated genes. The repressor Fur has been found to regulate expression of iron transport proteins, siderophore biosynthesis genes, and toxins in several different bacterial species (4, 6, 17, 18, 19, 20, 25, 32, 33). Thus, we hypothesized that *V. anguillarum* may also regulate its iron transport genes through a Fur-like protein. By using complementation experiments and subsequent cloning I was able to demonstrate that there is a Fur homologue in *V. anguillarum* (Chapters 1 and 2).

V. anguillarum Fur shows high amino acid homology with the Fur proteins in other organisms, in particular *V. cholerae*, *V. vulnificus*, and to a lesser extent, *E. coli*. Computer predictions revealed that the secondary structure predicted for *V. anguillarum* Fur is similar to that for the *E. coli* protein and that *V. anguillarum* Fur shares greater secondary structure homology to the Fur proteins from *V.cholerae* and *V. vulnificus* Fur (Chapter 2). Curiously, the least conserved region of the Fur proteins is within the putative metal binding domains in the carboxy terminus, while the DNA binding domains are more similar (10, 26, 27). Additionally, a cosmid clone, pMET67 carrying *V. anguillarum* Fur could complement an *E.coli* Fur deficient mutant and recognize the Fur reporter construct in *E.coli* (34). This work suggested that the pJM1 plasmid may have similar DNA recognition sequences upstream of iron-regulated genes such as the iron transport genes. However, I did not find such sequences immediately upstream of the *fatD* gene nor the *fatB* or *fatA* genes. Therefore it was not clear if Fur regulated these transcripts directly. To understand the nature of this negative regulator I attempted to distrupt the chromosomal *fur* gene by using marker exchange experiments in order to generate a null mutant. These

attempts were unsuccessful. The inability to generate null Fur mutants was also the case for *Pseudomas sp.* and *Yersinia sp.* (20, 25). Therefore it is possible that in some organisms Fur may be essential. Two mutant Fur strains (775MET9, and 775MET11) were finally isolated in *V. anguillarum* by using a $MnCl_2$ selection method (16) (Capter 1). Despite our success, the mutant strains we isolated were not as viable as wild type *V. anguillarum* and they quickly reverted to wild type if not grown under high concentrations of manganese (Chaper 2). These findings underscore the fact that Fur may indeed serve an essential function in *V. anguillarum* (Chapters 1 and 2). Our hypothesis is that a functional Fur protein may successfully bind or coordinate the metal ions or somehow maintain the delicate balance of intracellular iron. A recent report verified that Fur does play a role in minimizing free radical production in *E.coli* and that Fur mutants have lost this ability (31). Analysis of the mutant *V. anguillarum* Fur strains revealed that production of the iron transport protein FatA and catechols, were no longer repressed in iron rich conditions in the Fur-deficient background (Chapter 1). Thus, Fur regulates not only the transport of iron into the cell but also the ability to acquire iron from the host iron binding proteins. These mutants also revealed that Fur is essential for expression of RNA α, the other negative regulator of iron transport gene expression and that the transcription of the *angR* gene is also regulated by Fur (9).

Current reports suggest that Fur might belong to the LexA family of helical binding proteins and that helicity may be important for DNA contact (17b). However, there is controversy as to whether Fur is a true member of the helix-turn-helix family of DNA binding proteins. It is apparent though, that the typical features characteristic of the family are not present (8). My studies of the wild type and mutant *fur* genes revealed that mutant 775MET9 is affected in a region which is predicted to be helical. This mutation, D104 to G104 occurred within one such helical region and the predicted secondary structure ascribed would seriously perturb the helicity of the region (Chapter 2).

In many Fur regulated operons, iron regulated promoter regions have been identified or Fur has been shown to bind (14, 17, 26). To investigate the role of Fur in regulation, I began by exploring where *V. anguillarum* Fur could interact with DNA. Despite the fact that our mutants revealed that the iron transport genes are in some way controlled by the presence of Fur, we were not able to identify sequence homology with the Fur consensus. Consequently, I attempted to determine genetically, where Fur might act in regulating expression of iron transport genes under iron rich conditions by investigating the nature of the mRNA transcribed from the iron transport region and attemping to locate an iron-regulated promoter where Fur may interact. My studies using RT-PCR in combination with genetic evidence from insertion mutations demonstrated that *fatDCBA* is contained within a polycistronic message (Chapter 3). The transcript appears to be quite unstable, as Northern blots failed to detect it, and only RT-PCR was able to physically prove the existence of the large transcript spanning *fatD* through to *angR*.

To identify the promoter region for the *fatDCBA* genes, the upstream *fatDCBA* region was fused with a CAT reporter construct. Very little activity was detected within the first 600 bp upstream of *fatD*. The constructs which demonstrated strong promoter activity contained either the entire 2.1 kb region upstream of *fatD* or a 400 bp region just upstream of the *Cla*I site (Figure 3 Chapter 3). The activity from each of these promoters was comparable. Upon addition of 4 μM ferric ammonium chloride, I found nearly 90% less promoter activity from the construct containing the 2.1 kb region. I also initiated gel shift experiments to determine where precisely the Fur protein may be binding (Appendix I) Although extremly high protein were required, I consistently saw DNA band retardation with wild type Fur using the 200 bp region immediately upstream of *fatD*. When I used Mutant 9 Fur protein instead of the wild type no band retardation occurred. We are currently exploring how this binding correlates with the iron regulated expression from the promoter in the 2.1 kb upstream fragment.

The regulation of iron transport genes has also been recently shown to involve the AngR protein and anguibactin as well as the TAF products(s) for positive activation of the system (9). We also previously determined that AngR and TAF are both required for anguibactin production (22). The AngR protein has features consistent with roles in both anguibactin synthesis as well as regulation of gene expression.

Our earlier work demonstrating that AngR is involved in regulation (9, 22) and that AngR complements the *E.coli* 2,3-DHBA AMP-ligase mutant EntE-deficient (21) strain, prompted my analysis of the AngR protein. Examining the structure/function of AngR could illuminate critical aspects of the positive regulation mechanism employed in *V. anguillarum*. It is interesting that this single protein may be acting at the level of gene expression, or transcript stabilization, and be involved in producing a compound, anguibactin, which in turn also increases the expression of the iron transport genes. The approach I followed was to generate and analyze a broadly selected panel of mutants located throughout the AngR protein (Chapter 4). The analysis of these mutations revealed that the AngR regulatory function requires the first predicted helix-turn-helix motif (amino acid residues 290-312). It was particularly interesting that only the first 600 amino acids of AngR appear to be essential for regulatory activity. The biosynthetic motifs are located toward the carboxy terminus, just beyond amino acid 500. However, mutations in the carboxy terminus as well as mutations within the amino terminus led to a loss of biosynthetic activity. These data suggest that the entire molecule of AngR is actually required for anguibactin biosynthesis. The specific mutations may be critically altering structure, either at discreet regions or affecting general folding required for enzymatic activity.

I also determined that AngT, the putative thioesterase downstream of AngR is also required for anguibactin biosynthesis but not regulation of the *fatDCBA* transcript. Additionally, I found that *angT* and *angR* are contained on a single transcript. From these

experiments, we are beginning to understand the mechanism of iron regulation of the iron transport genes *fatDCBA* and the anguibactin biosynthetic pathway.

In conclusion, analysis of Fur has revealed that the iron transport proteins and catechols are indeed negatively regulated in the presence of Fur. I also discovered that Fur is critical for normal physiological function of *V. anguillarum*, which is similar to the findings in other systems (5, 6, 14-19, 25, 31, 32). Attempts to identify the specific Fur binding site led to discovery of a considerably larger upstream region of *fatDCBA* than we had anticipated, suggesting that the operon is more complex than we originally thought.

The characterization and cloning of the mutant and wild type *fur* genes provide a means to study Fur function *in vitro* while the actual Fur mutant strains provide a background to examine the complexity of Fur regulation *in vivo*. Based on CAT ELISA assays there is significant iron-regulated promoter activity within the *fatDCBA* upstream region suggesting a target-site for negative transcriptional regulation. In addition this fragment should have cis elements required for positive regulation, if indeed regulation by the positive regulators occurs at the transcriptional level. Finally my analysis of AngR has revealed that AngR does possess both regulatory as well as biosynthetic functions. I found a putative helix-turn-helix region was essential for regulatory activity, yet biosynthetic activity was severely diminished by nearly every modification of *angR*. With this new information, we are revealing a clearer picture of regulation and how the many known players interact. It is the long term goal of these studies to describe the direct interaction required to accomplish the regulation of the expression of the iron acquisition genes.

LITERATURE CITED

1. **Actis, L. A., W. Fish, J. H. Crosa, K. Kellerman, S. R. Ellenberger, F. M. Jauser, and J. Sanders-Loehr.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). Journal of Bacteriology. **167:**57-65.
2. **Actis, L. A., S. Potter, and J. H. Crosa.** 1985. Iron regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. Journal of Bacteriology. **161:**736-742.
3. **Alber, T.** 1992. Structure of the leucine zipper. [Review]. Current Opinion in Genetics & Development. **2:**205-10.
4. **Angerer, A., S. Enz, M. Ochs, and V. Braun.** 1995. Transcriptional regulation of ferric citrate transport in *Escherichia coli* K-12. FecI belongs to a new subfamily of sigma 70-type factors that respond to extracytoplasmic stimuli. Molecular Microbiology. **18:**163-74.
5. **Baumler, A., R. Koebnik, I. Stojiljkovic, J. Heesemann, V. Braun, and K. Hantke.** 1993. Survey on newly characterized iron uptake systems of *Yersinia enterocolitica*. Zentralblatt fur Bakteriologie. **278:**416-24.
6. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in *Enterobacteria* [Review]. FEMS Microbiology Letters. **147:**173-180.
7. **Borchert, S., S. S. Patil , and M. A. Marahiel.** 1992. Identification of putative multifunctional peptide synthetase genes using highly conserved oligonucleotide sequences derived from known synthetases. FEMS Microbiology Letters. **71:**175-80.
8. **Brennan, R.** 1991. Interactions of the helix-turn-helix binding domain. Curr. Opin. Struct. Biol. **1:**80-88.

9. **Chen, Q., A. M. Wertheimer, M. E. Tolmasky, and J. H. Crosa.** 1996. The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum*. Molecular Microbiology. **22:**127-134.

10. **Coy, M., and J. Neilands.** 1991. Structural dynamics and functional domains of the *fur* protein. Biochemistry. **30:**8201-8210.

11. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature. **284:**566-568.

12. **Crosa, J. H.** 1984. The relationship of plasmid mediated iron transport and bacterial virulence. Annual Review of Micriobiology. **38:**69-89.

13. **Crosa, J. H., L. Hodges, and M. Schiewe.** 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. Infection and Immunity. **27:**897-902.

14. **deLorenzo, V. L., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur (ferric uptake regulator) protein and CAP(catabolite activator protein) modulate transcription of *fur* gene in *Escherichia coli*. European Journal of Biochemistry. **173:**537-546.

15. **Furman, M., A. Fica, M. Saxena, J. L. Di Fabio, and F. C. Cabello.** 1994. *Salmonella typhi* iron uptake mutants are attenuated in mice. Infection & Immunity. **62:**4091-4.

16. **Hantke, K.** 1987. Selective procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12. Molecular and General Genetics. **210:**135-139.

17. **Hassett, D. J., P. A. Sokol, M. I. Howell, J. F. Ma, H. T. Schweizer, U. Ochsner , and M. I. Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-

mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. Journal of Bacteriology. **178:**3996-4003.

17b. **Holm L., C. Sander, H. Ruterjans, M. Schnarr, R. Fogh, R. Boelens, and R. Kaptein.** 1994. LexA repressor and iron uptake regulator from *Escherichia coli*: new members of the CAP-like DNA binding domain superfamily. Protein Engineering. **7:**1449-53.

18. **Lam M. S., C. M. Litwin, P. A. Carroll, and S. B. Calderwood.** 1994. *Vibrio cholerae* fur mutations associated with loss of repressor activity: implications for the structural-functional relationships of Fur. Journal of Bacteriology. **176:**5108-15.

19. **Ochsner U. A., A. I. Vasil, and M. I. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. Journal of Bacteriology. **177:**7194-201.

20. **Prince, R. W., C. D. Cox, and M. I Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. Journal of Bacteriology. **175:**2589-98.

21. **Rusnak, F., W. S. Faraci, and C. T. Walsh.** 1989. Subcloning, expression, and purification of the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP ligase: demonstration of enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. Biochemistry. **28:**6827-35.

22. **Salinas, P. C., M. E. Tolmasky, and J. H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: Evidence for a cooperative effect between two transcriptional activators. Proceedings of the National Acadamy of Sciences U.S.A. **86:**3529-3533.

23. **Stachelhaus, T., and M. A. Marahiel.** 1995. Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. Journal of Biological Chemistry. **270:**6163-9.

24. **Stachelhaus, T., and M. A. Marahiel.** 1995. Modular structure of genes encoding multifunctionall peptde synthetases required for non-ribosomal peptide sythesis. FEMS Microbiology Letters. **125:**3-14.

25. **Staggs, T. M., J. D. Fetherston, and R. D. Perry.** 1994. Pleiotropic effects of a *Yersinia pestis* fur mutation. Journal of Bacteriology. **176:**7614-24.

26. **Stojiljkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay [published erratum appears in Journal of Molecular Biology 1994 Jul 15;240(3):271]. Journal of Molecular Biology. **236:**531-45.

27. **Stojiljkovic I, and K. Hantke.** 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). Molecular & General Genetics. **247:**199-205.

28. **Tolmasky, M. E., L. A. Actis , and J. H. Crosa.** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyperproduction of anguibactin. Infection & Immunity. **61:**3228-33.

29. **Tolmasky, M. E., L. A. Actis, A. E. Toranzo, J. L. Barja, and J. H. Crosa.** 1989. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. Journal of General Microbiology. **131:**1989-97.

30. **Tolmasky, M. E., L. A. Actis, and J. H. Crosa.** 1993. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyperproduction of anguibactin. Infection and Immunity. **61:**3228-3233.

31. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta *fur*

mutants of *Escherichia coli:* protective role of superoxide dismutase. Journal of Bacteriology. **177:**2305-14.

32. **Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron.** 1995. Fur regulon of *Salmonella typhimurium:* identification of new iron-regulated genes. Journal of Bacteriology. **177:**4628-37.

33. **Venturi, V., P. Weisbeek, and M. Koster.** 1995. Gene regulation of siderophore-mediated iron acquisition in Pseudomonas: not only the Fur repressor. [Review]. Molecular Microbiology. **17:**603-10.

34. **Waldbeser, L. S., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1993. Mechanisms for negative regulation by iron of the fatA outer membrane protein gene expression in *Vibrio anguillarum* 775. Journal of Biological Chemistry. **268:**10433-9.

35. **Walter, M., S. Potter, and J. H. Crosa.** 1983. Iron uptake system mediated by Vibrio anguillarum plasmid pJM1. Journal of Bacteriology. **156:**880-887.

36. **Watnick, P. I., T. Eto, H. Takahashi, and S. B. Calderwood.** 1997. Purification Of Vibrio Cholerae Fur and Estimation Of Its Intracellular Abundance By Antibody Sandwich Enzyme-Linked Immunosorbent Assay. Journal of Bacteriology. **179:**243-247.

APPENDIX I

Analysis of pJM1 *Bam*HI restriction fragment #4: a required region for TAF activity in *V. anguillarum*

The process of iron uptake in *V. anguillarum* is positively regulated at the transcription level by the 110 kDa protein AngR and the products of the pJM1 TAF region, which act synergistically (3). The TAF region is non-contiguous with the iron uptake region of pJM1 (Figure 5, Introduction). Restriction mapping and deletion analysis of pJM1 revealed that the TAF region requires an unclonable *Bam*HI fragment for TAF activity (4). The pJM1 plasmid when digested with restriction endonuclease *Bam*HI generates 8 fragments. The fourth largest fragment of 6.5 kb was the only fragment which could not be subcloned (4). This inability to subclone, made characterization of the TAF element(s) very difficult. The smallest construct which confers TAF activity, plasmid pBP44 a c.a. 40kb plasmid, was generated using pBR325 as the vector and inserting a pJM1 partial *Bam*HI digest containing the 20kb segment: *Bam*HI 8, *Bam*HI 7, *Bam*HI 4 , and *Bam*HI 3. Several methods of transposition mutagenesis were also employed by using transposons such as, F1::Tn*5*.Tn*1331*, ColE1::Tn*5*, and F1::Tn10 ts. Despite these many attempts for mutation, resulted insertions resulted in extensive rearrangements.

In an attempt to further understand the nature of the TAF activity I became involved in analyzing the sequence of the TAF region. Manual sequencing was very difficult as multiple compressions were encountered. Only through PCR sequencing did we generate reliable sequence data. We sequenced a total of 6 kb identifying three open reading frames. These open reading frames are: ORF1, ORF 2 and ORF3 which potentially encode products of the predicted size 24K, 29K and 27K respectively (Figure 1).

I found that one of the open reading frames, ORF1, shows a considerable domain homology with certain positive regulators, phage Φ-CII(2), 56.4 %, similarity, incompatibility protein IncC, 50.7 % similarity, *Bacillus subtilis* division gene MinD, 46.9 % similarity, nitrogenase iron protein NifH2, 42.7 % similarity and 56.3 % similarity, with the as yet unclassified genes ORF253 and ORF263 from *B. subtilis* and *Pseudomanas putida* respectively, as determined by using a BLAST search at the NCBI (1). My homology search also revealed that there are three main areas of consensus within

ORF1. These regions are highly conserved among the variety of genes with which our ORF1 is homologous to (Figure 2). This first consensus region has been implicated in mononucleotide binding (GXGXXGKS/T) in NifH2. The other two open reading frames, ORF2 and ORF3 do not have any significant homology to any proteins in GenBank.

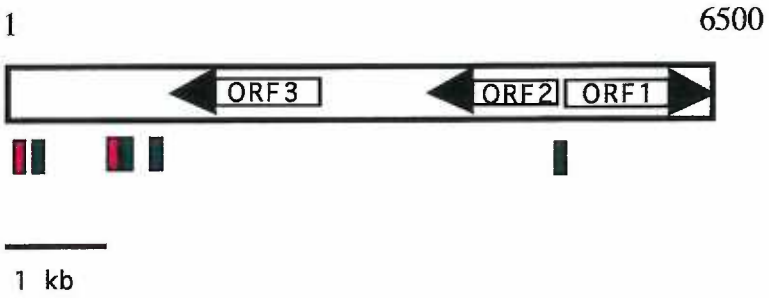
The sequence data also reveals multiple hairpin structures and highly repetitive sequences throughout the *Bam* HI #4 fragment. The largest repeat spans from bp 1100 to 1250, portions of this region are directly repeated at bp 60 to 136, from bp 1609 to 1651, and from bp 5449 to 5513 (see Figure1). These features partially explain why sequencing of this region has been so difficult.

In summary, we were able to sequence the entire *Bam*HI #4 segment and discovered three ORFs which may be essential for TAF activity. In addition we found a considerable amount of secondary structures which may serve roles in physiologic functions related to TAF activity, cell division, or plasmid maintenance. These findings will likely help explain the difficulties we have been experiencing in molecular manipulations of this region and provides us with the first insight into the nature of TAF. Future experiments using PCR to amplify sub regions will be pursued to further understand the TAF activity associated with this region.

Table 1: pJM1 *Bam*HI Banding pattern(4)

Bam HI	1	19.9kb
Bam HI	2	14.4kb
Bam HI	3	10.9kb
***Bam* HI**	**4**	**6.5kb**
Bam HI	5	4.9kb
*Bam*HI	6	3.2kb
*Bam*HI	7	2.7kb
*Bam*HI	8	2.1kb

Figure 1: Diagram of the *Bam*HI 4 fragment with arrows illustrating the position and direction of the three open reading frames. The repeated sequence has been color coded to show where each within BamHI #4 the repeats occur. The approximate location of each repeat is illustrated by a color coded cube.



REPEATED SEQUENCE

5' TACTCCATATTGTTCGGCGGGATTGCCT
AATCTCTGACTACCATATTAGCA GCAC
TCCTTTATAAAGTCAATAAAACAATATAAAGGGA

Figure 2: A pile up comparison (GCG) of seven proteins highly similar to ORF1 illustrating the three conserved consensus sequences. The shading represents highly conserved or identical residues. Amino acid residue column lists the total number of residues encoded by each open reading frame respectively. The bold numbers in parentheses denote at which amino acid the particular conserved consensus (either I, II, or III) begins in Orf1. Only IncC has a significantly different start for the conserved sequences that being at residue 100 instead of residue 18. The consensus sequences within the other proteins are all found within similar regions compared with Orf 1.

Amino acid Residues		I (18)	(50) II	(130) III
219	ORF 1	NEKGGVGKTT	DKKVLVVDLDPQFNL	NEYDYVLIDCPPSV...GNL
341	ϕ-CII	NHKGGVSKTT	GYKVLIVDADPQCNL	HEFDYVLIDMSPSV...GAL
364	IncC (100)	NQKGGVGKTS	GLRVAVIDLDPQGNA	QGFDVCLIDTAPTL...GVG
253	ORF 253	NQKGGVGKTS	GKRVLLVDIDPQGNA	QNYDYIIIDCPPSL...GLL
263	ORF 263	NQKGGVGKTT	KRRVLLIDLDPQGNA	DEYDYILIDCPPSL...SML
345	Lep	NQKGGVGKTT	GRRVLLVDIDPQGAL	DRYDYVLIDCQPSL...GLL
268	MinD	SGKGGVGKTT	GKRVCLVDTD.....	QEFDYVIIDCPAGI...EQG
292	NifH2	G.KGGIGKTT	GKKVMVVGCDPKHDC	LKLDIVLYDVLGDVVCGGFA

LITERATURE CITED

1. **Altschul, S., W. Gish, W. Miller, E. Myers, and D. Lipman.** 1990. Basic local alignment search tool. Journal of Molecular Biology **215:**403-410.

2. **J.Sun, M.Inouye, and S.Inouye.** 1991. Association of a Retroelement with a P4-like Cryptic Prophage (Retrophage phi-R73) Interated into the Selenocystyl tRNA Gene of *Escherichia coli.* Journal of Bacteriology. **173:**4171-4181.

3. **Salinas, P. C., M. E. Tolmasky, and J. H. Crosa.** 1989. Regulation of the iron uptake system in *Vibrio anguillarum*: Evidence for a cooperative effect between two transcriptional activators. Proceedings of the National Acadamy of Sciences U.S.A.. **86:**3529-3533.

4. **Tolmasky, M. E., P. C. Salinas, L. A. Actis, and J. H. CrosA.** 1988. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum.* Infection and Immunity. **56:**1608-1614.

APPENDIX II

Purification of *V. anguillarum* Fur and DNA Binding Studies with Sequences Upstream of the Iron Transport Genes and the *angR* Gene

Anne M. Wertheimer and Jorge H. Crosa

We proved that the *V. anguillarum* Fur protein recognizes the consensus sequence for Fur binding **GATAATGAT A ATCATTATC** *in vivo*, (Chapter 1 and 2)(2, 3). However, we have not found a definitive Fur binding site in the predicted promoter region of the iron transport genes. We initiated a search of potential Fur boxes upstream of the *fatB* and *fatA* genes as well as in other sequenced regions of the pJM1 iron uptake region. The potential Fur boxes that we found and a comparison with the palindromic consensus "Fur box" are shown below.

"Fur-Box"	**GATAATGAT**	**A**	**ATCATTATC**
UPSTREAM OF ORF8	**CTAAATGAT**	**A**	**ATCAATGC**
UPSTREAM OF *fatD*	**GATAATTCT**	**A**	**TTTTATGAG**
UPSTREAM OF *fatB*	**AAACATGAT**	**A**	**TGCATCAAG**
UPSTREAM OF *fatA* at SalI*	**CATAATCAA**	**A**	**CAGCACGT**
UPSTREAM OF *fatA*	**ATC ACTGAA**	**A**	**ATCGTGATT**
UPSTREAM OF *angR*	**GCCAATCAA**	**A**	**GTGTCAATT**

It is obvious that the sequence found upstream of ORF8 is the closest to the *E. coli* Fur box consensus. Further inspection indicates that all of the other putative boxes found upstream of the Fur-regulated iron transport genes and *angR*, although divergent, are very close to the consensus in the left arm (as shown above), while very much diverged on the other. I proceeded to purify both the wild type Fur and Fur9 in order to begin quantitative analysis of binding using the fragment from upstream of *fatD* (probe B, the 200bp *Eco*RI-*Hind*III fragment), as well as one from upstream of *angR* (probe A, the 800 bp fragment generated using PCR primers fatA.down and angR.up), and the control fragment containing the Fur consensus sequence, (probe 1 the 200bp fragment from pSC27.1 containing the Fur consensus sequence) (See Figure 1 for map of probes). Preliminary gel retardation experiments revealed that the purified WT Fur binds the 200bp fragment up stream of *fatD* , the 800bp region upstream of *angR* and the control region containing the Fur consensus, while Fur9 does not (Figure 2). Interestingly I also discovered that WT Fur recognized the T7 promoter in pBluescript (Figure 3).

Materials and Methods

Purification of Fur Protein: Purification of the Fur proteins first required cloning the *fur* and *fur9* genes into the pT7-5 overexpression vector so that production of the recombinant Fur proteins was inducable in the presence of T7 RNA polymerase and IPTG. For Fur, Fur9, as both contain multiple histidines which will interact with the divalent nickel, the nickel agarose column from Novagen was used. For gel shifts a 2 ml bed was used. The bed was charged and equilibrated (using the commercial protocol). The cell pellet was sonicated in one column volume of binding buffer with 10-30 sec pulses with cooling, on ice 5-10 min. The sonicated cells were spun @12,000 rpm to pellet debris 30 min. The supernatant was passed over a 0.45 μm filter, loaded onto the column, washed with 5 column volumes of binding buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.5), washed with 5 column volumes of wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCL pH 7.5). The Fur usually begins to elute in the second wash. Elution with 4-5 column volumes of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris pH7.5) was carried out to clean the column. Protein was concentrated in a Centriplus concentrator to 3ml volume then resuspend in 20 mM Hepes pH 7.5 to 15 ml. A total of 4 washes into Hepes was performed for use in the gel retardation experiments. The final 3 ml wash was checked on a 15% SDS-PAGE and absorbance at A $_{260}$ was measured for calculation of concentration and then stored at 4C until use.

Gel Retardation: The Gel shift protocol used was a modification of a method published by Ochsner (1). The probes used were: Probe 1, Probe B and Probe A. Probe 1 is the 250bp fragment (*Eco*RI-*Bam*HI) containing the *E.coli* Fur Box consensus sequence from the reporter plasmid pSC27.1. Plasmid pSC27.1 is the Lac Z reporter construct I used to assay Fur activity *in vivo* from wild type *V. anguillarum* and *V. anguillarum* Fur

mutant strain 9 (Chapter 1 and 2), digesting the construct with *Eco*RI and *Bam*HI releases the 250bp fragment from the remainder of the vector. Probe B is the putative promoter region of *Eco*RI- *Hind*III (See Figure 5 chapter 3) as amplified out of pBluescript multiple cloning site (MCS) with Universal and Reverse primers. Probe A is the putative promoter region upstream of *angR* PCR was used to generate an 800 bp fragment with primers fatA.down and angR.up (see Chapter 3 for primers) then digested with *Cla*I to generate an end for radiolabeling. For probe B the PCR fragment was digested via *Eco*RI-*Hind*III prior to labeling. There are 3 products after digestion (as seen in the gel Figure 3) at 200 bp is *Eco*RI-*Hind*III fragment, at 150 bp is the T3 flanking section of pBluescript's MCS and at 100 bp is the T7 flanking section of pBluescript's MCS. For my preliminary shifts I did not further purify the 200 bp insert. I used purified Fur protein from *V. anguillarum, V. anguillarum* Fur mutant strain 9, and *E.coli.* The proteins from *V. anguillarum* were purified as described. The probes were labeled using Klenow fragment of DNA polymerase I and α-dATP ^{32}P then purified using a spin column. The final reaction volume is 20 µl , 1 µl of probe was added together with varying concentrations of purified protein (from 0 to 91 pmoles per reaction (the final concentration approximately 5µM)). The buffer for the gel retardation is a Mn based buffer (10 mM Bis Tris Borate, 40 mM KCl, 0.1 mM $MnSO_4$, 1 mM $MgSO_4$, 2µg/ml BSA, 0.05µg/µl poly (dI-dC) and 10% glycerol. The reaction was incubated at room temperature 15 minutes, then 10 µl was electrophoresed in a native 4% PAGE gels buffered in a similar Bis/Tris and Mn buffer (20 mM Bis/Tris borate pH 7.9, 0.1 mM $MnSO_4$). Gels were run 1 hr, dried 1hr and exposed to film overnight.

Results and Discussion: *V. anguillarum* Fur can be easily purified using the Nickel agarose method (Figure 2). Figure 3 illustrates that *V. anguillarum* Fur can bind the *E.coli* Fur box *in vitro* (probe 1), the *Eco*RI-*Hind*III fragment (probe B), and the upstream region of *angR* (probe A). However the Fur9 does not bind well. In probe B, a second

fragment is shifted by the *V. anguillarum* Fur that was found to contain the T7 promoter. I was curious why this second fragment was shifting so I compared the two regions and found that there is a region of homology between *Eco*RI and *Hind*III and the T7 promoter from pBluescript. This region has an interesting similarity to the "Fur-Box"...

GATAATGATAATCATTATC	FUR CONSENSUS
TGAATTGTAATACGACTCACTATT	T7
TGAATTTAGATGCGTCTCAATATT	upstream *fatDCBA*

I also found that the *E.coli* Fur protein only shifted the upper band which corresponds to the upstream region of *fatDCBA* not the T7 fragment.

These finding suggest that *V. anguillarum* Fur protein binds at regions upstream of the genes for *fatD* and *angR*. These are preliminary data and quantification is required for both the DNA probe and the protein to determine whether these interactions are physiologically relevant. It is curious that neither of these regions have significant promoter activity as determined by cloning the regions up stream of a promoterless *cat* gene (data not shown). These conflicting results suggest the mechanism of both promoter activity as well as regulation of the iron transport gene expression, may be quite complex and involve multiple promoter elements as well as require regulatory regions in *cis*. As I have now identified an iron-regulated promoter region (Chapter 3) efforts to identify how Fur is interacting throughout the region are being conducted.

Figure 1: Map of probes A and B isolated from the pJM1 iron uptake region. The dark arrows represent the direction of the transcipt, the genes are listed below each arrow. The pertinent restriction sites are marked E, for EcoRI, H, for HindIII, and C for ClaI. The location(noted with a small box), and direction (noted with a small arrow) of the PCR primers (fatA.down, and angR.up) used to generate probe A are also included.

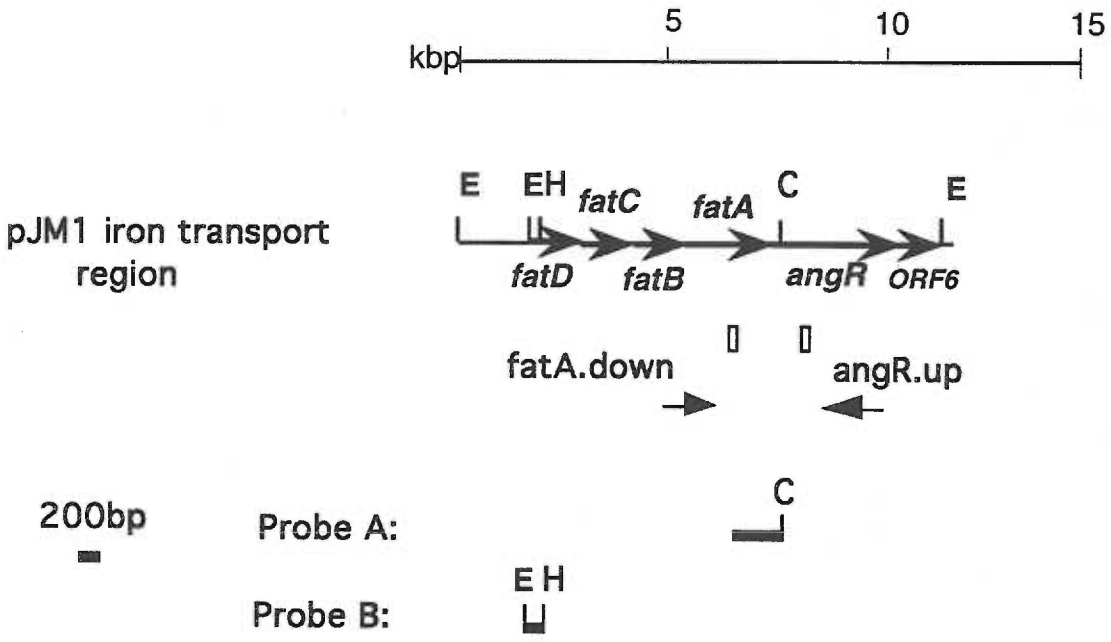
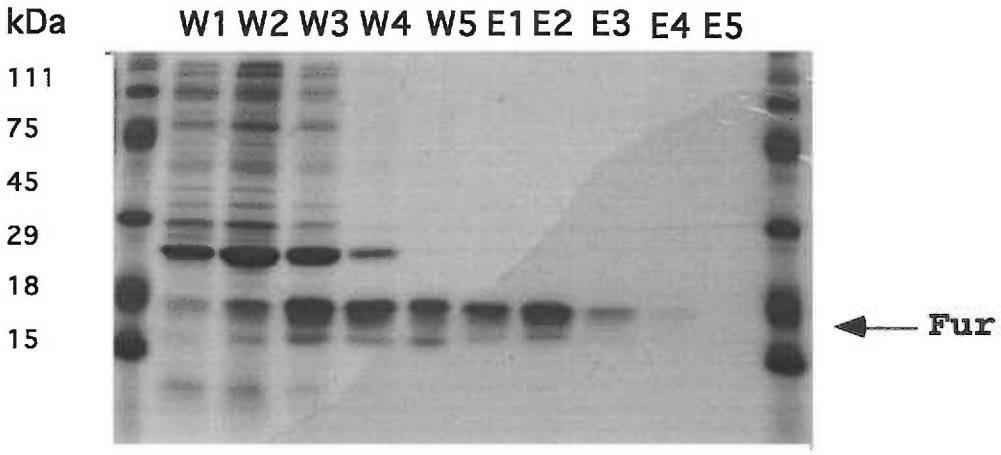

kbp
5
10
15
pJM1 iron transport region
E
EH
fatC
fatA
C
E
fatD
fatB
angR
ORF6
fatA.down
angR.up
200bp
Probe A:
C
Probe B:
E H

Figure 2: Purification of Fur (top panel) and Fur9 (bottom panel). Coomasie Stained 15% SDS -PAGE gel showing elution fractions. Fractions are as follows, W, wash, and E, is eluate. Fractions were roughly 2 ml column volumes. Arrows indicate Fur or Fur9.

PANEL A



PANEL B:

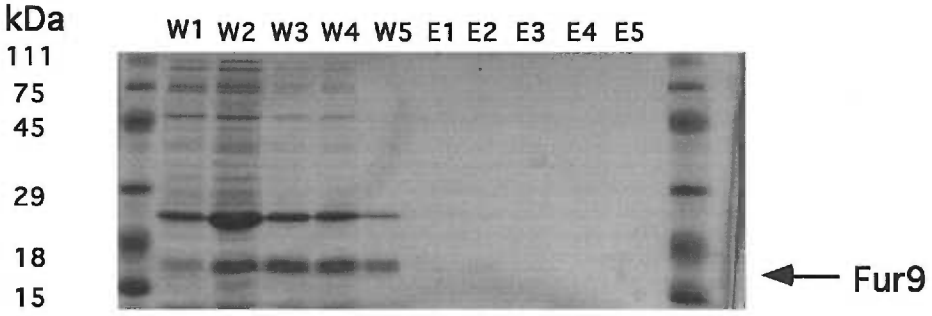
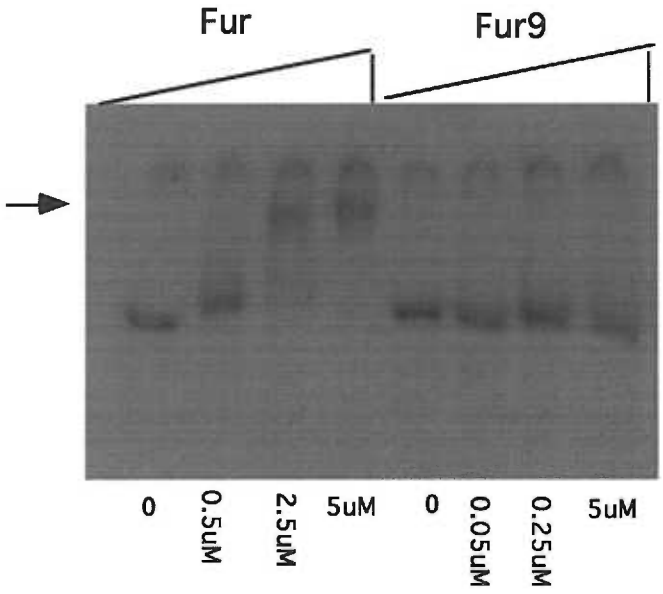
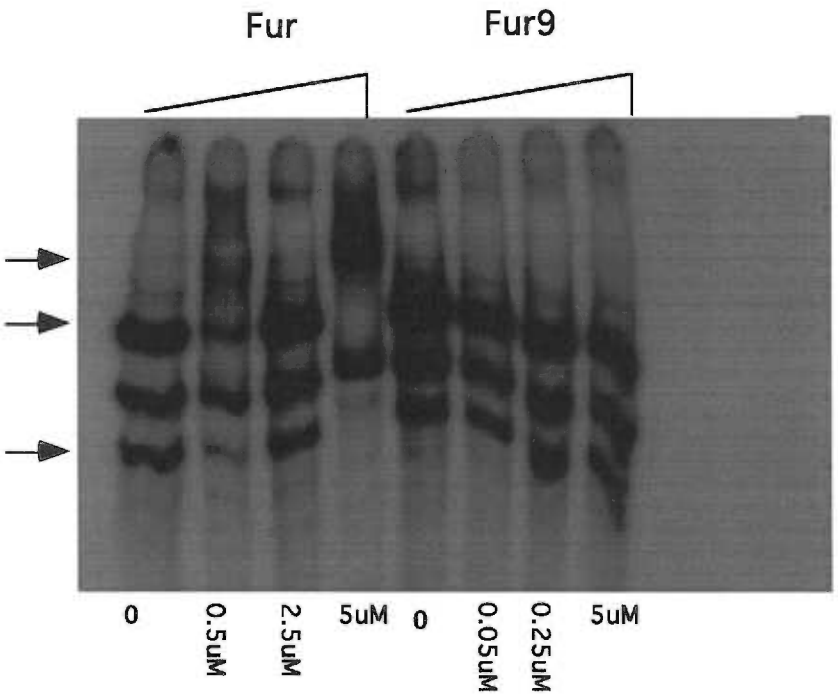


Figure 3: Mobility shift 6% PAGE using purified *V. anguillarum* Fur and Fur9. Probe used as illustrated under the figure. Probe B, the *Eco*RI-*Hind*III fragment upstream of *fatD*; Probe 1 the 200 bp fragment containing the Fur consensus site. Proteins used and their concentrations are indicated above and below the lanes, respectively. Arrows indicate shifted probe.

Probe 1

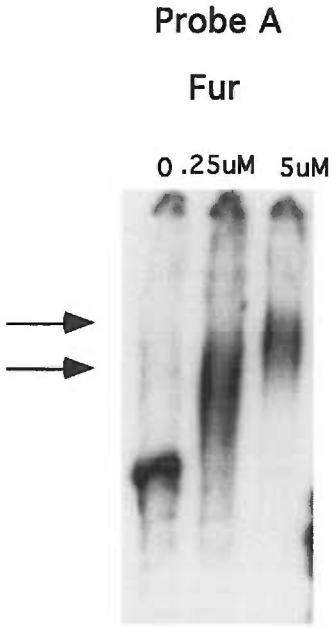


Probe B



Conclusions: ***V.anguillarum*** **WT Fur recognizes both probe 1 and probe B Fur9 appears to only weakly recognize either probe.**

Figure 4: Mobility shift 6% PAGE using purified *V. anguillarum* Fur. Probe used is Probe A, the 800 bp region upstream of *angR*. Proteins used and their concentrations are indicated above the lanes. Arrows indicate shifted probe.


Probe A
Fur
0
.25uM
5uM

LITERATURE CITED

1. **Ochsner U.A., A. I. Vasil , and M. l. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. Journal of Bacteriology. **177:**7194-201.

2. **Tolmasky M. E., A.M. Wertheimer, L.A. Actis, and J. H. Crosa.** 1994. Characterization of the *Vibrio anguillarum fur* gene: role in regulation of expression of the FatA outer membrane protein and catechols. Journal of Bacteriology. **176:**213-20.

3. **Wertheimer, A. M., M. E. Tolmasky, L. A. Actis, and J. H. Crosa.** 1994. Structural and Functional Analyses of Mutant Fur Proteins with Impaired Regulatory Function. Journal of Bacteriology. **176:**5116-5122.