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Characterization of a Gene Required for Catecholate Siderophore Biosynthesis in *Azotobacter vinelandii*

by

Anne Sharpe 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta
Spring, 1999

University of Alberta

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Degree: Master of Science

Year this Degree Granted: 1999

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"Characterization of a Gene Required for Catecholate Siderophore Biosynthesis in *Azotobacter vinelandii*"** submitted by **Anne Sharpe** in partial fulfillment of the requirements for the degree of **Master of Science** in **Microbiology and Biotechnology.**

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Jan 14/99
Date

To my parents

For all of their encouragement and support

ABSTRACT

Azotobacter vinelandii strain F196 is deficient in catecholate siderophore production as a result of a Tn*5luxAB* insertion. In order to characterize this mutation, a genomic library was constructed. Sequencing revealed the identity of the gene into which Tn*5luxAB* had inserted to be isochorismate synthase, the product of which is required to synthesize 2,3-dihydroxybenzoic acid, a precursor common to catecholate siderophores. This gene was designated as *csbC* for catecholate siderophore biosynthesis. Promoter mapping experiments identified the *csbC* promoter. Northern analysis revealed that *csbC* is expressed only under iron-limited conditions. This is likely due to Fur repression, as an iron-box was identified and the *E. coli* Fur protein did bind to the promoter region. The level of iron required to repress *csbC* was determined and the possibility that it is regulated by O_2-stress investigated. These results were used to propose a model for the sequential control of siderophore production observed in *A. vinelandii*.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. W. J. Page for giving me the opportunity to pursue graduate studies. I appreciate the independence he gave me with this project as well as the fact that he was always very approachable whether it was to discuss an exciting result or when experimental problems were encountered.

I would also like to thank the members of my supervisory committee, Dr. J. Foght, and Dr. B. Leskiw who provided excellent guidance over the course of this project. Special thanks also to Dr. Leskiw who allowed me not only to use hot-lab space but also lab equipment without which this work would have been impossible.

Without the help of many other people, completion of this thesis would have been very difficult and much less enjoyable. I really appreciate the patience and help of two people in particular, Manisha Mehrotra and Sonya Kujat, who both taught me a great deal and were always there answer my many questions. Manisha introduced me into the world of molecular biology, and really helped me through the first stage of my work. Without Sonya's help, I don't think I would have survived the RNA experiments. Thanks also to Tony Cornish for his many helpful hints, especially with computer related problems.

Thanks also to members of the Frost, Leskiw and Owttrim labs for providing advice with protocols, as well as sharing various reagents and enzymes.

Finally, thanks to Sean Tindale for his understanding and support during challenging and frustrating times.

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LIST OF ABBREVIATIONS

ACE	sodium acetate in ethanol
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
BBGN	Burk's buffer with glucose and nitrogen source
Bis-Tris	bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
bp	base pair(s)
BSA	bovine serum albumin
CAS	chrome azurol-S
cfu	colony forming units
cpm	counts per minute
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDDHA	ethylenediamine-di-(o-hydroxyphenyl) acetic acid
EDTA	ethylene diamine tetra acetic acid
EtOH	ethanol
HDTMA	hexadecyltrimethyl ammonium bromide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	hour(s)
IPTG	isopropyl-ß- D-thiogalatoside
kbp	kilo base pair (s)
LB	Luria-Bertani
MOPS	3-[N-Morpholino]propanesulfonic acid
Milli-Q H_2O	deionized water
NaOAc	sodium acetate
NH_4OAc	ammonium acetate
OD	optical density
0FeBBGN	Burk's buffer with glucose and nitrogen source, but no iron
ORF	open reading frame
PAG	polyacrylamide gel
PCR	polymerase chain reaction
PEM	potassium phosphate/EDTA/magnesium
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
rbs	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
sec	second(s)
SDS	sodium dodecyl sulfate
SSC	sodium chloride in tri-sodium citrate
SSPE	sodium chloride in sodium phosphate/EDTA
TAE	Tris/acetate/EDTA
TBE	Tris/borate/EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
µCi	microCurie
UV	ultra violet light

v/v	volume per volume
W	Watt(s)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

CHAPTER 1. INTRODUCTION

1.1 Physiological Importance of Iron

Iron (Fe) is a requirement for the survival of almost all organisms. The exception in the microbial world is *Lactobacillius* spp, which have evolved to utilize manganese and cobalt in place of iron (Guerinot, 1994). For the vast majority of organisms, iron plays an important role in many essential processes in the cell. It serves as a transporter of oxygen and as catalyst in energy-yielding electron transport processes (Payne, 1993). Iron is a cofactor of many important enzymes, including ribonucleotide reductase (for reduction of ribonucleotides to deoxyribonucleotides), nitrogenase (for N_2 fixation), peroxidase, catalase and superoxide dismutase (for protection against toxic products of oxygen metabolism), cytochromes, succinate dehydrogenase and ferredoxin which are used in electron transport chains (Weinberg, 1989; Litwin and Calderwood, 1993). The fact that this metal is indispensable is due to the nature of the iron atom, which can undergo reversible changes in its oxidation state. Both Fe(II) and Fe(III) are relatively small ions, and are able to form six-coordinate complexes with ligands containing oxygen, nitrogen or sulphur atoms (Neilands, 1984). The reduction potential of iron containing compounds varies depending on the ligand to which it is bound, but spans approximately 1 volt, making iron a very versatile biocatalyst (Guerinot, 1994; Litwin and Calderwood, 1993).

1.2 Availability of Iron in the Microbial World

Iron is plentiful, and is in fact the fourth most abundant element in the earth (Guerinot, 1994). In anaerobic environments, Fe(II) is the predominant form, which is quite soluble and thus easily acquired by microorganisms (Guerinot, 1994). However in

aerobic environments at neutral pH, iron is biologically unavailable. This is because Fe(II) is spontaneously oxidized to Fe(III) which is found most commonly complexed with oxide and oxyhydroxide polymers, forming insoluble mineral complexes such as goethite, hematite and magnetite (Hartwig and Loeppert, 1993). In vertebrate hosts, free iron is limiting, as it is either complexed with the glycoproteins transferrin (found in serum) and lactoferrin (found on mucosal surfaces) or is contained in heme, iron-sulphur proteins or stored in ferritin (Crosa, 1989; Guerinot, 1994). Iron must be supplied at a minimum level of between 0.01 to 1 μM for optimal growth of most microbes. However, free Fe(III) in an aerobic environment at neutral pH exists at levels of approximately 10^{-17} M (Neilands, 1984). A similar situation is encountered in mammalian hosts, where the amount of free iron in equilibrium with transferrin-bound iron is roughly 10^{-18} M (Litwin and Calderwood, 1993). Therefore, microorganisms require some mechanism to solubilize iron if they are to survive in such environments (Guerinot, 1994).

1.3 Production of Siderophores - A Means of Obtaining Iron

The word siderophore is derived from the Greek "sideros" meaning "iron", and "phorous" meaning "bearer". Siderophores are small molecules (between 500 and 1000 Da), excreted into the surrounding environment which specifically bind Fe(III) (Neilands, 1981). They have a considerably lower affinity for the more soluble Fe(II) (Neilands, 1995). Siderophores are unique to bacteria and fungi, and function to provide iron to the cell under conditions where it is insoluble and not in a readily accessible form. Siderophores are able to solubilize iron from mineral complexes. Most have a higher affinity for Fe(III) than do lactoferrin and transferrin and therefore are capable of scavenging iron from their host (Litwin and Calderwood, 1993; Guerinot, 1994). Aerobes and facultative anaerobes are thought to synthesize at least one siderophore, as they have been detected in all species tested (Neilands, 1981; 1984). Anaerobes do not

require any means to solubilze Fe, but likely have transport systems for Fe(II) (Guerinot, 1994). Similarly, facultative anaerobes like *E. coli* have a ferrous iron transport system which is expressed when this organism is grown under anaerobic conditions (Crosa, 1989).

Most siderophores can be classified according to their main chelating groups into either of two major classes: hydroxamates or catecholates. However, other siderophores of the mixed-ligand type have also been identified and these do not contain three identical bidentate chelating groups (Höfte, 1993). An example of a siderophore from each of these groups is shown in Figure 1.1, where a typical hydroxamate siderophore is illustrated by ferrichrome, enterobactin represents a catecholate, and the pyoverdin pseudobactin is an example of a siderophore containing three different chelating groups. Siderophores can be quite varied in structure, as seen in Figure 1.1. Despite these differences, it is most commonly the hydroxamate or catechol functional group of the siderophore that provides the oxygen atoms which form six-coordinate octahedral complexes with Fe(III) (Guerinot, 1994; Neilands, 1984). The high affinity of siderophores for Fe(III) is due to the pairing of the "hard" acid ion (ferric iron) with a correspondingly hard base atom, such as oxygen (Neilands, 1984).

Some microbes have evolved systems to avoid production of siderophores, but instead they are able to use siderophores synthesized by other organisms. This is done simply by producing a specific receptor protein and its cognate transport system, thus enabling the bacteria to "steal" iron intended for another organism. For example, in addition to producing enterobactin and aerobactin, *E. coli* has a transport system for the utilization of fungal siderophores such as ferrichromes (hydroxamates produced by *Penicillium* spp. and many other fungi), as well as using citrate (which is not a growth substrate) as an alternative Fe chelator (Neilands, 1995).

It should be noted that, although siderophores are probably the most common and efficient means of obtaining iron, other methods have evolved. Other strategies include:

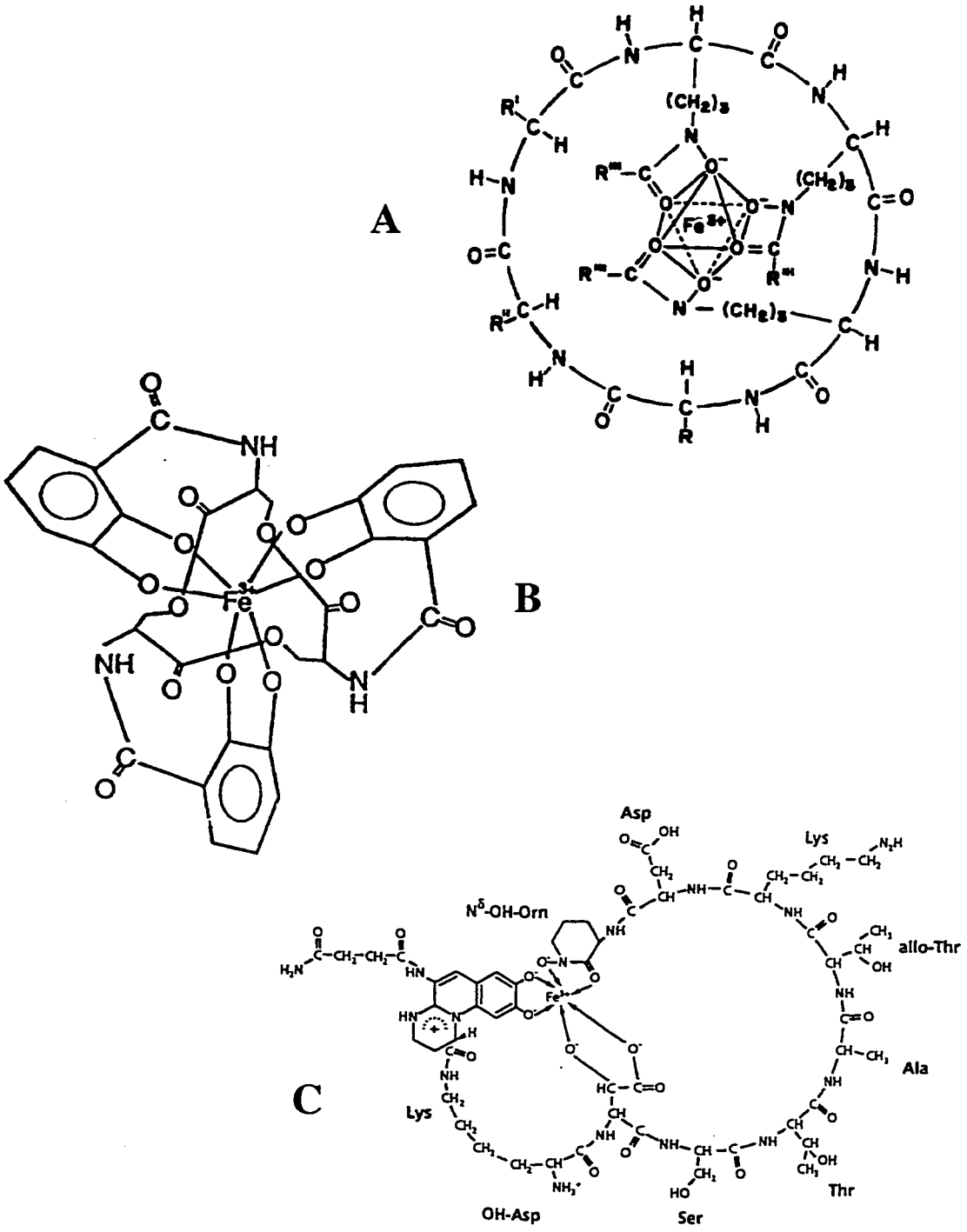


Figure 1.1 Siderophore structures. A: Ferrichrome, a typical hydroxamate siderophore produced by many fungal species (Neilands, 1995) B: Enterobactin, a typical catecholate siderophore produced by *E. coli* (Nikaido, 1993) C: Pseudobactin 358, a pyoverdine siderophore produced by *Pseudomonas putida* (Venturi *et al.*, 1995).

surface reduction of Fe(III) to the more soluble Fe(II) form, lowering the surrounding pH (since iron exists in soluble form at low pH), and direct utilization of host iron compounds such as heme, transferrin or lactoferrin (Neilands, 1995; Guerinot, 1994; Litwin and Calderwood, 1993). Although little is known about them, low-affinity uptake systems are also thought to exist, since it has been observed that isolates with mutations in the high affinity system are still viable in minimal media supplied with iron (Guerinot, 1994). Low affinity iron uptake is thought to function non-specifically and without carriers. High levels of soluble iron are required for growth independent of the high affinity uptake system and is successful only if Fe is not chelated (for example to citrate) so that it is readily available to the cell (Neilands, 1984; Guerinot, 1994).

1.4 Iron Contributes to Oxidative Stress

In addition to the many beneficial roles that iron plays in biological systems, it can also be deleterious to a cell. A result of aerobic respiration is the generation of partially reduced oxygen species; superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) which are the consequence of the inefficient reduction of oxygen to water (Neilands, 1989). As seen below, the Fenton reaction can catalytically promote hydroxyl radical ($\cdot OH$) production. Free Fe(III) is reduced by superoxide, generating ferrous iron which reacts with hydrogen peroxide to produce the hydroxyl radical, with regeneration of Fe(III) which is available to begin the cycle again. The hydroxyl radical is the most damaging of these oxidizing agents since it can oxidize and combine with any organic molecule (Neilands, 1989). Therefore, excess free iron in the cell in the presence of oxygen can generate oxidative stress which can result in cellular damage, mutation and death (Wai *et al.*, 1996).

The Fenton Reaction:

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH$$

In *E. coli*, there are at least two regulons that respond to oxidative stress. These include OxyR, which responds to H_2O_2 and SoxRS which is induced by $\cdot O_2^-$. After exposure to oxidizing conditions, the OxyR protein undergoes a conformational change and is able to activate transcription of several genes such as those encoding for catalase and alkyl hydroperoxidase (Demple and Amábile-Cuevas, 1991). The SoxRS system is a two component regulatory system where SoxR is the sensor of oxidative stress and activates transcription of *soxS*. SoxS is a DNA binding protein and a transcriptional activator of the regulon genes (Li and Demple, 1994). A consensus "sox-box" sequence has been determined (as seen below), version 1 proposed by Fawcett and Wolf (1994) and version 2 proposed by Li and Demple (1996). The SoxS binding site generally overlaps the -35 region of SoxS regulated promoters (Fawcett and Wolf 1994). Examples of genes under control of SoxRS system include those for Mn-superoxide dismutase (Mn-SOD), endonuclease IV and glucose-6-phosphate dehydrogenase (Demple, 1996).

1 AYNGCAYNRRNNRNYANNNNNWNNNNNNYW

2 ANNGCAYNNNNNNNCWA

Where Y = C or T, R = A or G, W = A or T and N= any nucleotide.

After assimilation, iron can be used to supply metabolic requirements or is stored internally in various forms (Briat, 1992). Because of the toxicity of iron, in an aerobic environment, it is thought that all aerobes likely produce ferritin, a protein able to bind iron in a soluble, non-toxic and bioavailable form (Briat, 1992). However, relatively little is known about the intracellular metabolism of iron as compared to the extensive

knowledge we have of iron-uptake mechanisms (Wai *et al.*, 1996). The importance of regulating the internal iron concentration is obvious, and since mechanisms for excreting iron from the cell do not exist, it must be regulated at the level of uptake (Bagg and Neilands, 1987).

1.5 The Fur Protein - Regulator of Iron Uptake

Fur (ferric uptake regulator) was first identified in *Salmonella typhimurium,* when mutants constitutively derepressed in the production of all systems for siderophore synthesis and transport were isolated (Ernst *et al.*, 1978). An *E. coli fur* mutant was isolated (Hantke, 1981) and the *fur* gene was subsequently cloned and sequenced (Hantke, 1984; Schaffer *et al.*, 1985) and has been intensively studied. Fur is a 148 amino acid, 17 kDa protein, rich in histidines (Hantke, 1984). This protein is a classical transcriptional repressor and, although it lacks the classic helix-turn-helix motif, it has been shown to bind DNA in the presence of Fe(II) as a co-repressor. It has been shown that any of the first row divalent cation transition metals (Mn, Co, Cu, Zn and Cd) can also act as Fur's co-repressor *in vitro* (Bagg and Neilands, 1987; De Lorenzo *et al.*, 1987). The Fur-Fe(II) complex recognizes a nucleotide sequence called the "fur-box" or "iron-box" which is a 19 bp hyphenated dyad repeat (Litwin and Calderwood, 1993). A consensus iron-box sequence (shown in Figure 1.2) for Fur binding was proposed by several researchers (Calderwood and Mekalanos, 1987; De Lorenzo *et al.*, 1987) based on the results of DNA footprinting of the aerobactin promoter and comparison to published sequences of other iron-regulated genes. Calderwood and Mekalanos (1988) demonstrated that by simply introducing an oligonucleotide with the iron-box consensus sequence just downstream of the *ompF* promoter, cloned in front of *lacZ* , this rendered β-galactosidase activity iron-regulated. As seen in Figure 1.2, the iron-box overlaps the promoter of iron regulated genes, and when ferrous iron is readily available, Fur is

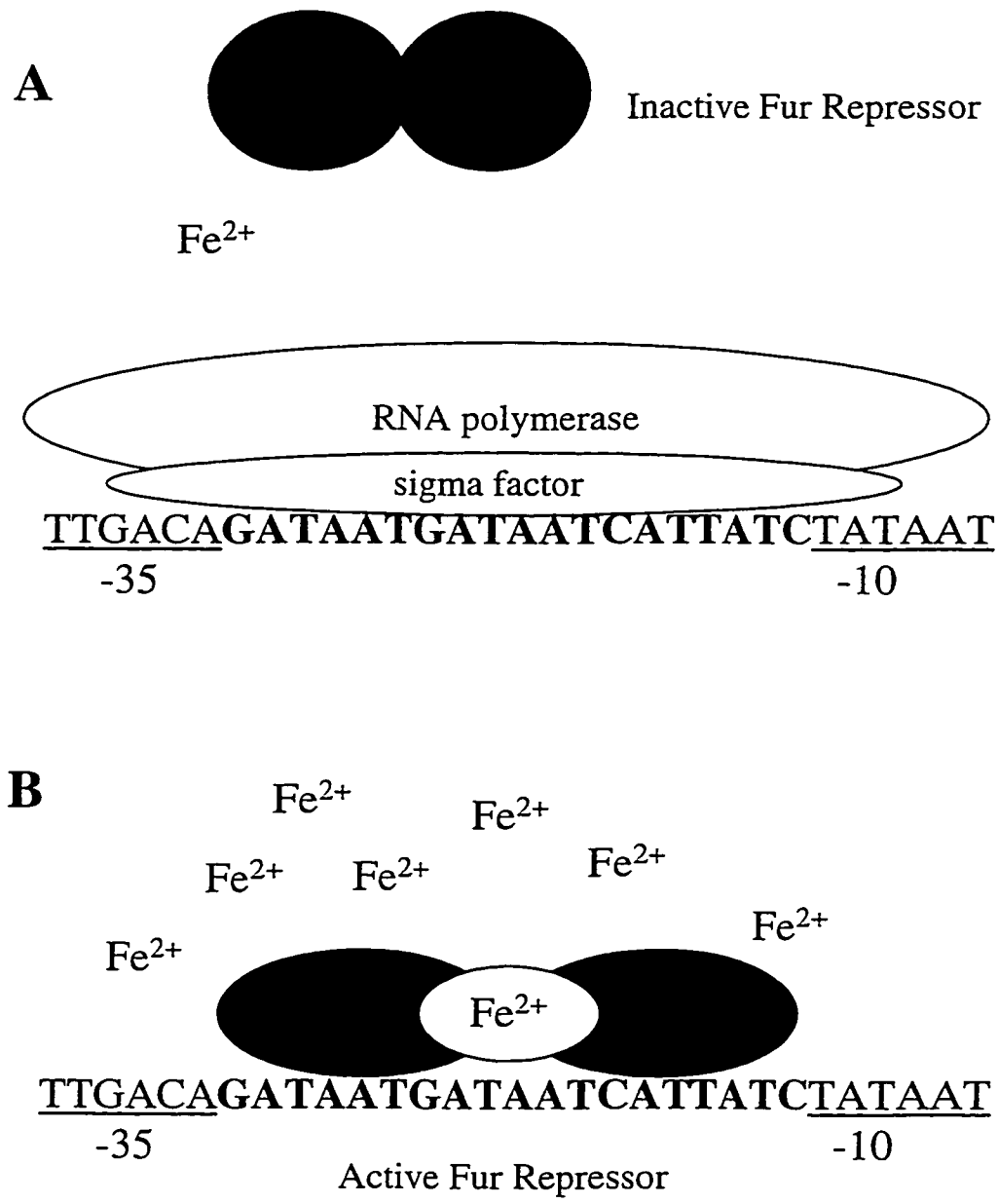


Figure 1.2 Regulation of an iron regulated promoter by the *E. coli* Fur protein. The iron box is shown in bold. A: Transcription occurs when Fe(II) levels are low, Fur is inactive and dissociates from the promoter. B: Repression, in the presence of Fe(II) by the active Fur protein (adapted from Neilands, 1989; Hennecke, 1990; Litwin and Calderwood 1993).

activated, and binds to the iron-box preventing transcription from that promoter. When intracellular Fe(II) concentrations are low, Fur is unable to bind to the DNA, and dissociates from the promoter, allowing expression of that gene. It is thought that a Fur dimer contacts one face of the DNA across three successive major grooves (Coy, 1995). It is believed that increased amounts of Fur lead to repressor polymerization through the operator region (De Lorenzo *et al.*, 1988a). The alpha helices in the N-terminal region of Fur are thought to recognize DNA and are thus involved in DNA binding, while the C-terminus mediates dimerization and is thought to be involved in Fe(II) binding to the repressor (Saito *et al.*, 1991a, b, c; Coy *et al.*, 1994; Stojiljkovic and Hantke, 1995). The same authors speculate that the binding of Fe(II) to Fur causes a conformational change in the N-terminus, allowing binding to the operator of Fur-regulated genes. However, it is unknown whether Fur dimerizes upon Fe(II) binding or occurs as a dimer independent of iron. It is in this way that the production of siderophores and their cognate uptake systems are regulated, such that the absorption of iron is tightly regulated by the availability of iron in the cell. This presents a model that can be used to study the molecular mechanisms of iron-regulation in other bacterial species (Litwin and Calderwood, 1993).

In fact, this mechanism for regulation of genes by iron including those required for iron uptake is thought to be widespread, as Fur homologs have been identified in several genetically diverse bacteria. These include *Yersinia pestis* (Staggs and Perry 1991), *Vibrio cholerae* (Litwin *et al.*, 1992), *Pseudomonas aeruginosa* (Prince *et al.*, 1993), *Neisseria meningitidis* (Thomas and Sparling, 1994), *Legionella pneumophila* (Hickey and Cianciotto, 1994), *Synechococcus* sp. strain PCC 7942 (Ghassemian and Straus, 1996) and *Klebsiella pneumoniae* (Achenbach and Yang, 1997).

The *fur* gene in *E. coli* is thought to be under dual regulation of the catabolite gene activator protein (CAP) as well as being auto-regulated by its own product. A binding site for CAP was identified in the promoter region of *fur*, and analysis of *E. coli*

strains containing a *fur-lacZ* fusion showed that *fur* expression is regulated by the CAP-cAMP system (De Lorenzo *et al.*, 1988b). Identification of an iron-box as well as DNA footprinting with Fur demonstrated that the Fur protein does bind to its own promoter, however relatively high levels were required to protect the DNA (De Lorenzo *et al.*, 1987). This indicates that there is a correlation between the modulation of Fe absorption and the metabolic status of the cell (De Lorenzo *et al.*, 1988b).

Fur has the ability to coordinate the regulation of more than just iron acquisition systems, but actually regulates numerous genes in *E. coli* and several other microbes (Stojiljkovic *et al.*, 1994; Litwin and Calderwood, 1993). These include various toxins and other virulence factors apparently unrelated to iron metabolism. For example, Fur appears to influence the production of several pH-regulated gene products in *S. typhimurium*, since *fur* mutants failed to induce effective acid tolerance response and were thus found to be extremely acid sensitive (Foster and Hall, 1992). It has also been shown that the Fe-limited conditions of a host are used as an environmental signal to the invading pathogen that they have entered their host. Thus, not only are the genes required for iron acquisition derepressed, as a result of Fur dissociating from the iron-boxes, but other virulence factors such as hemolysins and toxins appear to be under control of the same regulator and are simultaneously expressed. This is the case in *V. cholerae* where Fur acts as a global regulator, modulating the expression of a hemolysin, an outer membrane protein required for virulence, the siderophore vibriobactin and possibly an outer membrane protein required for heme utilization (Litwin and Calderwood, 1994; Payne, 1993). Other examples of Fe-regulated virulence factors include production of the diphtheria toxin in *Cornebacterium diphtheriae*, synthesis of exotoxin A in *P. aeruginosa,* and Shiga toxin in *Shigella dysenteriae* (Payne, 1993; Litwin and Calderwood, 1993).

1.6 Enterobactin - the Prototypical Catecholate Siderophore

For the purpose of this thesis, focus will be on the biosynthesis of catecholate-type siderophores, using the well characterized enterobactin system of *E. coli* as a model for catecholate siderophore biosynthesis in other organisms. A common intermediate in the biosynthesis of catecholate siderophores is 2,3-dihydroxybenzoic acid (DHBA), which provides the catechol moiety of the siderophore. The synthesis of catecholate siderophores is generally a two-stage process, first requiring the formation of DHBA from the precursor chorismate and the second involving the final assembly of the active siderophore, complexing DHBA, often with amino acids, as is the case with enterobactin which involves serine.

The catecholate siderophore which has been best characterized in terms of both biosynthesis, and transport is enterobactin produced by *E. coli*. It has been found that enterobactin is also synthesized by other members of the family *Enterobacteriaceae* such as *Salmonella*, *Klebsiella* and *Shigella* spp., and also *Aeromonas* spp. (family *Vibrionaceae*) which explains the interest in understanding the genetics and metabolism of this particular siderophore (Crosa, 1989).

1.6.1 Biosynthesis of Enterobactin

Enterobactin biosynthesis begins with the synthesis of DHBA from chorismic acid (the general intermediate in aromatic amino acid biosynthesis) by the products of three genes: *entA*, *entB* and *entC* (Young *et al.*, 1971) as seen in Figure 1.3. EntC, isochorismate synthase, catalyzes the conversion of chorismate to isochorismate. The *entC* gene has been sequenced (Ozenberger *et al.*, 1989), and the protein has been overexpressed and characterized (Liu *et al.*, 1990). This gene shares homology to *trpE* and *pabB* which encode chorismate-utilizing proteins (Elkins and Earhart, 1988). An

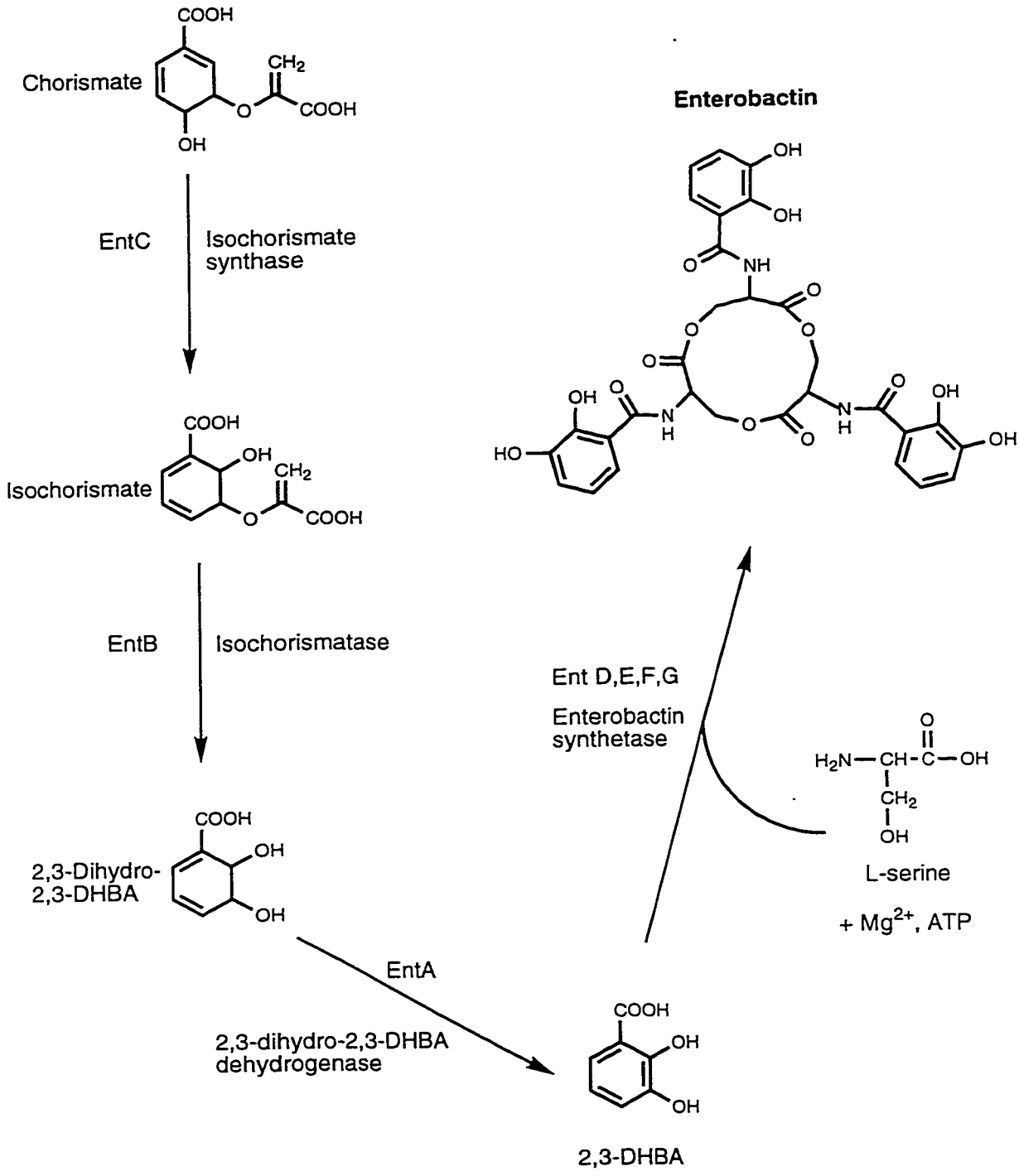


Figure 1.3 Biosynthesis of enterobactin in *E. coli*.
Adapted from Silver and Walderhaug, 1992.

iron-box has been identified overlapping the *entC* promoter region (Brickman *et al.*, 1990). The next step is catalyzed by isochorismatase, encoded by *entB*. This protein is bi-functional, where the C-terminal region encodes EntG activity, required in enterobactin synthetase activity, as discussed below (Staab and Earhart, 1990). The *entB* gene also has been sequenced (Nahlik *et al.*, 1989), overexpressed and characterized (Rusnak *et al.*, 1990). EntA, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, catalyses a NAD^+-dependent oxidation, the final step in the formation of DHBA (Liu *et al.*, 1989; Sakaitani *et al.*, 1990). The gene for this protein has also been sequenced, overexpressed and characterized (Nahlik *et al.*, 1989; Liu *et al.*, 1989).

Enterobactin synthesis is completed by coupling three molecules each of DHBA and L-serine in an ATP-dependent manner to create a cyclic trimer of 2,3-dihydroxybenzoyl-serine (DHBS) (Bryce and Brot, 1972). The reactions required for the final assembly are catalysed by the products of the *entDEFG* genes (Luke and Gibson, 1971; Woodrow *et al.*, 1975). Questions surrounding the final steps in enterobactin biosynthesis involving the conversion of DHBA and serine to enterobactin have just recently been answered. In fact Gehring *et al.* (1998) have demonstrated the *in vitro* reconstitution of enterobactin synthetase activity using purified enzyme components. The process is similar to the reactions involved in the synthesis of peptide antibiotics, where the peptide (amide) bonds linking one DHBA molecule to one L-serine to generate DHBS are made non-ribososomally. Two DHBS monomers are coupled to form a dimer, by ester bond formation between the carboxyl group of one serine and the hydroxyl group of a second serine residue. A third monomer is added and the resulting trimer is cyclized, thus releasing enterobactin. EntE, 2,3-dihydroxybenzoate-AMP ligase, plays the role of activating DHBA by forming a DHBA-adenylate complex which is available for subsequent amide bond formation with serine (Bryce and Brot, 1972; Rusnak *et al.*, 1989). It has recently been shown that EntD phosphopantetheinylates EntG and EntF for their roles in enterobactin assembly (Gehring *et al.*, 1997). After priming of EntG by

EntD, EntE transfers the activated DHBA onto EntG in preparation for amide bond formation with L-serine (Gehring *et al.*, 1997). EntF is responsible for activating and binding serine (Rusnak *et al.*, 1991; Reichert, 1992). It has been suggested that the transamidation reaction between activated DHBA and serine to generate DHBS occurs on EntF and that this complex remains covalently attached to EntF (Bryce and Brot, 1972; Rusnak *et al.*, 1991). Gehring *et al.* (1998) propose that EntF also catalyses the formation of the three ester bonds in enterobactin. The C-terminus of the EntB protein (which has EntG activity) was found to have homology to a consensus substrate amino acid binding site for enzymes engaged in non-ribosomal peptide synthesis (Hantash *et al.*, 1997). It has been proposed that EntD, E, F and G form a multi-enzyme complex and that this complex is associated with the cytoplasmic membrane by the association of EntD, which is proposed to anchor the complex to the membrane (Greenwood and Luke, 1976). Recently, Hantash *et al.* (1997) have provided indirect evidence that the multi-enzyme complex is membrane associated *in vivo*. They determined that EntE, F and G belong to the Beacham group D class of proteins, which have a loose or partial association with the inner surface of the cytoplasmic membrane. The mechanism for exporting enterobactin, or for that matter, any other siderophore from the cell is not well understood. It has been suggested that due to the fact that the enzyme complex is associated with the membrane, that enterobactin synthesis might be coupled with its export from the cell.

As shown in Figure 1.4, the genes required for enterobactin biosynthesis and transport are conveniently clustered in several transcriptional units. Each operon is negatively regulated by the Fur protein, due to the presence of an iron-box which overlaps the promoter region. The genes required for the first stage of biosynthetic pathway are transcribed as a polycistronic operon controlled by the *entC* promoter, along with a gene encoding a 15 kDa protein (*orf15*), the function of which is unknown, but is not required for enterobactin biosynthesis (Nahlik *et al.*, 1989). As can be seen in Figure

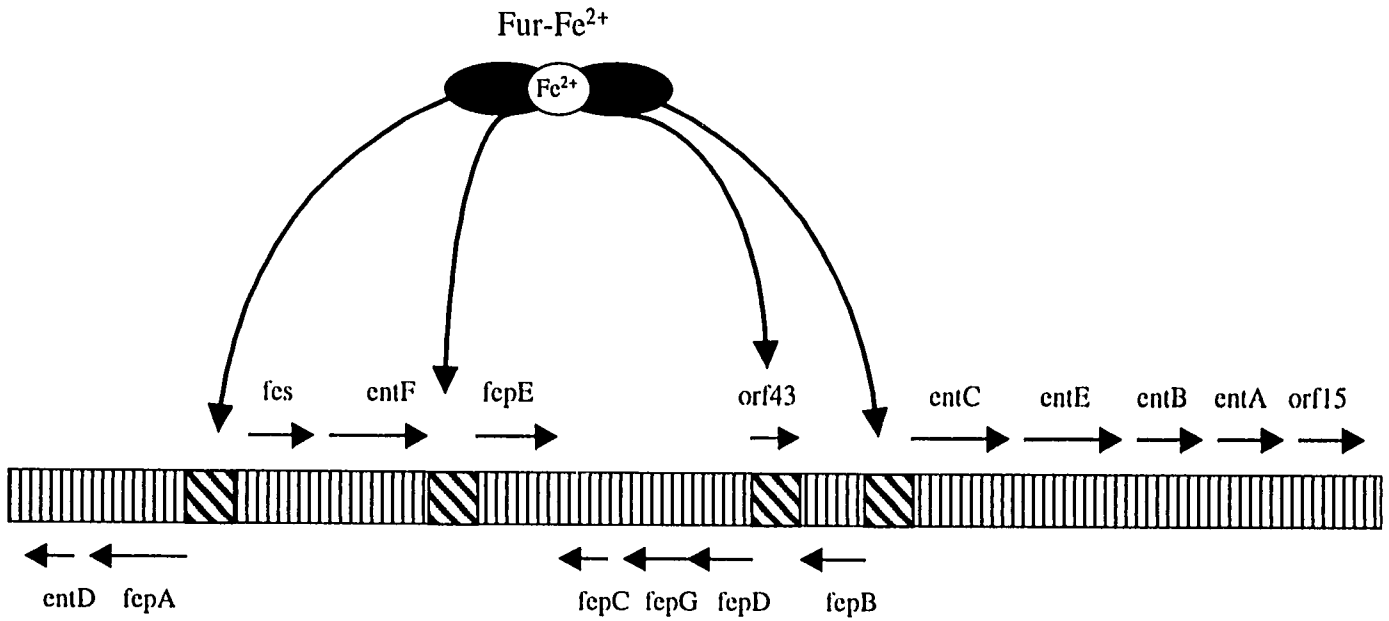


Figure 1.4 Organization of the cluster of *E. coli* genes required for enterobactin biosynthesis and transport. Reproduced from Crosa, 1989; Silver and Walderhaug, 1992.

▨ Indicates an iron box

1.4, there are many other genes in this cluster. The genes required for the second stage of enterobactin biosynthesis are scattered among the *fep* genes, which are required for transport of the ferric-enterobactin complex into the cell, as well as *fes*, which is required for iron release from ferric enterobactin (Pettis and McIntosh, 1987; Chenault and Earhart, 1991). Shea *et al.* (1991) identified an open reading frame (termed *orf43*), predicted to encode a 43 kDa protein, that is found within the cluster of genes required for enterobactin synthesis and transport, but is not essential for either of these processes.

1.6.2 Transport of the Ferric-Enterobactin Complex

The means by which iron is taken up by the cell once bound to a siderophore has been well characterized in *E. coli.* The molecular weight of siderophores exceeds the free diffusion limit of the outer membrane (for example, enterobactin is 669 Da) since porins generally allow diffusion of substrates up to 600 Da (Neilands, 1995). Therefore, *E. coli* has evolved a transport system which includes a receptor protein which is highly specific for each individual siderophore type that it uses. It is likely that a similar mechanism exists in other bacteria, although some microbes have receptors that are less specific and function to recognize several siderophore types. This suggests that microorganisms have at least two strategies for using siderophores; one employing highly specific receptors and a second that requires one or a few receptors that have broad specificity (Crowley *et al.*, 1991). These receptors have been found to also be used by a variety of lethal agents such as bacteriophage, bacteriocins and antibiotics (Neilands, 1995).

All systems in gram negative bacteria for the uptake of ferric-siderophore complexes into the cytoplasm involve active transport across the outer membrane. Active transport across the outer membrane commonly involves the participation of the TonB, ExbB and ExbD proteins which are involved in energizing the outer membrane receptor (Guerinot, 1994). TonB couples the electrochemical potential of the

cytoplasmic membrane to the active transport of ferric-siderophore complexes across the outer membrane by opening channels in outer membrane siderophore receptor proteins (Guerinot, 1994; Braun *et al.*, 1996). ExbB and ExbD are found in the cytoplasmic membrane and are involved in stabilizing TonB (Guerinot, 1994). Subsequent steps involve a periplasmic binding protein, an integral membrane component and an energy providing membrane bound ATPase which together facilitate Fe or Fe siderophore passage to the cytoplasm. It is evident that translocation of the iron-siderophore compound across the cytoplasmic membrane is less specific than transport across the outer membrane (Braun, 1985).

As shown in Figure 1.5, ferric enterobactin is transported into the cytoplasm through a typical periplasmic binding protein-dependent system. FepA is an outer membrane receptor protein that is highly specific for ferric-enterobactin. Binding induces a conformational change (Moeck and Coulton, 1998) and the siderophore receptor interacts with the TonB protein. There is some debate as to whether TonB remains anchored in the cytoplasmic membrane, or if it is released and associates entirely with the outer membrane (Moeck and Coulton, 1998; Letain and Postle, 1997). TonB-dependent outer membrane proteins have a common amino acid sequence (a TonB box), which is important for interaction between the two proteins. The FepA protein has a TonB box in its N-terminus, and when FepA undergoes conformational change due to ligand binding, this provides a signal causing a physical interaction between FepA and TonB (Moeck and Coulton, 1998). This interaction allows the transduction of energy from the cytoplasmic membrane through TonB and induces a conformational change in FepA which is a gated pore. Opening the pore allows uptake of ferric-enterobactin (Braun *et al.*, 1996). After transport across the outer membrane, the next step in the internalization of ferric-enterobactin involves binding to the periplasmic binding protein FepB (Pierce and Earhart, 1986; Elkins and Earhart, 1989; Stephens *et al.*, 1995). FepB presents ferric-enterobactin to a complex of inner membrane proteins FepD, FepG (Shea

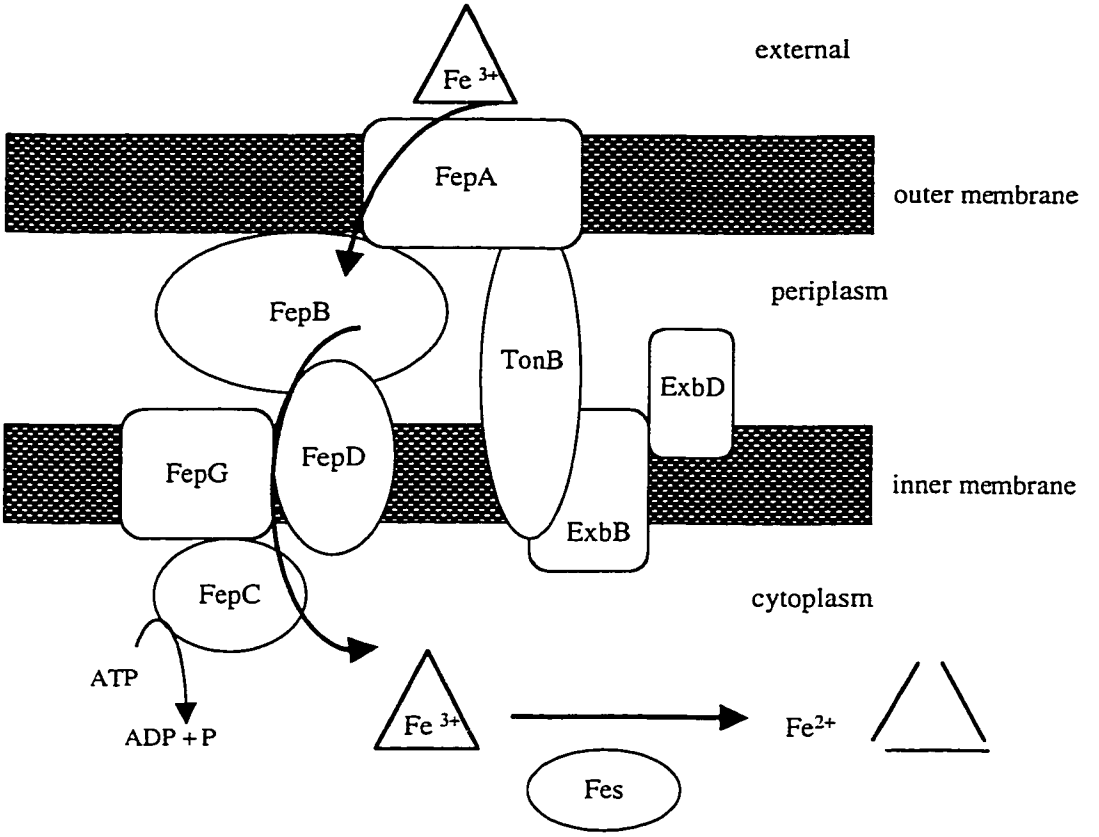


Figure 1.5 Transport of ferric-enterobactin. Enterobactin is represented by the triangle. Based on Silver and Walderhaug, 1992; Guerinot, 1994.

and McIntosh, 1991), and FepC, an ATP-binding protein that is membrane associated (Pierce and Earhart, 1986; Ozenberger *et al.*, 1987). This complex uses ATP to transport ferric-enterobactin through the cytoplasmic membrane (Silver and Walderhaug, 1992). FepE also has been identified and is required in this process, but its function is unknown (Ozenberger *et al.*, 1987).

Once in the cytoplasm, the product of the *fes* gene, an esterase, cleaves the ester backbone of enterobactin, which results in an increase of the dissociation constant from 10^{-52} M to 10^{-8} M (Guerinot, 1994). This is coupled with iron reduction, to release Fe(II) (Brickman and McIntosh, 1992). From here, Fe(II) is incorporated by ferrochelatase (which is not a specific *ent*-system gene product) into heme and non-heme iron proteins (Silver and Walderhaug, 1992). Once the Fe is released, the siderophore is either transported out (recycled) or modified in order to prevent it from chelating intracellular Fe (Guerinot, 1994).

1.7 Overview of Catecholate Siderophore Production by Other Microbes

A common intermediate of catecholate siderophore biosynthesis is DHBA and it is believed that the synthesis pathways for DHBA are strongly conserved among many gram negative and gram positive bacteria (Wyckoff *et al.*, 1997). Similar mechanisms for the formation of DHBA have been demonstrated among many bacteria, including *E. coli*, *V. cholerae*, *Aeromonas hydrophila*, *Bacilus subtilis,* and *Erwinia chrysanthemi.* Among the enterobactin producing enteric bacteria, such as *Salmonella*, *Klebsiella* and *Shigella* spp., it is expected that there would be some similarity in all aspects of the biosynthetic pathway. The final assembly of the siderophore involves complexing DHBA with various amino acids such as lysine (in *Azotobacter vinelandii*, and *A. hydrophila*) and glycine as well as either tryptophan or phenylalanine (in the case of amonabactin produced by *A. hydrophila*) or other molecules like norspermidine (in the

case of vibriobactin produced by *V. cholerae*) and therefore these steps are thought to require enzymes which are specific to production of each siderophore. It has been shown that among many microbes, there is functional homology between the gene products involved in the early steps of catechol biosynthesis particularly leading to the production of DHBA. The enzymes involved in final assembly are unique to the particular siderophore being produced, and therefore, much less similarity is expected among the genes coding for these enzymes.

Among the genes required for DHBA synthesis, there does appear to be a great deal of similarity in terms of gene organization, sequence and function. This suggests evolutionary relatedness of these systems, with the possibility that they evolved from a single ancestral group of DHBA genes (Massad *et al.*, 1994). Another common feature of the genes required for DHBA synthesis is that they tend to be clustered on the chromosome, however, there is some variation in the organization of the genes within the clusters (See Figure 1.6). It generally has been found that there is strong functional and amino acid homology, but weak homology at the DNA level among these genes. Not surprisingly, a common means of identifying catecholate biosynthetic genes from various catecholate siderophore producing bacteria has been to generate genomic libraries and screen them in *E. coli ent* mutants for complementation of enterobactin production. Massad *et al.* (1994) suggest that DHBA may be the archetypical catecholate and that more complex and varied structures have evolved as a result of the different environmental niches in which bacteria are found.

1.8 *Azotobacter vinelandii* - General Introduction

Azotobacter vinelandii is a large, pleomorphic, gram negative rod that is an obligately aerobic heterotroph. This organism is a common inhabitant of the soil and forms desiccation-resistant cysts when environmental conditions are unfavorable

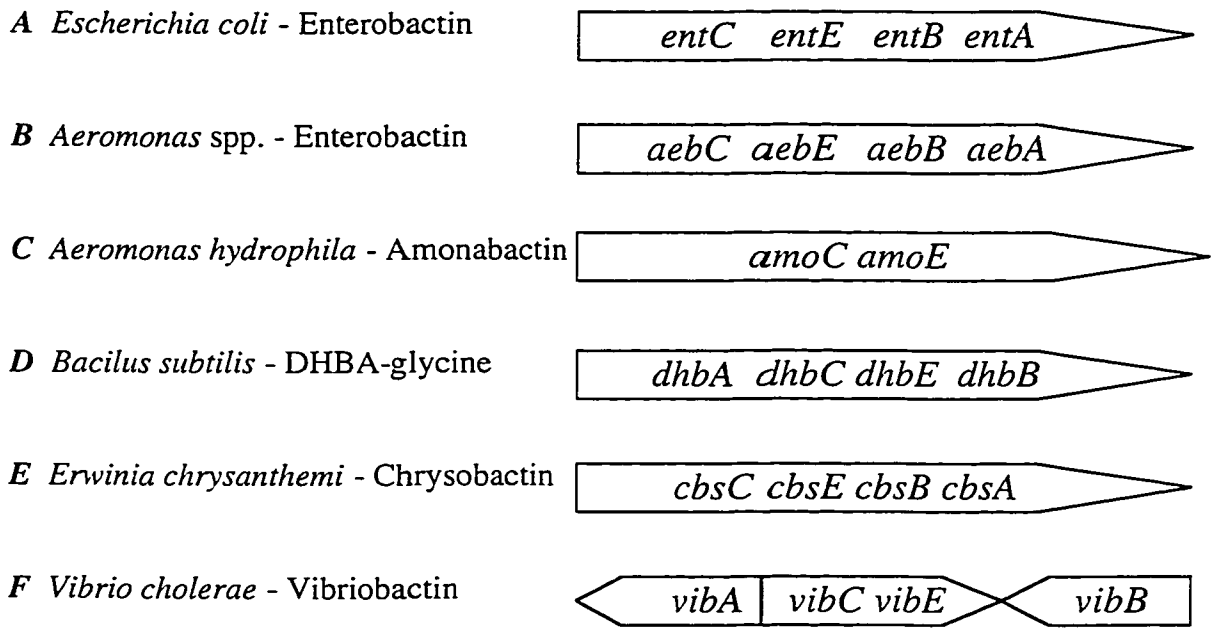


Figure 1.6 Organization and positions of the DHBA biosynthetic genes of various microorganisms. Arrows indicate direction of transcription. Adapted from: A. Nahlik *et al.*, 1989; B. Massad *et al.* , 1994; C. Barghouthi *et al.*, 1991; D. Rowland *et al.*, 1996; E. Franza and Expert, 1991; F. Wyckoff *et al.*, 1997.

(Becking, 1974). Phylogenetically, *Azotobacter* belongs to the gamma group of purple bacteria.

Another unique feature of *A. vinelandii* is that it is capable of aerobic, non-symbiotic N_2-fixation. The nitrogenases of *A. vinelandii* have either Fe-Mo, Fe-V or Fe-only active centers (Bishop, 1993). As a result of its ability to fix nitrogen under aerobic conditions, *Azotobacter vinelandii* has the highest respiratory rate of any living organism (Robson and Postgate, 1980). This extremely high rate of respiration is essential in order to scavenge cytoplasmic O_2 so as to protect nitrogenase from inhibition by O_2. In order to do this, *A. vinelandii* has a branched electron transport system. From quinone-cytochrome *b*, electrons are passed on to either of two terminal terminal oxidases: cytochrome *d* (which is uncoupled to ATP synthesis) or to cytochrome c_{4+5} (Robson and Postgate, 1980). Under N_2 fixing conditions and high aeration, levels of cytochrome *d* are elevated (Moshiri *et al.*, 1991). Thus, cytochrome *d* offers "respiratory protection" to the cells by depleting O_2 (Robson and Postgate, 1980). When O_2 tension is low, more electrons are passed to cytochrome c_{4+5}, which is coupled to ATP synthesis, and thus N_2 fixation is more efficient (Robson and Postgate, 1980).

The *b*-type cytochrome of *A. vinelandii* was reported in 1979 (Steifel and Watt) to be a bacterioferritin. The protein was found to contain heme as well as large amounts of non-heme iron and information obtained by examination by electron microscopy, amino acid composition, and molecular weight revealed characteristics nearly identical to mammalian ferritins. This led the authors to conclude that this protein was indeed a ferritin and likely served as an iron storage protein in *A. vinelandii*.

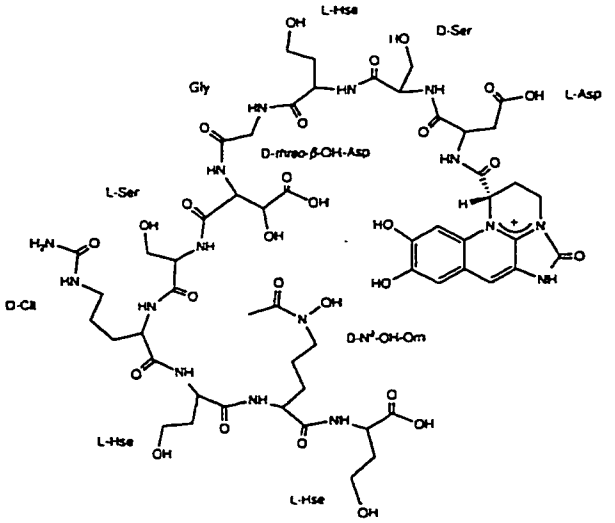
The *A. vinelandii* chromosome is approximately 4700 kbp and has a mol% guanosine + cytosine content of 65 (Sadoff *et al.*, 1979; Maldonado *et al.*, 1994). It has been suggested that *A. vinelandii* possesses approximately 40 copies of its chromosome (Sadoff *et al.*, 1979). Maldonado *et al.* (1994) compared DNA contents of *A. vinelandii* cells by flow cytometry and their results suggested that chromosome numbers varied

according to growth stage. Early exponential-phase cells have approximately 4 chromosomes per cell, whereas late-exponential-phase have 40 copies and this increases to at least 100 copies in stationary-phase cells, and is decreased to 4 chromosomes in cysts. The fact that it is possible to generate homozygotes is explained by the fact that at the start of every growth cycle there is a drastic decrease in ploidy (Maldonado *et al.*, 1994).

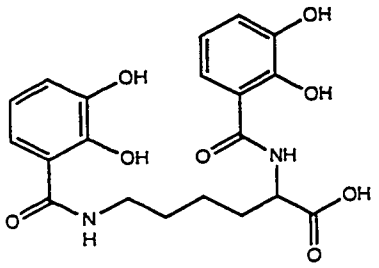
A by-product of aerobic respiration is the generation of toxic O_2 radicals. *A. vinelandii* has evolved ways to destroy these damaging molecules by production of catalase and Fe-containing superoxide dismutase. The fact that iron is indispensable for the survival of *A. vinelandii* is obvious, as Fe is required not only for respiratory enzymes and nitrogenases, but also for the enzymes that aid in protecting the cell from oxidative damage.

1.9 Siderophore Production in *A. vinelandii*

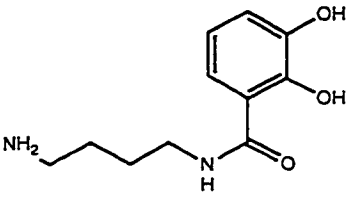
A. vinelandii produces four siderophores: three catecholates; azotochelin (Corbin, 1969), aminochelin (Page and Von Tigerstrom, 1988), and protochelin (Cornish and Page, 1995) as well as the fluorescent peptide (pyoverdin-like) siderophore, azotobactin (Demange *et al.*, 1986). The structures of these siderophores are shown in Figure 1.7. Under normal laboratory conditions, protochelin is produced in very low levels, but formation is seen to increase in the presence of millimolar levels of molybdate (Cornish and Page, 1995). As can be seen in Figure 1.7, protochelin is a product of the condensation of aminochelin and azotochelin (Cornish and Page, 1995). A unique feature seen in *A. vinelandii* is the sequential regulation of siderophores, where the catecholates are synthesized under iron limiting levels of less than 7 μM Fe but production of azotobactin does not occur until iron levels are less than 3 μM (Page and Von Tigerstrom, 1988). This is intriguing as this unique pattern has not been described



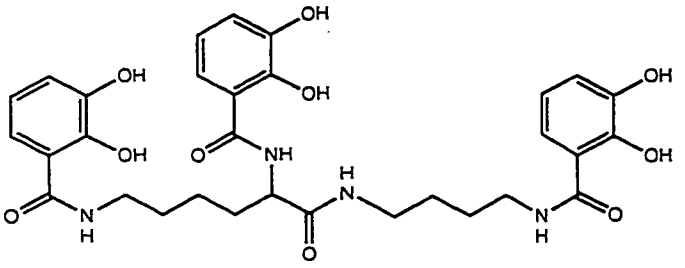
Azotobactin



Azotochelin



Aminochelin



Protochelin

Figure 1.7 Siderophores produced by *A. vinelandii*.
(Page *et al.*, 1991; Cornish and Page, 1995)

in other bacteria studied. The catecholate siderophores produced by *A. vinelandii* have a varied affinity for Fe, where aminochelin forms bidentate coordination of iron, azotochelin forms tetradentate coordination and protochelin is the best chelator of the three (Cornish and Page, 1998), owing to the fact that it certainly forms tetradentate coordination and likely forms hexadentate coordination of iron by the catechol moiety on the putrescine arm. In addition to the elaborate high-affinity iron uptake system, it is thought that DHBA is synthesized as part of the low affinity uptake system as production is seen even when iron levels are greater than 7 μM (Page and Huyer, 1984). The role of DHBA is thought to be the mobilization of cell surface-complexed iron, in cases where iron is readily available (Page and Huyer, 1984).

In addition, *A. vinelandii* derepresses high-molecular weight outer membrane proteins in response to iron limitation (Page and Huyer, 1984). These proteins are believed to function as ferric-siderophore receptors. It has also been shown that *A. vinelandii* produces a ferric reductase which may play a role in siderophore-mediated iron uptake, as it was shown that this enzyme has the ability to reduce siderophore bound iron. This ferric reductase was found to be located in the cytoplasm and is produced constitutively (Huyer and Page, 1989).

1.10 Strain F196 - General Introduction

A logical approach to studying siderophore biosynthesis in *A. vinelandii* would be to generate mutants deficient in siderophore production. This was indeed undertaken in our laboratory by transposon mutagenesis of the capsule-negative wild-type strain UW and subsequent screening of mutants for unusual siderophore phenotypes (Sevinc and Page, 1992). This method was particularly convenient, since a Tn*5* derivative containing promoterless *luxAB* was used. This allowed identification of mutants where the transposon had inserted into an iron-regulated gene, and permitted a simple means of

following the gene's activity, using Lux (bioluminescence) as a reporter (Sevinc and Page, 1992).

Siderophore deficient mutants were identified by spectrophotometrically scanning the culture supernatant of cultures grown under iron-limited and iron-sufficient conditions (Sevinc and Page, 1992). Mutants such as F196 (deficient in production of all catecholates) and D27 (azotobactin negative) are examples of strains generated by transposon mutagenesis. Both have Fe-repressible Lux activity, such that *luxAB* is under the control of *A. vinelandii* Fe- regulated promoters (Figure 1.8). Due to the fact that F196 and D27 were identified, it is clear that the genes involved in the synthesis of azotobactin and the catecholate siderophores are independent since each of these mutants is unable to produce one class of siderophore, but this has no effect on the ability to produce the other type of siderophore (Sevinc and Page, 1992).

Initial study of mutant F196 involved physiological analysis (Sevinc and Page, 1992). It was clear that this mutant was unable to produce any of the catecholate siderophores, and to compensate for this deficiency it produced 2.5 times the amount of azotobactin as wild-type UW. When grown in the presence of the catecholate siderophore precursor (DHBA), F196 was still unable to produce catecholate siderophores. It was hypothesized therefore that the insertion of the Tn*5luxAB* had a polar effect on an operon containing several genes required for synthesis of these siderophores. Failure of DHBA to restore catecholate biosynthesis could have also been due to the fact that it could not enter the cell.

Other recent work in our laboratory has shown that *A. vinelandii* produces a Fur-like protein (Mehrotra, 1997). This was shown by Western blotting, using antiserum for both the *E. coli* and *P. aeruginosa* Fur proteins. The Fur protein in *A. vinelandii* is thought to play a important role in the regulation of many genes (as described for other organisms). This idea is supported by the finding of Sevinc and Page (1992) that when UW mutants (generated by insertion of Tn*5luxAB*) were screened for iron-repressible

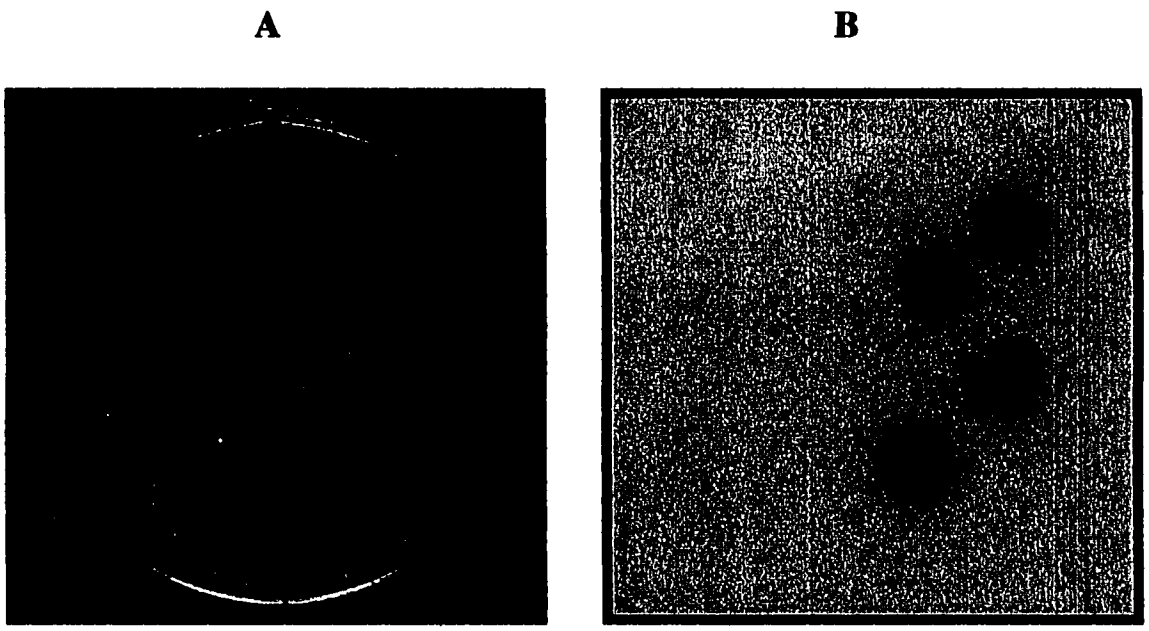


Figure 1.8 Iron repressible Lux activity in strain F196. A: The photograph shows a split plate which contains iron limited media on the right hand side and iron sufficient media on the left side of the plate. B: The same plate was exposed to X-ray film, and Lux activity is expressed only under iron limited conditions.

bioluminescence, many mutants that did not seem to have mutated Fe uptake systems demonstrated either moderately or strongly regulated bioluminescence.

1.11 An Additional Role for the Catecholate Siderophores of *A. vinelandii*

Recent studies by Cornish and Page (1998) demonstrated that, in addition to serving as iron solubilizing agents, the catecholate siderophores of *A. vinelandii* have a role in protecting the cell from oxidative stress. It was first observed that catecholate siderophores are over-produced when UW and the isogenic FdI-negative strain, LM100, are cultured under conditions of oxidative stress and this was seen to an even greater extent when the cells were fixing nitrogen. It is thought that the cells detect $\cdot O_2^-$ as a regulatory signal to induce protective mechanisms against oxidative stress. This is because *A. vinelandii* lacks a Mn-SOD and only has a Fe-SOD, which is inactive under iron-limited conditions (Cornish and Page, 1998). If $\cdot O_2^-$ is generated in the cell there is a risk of Fenton mediated ·OH formation. It was determined that protochelin and azotochelin both have the ability to withhold iron from participating in the Fenton reaction, thus preventing the formation of ·OH. It is thought that only low levels (4-5 μM) of protochelin are required to afford protection against oxidative stress. The results of Cornish and Page (1998) suggested that the catecholate biosynthetic genes may be under dual control, such that they are repressed by iron and induced by oxidative stress.

1.12 Thesis Objectives

In this thesis, I report progress towards a better understanding of the genetic organization of the genes required for biosynthesis of catecholate siderophores in *A. vinelandii*. I was interested in taking a molecular approach to studying F196 so as to determine the exact nature of the mutation, by identifying the gene which had been

disrupted by Tn*5luxAB*. This work was undertaken because very little was known about the biosynthesis of catecholate siderophores in *A. vinelandii*. Also, I was interested in gaining more insight into the nature of the unique pattern of sequential Fe regulation of siderophore expression in *A. vinelandii*, as well as learning more about the possibility that the catecholate biosynthetic genes are under the dual control by iron and superoxide. Attempts have been made to identify and characterize the iron-regulated promoter upstream of Tn*5luxAB* so as to gain some understanding about the Fe-regulated gene expression in *A. vinelandii*.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

Azotobacter vinelandii and *Escherichia coli* strains used in this study as well as cloning vectors and plasmid constructs are listed in Tables 2.1, 2.2, 2.3 respectively.

2.1.2 Growth Conditions and Media

A. vinelandii strains were maintained on slants or plates composed of Burk's Buffer (BB): 0.81 mM $MgSO_4$, 0.58 mM $CaSO_4$, 5 µM Na_2MoO_4, and 12.5 µM $FeSO_4 \cdot 7H_2O$ in 5 mM potassium phosphate buffer (pH 7.2) (Page and Sadoff, 1976) with the addition of 1% w/v glucose (G), the fixed nitrogen source 0.11% ammonium acetate (N); abbreviated as BBGN; 1.5% w/v Difco Bacto-agar was added for solid medium. A variation of this growth medium was 0FeBBGN, which contained no added iron. When 0FeBBGN was used, cultures were grown in acid-washed glassware (flasks were rinsed first with 4 N HCl followed by a rinse with Milli-Q H_2O and then 50 mM EDTA [pH 7.0] to chelate any ions, and finally thoroughly rinsed with Milli-Q H_2O). When required, iron was added to the medium in varying amounts by the addition of ferric citrate (25 mM sterile stock, added to sterile media). All strains were grown at 28 to 30°C. Liquid cultures were aerated on a platform shaker (New Brunswick Scientific gyrotory water bath shaker, model G-76) at 225 rpm. Kanamycin was added to the medium as required, to a final concentration of 12.5 µg/mL.

E. coli strains were grown in Luria-Bertani (LB) medium (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and 10 g/L NaCl, made in Milli-Q H_2O) solidified with 1.5 w/v Difco Bacto-agar if required. All strains were incubated at 37°C. For cultures grown in

Table 2.1 List of bacterial strains used in this study.

STRAIN	RELEVANT CHARACTERISTICS	USE	SOURCE	REFERENCE
A. vinelandii UW	Wild Type	Subject of Study	W. Brill University of Wisconsin	ATCC
A. vinelandii F196	W Tn5*luxAB* mutant, catechol negative	Subject of Study	W. J. Page University of Alberta	Sevinc and Page, 1992
E. coli DH5α	*supE*44 ΔlacU169 (ϕ80 *lac*ZM15) *hsdR*17 *recA*1 *end A*1 *gyrA*96 *thi*-1 *rel*A1	Cloning	Lab stocks	Sambrook *et al.*, 1989
E. coli VCS257	DP50 *sup* F[*supE44 supF58 hsd53*(r_Bm_B) *dapD8 lacY1 glnV44* Δ(*gal-uvrB*)*47 tyrT58 gyrA29 tonA53* Δ(*thyA57*)]	Host for Genomic Library	Stratagene	Stratagene
E. coli HB101	*supE*44 *hsdS*20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	study Fe regulation of *luxAB*	Lab stocks	Sambrook *et al.*, 1989
E. coli JM106	*end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 λ⁻Δ (*lac-pro*AB)	β-galactosidase assays	Lab stocks	Yanish-Perron *et al.*, 1985
E. coli AN 193-60	*entA*⁻Δ*recA*	Complementation assays	M. McIntosh University of Missouri	Nahlik *et al.*, 1987
E. coli AN 192-60	*entB*⁻ Δ*recA*	Complementation assays	M. McIntosh	Nahlik *et al.*, 1987
E. coli MT 147	*entC*::kan	Complementation assays	M. McIntosh	Ozenberger *et al.*, 1989

STRAIN	RELEVANT CHARACTERISTICS	USE	SOURCE	REFERENCE
E. coli AN 90-60	*entD*$^-$ Δ*recA*	Complementation assays	M. McIntosh	unpublished
E. coli AN 93-60	*entE*$^-$ Δ*recA*	Complementation assays	M. McIntosh	Nahlik *et al.*, 1987
E. coli AN 117-60	*entF*$^-$Δ*recA*	Complementation assays	M. McIntosh	unpublished
E. coli MFT5-0	*entG*$^-$	Complementation assays	M. McIntosh	unpublished

Table 2.2 List of cloning vectors used in this study.

CLONING VECTOR	SELECTIVE MARKERS	USE	REFERENCE
pLAFR3	Tetracycline	Cosmid, library	Staskawicz *et al.*, 1987
pK184	Kanamycin	cloning vector	Jobling *et al.*, 1990
pUC119	Ampicillin	cloning vector	Vieira *et al.*, 1987
pBluescriptKS+	Ampicillin	cloning vector	Stratagene
pQF50	Ampicillin	promoter probe vector	Farinha *et al.*, 1990

Table 2.3 List of plasmid constructs used in this study.

PLASMID NAME	CLONING VECTOR	SELECTIVE MARKERS	INSERT	USE	REFERENCE
pMH15	pACYC184	Chloramphenicol	*E. coli fur* gene	Fe regulation of *luxAB* on pAS1 and pAS20	Hantke, 1984
pAS1	pLAFR3	Tetracycline, Kanamycin	23 kbp *Sau*3A F196 chromosomal fragment	original clone	This study
pAS20	pK184	Kanamycin	11.5 kb *Sal*I fragment from pAS1	Fe regulation of *luxAB*	This study
pAS40	pUC119	Ampicillin, Kanamycin	11.5 kb *Sal*I fragment from pAS20	sequencing	This study
pAS50	pQF50	Ampicillin	250 bp PCR fragment cloned into *Bgl*II-*Sal*I site of vector	β-gal assays, source of DNA target for gel shift assays	This study
pAS60	pQF50	Ampicillin	250 bp PCR fragment cloned into *BamH*I-*Sal*I site of vector	β-gal assays	This study
pCON	-	Ampicillin	*E. coli iucA*	target for gel shift assays	De Lorenzo *et al.*, 1988a
pMVW97	pUC119	Ampicillin	*luxA*	330 bp *Pst*I fragment - probe for Southern blots	M. Mehrotra, 1997
pAS310	pBluescriptKS+	Ampicillin	310 bp *Not*I-*Pst*I fragment from *A. vinelandii csbC*	probe for Northen blots	This study

liquid medium, small volumes (5 mL or less) were incubated in test tubes on a tube roller, while larger volumes were incubated in flasks on a platform shaker at 225 rpm. Antibiotics were added to the medium, as required, at the following final concentrations: ampicillin 80 μg/mL, chloramphenicol 50 μg/mL, kanamycin 50 μg/mL, tetracycline 50 μg/mL.

Chrome Azurol-S (CAS) plates, used to screen for siderophore production by *E. coli* transformants, were prepared by the addition of necessary supplements to *E. coli* growth medium following the procedure described by Schwyn and Neilands (1987). All glassware was acid washed. A CAS stock was made by dissolving 60.5 mg CAS in 50 mL dH_2O and adding 10 mL of 1 mM $FeCl_3 \cdot 6\ H_2O$ (made in 10 mM HCl) with stirring. This solution was then added dropwise to 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) in 40 mL H_2O, and sterilized by autoclaving. The medium was prepared by mixing 30.24 g PIPES, 100 mL 10X salts (3 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl), 15 g Difco Bacto agar, brought to a final volume of 1 L with dH_2O and adjusted to pH 7.0 with 50% w/v NaOH. After the medium was autoclaved and had cooled to approximately 50°C, the CAS stock was added, along with the remaining components of the medium from sterile stocks, to give a final concentration of: 0.3% casamino acids, 0.2% glucose, 0.0002% thiamine-HCl and 0.003% tryptophan.

2.2 Isolation of DNA

2.2.1 Isolation of Chromosomal DNA from *A. vinelandii*

Chromosomal DNA was extracted from *A. vinelandii* following the procedure described by Robson *et al.* (1984). Three hundred millilitres of an 18 hour culture were harvested by centrifugation in a RC-5 super-speed refrigerated centrifuge (Dupont Instruments), at 10400 x g for 10 min. The cell pellet was resuspended in 10 mL of PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0) and the cells were collected by

centrifugation for 10 min at 10400 x g. The cell pellet was resuspended in 12 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 1.5% SDS (w/v) and was transferred to a 30 mL Corex tube and incubated at 30°C for about 10 min. Proteinase K (Boehringer Mannheim, stock made fresh in TE buffer) was added to a final concentration of 1 mg/mL and protein digestion was carried out at 37°C for about 3 hr, with occasional gentle mixing until the solution was clear and viscous. DNA was precipitated by addition of 2 volumes of ACE (0.2 M NaOAc made in 90% v/v EtOH) and collected by spooling gently onto a sterile glass rod. Excess EtOH was allowed to drain and the DNA was transferred into 10 mL of TE buffer, where it was left to solubilize overnight at RT. This solution was extracted three times with half a volume of phenol saturated with STE (100 mM NaCl, 1 mM Na_2 EDTA, 10 mM Tris-HCl, pH 8.0) by gentle shaking for 3 min, followed by centrifugation at 10400 x g for 5 min, and the aqueous phase was transferred to a sterile tube with a pasteur pipette. The aqueous phase was then extracted twice (as above) with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was reprecipitated with 2 volumes of ACE, then the DNA was spooled onto a glass rod, and transferred to 3 mL of TE buffer where it was left to solubilize overnight at RT, and was stored thereafter at 4 °C.

2.2.2 Isolation of Plasmid DNA from *Escherichia coli*

Small-scale preparations of plasmid DNA were carried out using the alkaline lysis method described by Sambrook *et al.* (1989). Approximately 1.5 mL of an overnight culture was transferred to an Eppendorf tube and cells were harvested by microcentrifugation at 13 000 rpm in a benchtop Eppendorf centrifuge (model 5415C) for about 20 sec. As much of the supernatant as possible was removed by aspiration. The cell pellet was completely resuspended in 100 µL of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) on a vortex mixer. The tubes were inverted quickly

several times after addition of 200 µL of solution II (0.2 N NaOH, 1% w/v SDS, freshly made), ensuring that the entire surface of the tube came into contact with solution II, and the tube was placed on ice. After addition of 150 µL of cold solution III (60 mL of 5 M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL dH_2O), the tubes were mixed on a vortex in an inverted position for 10 seconds so that the solution III was dispersed through the bacterial lysate. Tubes were incubated on ice for 3-5 min then cell debris was removed by microcentrifugation in a MSE benchtop centrifuge for 5 min at 13 000 rpm at 4°C. An equal volume of phenol:chloroform was added to the supernatant in a clean Eppendorf tube, mixed on a vortex, and the aqueous phase was separated from the organic phase by microcentrifugation for 5 min at 13 000 rpm. The supernatant was transferred to a new Eppendorf tube and DNA was precipitated by addition of 900 µL of 95% v/v EtOH and incubated on ice for at least 10 min. DNA was collected by centrifugation at 12 000 rpm for at least 5 min at 4°C. The supernatant was removed by aspiration, the DNA pellet was washed with 1 mL 70% v/v EtOH, and allowed to air-dry for about 15 min. Once dry, the DNA was resuspended in 25 µL of TE buffer containing 40 µg/mL DNase-free RNase (Sigma) and incubated for about 30 min at 37°C, and stored at -20°C.

2.3 Restriction Endonuclease Digestion of DNA

Restriction enzymes were purchased from either Boehringer Mannheim or New England Biolabs, Inc. DNA was digested by restriction endonucleases according to manufacturers instructions in a reaction using the provided buffer and containing an amount of restriction enzyme that did not exceed 0.1 volume of the total reaction mixture, in order to prevent restriction enzyme inhibition by glycerol.

2.4 Gel Electrophoresis of DNA

DNA digests were analysed on either 0.8-2.0% w/v agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or 5-12% polyacrylamide gels, in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0), either immediately following digestion, or after inactivation of the restriction endonuclease by heat or by extraction with one volume of phenol:chloroform. Prior to loading the sample on the gel, 6X loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF in a 30% v/v glycerol solution made in water) was added to a final concentration of 1X. In order to determine the size of DNA fragments generated by restriction enzyme digestion, molecular weight standards were also run on each gel: 300 - 500 ng λ phage DNA digested with *Hin*dIII or *Pst*I, and/or commercially available markers such as the Boehringer Mannheim DNA molecular weight marker XIII (a 50 bp ladder) or the Bio-Rad 100 bp molecular ruler. Electrophoresis was carried out at constant voltage and gels were stained with ethidium bromide (10 μg/mL in dH_2O) and DNA was visualized on a UV transilluminator.

2.5 Crush and Soak Method for Eluting DNA from Polyacrylamide Gels

This method, following the protocol described by Sambrook *et al.*, 1989, was used for isolating and purifying DNA fragments to be used in cloning, as targets in gel shift assays, or as DNA probes for Southern or Northern hybridization.

The DNA of interest was run on 5-12% polyacrylamide gel (as appropriate) and the gel was stained with ethidium bromide. The DNA band of interest was located and carefully cut out of the gel with a scalpel. The slice of polyacrylamide was crushed with a 1 mL pipette tip in an Eppendorf tube. To this tube, 500 μL of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0) was added, and the tube was placed on a tube

roller overnight at 37°C. The next day, the polyacrylamide fragments were removed by microcentrifugation for 15 min at 4°C. As much of the supernatant as possible was removed and transferred to a clean Eppendorf tube. To the remaining acrylamide, another 300 µL of elution buffer was added, the tube was mixed on a vortex and the supernatant collected as above. Both supernatants were pooled and passed through a 1 mL syringe filled with silanized glass wool in order to remove any small pieces of polyacrylamide gel. Two volumes of 95% v/v EtOH were added and the DNA was precipitated for at least 30 min on ice or overnight at -20°C. DNA was collected by microcentrifugation for 15 min at 4°C and resuspended in 200 µL of TE buffer, then precipitated once again, by the addition of 1/10 volume of 3 M NaOAc, and 2 volumes of 95% v/v EtOH on ice for 30 min, then collected by microcentrifugation for 10 min. The DNA pellet was washed in 1 mL 70% v/v EtOH and finally resuspended in either dH_2O or TE buffer and stored at – 20°C.

2.6 Quantitation of DNA

2.6.1 Oligonucleotides

The Σ_0 (extinction coefficient) for each oligonucleotide was calculated by the sum of (#T x 9.7 + #G x 11.4 + #C x 9.2 + #A x 15.4). The A_{260} of the oligonucleotide stock was divided by Σ_0 to give the concentration in mM, and was converted to µM, which is equivalent to pmol/µL.

Alternatively oligonucleotides could be quantitated in terms of µg, given that an A_{260} value of 1 is equivalent to 30-35 µg/mL.

2.6.2 DNA Fragments

The concentration of larger DNA fragments was determined by running a sample on an agarose gel along with known amounts of DNA molecular weight markers (Section 2.4) and comparing the band intensities of similarly sized, ethidium bromide stained DNA fragments. Alternatively, the A_{260} was measured, where a value of 1 corresponds to 50 µg/mL (Sambrook *et al.*, 1989).

Where necessary, the DNA concentration in terms of pmol per µL was calculated, based on the molecular weight of one bp being 660 Daltons. Therefore, the number of bp in the fragment was multiplied by 660, giving the weight of the fragment in terms of g/mol, which is easily converted to ng/pmol. This value was then used to calculate pmol equivalents by substituting the concentration (as determined above) in terms of ng.

2.6.3 Chromosomal DNA

Chromosomal DNA was quantified in a fluorometric assay (Morgan *et al.*, 1979). The standard curve consisted of 0 to 500 ng calf thymus DNA in 950 µL 5 mM Tris-HCl (pH 8.1) containing 0.5 mM EDTA. Fifty µL of a 10 µg/mL ethidium bromide stock (made in the same buffer) was added and the emission at 600 nm was measured in a Hitachi F-2000 Fluorescence Spectrophotometer with the excitation wavelength set to 525 nm.

Chromosomal DNA concentration also was determined by first diluting the DNA sample 1:100 and then measuring A_{260}, where an A_{260} value of 1 corresponds to 50 µg/mL (Sambrook *et al.*, 1989).

2.7 DNA Ligation

DNA fragments and digested cloning vectors were resuspended in Milli-Q H_2O, and were mixed in a 1:1 molar ratio so that the total DNA in the ligation reaction was between 20 to 60 ng/μL. The DNA was mixed and heated to 45°C for 5 min in order to denature any cohesive ends and reduce concatamer formation. Finally, 1 unit of T4 DNA ligase (Boehringer Mannheim) and ligase buffer to a final concentration of 1X (provided with the enzyme) were added in a total volume of no more than 20 μL. The reaction was incubated overnight at 15°C, and was used to transform the desired competent host.

2.8 Genomic Library

2.8.1 Construction of *A. vinelandii*, Strain F196 Genomic Library

F196 chromosomal DNA was isolated as described in Section 2.2.1, and was partially digested with *Sau*3A and conditions were optimized to generate fragments of approximately 23 kbp, so that size fractionation was not required. Cosmid vector pLAFR3 was digested with *Bam*H1 and was treated with calf intestine alkaline phosphatase (Boehringer Mannheim) according to manufacturer's instructions, using 1 unit per 50 pmol DNA to dephosphorylate the 5' termini, followed by a phenol:chloroform extraction to remove the alkaline phosphatase. Ligation reactions were set up with vector and insert DNA mixed in a 1:1 ratio. Approximately 0.1 μg of ligated DNA was used in the packaging reaction.

A Gigapack® II Gold packaging extract (Stratagene) was used to package the recombinant DNA into lambda phage heads with high efficiency following the manufacturer's instructions. The packaged cosmid library was then titered by making 1:10 and 1:50 dilutions (done in duplicate) of the packaged library in SM buffer [5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 mL of 1 M Tris-HCl (pH 7.5), 5 mL of 2% w/v gelatin and

dH_2O to 1L]. An overnight culture of host *E. coli* VCS257 was prepared by inoculating an isolated colony into 50 mL LB containing 10 mM $MgSO_4$ and 0.2% maltose which was incubated overnight at 30°C with shaking at 200 rpm. The next day, the culture was spun at approximately 500 x *g* (setting 4, in a clinical centrifuge) for 10 min at 4°C, resuspended in 12.5 mL 10 mM $MgSO_4$, and then diluted to an OD_{600} of 0.5 in 10 mM $MgSO_4$. Each dilution of the packaged library (25 µL) was then mixed with 25 µL of diluted host cells and incubated in an Eppendorf tube at RT for 30 min. To allow the expression of tetracycline resistance genes on the cosmid, 200 µL of LB broth was added to each sample and incubated for 1 hr at 37°C, with gentle shaking every 15 min. The cells were collected by microcentrifugation for 1 min at RT, and the cell pellet was resuspended in 50 µL LB broth which was spread onto LB plates containing 12.5 µg/mL tetracyline. After overnight incubation at 37°C, colonies on the plates were counted, and the cfu/original packaging mix was determined. The volume of packaged recombinant cosmid DNA required to give 5 x 10^4 cfu was mixed with an equal volume of VCS257 cells at OD_{600} of 0.5. The mixture was incubated at RT for 30 min, after which 4 volumes of LB broth were added and the tube was incubated at 37°C for 1 hr on a tube roller. The cells were then collected by centrifugation in a clinical centrifuge, resuspended in 500 µL of LB broth, and then spread onto LB plates containing 12.5 µg/mL tetracycline. After an overnight incubation at 37°C, a lawn of growth appeared on the plates. This amplified library was pooled by pouring 3 mL of LB broth onto the plate, and scraping the cells into the broth with a sterile spreader. Each plate was then rinsed once more with 3 mL LB broth, the broth was transferred to cryo-tubes, and flash frozen in EtOH-dry ice. This was stored at - 70°C until needed.

2.8.2 Isolation and Preliminary Characterization of a Kan^R, Lux$^+$ Clone

Once the library had been amplified, clones were screened first for Kan^R to select clones carrying the Tn*5luxAB* by taking a 2 mL aliquot of the amplified library, spinning it in a microcentrifuge for 5 min at 4°C and resuspending the cells in LB broth containing 50 μg/mL Kan. This suspension was plated onto the same medium. The Kan^R clones were then screened for Lux activity by addition of 20 μL n-decanal onto the lid of the petri plate and then exposing the plate, agar side down, to a piece of X-ray film for approximately 15 min. Alternatively, the petri plate was placed agar side up in a low-light imager (Siemens) and bioluminescence observed.

Fe-repressible Lux activity was then determined by spotting isolated Lux$^+$ colonies (with a toothpick) onto high/low Fe-split plates. These plates were made using petri dishes that have a plastic barrier which divides the plate down the middle. Each half of the plate contained 10 mL LB agar with 50 μg/mL Kan and either 300 μM Fe citrate or without any additional Fe. Lux activity was then assayed by either of the methods described above. By this process, pAS1, a Kan^R, bioluminescent clone was selected.

pAS1 was also transformed (Section 2.9) into 7 different *E. coli ent* mutants, AN 193-60, AN 192-60, MT 147, AN 90-60, AN 93-60, AN 117-60 and MFT 5-0 (Table 2.1). These strains were made competent by: $CaCl_2$, Section 2.9.1 (AN 193-60, AN 192-60, AN 90-60 and AN 117-60); RbCl/$CaCl_2$, Section 2.9.2 (MT 147, AN 93-60); and MFT5-0 was transformed by electroporation. Transformants were plated on CAS medium, incubated overnight at 37°C, and checked for orange halos around the colonies, which would indicate complementaion of the *E. coli ent* mutants by genes on pAS1.

2.9 Bacterial Transformation

Transformation was carried out using 100 µL competent cells with approximately 1 µg DNA. This mixture was incubated on ice for 30 min to allow DNA uptake to occur. The cells were then heat shocked at 42°C for 1 min and 1 mL LB broth without antibiotic was added. The cells recovered for 1 hr at 37°C and were then plated (150 µL) onto LB plates with the appropriate antibiotic selection and incubated overnight at 37°C. When it was necessary to detect alpha β-galactosidase activity (also known as α-complementation or blue-white selection), 40 µL of X-gal (20 mg/mL) and 4 µL IPTG (100 mM) were spread over the plates prior to plating the cells.

2.9.1 Calcium Chloride Competent *E. coli*

Cells for bacterial transformation were made competent by treatment with $CaCl_2$. The required *E. coli* strain was grown overnight from a single colony with the appropriate antibiotic in LB medium. The next day, this culture was diluted 1:100 into 20 mL of fresh medium with antibiotics (as required), and incubated until the OD_{600} reached approximately 0.4. At this time, the cells were harvested by centrifugation for 5 min at 480 x g. The cells were washed once with 5 mL 100 mM $MgCl_2$, harvested as above and then washed once in 5 mL 100 mM $CaCl_2$, harvested once again, and finally resuspended in 1 mL 100 mM $CaCl_2$. At this point, cells were either used for transformation immediately, or could be flash frozen (using an EtOH-dry ice bath) in 20% v/v glycerol and kept at - 70°C until needed.

2.9.2 Rubidium Chloride/Calcium Chloride Competent *E. coli*

Where the above method was unsuccessful for transformation, *E. coli* was made competent by treatment with both RbCl and $CaCl_2$. The required *E. coli* strain was grown overnight from an isolated colony in LB broth with the appropriate antibiotic. This culture was diluted 1:100 into 20 mL of fresh medium and incubated until OD_{600} was approximately 0.6. The cells were harvested by centrifugation at 1935 x g at 4°C for 5 min, resuspended in 10 mL buffer A (10 mM RbCl, 10 mM MOPS, pH 7.0) and incubated on ice for 10-20 min. The cells were then harvested as above, resuspended in 10 mL buffer B (10 mM RbCl, 0.1 M MOPS, 50 mM $CaCl_2$, pH 6.5) and incubated on ice for 20-30 min. The cells were harvested, resuspended in 1 mL of buffer B, and DMSO was added to a final concentration of 7%. Competent cells were used directly for transformation (Section 2.9), or stored at -70°C (Section 2.9.1).

2.9.3 Electroporation of *E. coli*

The required *E. coli* strain was grown overnight from a single colony with the appropriate antibiotic in LB medium. Cells were diluted 1:100 into 3 mL LB broth with the appropriate antibiotic and incubated at 37°C on a tube roller until the culture reached an OD_{600} of 0.4. Cells were harvested by microcentrifugation for 2 min at 8000 rpm and washed twice in 1 mL sterile Milli-Q H_2O. The cell pellet was then washed twice in 1 mL 15% v/v glycerol and harvested by microcentrifugation for 4 min at 8000 rpm. Cells were resuspended in 250 µL 15% glycerol, and were used immediately for electroporation.

Approximately 1 µg of plasmid DNA (resuspended in water) and 40 µl of electrocompetent cells were mixed gently in an Eppendorf tube and then transferred to a pre-chilled cuvette (2 mm gap, BTX Electroporation Cuvettes Plus™) and electroporated

in a Bio-Rad Gene Pulser® II using the following settings: 200 Ohms resistance, 25 μF capacitance, 2500 Volts. Immediately after electroporation, the cuvette was removed and 1 mL LB broth was added to the cells. This suspension was transferred to an Eppendorf tube and incubated for 1 hr at 37°C before plating onto selective medium.

2.10 Southern Transfer and Hybridization

2.10.1 Southern Transfer

DNA samples were separated by agarose gel (0.8%) electrophoresis overnight at approximately 25-30 Volts. Shortly before the end of electrophoresis, 2 μL of 6X loading dye was loaded to one lane of the gel, to aid in further steps where a change in dye colour needed to be observed. Electrophoresis was continued until the bromophenol blue and xylene cyanol had entered the gel and were easily distinguished. The gel was placed in a glass dish, on top of a piece of Saran Wrap (to make moving the gel easier), and 0.25 M HCl was added to cover the gel. This step was to depurinate the DNA, and once the bromophenol blue had changed colour to yellow, the gel was allowed to soak for 10 min longer. The gel was then rinsed three times with dH_2O and covered with denaturing solution (1.5 M NaCl, 0.5 M NaOH). The gel was left to soak, with gentle constant mixing on a platform shaker for 30 min or for 15 min after the dye colours returned to normal (whichever was longer). The gel was then rinsed in dH_2O and placed in neutralizing solution (1.5 M NaCl, 1 mM EDTA, 0.5 M Tris-HCl, pH 7.2) for at least 15 min with gentle mixing. This step was repeated and after a final wash in neutralizing solution, the gel was transferred to the capillary blot. This was set up using a glass baking dish half filled with 20X SSC (3 M NaCl, 0.3 M trisodium citrate, adjusted to pH 7.0 with 1M HCl), with a glass plate placed on top, using a piece of Whatman 3MM paper as a wick placed across the glass plate, with ends resting in the 20X SSC. The gel was placed on the saturated wick, and was surrounded with parafilm. A piece of (pre-

wet) 0.45 micron Hybond™-N nylon membrane (Amersham Life Sciences) was placed on the gel, followed by 3 sheets of Whatman 3 MM paper and a 5 cm stack of paper towels, all cut to the exact size of the gel. Any air bubbles were squeezed out using a glass pipette. Finally, a glass plate with a 1 kg weight was placed on top of the paper towels and transfer proceeded overnight. The next day the apparatus was dismantled, and before removing the membrane, the wells of the gel were marked using a pencil for later identification. The DNA was then UV cross-linked to the membrane using a Bio-Rad GS Gene Linker™, according to manufacturer's instructions.

2.10.2 Southern Hybridization

The membrane was transferred to a glass hybridization bottle. One mL of prewarmed pre-hybridization solution [5X SSC, 5X Denhardts (0.1% w/v BSA, 0.1% Ficoll, and 0.1% polyvinylpyrrolidone), 0.5% w/v SDS] per 10 cm^2 membrane was added, and 10 µL per cm^2 membrane of sonicated calf-thymus DNA (10 mg/mL in dH_2O which had been denatured by heating to 100°C for 5 min, and rapidly cooled on ice) was added. Pre-hybridization was carried out for at least 4 hr at 65°C, following instructions provided with Hybond™-N nylon membrane. When using oligonucleotide probes, the Tm was calculated using the formula:

$$Tm = 4(\#G+C) + 2\ (\#A+T)$$

and hybridization was done at 10°C below the Tm. After pre-hybridization, the labeled probe (Section 2.10.3; approximately 3 million cpm) was added and hybridization was carried out at the same temperature for at least 12 hr. After hybridization, the membrane was washed, first in a 2X SSC, 0.1% w/v SDS solution twice for 15 min at RT, followed by a 30 min wash in 1X SSC, 0.1% w/v SDS at a temperature which was 10°C less than the hybridization temperature. If necessary, a more stringent wash of the membrane was done in 0.5X SSC, 0.1% w/v SDS for as long as was required. The membrane was then

placed onto a piece of Whatman 3MM paper, wrapped in Saran Wrap and autoradiography was carried out using Kodak X-OMAT™ AR X-ray film, with an intensifying screen, at -70°C for up to 1 week, and developed in a Fuji RG II X-Ray Film processor.

2.10.3 Preparation of Probes

2.10.3.1 End-Labeling

When an oligonucleotide was used as the probe, it was end-labeled in a reaction with 20 pmoles of oligonucleotide in 3 µL dH_2O, with 1 µL 10X polynucleotide kinase buffer (supplied with the polynucleotide kinase), 5 µL [γ^{32}P] ATP (10 µCi/µL, ICN) and 1 µL diluted polynucleotide kinase [polynucleotide kinase 10 units/µL (purchased from Boehringer Mannheim) was diluted 1:10 in 1X polynucleotide kinase buffer]. The reaction was incubated at 37°C for 30 min, then an additional 1 µL of diluted polynucleotide kinase was added, and the mixture was incubated for an additional 30 min at 37°C. The reaction was stopped by heating to 75°C for 10 min. Unincorporated nucleotides were removed after adding 3 µL Blue Dextran (in 0.5 M EDTA, pH 8.0) by passage through a Sephadex G-50 (in TE buffer) column, made in a 1 mL syringe. Radioactivity was quantified by Cerenkov counting.

2.10.3.2 Random Primer Labeling

When DNA fragments were used as probes, they were labeled using the random primer method (Feinberg *et al.*, 1983) in which 25 ng DNA in 9 µL dH_2O was first denatured by heating at 100°C for 10 min and then rapidly cooled on ice. The following components were then added to the DNA: 3 µL dNTP mix (0.5 mM each of dATP, dGTP, dTTP; Boehringer Mannheim), 5 µL [α^{32}P] -dCTP (10 µCi/µL, Amersham), 2 µL

10X hexanucleotide mix (Boehringer Mannheim) and finally 1 µL Klenow fragment of DNA polymerase (2 units/µL, Boehringer Mannheim). The reaction was left overnight at RT, after which time the reaction was stopped by heating to 65°C for 10 min. Blue Dextran (6 µL) was added and unincorporated nucleotides were removed as described above (Section 2.10.3.1). In this case the probe was denatured by heating to 100°C for 5 min before use in hybridization.

2.11 DNA Sequencing

2.11.1 Plasmid Denaturing and Primer Annealing

pAS40 was used as the template for sequencing, and primers (listed in Table 2.4) were synthesized by Molecular Biology Services, Dept. Biological Sciences, University of Alberta as required. Approximately 3-6 µg of plasmid DNA (in 18 µL of Milli-Q H_2O) was denatured in an Eppendorf tube by addition of 2 µL denaturing buffer (2N NaOH, 2mM EDTA) and heating at 50°C for 5 min. DNA was then precipitated by addition of 14 µL 5 M NH_4OAc and 100 µL 95% v/v EtOH at -70°C for 5 min. The DNA was collected by microcentrifugation for 15 min at 4°C and rinsed twice in 1 mL of 70% v/v EtOH. The DNA pellet was allowed to air-dry for approximately 20 min and was resuspended in 7 µL Milli-Q H_2O. Two µL Sequenase™ buffer and 1 µL primer (approximately 3.5 pmol) were added and the tube was incubated at 37°C for 12 min, then allowed to cool to RT for at least 15 min. DNA sequencing followed immediately or after storage at -20°C.

2.11.2 Dideoxy Chain Termination Sequencing

Sequencing using chain-terminating nucleotide analogs, 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs), was performed using the T7 Sequenase 7-deaza-dGTP

Table 2.4. List of oligonucleotides used in this study. The positions of the primers from *csbC* and *orfA* corresponds to the sequence in Figure 3.5 and Appendix 1.

NAME	SEQUENCE 5' → 3'	ORIGIN	USE
WJP 11	CGGTCTGAGATAGCTCAGG	*luxA* bp 191-173 (antisense strand)	Sequencing and Southern probe
WJP 14	GCTCACGTTCTGCTCGTAG	*csbC* bp 687 – 669 (antisense strand)	Sequencing and Southern probe
WJP 15	GCATGTCGAACTGGCTGGC	*csbC* bp 573 to 591 (sense strand)	Sequencing
WJP 17	GTCAGATCCTGGAAAACGG	Tn5 right border bp 5759-5777 (sense strand)	Sequencing
WJP 18	GGTAATGGGTGAACAGCAC	*csbC* bp 358-340 (antisense strand)	Sequencing and PCR
WJP 19	CTAGGTGGTCCTGCTCGTC	*csbC* bp 240-258 (sense strand)	Sequencing and Gel Shift
WJP 20	GACCAATCCCTGCAATCGG	*csbC* bp 59-41 (antisense strand)	Sequencing
WJP 21	CTCTGTCGACGCTTCCGATTGCAGGGATTG	*csbC* bp 37-56 (sense strand)	PCR
WJP 22	CTACAGATCTTTACGGCTAGAGGACGAGCA	*csbC* bp 270-251 (antisense strand)	PCR
WJP 23	CTTCGTGCTGGTGGCTGTG	*orfA* bp 716-698 (sense strand)	Sequencing
WJP 24	CAGCCTGCTGCCGTTCTTC	*orfA* bp 358-340 (sense strand)	Sequencing
WJP 27	GGTGCTGTTCACCCATTACC	*csbC* bp 339-358 (sense strand)	Sequencing
WJP 28	AATGTTGATACTTATTATC	*csbC* bp 173-191 (sense strand)	Gel Shift
WJP 29	GATAATAAGTATCAACATT	*csbC* bp 191-173 (antisense strand)	Gel Shift

NAME	SEQUENCE 5'→ 3'	ORIGIN	USE
WJP 30	GAGTATTGATGTCATTTTG	*csbC* bp 152-134 (antisense strand)	Gel Shift
WJP 31	CAAAATGACATCAATACTC	*csbC* bp 134-152 (sense strand)	Gel Shift
WJP 32	GGCGCCAGTGCCTTCCCCT	*csbC* bp 223-241 (sense strand)	Gel Shift
WJP 33	AGGGGAAGGCACTGGCGCC	*csbC* bp 241-223 (antisense strand)	Gel Shift
WJP 34	GACGAGCAGGACCACCTAG	*csbC* bp 258-240 (antisense strand)	Gel Shift
WJP 36	GTCGCTGCTATGCGGGATG	*csbC* bp 954-936 (antisense strand)	Sequencing
rRNA2	CCGTCAATTC(AC)TTT(AG)AGTTT	16S rRNA gene bp 412-393 (antisense strand)	Northern probe
forward (-40 primer)	GTTTTCCCAGTCACGAC	pUC18	Sequencing
forward (-40, primer)	GTTTTCCCAGTCACGACGTTGTA	pUC18	Sequencing

sequencing kit purchased from Amersham Life Sciences, Inc. Four Eppendorf tubes were filled, each one with 2.5 μL of one of the termination mixtures (ddATP, ddCTP, ddGTP and ddTTP) and pre-warmed in a 37°C temperature block. Labeling was carried out by addition to the Eppendorf tube containing the primer annealed DNA template, the following components: 1 μL 0.1 M DTT, 2 μL undiluted labeling mix, 0.5 μL [α^{35}S]-dATP (10 μCi/μL, Amersham), and 2 μL diluted T7 Sequenase DNA polymerase (diluted 1:8 in Sequenase™ dilution buffer). This reaction was carried out for 2 min at RT. If sequence close to the primer was required, 1 μL of the Mn buffer (provided with the kit) was added to the labeling reaction. Termination reactions were carried out by transferring 3.5 μL of the labeling reaction to each termination tube, mixing gently and incubating at 37°C for 3 min. To stop the reactions, 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05% xylene cyanol FF) was added. The sequencing reactions were either stored at -20°C or heated to 75°C for 2 min, followed by quick cooling on ice, and then 4 μL of each reaction was loaded onto a sequencing gel.

Sequencing gels were cast in a Bio-Rad Sequi-Gen™ sequencing cell. First a plug was cast which consisted of approximately 20 mL 6% acrylamide stock [460 g ultrapure urea, 150 mL 40% acrylamide:bisacrylamide (19:1, purchased from Bio-Rad), in 1X TBE buffer in a total volume of 1 L] with 75 μL of each TEMED and 25% w/v APS added immediately prior to pouring the plug. Once the plug had polymerized, the gel was cast using approximately 50 mL 6% acrylamide stock with 75 μL of TEMED and 25% w/v APS added immediately prior to casting the gel. The gel was allowed to polymerize for at least 1 hr before beginning electrophoresis. Gels were pre-run at 40 W in 1X TBE buffer for at least 30 min prior to loading. Gels were run at a constant power of 40 W for varying amounts of time, depending on how much sequence was required. Gels were transferred to Whatman 3MM paper and dried on a Bio-Rad Gel Dryer (model 583) at 80°C for at least 90 min. Autoradiography was carried out by exposing the dried

gel directly to Kodak X-OMAT™ AR X-ray film at RT, for at least 20 hr. The film was developed using a Fuji RG II X-ray film processor.

2.11.3 Cycle Sequencing with Radiolabeled Dideoxy Terminators

Cycle sequencing with ^{33}P labeled dideoxynucleotide (ddNTP) terminators was performed using the Thermo Sequenase™ radiolabeled terminator cycle sequencing kit purchased from Amersham Pharmacia Biotech, Inc. First, 4 termination mixes were prepared by combining 2 µL of the nucleotide master mix with 0.5 µL of labeled [α-^{33}P]ddGTP per sequencing reaction, and this was repeated for [α-^{33}P]ddATP, [α-^{33}P]ddTTP and [α-^{33}P]ddCTP. After mixing, 2.5 µL of each termination mix was dispensed into 4 separate 0.5 mL Eppendorf tubes. The reaction mixture was prepared by mixing 2 µL of reaction buffer (supplied with the kit), approximately 400 ng DNA (pAS40), 2.5 pmol primer and 2 µL Thermo Sequenase™ DNA polymerase. To each tube containing the termination mix, 4.5 µL of the reaction mixture was added, mixed thoroughly and overlaid with approximately 20 µL of mineral oil. The tubes were then transferred to a Techne PHC-2 thermocycler which was pre-heated to 95°C, incubated at this temperature for 5 min and then the cycling program commenced, which included 30 cycles of the following: 30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C. After completion of the cycling, the reactions were stopped by addition of 4 µL of stop solution (as described in Section 2.11.2). At this point, the reactions could be stored at -20°C, or 3 µL could be loaded onto a 6% sequencing gel. Immediately prior to loading on the gel, the samples were denatured at 70°C for at least 2 min, and then cooled quickly on ice. Gels were prepared and run as described above (Section 2.11.2).

2.12 Polymerase Chain Reaction (PCR)

All PCR reactions were set up in a laminar flow hood equipped with UV lights, and pipettors, Eppendorf tubes, and pipette tips were all placed in the hood with the UV lights on for at least 30 min prior to setting up the reactions. All components added to the PCR reactions were sterile.

2.12.1 PCR using Taq DNA Polymerase

The Taq DNA polymerase used was a gift from Dr. Pickard (University of Alberta) and buffer conditions used were those which had been optimized for this enzyme by Dr. Stemke (University of Alberta). In a 0.5 mL Eppendorf tube, the following components were thoroughly mixed: 10 µL of 10X Stemke buffer (0.7 M Tris, pH 8.8, 40 mM $MgCl_2$, 1% Triton X-100, 1 mg/mL BSA), 10 µL dNTPs (1 mM), approximately 30 pmol each of primers WJP21 and WJP22, 20 ng pAS40 template and 5 µL of Taq (last component added to the mixture, diluted 1:10 in 1X strength Stemke buffer), and enough Milli-Q H_2O to bring the total volume to 100 µL. Each reaction was overlaid with approximately 100 µL sterile mineral oil, and placed immediately in a thermocycler (Minicycler™, MJ Research), set to the following program: 5 min denaturation at 95°C, followed by 30 cycles of 30 sec denaturing step at 95°C, 30 sec annealing at 52°C and 1 min extension at 72°C, and finally one 5 min extension at 72°C.

The mineral oil was removed by freezing the sample at -70°C, and subsequently pipetting off the oil from the frozen aqueous layer below. What remained in the tube was extracted once with phenol:chloroform, and then precipitated with 1/10 volume of 3M NaOAc and 2 volumes of 95% v/v EtOH, resuspended in Milli-Q H_2O, run on a 5% TBE-polyacrylamide gel and the 250 bp fragment was eluted following the method described in Section 2.5.

2.12.2 PCR using Expand™

Expand™ Long Template PCR system was purchased from Boehringer Mannheim, and used following manufacturer's instructions. In a 0.5 mL Eppendorf tube, the following components were thoroughly mixed: 5 µL of buffer #1, approximately 100 pmol each of primers WJP18 and WJP21, 17.5 µL dNTPs (1 mM), 20 ng pAS40 template and 0.5 µL of enzyme mixture (last component added) and enough Milli-Q H_2O to bring the total volume to 50 µL. Each reaction was overlaid with approximately 50 µL sterile mineral oil and placed immediately in a thermocycler (Minicycler™, MJ Research) set to the following program: 2 min denaturation at 95°C followed by 30 cycles of: 30 sec denaturing at 94°C, 30 sec annealing at 52°C and 1 min extension at 68°C, and ending with a 5 min extension step at 68°C.

The mineral oil was removed as above and the PCR product was purified by running on an 8% TBE-polyacrylamide gel, and eluting the 330 bp fragment from the gel following the method described in Section 2.5.

2.13 β-Galactosidase Assays

The procedure used for assaying β–galactosidase activity was that described by Miller (1972). The required *E. coli* strains were grown overnight from a single colony in LB medium with ampicillin. The next day cultures were diluted 1:100 into 50 mL LB medium containing ampicillin as well as 250 µM deferrated ethylenediamine-di-(o-hydroxyphenyl) acetic acid (EDDHA) (iron limited conditions) or 50 µM Fe citrate (iron sufficient conditions) and incubated until OD_{600} reached approximately 0.3, after which time samples were removed every hr. Approximately 1.5 mL samples were stored on ice for 20 min to prevent further growth, and at this time OD_{600} was determined. The assay involved mixing 0.5 mL culture with 0.5 mL Z buffer (60 mM $Na_2HPO_4 \cdot 7\ H_2O$, 40 mM

$NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β–mercaptoethanol, adjusted to pH 7.0). To lyse the cells, 2 drops of chloroform and 1 drop of 0.1% w/v SDS were added and the tubes were mixed on a vortex for 10 sec. The tubes were then placed in a 28°C water bath for 5 min and the reaction was started by addition of 0.2 mL o-nitrophenyl β–D-glucopyranoside (ONPG) which was used as the substrate (4 mg/mL made fresh in 100 mM phosphate buffer pH 7.0). Once sufficient yellow colour had developed, the time of the reaction was recorded, and the reaction was stopped by addition of 0.5 mL of a 1M Na_2CO_3 solution. For each tube, the OD_{420} and OD_{550} were determined and enzyme units were calculated using the following formula:

$$\frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}}$$

Where, v is the volume of culture (in mL) used in the assay, t is the time of the reaction (in min) and 1.75 is the correction factor for light scattering by cell debris, so that the true absorbance of o-nitrophenol can be determined.

2.14 Gel Shift Assays

DNA gel shift analysis with the *E. coli* Fur protein (a gift from Dr. J. Neilands, University of California at Berkeley) was carried out following the procedure described by De Lorenzo *et al.* (1988a). As described in Section 2.12.1, a 250 bp fragment was generated by PCR, using oligonucleotide primers WJP21 and WJP22. These primers are both 30-mers that contain 10 base non-homologous extensions at the 5' end. These extensions contain *SalI* (WJP21) and *BglII* (WJP22) restriction endonuclease sites which made it possible to clone this DNA fragment into the *SalI* and *BglII* sites of pQF50, generating clone pAS50. Instead of using the PCR product directly (as the target for gel shift assays), which may consist of a population of DNA fragments, pAS50 was used as a source of DNA target since the insert DNA had been sequenced and was known to

contain no errors. This DNA fragment was obtained by isolation of plasmid DNA (Section 2.2.2), followed by digestion with *Bgl*II and *Sal*I, and then running the entire DNA digest on a 5% TBE-PAG, and isolating the 250 bp fragment by the crush and soak method (Section 2.5). The DNA was dephosphorylated with calf intestine alkaline phosphatase, according to manufacturer's instructions. This was followed by a phenol:chloroform extraction to remove the alkaline phosphatase. The DNA (90 ng) was then end-labeled with [γ ^{32}P] ATP (as described in Section 2.10.3.1), purified on a G-50 column and diluted in TE buffer to a final concentration of 0.25 ng/µL.

The DNA binding reactions were set up in an Eppendorf tube by first adding 10 µL binding buffer which contained 10 mM Bis-Tris (made from a 1M stock of bis [2-Hydroxyethyl] imino-tris [hydroxymethyl] methane [Bis-Tris] which was adjusted to pH 7.5 with a saturated solution of boric acid), 5 µg/mL single stranded herring sperm DNA, 5% v/v glycerol, 100 µM $MnCl_2$, 100 µg/mL bovine serum albumin, 1 mM $MgCl_2$, 40 mM KCl and varying concentrations of *E. coli* Fur protein (in 10 mM Tris-HCl, pH 7.5). The volume was brought up to 14 µL with Milli-Q H_2O and finally 1 µL of the labeled DNA target was added. The solution was mixed and allowed to equilibrate for 10 min at 37°C. After this incubation, 10 µL of each sample was loaded onto a 5% PAG made in 20 mM Bis-Tris buffer (pH 7.5) and 100 µM $MnCl_2$. Gels were run in 20 mM Bis-Tris buffer (pH 7.5) containing 100 µM $MnCl_2$ at a constant voltage of 200 Volts, and were pre-run for at least 30 min prior to loading. Samples were loaded onto the gel without any loading dye and were run for 3 hr, after which time they were dried on a gel drier for at least 1 hr and exposed to X-ray film as described in Section 2.10.2.

This procedure was repeated exactly as described above except that $MnCl_2$ was omitted from the binding buffer, gel and running buffer in order to confirm that the Fur protein bound to the DNA in a metal ion-dependent manner.

Other modifications of this procedure include a competition gel shift assay in order to confirm the specificity of the Fur protein binding to the target DNA fragment. In

this case, instead of adding only radioactively labeled DNA target, various amounts of unlabeled DNA fragment (the 250 bp fragment containing the promoter region) or non-homologous DNA (poly dI-dC) were mixed with the labeled DNA and this mixture was then added to the binding buffer-Fur mixture which contained 150 nM Fur, and allowed to equilibrate as described above.

2.15 Isolation of RNA

All solutions used for RNA isolation and manipulation were made with Milli-Q H_2O that had been autoclaved for 40 min. All glassware used was baked at 180°C for at least 16 hr. Clean gloves were always worn when working with RNA.

2.15.1 Hot Phenol Extraction for RNA Isolation from *E. coli* and *A. vinelandii*

A. vinelandii cultures were grown for 16 to 18 hr before RNA was harvested. An overnight *E. coli* culture was sub-cultured into fresh medium and RNA was extracted once cultures had reached OD_{600} of at least 0.6. Approximately 1.5 mL of culture was transferred to an Eppendorf tube and then microcentrifuged for approximately 20 sec at 13 000 rpm. The supernatant was removed and, for *A. vinelandii* cultures, was saved for spectrophotmetric scanning. The cell pellet was resuspended in 400 µL of a fresh 1:1 mixture of phenol (saturated with Tris-HCl, pH 8.0) and lysis buffer (0.5% w/v SDS, 1mM EDTA in 10mM Tris-HCl, pH 7.0) by mixing on a vortex. Samples were incubated at 65°C for 10 min during which time they were mixed on a vortex every 2 min, and then microcentrifuged at 14 000 rpm for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and extracted once with an equal volume of phenol:chlorform:isoamyl alcohol (25:24:1) and nucleic acids were precipitated on ice for 30 min after addition of 1/10 volume of 3 M NaOAc and one volume of isopropanol. Nucleic acid was collected

by microcentrifugation at 14 000 rpm at 4°C for 15 min. The supernatant was removed and the nucleic acid pellet was allowed to air-dry for approximately 20 min. The pellet was resuspended in 180 µL of water and 20 µL of 10X DNase buffer (1 M NaOAc, 50 mM $MgSO_4$, pH 5.0), and 2 µL (7500 Units/µL) of RNase-free DNase (Pharmacia Biotech) was added, and DNA was digested for 30 min at 30°C. Each sample was subjected to two phenol:chloroform extractions, and finally, RNA was precipitated by addition of 1/10 volume of 3 M NaOAc and one volume of isopropanol, and was stored at -70°C until needed. RNA was recovered by microcentrifugation for 15 min at 4°C at 14 000 rpm, then rinsed with 1 mL 80% v/v EtOH, and allowed to air-dry for approximately 20 min. It was then resuspended in an appropriate volume of Milli-Q H_2O and quantified by measuring A_{260}, where a value of 1 corresponds to 40 µg/mL RNA. Prior to using RNA samples in further experiments, 5 µg of each RNA sample was run on a 1% TAE agarose gel to confirm that the RNA was not degraded and that there was no DNA contamination.

2.16 Northern Transfer and Hybridization

2.16.1 Northen Transfer

RNA was denatured by treatment with freshly deionized glyoxal [glyoxal was deionized using a mixed-bed resin of Bio-Rad AG® 501-X8 (D) resin until a colour change from blue to gold had occurred]. Deionized glyoxal was stored in small aliquots at -20°C until needed. Additional preparations included washing the electrophoresis tank, gel caster and comb first with soap and water, followed by a rinse with 95% v/v EtOH and then filling the tank with a 3% v/v solution of hydrogen peroxide for at least 10 min, and finally, rinsing several times with Milli-Q H_2O.

RNA (10 µg in 3.7 µL dH_2O) was mixed with 2.7 µL freshly deionized 6 M glyoxal, 8 µL DMSO and 1.6 µL 100 mM sodium phosphate buffer (pH 7.0) and

incubated at 50°C for 1 hr. During this time, 1% w/v agarose was dissolved by melting in 10 mM sodium phosphate buffer (pH 7.0), cooled slightly and cast into an 11 x 17 cm gel. After the denaturing step, the RNA samples were cooled on ice and 2 µL 6X loading dye was added. The samples were loaded onto the gel and separated by electrophoresis at 55-60 Volts for at least 4 hr with constant buffer recirculation.

This gel was used directly for Northern transfer, which used the same apparatus and procedure as described for Southern transfer (Section 2.10.1), except that all solutions and glassware were RNase-free. After transfer, the membrane was carefully removed and the RNA was UV cross-linked to the membrane.

2.16.2 Northern Hybridization

Pre-hybridization was done in a Seal-a-Meal® (Dazey) bag in the same way as described in Section 2.10.2, except that 20X SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.7) was used instead of 20X SSC. In this case, hybridization was carried out at 60°C except that when using an oligonucletoide probe, 45°C was used. Before the probe was added, the pre-hybridization solution was replaced with fresh solution and a second aliquot of non-homologous DNA was added at the same time as the probe. The probe used to detect the *csbC* transcript was a 310 bp *Not*I - *Pst*I fragment cut from pAS310 and labeled using the random primer method, as described in Section 2.10.3.2. After hybridization, the membrane was washed twice in a solution of 2X SSPE containing 0.1% w/v SDS for 15 min at RT, followed by a wash in 1X SSPE containing 0.1% w/v SDS for 30 min at 37°C and finally washed once in 0.1X SSPE containing 0.1% w/v SDS for 30 min at 50°C. Autoradiography was carried out as described in Section 2.10.2.

2.17 RNA Dot Blot

A Hybri-Dot manifold (BRL) was used for RNA dot blotting and was cleaned with 0.1 M NaOH and rinsed several times with Milli-Q H_2O immediately before use. Hybond™-N 0.45 micron nylon membrane was used and was soaked in 20X SSC in a glass dish for at least 10 min. The apparatus was assembled by placing 2 pieces of Whatman 3 MM paper (soaked in 20X SSC) on the bottom platform, followed by the membrane on top and then the top platform of the apparatus, which was securely fastened ensuring that there were no trapped air bubbles or air leaks. Meanwhile, 5 µg of each RNA sample was denatured by heating at 65°C for 15 min in 3 volumes of denaturing solution [1X MOPS (10X stock is 0.2 M MOPS, 50 mM NaOAc, 10 mM EDTA adjusted to 7.0), 50% formamide and 6% formaldehyde]. The sample was placed on ice and 2 volumes of ice cold 20X SSC were added to each sample before loading onto the dot blot apparatus. With the vacuum turned on each dot was washed twice with 10X SSC and then the samples were loaded. This was followed by washing each dot at least once with 1 mL of 10X SSC to ensure that no RNA had adhered to the walls of the holes in the top platform. Suction was continued for approximately 5 min, thus ensuring that the filter was dry. The membrane was then removed, placed on a piece of Whatman 3 MM paper and the RNA was UV cross-linked onto the membrane using a Bio-Rad GS Gene Linker™ with at least 150 mJoule UV light.

Hybridization was carried out exactly as described for Northern analysis (Section 2.16.2).

2.18 S1 Nuclease Mapping

The DNA probe was generated by PCR using primers WJP21 and WJP18, as described in Section 2.12.2. This generated a 330 bp double stranded DNA fragment that

had a 10 bp non-homologous overhang at the 5' end. Approximately 1 pmol of the probe was end-labeled in a reaction containing 1 µL 10 polynucleotide kinase buffer, 2.5 µL [γ^{32}P] ATP (10 µCi/µL, ICN) and 1 µL polynucleotide kinase (1 unit in 1X polynucleotide kinase buffer), and incubated for 30 min at 37°C, at which time an additional 1 µL of polynucleotide kinase was added and incubated for 30 min longer at 37°C. The reaction was stopped by heating to 75°C for 10 min, and then 2 µL glycogen (20 mg/mL, Boehringer Mannheim), 87.5 µL water, 10 µL 5 M NH_4OAc and 250 µL 98% v/v EtOH were added. The DNA was left to precipitate overnight at -20°C. The next day, the DNA was collected by microcentrifugation at 13 000 rpm for 30 min at 4°C. The DNA pellet was rinsed with 1 mL 98% v/v EtOH followed by 5 min microcentrifugation at 4°C. The pellet was left to air-dry for 5 min, then the Eppendorf tube was placed in a scintillation vial, and the radioactivity of the entire sample was quantified by Cerenkov counting. The probe was resuspended in dH_2O to a final concentration of 10^5 cpm/µL.

Hybridization of RNA to the DNA probe was carried out in a 30 µL reaction consisting of 50 µg total RNA in 17 µL dH_2O, 10 µL 3X hybridization buffer (3M NaCl, 0.5 M HEPES, pH 7.5, 1 mM EDTA, pH 8.0), and 3 µL probe. The DNA and RNA in this mixture were denatured by heating each tube to 85°C for 10 min, the samples were then cooled to 42°C, then microcentrifuged for a few seconds and hybridization followed during incubation of the samples for 1 hr at 42°C.

Single stranded nucleic acid was digested in a reaction containing 165 µL 2X S1 nuclease buffer (0.56 M NaCl, 9 mM $ZnSO_4$, 0.1 M NaOAc, pH 4.5), 3 µL single stranded herring sperm DNA (2 mg/ml in dH_2O), 131 µL dH_2O and 1 µL of *Aspergillus oryzea* S1 nuclease (Pharmacia; diluted to 33 units/µL in 2X S1 nuclease buffer), added to each of the 30 µL annealing reactions. These were incubated for 30 min at 37°C, and the reaction was stopped by the addition of 80 µL stop solution (4M NH_4OAc, 20 mM EDTA, pH 8.0, 40 µg/ml baker's yeast tRNA) and 1 mL 98% v/v EtOH. Nucleic acid

was precipitated over 30 min at -70°C and collected by microcentrifugation for 30 min at 4°C. The pellet was rinsed once in 98% v/v EtOH and allowed to air-dry for 5 min. It was then resuspended in 5 µL of stop solution (from the Sequenase kit) and was heated to 100°C for 3 min immediately before loading onto a 6% sequencing gel prepared as in Section 2.11.2. These reactions were run for 3 hr at constant power of 40 W, alongside the sequencing reaction products (Section 2.11.2), using WJP18 as the primer and pAS40 DNA template. After the gel was dried (Section 2.11.2) the bands were visualized by autoradiography, using an intensifying screen, and film exposure at -70°C for at least 36 hr.

2.19 Primer Extension

WJP18 was chosen as the probe, as it is approximately 140 bp from the putative 5' start of *csbC*. This oligonucleotide was end-labeled in a reaction containing 2 pmoles of WJP18, 1 µL 10X polynucleotide kinase buffer, 5 µL [γ^{32}P] ATP (10 µCi/µL, ICN), 1 µL dH_2O and 1 µL of polynucleotide kinase (1 unit/µL in 1X PNK buffer). This was incubated for 30 min, at 37°C after which time, 1 µL more polynucleotide kinase was added and incubated for 30 min longer. At the end of the incubation, the entire reaction was loaded onto a Bio-Rad Micro Bio-Spin® 6 chromatography column and used according to manufacturer's instructions, so that the labeled oligonucleotide was separated from the unincorporated radioactive nucleotide. The entire probe was placed in a scintillation vial and amount of radioactivity was determined by Cerenkov counting.

The probe and RNA were then annealed in a reaction consisting of 50 µg total RNA, 0.5 µL RNA Guard® (33.7 Units/µL, Pharmacia Biotech), 10 µL 3X hybridization buffer, 6 x10^5 cpm ^{32}P-end-labeled WJP18, and enough dH_2O to bring the final volume to 30 µL. This mixture was placed in an 85°C temperature block for 10 min to denature the RNA, after which time the temperature block was cooled to 45°C, the samples were

collected by brief microcentrifugation, and incubated for 1 hr at 45°C to allow for the primer to anneal to the target RNA. The RNA was then precipitated by addition of 2 volumes of 98% v/v EtOH and stored at -70°C for 20 min. The RNA was collected by microcentrifugation at 13 000 rpm for 10 min at 4°C, followed by a wash with 98% v/v EtOH, and the sample was allowed to dry before it was resuspended in the reverse transcriptase mixture. This mixture consisted of 7 µL 2 mM dNTPs, 5 µL 5X buffer (supplied with the reverse transcriptase), 0.5 µL RNA Guard®, made up to a final volume of 25.5 µL with RNAse-free dH_2O. When thoroughly resuspended, 25 Units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) were added, mixed well and incubated for 1 hr at 42°C. To precipitate the cDNA (the product of this reaction), 1 µL of glycogen (20 mg/mL Boehringer Mannheim), 30 µL 3M NaOAc and 243 µL TE buffer were added, followed by 2 volumes of 98% v/v EtOH and storing the tube for 20 min at -70°C. The DNA was collected by microcentrifugation for 10 min at 4°C, followed by a wash with 1 mL 98% v/v EtOH and then left to air dry before resuspending in 10 µL of loading buffer (from the Sequenase kit). The sample was either stored at -20°C or 5 µL was denatured to 100°C for 2 min and loaded immediately on a 6% sequencing gel prepared as in Section 2.11.2. Standard sequencing reactions were generated with primer WJP18 (as described in Section 2.11.3). The gel was run at 40 W for 3 hr, dried and the bands visualized by autoradiography (Section 2.11.2).

CHAPTER 3. RESULTS

3.1 Isolation of a Lux$^+$ Clone From the F196 Genomic Library

In order to locate the clone containing the iron regulated promoter and hopefully part of the gene disrupted by the Tn*5luxAB*, the F196 genomic library was screened for Lux activity and Kan^R. Many such clones were identified and determined to represent 0.04% of the library.

By inoculating a split plate (containing low Fe medium on one half of the plate, and Fe-sufficient medium on the other half, and supplying the substrate n-decanal on the lid of the petri plate), repression of *luxAB* by Fe was investigated (Section 2.7.2). Kan^R, Lux positive clones were screened for Fe-repressible Lux activity and one clone, pAS1, was used for further study. However, the study of Fe repressible Lux activity showed that there was very poor regulation of *luxAB* by Fe in *E. coli* VCS257 (pAS1) (Figure 3.1). To confirm that the Fe-repressible promoter was contained within the insert DNA, *E. coli* VCS257 (pAS1) was transformed with pMH15 which encoded the *E. coli fur* gene on a plasmid with a copy number of 10-12. In addition, pAS1 was transformed into *A. vinelandii* UW and into the Fur-overproducing *E. coli* strain HB101 (pMH15). Iron-repressible Lux activity was observed when pAS1 was introduced into *A. vinelandii* UW and *E. coli* HB101(pMH15), and in *E. coli* VCS257 (pMH15). Therefore it was concluded that the Fe-repressible promoter was contained on this clone, since regulation of *luxAB* on pAS1 was evident in UW and that *luxAB* were also regulated in a similar way in *E. coli.* Increasing the copies of *fur*, by introduction of pMH15 resulted in further repression in both of the *E. coli* strains, thus indicating that Fur likely does regulate *luxAB* on pAS1.

In order to determine whether any functional gene(s) involved in catecholate

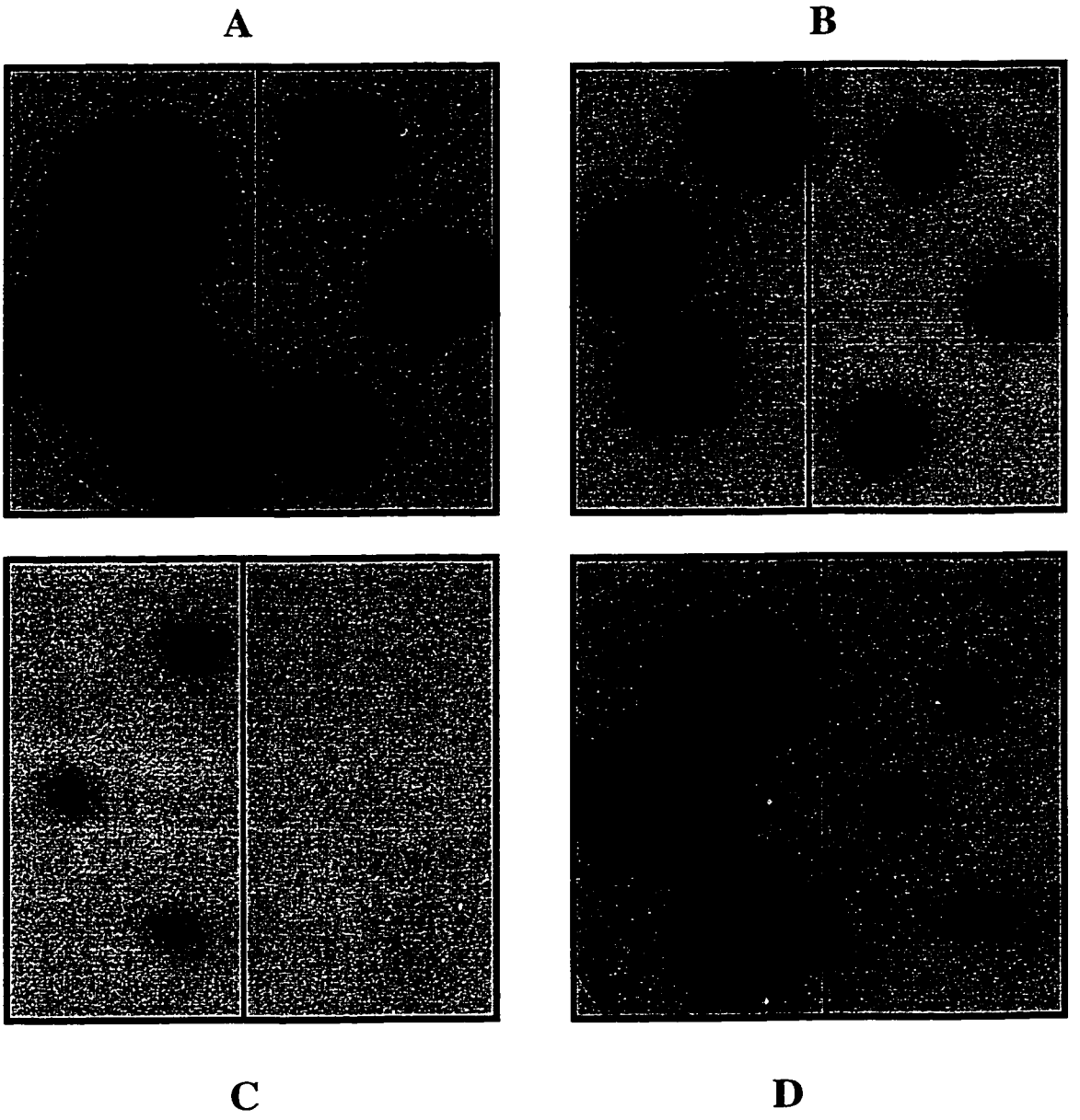


Figure 3.1 Fe-repressible Lux activity. The left hand side of each replica plate is Fe limited, and the right hand side of each plate is Fe sufficient. A: *E. coli* VCS257 (pAS1) B: *E. coli* VCS257 (pAS1, pMH15), C: *E. coli* HB101 (pAS1, pMH15), D: *A. vinelandii* UW (pAS1). The dark spots represent light production by exposure of X-ray film.

siderophore synthesis were encoded on this clone, pAS1 was used to transform *E. coli* mutants, that were defective in enterobactin biosynthesis because of mutations in *entA*, *entB*, *entC*, *entD*, *entE*, *entF* or *entG*. Transformants were spotted on CAS plates to determine whether the mutation had been complemented by pAS1. If this were the case, the restoration of enterobactin biosynthesis would result in an orange halo around the colony. pAS1 was unable to complement any of the 7 mutants, indicating that no functional gene was encoded on pAS1.

3.2 Subcloning pAS1

pAS1 was digested with restrictions endonucleases *Bgl*II, *Sal*I and a double digest using both of these enzymes, which are known not to have sites in Tn*5luxAB*, and the resulting DNA fragments were separated by agarose gel electrophoresis. The DNA fragment containing *luxA* (and hopefully the iron-regulated promoter) was identified by Southern hybridization using a random primer labeled 330 bp *Pst*I fragment from within the *luxA* gene of pTn*5luxAB* as a probe (DNA fragment prepared and kindly provided by M. Mehrotra, University of Alberta). An 11.5 kbp *Sal*I fragment, an approximately 31 kbp *Bgl*II fragment and a 10 kbp *Bgl*II-*Sal*I fragment were all found to hybridize to *luxA*. Hybridizing bands were also seen in F196 genomic DNA which had been digested with *Bgl*II or *Sal*I (Figure 3.2). A partial restriction map of pAS1 was determined as shown in Figure 3.3. All three fragments from pAS1 were subcloned into the low copy number vector, pK184, so that Fe-repressible Lux activity would be easier to assess. All three clones were luminescent, indicating that the entire Tn*5luxAB* was likely contained within each fragment. The smaller 10 kb fragment likely contained less surrounding sequence (just 2 kb of *A. vinelandii* DNA since the size of Tn*5luxAB* is approximately 8 kb). The *Bgl*II fragment was considered too large and as well it was determined by mapping to contain a large amount of pLAFR3 sequence. Because we wanted to obtain sequence of

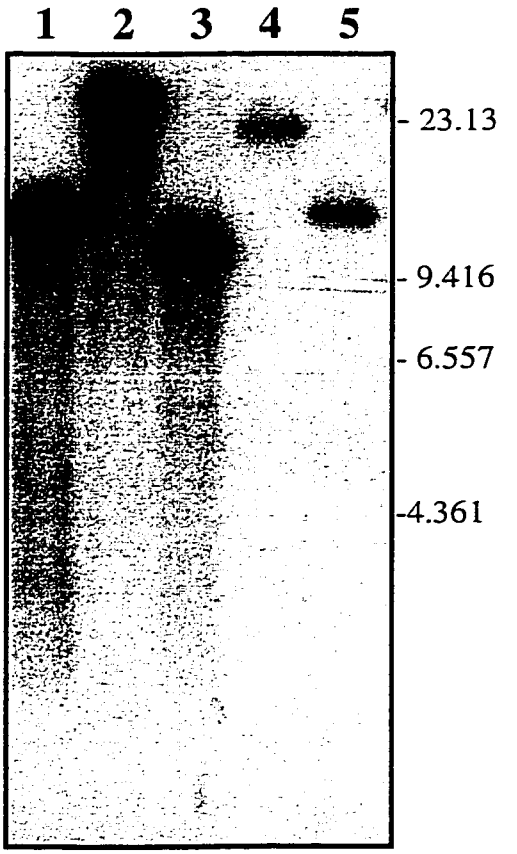
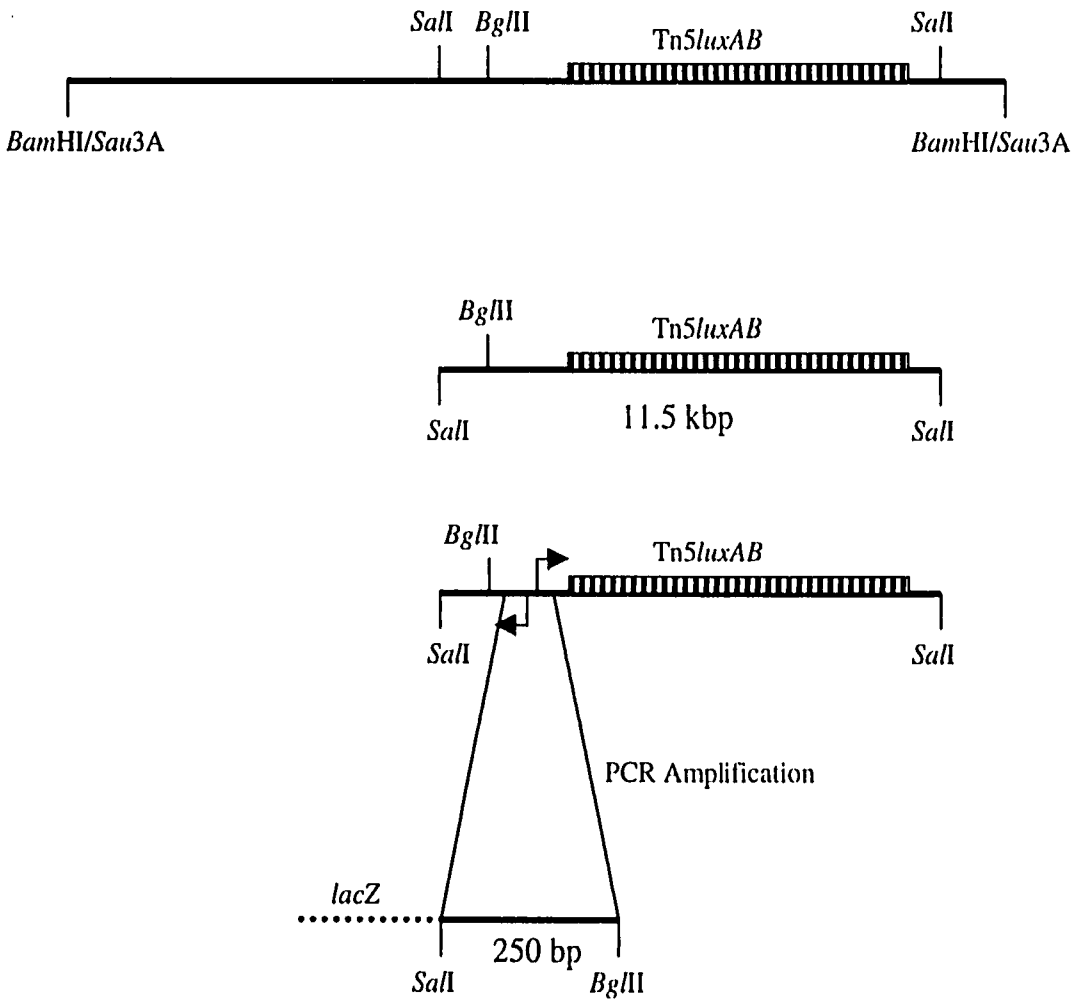


Figure 3.2 Southern hybridzation of digested pAS1 and F196 genomic DNA with *luxA*. Lane1: pAS1, *Sal*I digest, Lane 2: pAS1, *Bgl*II digest, Lane 3: pAS1, *Bgl*II, *Sal*I double digest, Lane 4: F196 Chromosomal DNA, *Bgl*II digest, Lane 5: F196 Chromosomal DNA, *Sal*I digest. Size markers (*Hin*dIII digest of λ DNA) are indicated in kbp.



pAS1 (cosmid pLAFR3 + 23 kbp F196 chromosomal DNA fragment)

pAS20 (low copy number cloning vector with Kan^R, pK184)

pAS40 (high copy number cloning vector with Amp^R, pUC119)

pAS50 (promoter probe vector with Amp^R, pQF50)

Figure 3.3 Partial map of cosmid clone pAS1 and strategy used to subclone the *csbC* iron regulated promoter from pAS1. Tn*5luxAB* is approximately 8 kb.

the DNA upstream of *luxAB*, the clone containing the 11.5 kb *SalI* fragment, pAS20, was chosen for further study as it contained approximately 3.5 kb of *A. vinelandii* DNA. This clone, pAS20, was Lux positive and when transformed into *E. coli* DH5α(pMH15) showed Fe-regulated Lux activity when grown on a split plate containing low and high Fe media. This confirmed that the Fe-regulated promoter from pAS1 was contained within the 11.5 kbp *SalI* fragment.

In order to confirm that the entire Tn*5luxAB* was contained within this insert DNA, the 11.5 kbp *SalI* fragment was cloned into pUC119 (since pK184 carries Kan^R gene, pAS20 could not be used to check for sensitivity to Kan), generating pAS40. pAS40 conferred Kan^R, which suggested that the 11.5 kbp fragment was comprised of 8kb of Tn*5luxAB* DNA and approximately 3.5 kbp of *A. vinelandii* DNA (Figure 3.3).

3.3 Sequencing

Clone pAS40 was used as the template for double-stranded sequencing, starting with primer WJP11, which was complementary to *luxA* sequence. Once initial sequence immediately upstream of the transposon was obtained, primers were designed and synthesized in order to sequence both further upstream and also to obtain the sequence of the complementary strand of DNA (Figure 3.4). A total of 874 bp of double stranded sequence upstream of the transposon was obtained (Figure 3.5). In a similar fashion, sequence downstream of the transposon was obtained, starting with a primer WJP17 which is complementary to the right border of Tn5 (Auerswald *et al.*, 1981). This generated 489 bp of double-stranded sequence. Initially sequence was obtained by chain termination sequencing using [α^{35}S]-dATP, but as a result of *A. vinelandii* having very high %G+C, difficulties were experienced in determining the sequence of certain regions of the DNA. To obtain the accurate sequence, cycle sequencing using ^{33}P-labeled dideoxy terminators was done using the same primers as before. Using this method,

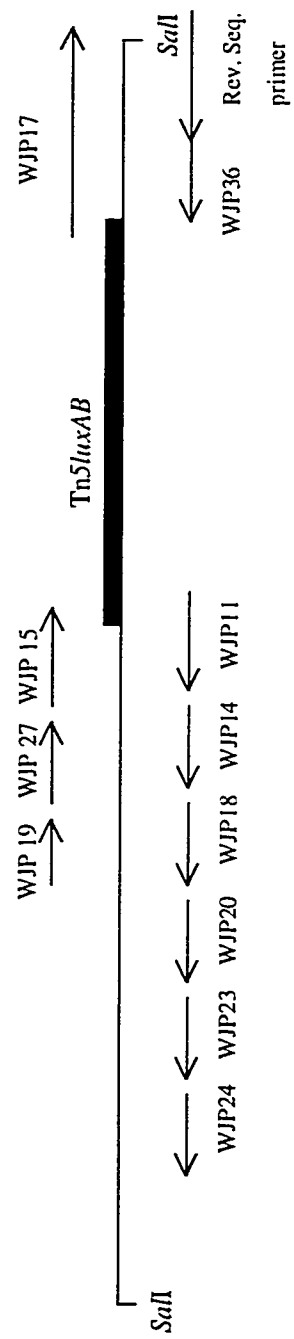
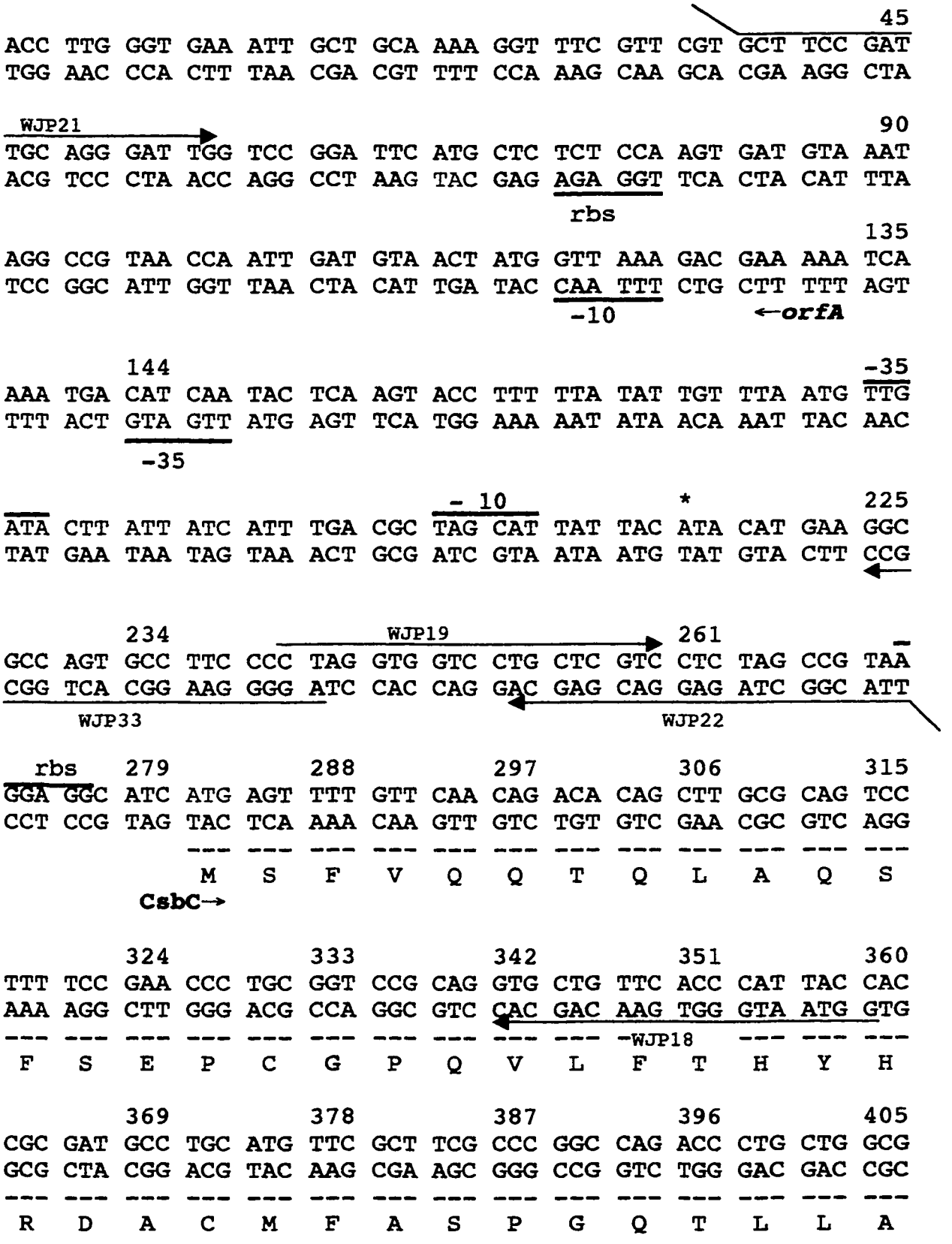


Figure 3.4 Sequencing strategy using pAS40 template.
Arrows indicate primers used and the direction of sequencing. Diagram is not drawn to scale.


45
ACC TTG GGT GAA ATT GCT GCA AAA GGT TTC GTT CGT GCT TCC GAT
TGG AAC CCA CTT TAA CGA CGT TTT CCA AAG CAA GCA CGA AGG CTA
WJP21
90
TGC AGG GAT TGG TCC GGA TTC ATG CTC TCT CCA AGT GAT GTA AAT
ACG TCC CTA ACC AGG CCT AAG TAC GAG AGA GGT TCA CTA CAT TTA
rbs
135
AGG CCG TAA CCA ATT GAT GTA ACT ATG GTT AAA GAC GAA AAA TCA
TCC GGC ATT GGT TAA CTA CAT TGA TAC CAA TTT CTG CTT TTT AGT
-10
←orfA
144
-35
AAA TGA CAT CAA TAC TCA AGT ACC TTT TTA TAT TGT TTA ATG TTG
TTT ACT GTA GTT ATG AGT TCA TGG AAA AAT ATA ACA AAT TAC AAC
-35
- 10
*
225
ATA CTT ATT ATC ATT TGA CGC TAG CAT TAT TAC ATA CAT GAA GGC
TAT GAA TAA TAG TAA ACT GCG ATC GTA ATA ATG TAT GTA CTT CCG
234
WJP19
261
GCC AGT GCC TTC CCC TAG GTG GTC CTG CTC GTC CTC TAG CCG TAA
CGG TCA CGG AAG GGG ATC CAC CAG GAC GAG CAG GAG ATC GGC ATT
WJP33
WJP22
rbs
279
288
297
306
315
GGA GGC ATC ATG AGT TTT GTT CAA CAG ACA CAG CTT GCG CAG TCC
CCT CCG TAG TAC TCA AAA CAA GTT GTC TGT GTC GAA CGC GTC AGG
M S F V Q Q T Q L A Q S
CsbC→
324
333
342
351
360
TTT TCC GAA CCC TGC GGT CCG CAG GTG CTG TTC ACC CAT TAC CAC
AAA AGG CTT GGG ACG CCA GGC GTC CAC GAC AAG TGG GTA ATG GTG
-WJP18
F S E P C G P Q V L F T H Y H
369
378
387
396
405
CGC GAT GCC TGC ATG TTC GCT TCG CCC GGC CAG ACC CTG CTG GCG
GCG CTA CGG ACG TAC AAG CGA AGC GGG CCG GTC TGG GAC GAC CGC
R D A C M F A S P G Q T L L A

```
          414          423          432          441          450
CAT GGC GTG GCC GCG ACC CTG CCG CTG GTC ATG AGC GGT TCG CCC
GTA CCG CTC CGG CGC TGG GAC GGC GAC CAG TAC TCG CCA AGC GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   G   V   A   A   T   L   P   L   V   M   S   G   S   P

          459          468          477          486          495
GCC GCC CTG GCC GGG CAT GCG GCC GCT TTT CTG CGT CAG GCC AGC
CGG CGG GAC CGG CCC GTA CGC CGG CGA AAA GAC GCA GTC CGG TCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   A   L   A   G   H   A   A   A   F   L   R   Q   A   S

          504          513          522          531          540
GAG GCC GGG CAT GCC GGC TGG CTG ATC GGC GCC ATC CCC TTC CTG
CTC CGG CCC CTA CGG CCG ACC GAC TAG CCG CGG TAG GGG AAG GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   A   G   H   A   G   W   L   I   G   A   I   P   F   L

          549          558          567          576          585
CCG GGC GCT GCG GCC CAT CTG TTC ATC CCC GAG CAT GTC GAA CTG
GGC CCG CGA CGC CGG GTA GAC AAG TAG GGG CTC GTA CAG CTT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   G   A   A   A   H   L   F   I   P   E   H   V   E   L

          594          603          612          621          630
GCT GGC GGC GGT CGC GCG GCG CTG GTC GGC GGG CCG CGT CCG GTA
CGA CCG CCG CCA GCG CGC CGC GAC CTG CCG CCC GGC GCA GGC CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   G   G   G   R   A   A   L   V   G   G   P   R   P   V

          639          648          657          666          675
CGC ACG GTC GCG GCG CGC AGC GAA CCG GCC GAG GCG GTC TAC GAG
GCG TGC CAG CGC CGC GCG TCG CTT GGC CGG CTC CGC CAG ATG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   T   V   A   A   R   S   E   P   A   E   A   V   Y   E

          684          693          702          711          720
CAG AAC GTG AGC CGG GCC TTG GAG CGG ATC GCC GAC GGG AAG TTG
GTC TTG CAC TCG GCC CGG AAC CTC GCC TAG CGG CTG CCC TTC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   N   V   S   R   A   L   E   R   I   A   D   G   K   L

          729          738          747          756          765
CAG AAA GTG GTG CTG TCG CGC TCG CTG CAC ATC CAG GCG GAG CTG
GTC TTT CAC CAC GAC AGC GCG AGC GAC GTG TAG GTC CGC CTC GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   K   V   V   L   S   R   S   L   H   I   Q   A   E   L
```

```
         774          783          792          801          811
GAT CAG GCC GAA CTG CTG CAG ACC CTG GCC AGC CGC AAC CCG CTG
CTA GTC CGG CTT GAC GAC GTC TGG GAC CGG TCG GCG TTG GGC GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   Q   A   E   L   L   Q   T   L   A   S   R   N   P   L

         819          828          837          846          855
GGC TAC ACC TAT GCC ATT CCG CTG CCG ACC GCC AAC GGC GAG CGC
CCG ATG TGG ATA CGG TAA GGC GAC GGC TGG CGG TTG CCG CTC GCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   Y   T   Y   A   I   P   L   P   T   A   N   G   E   R

                        Tn5luxAB
                          ▽
         864                                   891          900
CGC AGC CTG ATC GGC GCC AGC CCC GAG CTG CTG CTG GCC CGG CAC
GCG TCG GAC TAG CCG CGG TCG GGG CTC GAC GAC GAC CGG GCC GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   S   L   I   G   A   S   P   E   L   L   L   A   R   H

         909          918          927          936          945
GGC AAC CGG GTG ATT TCC AAC CCG CTG GCC GGC TCC ATC CCG CAT
CCG TTG GCC CAC TAA AGG TTG GGC GAC CGG CCG AGG TAG GGC GTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   N   R   V   I   S   N   P   L   A   G   S   I   P   H

         954          963          972          981          990
AGC AGC GAC CCG GCC GAG GAC CGC CAG CGC GCC GAG AAC CTG CTG
TCG TCG CTG GGC CGG CTC CTG GCG GTC GCG CGG CTC TTG GAC GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   S   D   P   A   E   D   R   Q   R   A   E   N   L   L

         999         1008         1017         1026         1035
CGC TCG CCC AAG GAC CTG CAC GAG CAC GCG CTG GTG GTG GAA GCC
GCG AGC GGG TTC CTG GAC GTG CTC GTG CGC GAC CAC CAC CTT CGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   S   P   K   D   L   H   E   H   A   L   V   V   E   A

        1044         1053         1062         1071         1080
GTC GCC GAG GCC CTG CGC CCC TAT TGC ACC GAT CTG CTG GTC CCC
CAG CGG CTC CGG GAC GCG GGG ATA ACG TGG CTA GAC GAC CAG GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   A   E   A   L   R   P   Y   C   T   D   L   L   V   P

        1089         1098         1107         1116         1125
GAA GCG CCG TCG CTG CTC TCG ACC CCG ACC ATG TGG CAC CTG TCC
CTT CGC GGC AGC GAC GAG AGC TGG GGC TGG TAC ACC GTG GAC AGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
E   A   P   S   L   L   S   T   P   T   M   W   H   L   S
```

```
        1134          1143          1152          1161          1170
ACC GAA GTC ACC GGC ACG CTG CGC GAC CCG GCC ACG ACC TCG CTG
TGG CTT CAG TGG CCG TGC GAC GCG CTG GGC CGG TGC TGG AGC GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   E   V   T   G   T   L   R   D   P   A   T   T   S   L

        1179          1188          1197          1206          1215
GAA CTG GCG CTG GCG CTG CAT CCG ACT CCG GCC GTG GGA GGC TAT
CTT GAC CGC GAC CGC GAC GTA GGC TGA GGC CGG CAC CCT CCG ATA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   L   A   L   A   L   H   P   T   P   A   V   G   G   Y


        1224          1233          1242          1251          1260
CCG ACC GCC GAG GCG CGG GAG TTC ATC CAG GCG TTC GAA GGC TTC
GGC TGG CGG CTC CGC GCC CTC AAG TAG GTC CGC AAG CCT CCG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   T   A   E   A   R   E   F   I   Q   A   F   E   G   F

        1269          1278          1287          1296          1305
GAC CGG GGC TTC TTC ACC GGC CTG GTC GGC TGG TGC AAC GCG CAG
CTG GCC CCG AAG AAG TGG CCG GAC CAG CCG ACC ACG TTG CGC GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   R   G   F   F   T   G   L   V   G   W   C   N   A   Q

        1314          1323          1332          1341          1350
GGC GAT GGC GAA TGG GCG GTG ACC ATC CGC TGC CGA AGT CGG CGA
CCG CTA CCG CTT ACC CGC CAC TGG TAG GCG ACG GCT TCA GCC GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   D   G   E   W   A   V   T   I   R   C   R   S   R   R

        1359
GAC AGT CGT CGA C
CTG TCA GCA GCT G
--- --- --- --- -
 D   S   R   R
```

Figure 3.5 Partial nucleotide sequence and deduced amino acid sequence of the *A. vinelandii csbC* gene. Regulatory signals of the putative *orfA-csbC* bidirectional promoter-operator region are indicated. Putative promoter determinants (-35 and -10) are shown in blue, iron boxes (shown in red text), sox boxes (shown in green text) and ribsome binding sites (shown in purple) are indicated. Transcriptional initiation site as defined by primer extension is designated by an asterisk. Oligonucleotide primers are indicated by an arrow. The site of Tn5*luxAB* insertion is indicated by a triangle.

regions of sequence compression were resolved as accurately as possible.

As well, 1185 bp sequence further upstream was obtained by automated sequencing, but here only one strand of DNA was sequenced (shown in Appendix 1). Altogether, this gave a total of 2548 bp of sequence data, which was analysed for open reading frames using FramePlot version 2.1 (Bibb *et al.*, 1984). This revealed two open reading frames in opposite orientation (Figure 3.6).

3.3.1 Sequence Analysis: Homology Search, Identification of a Putative Promoter

Once compiled, the 1363 bp of sequence, as shown in Figure 3.5 was subjected to a homology search, using the Gapped BLAST GenBank program version 2.0 (Altschul *et al.*, 1997)). This revealed that the sequence shared greatest similarity with the isochorismate synthase gene from *Bacillus subtilis* (44% identity and 55% similarity at the amino acid level with a probability of 3×10^{-65}). This sequence also showed significant homology to the isochorismate synthase gene from *Pseudomonas fluorescens* (42% identity and 57% similarity at the amino acid level with a probability of 2×10^{-56}), *Mycobacterium tuberculosis* (42% identity and 55% similarity at the amino acid level with a probability of 2×10^{-51}), *Vibrio cholerae* (39% identity and 50% similarity at the amino acid level, with a probability of 8×10^{-51}), *A. hydrophila* (38% identity and 57 % similarity at the amino acid level, with a probability of 3×10^{-48}) and *E. coli,* (38% identity and 53% similarity at the amino acid level, with a probability of 2×10^{-44}). In addition, this sequence showed homology to other chorismate utilizing proteins, like *trpE* which is involved in aromatic amino acid biosynthesis. A comparison of amino acid sequences of various isochorismate synthase proteins is shown in Figure 3.7. The gene encoding the isochorismate synthase of *A. vinelandii* was named *csbC*, for catecholate siderophore biosynthesis *C,* in keeping with the *E. coli* lettering system, where C denotes isochorismate synthase.

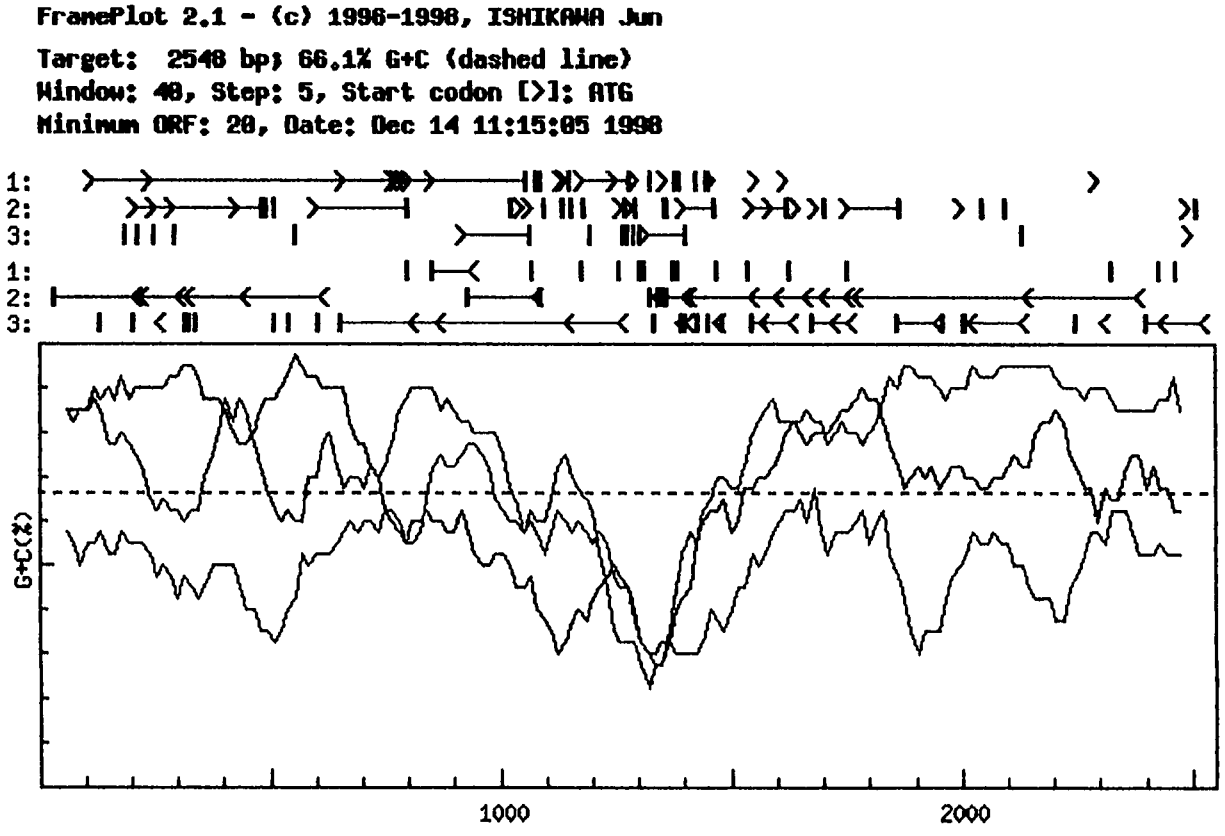
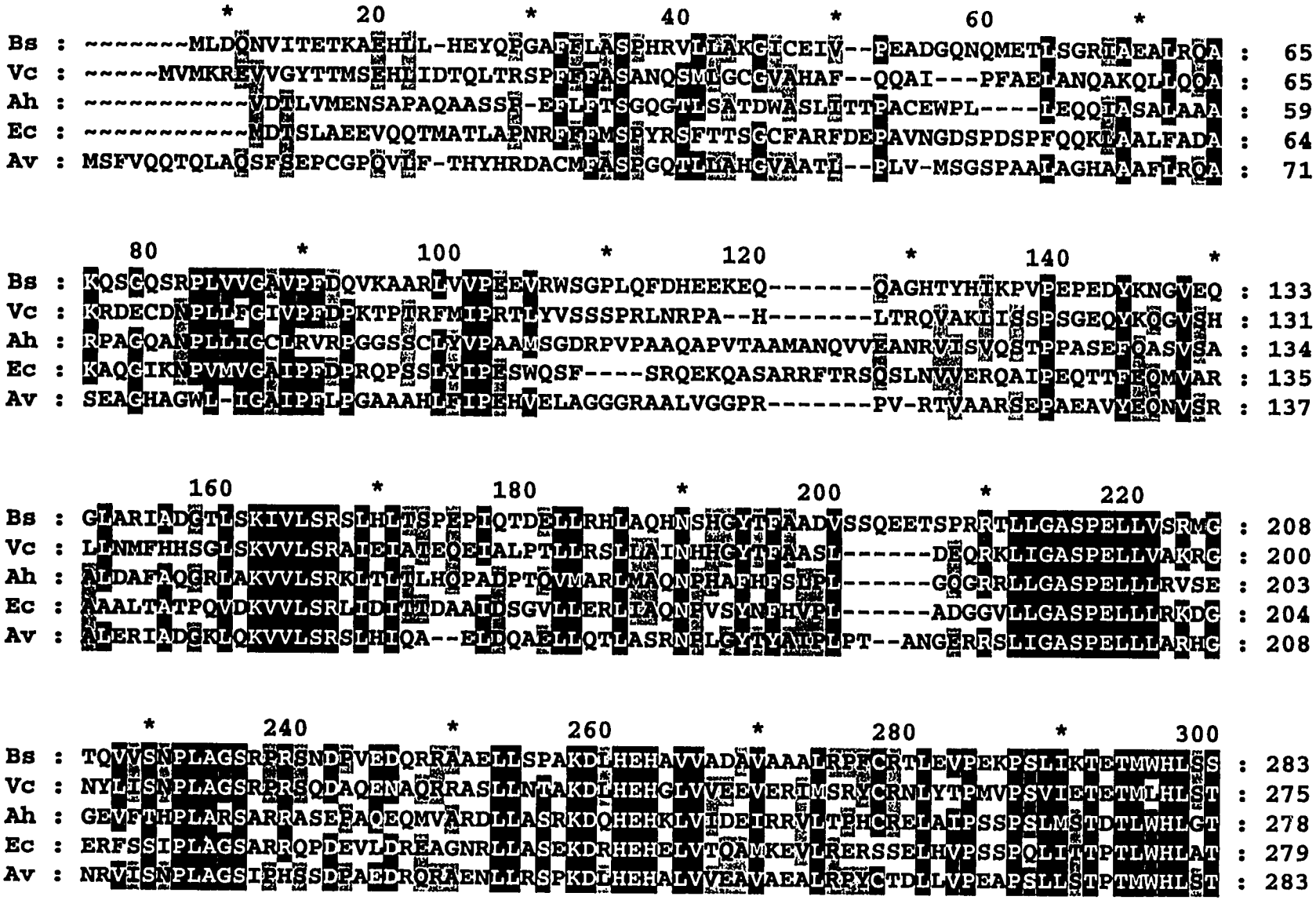


Figure 3.6 Identification of protein coding regions by FramePlot with the 2548 bp of *A. vinelandii* DNA sequence. At the top of the graph 1, 2 and 3 represent reading frames in register with the first, second and third bases respectively, of the sequence read in either direction (> for left to right and < from right to left).


* 20 * 40 * 60 *
Bs : ~~~~~~~MLDQNVITETKAEHLL-HEYQPGAFFLASPHRVLLAKGICEIV--PEADGQNQMETLSGRLAEALRQA : 65
Vc : ~~~~~MVMKREVVGYTTMSEHLIDTQLTRSPFFFASANQSMLGCGVAHAF--QQAI---PFAELANQAKQLLQQA : 65
Ah : ~~~~~~~~~~~VDTLVMENSAPAQAASSR-EFLFTSGQGTLSATDWASLITTPACEWPL----LEQQIASALAAA : 59
Ec : ~~~~~~~~~~~MDTSLAEEVQQTMATLAPNRFFFMSPYRSFTTSGCFARFDEPAVNGDSPDSPFQQKLAALFADA : 64
Av : MSFVQQTQLAQSFSEPCGPQVLF-THYHRDACMFASPGQTLLAHGVAATL--PLV-MSGSPAALAGHAAAFLRQA : 71
80 * 100 * 120 * 140 *
Bs : KQSGQSRPLVVGAVPFDQVKAARLVVPEEVRWSGPLQFDHEEKEQ-------QAGHTYHIKPVPEPEDYKNGVEQ : 133
Vc : KRDECDNPLLFGIVPFDPKTPTRFMIPRTLYVSSSPRLNRPA--H-------LTRQVAKIISSPSGEQYKQGVSH : 131
Ah : RPAGQANPLLIGCLRVRPGGSSCLYVPAAMSGDRPVPAAQAPVTAAMANQVVEANRVISVQSTPPASEFQASVSA : 134
Ec : KAQGIKNPVMVGAIPFDPRQPSSLYIPESWQSF----SRQEKQASARRFTRSQSLNVVERQAIPEQTTFEQMVAR : 135
Av : SEAGHAGWL-IGAIPFLPGAAAHLFIPEHVELAGGGRAALVGGPR-------PV-RTVAARSEPAEAVYEQNVSR : 137
160 * 180 * 200 * 220
Bs : GLARIADGTLSKIVLSRSLHLTSPEPIQTDELLRHLAQHNSHGYTFAADVSSQEETSPRRTLLGASPELLVSRMG : 208
Vc : LLNMFHHSGLSKVVLSRAIEIATEQEIALPTLLRSLLAINHHGYTFAASL------DEQRKLIGASPELLVAKRG : 200
Ah : ALDAFAQGRLAKVVLSRKLTLTLHQPADPTQVMARLMAQNPHAFHFSLPL------GQGRRLLGASPELLLRVSE : 203
Ec : AAALTATPQVDKVVLSRLIDITTDAAIDSGVLLERLIAQNHVSYNFHVPL------ADGGVLLGASPELLLRKDG : 204
Av : ALERIADGKLQKVVLSRSLHIQA--ELLQAELLQTLASRNRLGYTYATPLPT--ANGERRSLIGASPELLLARHG : 208
* 240 * 260 * 280 * 300
Bs : TQVVSNPLAGSRPRSNDPVEDQRRAAELLSPAKDLHEHAVVADAVAAALRPFCRTLEVPEKPSLIKTETMWHLSS : 283
Vc : NYLISNPLAGSRPRSQDAQENAQRRASLLNTAKDLHEHGLVVEEVERIMSRYCRNLYTPMVPSVIETETMLHLST : 275
Ah : GEVFTHPLARSARRASEPAQEQMVARDLLASRKDQHEHKLVIDEIRRVLTPHCRELAIPSSPSLMSTDTLWHLGT : 278
Ec : ERFSSIPLAGSARRQPDEVLDREAGNRLLASEKDRHEHELVTQAMKEVLRERSSELHVPSSPQLITTPTLWHLAT : 279
Av : NRVISNPLAGSIPHSSDPAEDRQRAENLLRSPKDLHEHALVVEAVAEALRPYCTDLLVPEAPSLLSTPTMWHLST : 283

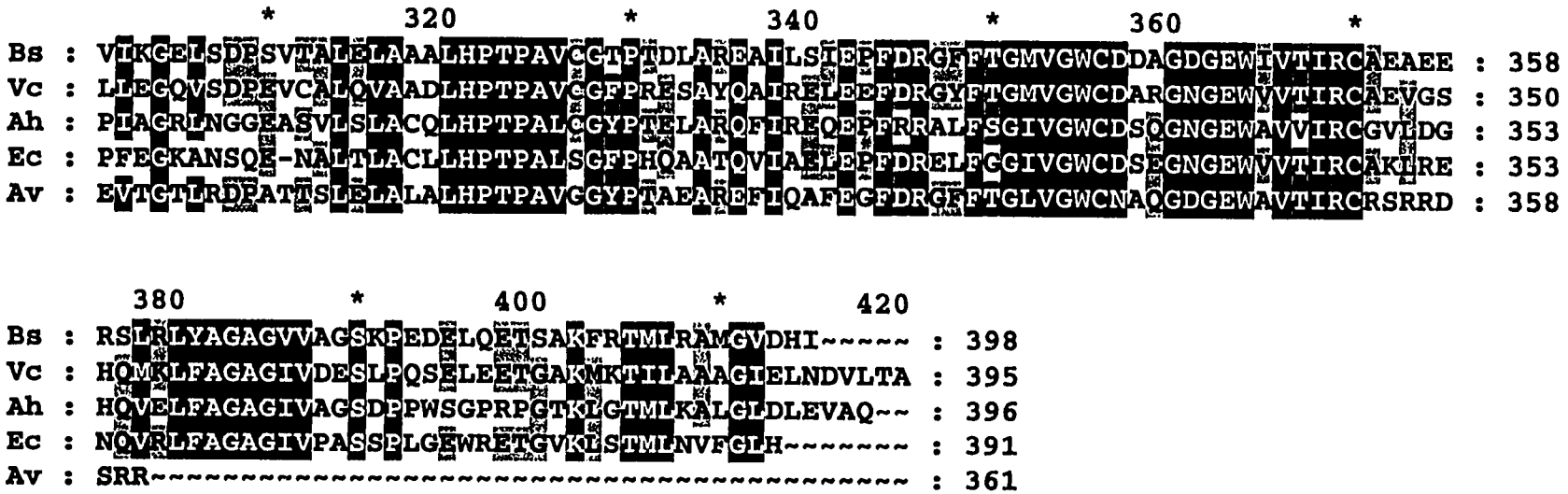


Figure 3.7 Alignment of amino acid sequences of Isochorismate Synthase from different bacteria. Sequences were obtained from the GenBank and were aligned using the PILEUP program of the Univeristy of Wisconsin Genetics Computer Group Sequence Analysis Software program version 8.1. Sequences shown include Bs, *B. subtilus*; Vc, *V. cholerae*; Ah, *A. hydrophila*; Ec, *E. coli*; Av, *A. vinelandii*. Black highlighting indicates that the residue is conserved among all five proteins, grey highlighting with white letters indicates the reisdue is conserved among 4 out of the 5 proteins (80%) and grey highlighting with black letters indicates that the residue is conserved among 3 out of the 5 proteins (60%).

A putative promoter as well as a putative iron-box, having 14 out of 19 bp identical to the consensus sequence was identified overlapping the -35 sequence. As well, two putative Sox-boxes were identified using the consensus sequence proposed by Fawcett *et al.* (1994): one upstream of the promoter having 28 out of 30 bp identical to the consensus sequence and the other overlapping the -10 region having 25 out of 30 bp identical to the consensus sequence.

Upstream of *csbC*, a second open reading frame (*orfA*) was identified (Figure 3.6, frame 3, reading right to left). Homology searches done with this sequence indicate that there is some similarity to antibiotic transporters. A putative promoter and iron-box having 11 out of 19 bp identical to the consensus sequence was also identified (Figure 3.5).

3.4 Cloning the Putatitve Promoter into a Promoter Probe Vector

A simple way to assess promoter activity is to clone a DNA fragment thought to contain a promoter into a promoter probe vector. In this study, we used pQF50 which has a multi-cloning site upstream of a promoterless *lacZ* gene. Once pAS50 had been generated (Section 2.14), the clone was sequenced to confirm that there were no errors as a result of Taq DNA polymerase, and a clone with the correct sequence was used in further studies. The pAS50 insert was also excised and cloned in the opposite orientation by ligating into pQF50 digested with *Bam*HI and *Sal*I to create pAS60.

pAS50 and pAS60 were used for further study of the activity of the putative promoters upstream of *csbC* and *orfA*. On plates with X-gal, *E. coli* JM106 colonies containing pAS50 or pAS60 were blue, indicating that a functional promoter was present in both clones. β-galactosidase assays were done using JM106 (pAS50), JM106 (pAS60) and as a negative control, JM106 (pQF50). However, only very low levels of β-galactosidase were detected (data not shown) and it was concluded that assaying *A.*

vinelandii promoter activity in *E. coli* was of questionable value. It was likely that a better way to study *csbC* expression in *A. vinelandii* would be by Northern hybridization and/or promoter mapping experiments.

3.5 Analysis of Mutant and Wild-Type Copies of *csbC* by Southern Hybridization

In order to determine whether all the copies of *csbC* in strain F196 had been mutated by the Tn5 insertion (as would be expected by the phenotype of F196, which is absolutely catecholate negative), Southern hybridization was carried out. WJP14 was used as a probe for *csbC* and WJP11 was used as a probe for *luxA* on the stripped blot. Chromosomal DNA from wild-type UW and F196 was digested with both *Sal*I and *Bgl*II. As a positive control pAS40 was digested with *Sal*I and as a negative control, pUC119 was digested with *Sal*I. The blot was first probed for *csbC* using end-labeled WJP14 and the probe hybridized to a 3.5 kbp *Sal*I fragment and an approximately 15 kbp *Bgl*II fragment in UW, a 23 kbp *Bgl*II fragment in F196 and an 11.5 kbp *Sal*I fragment in both F196 and pAS40 (Figure 3.8A). In both cases the size difference between the hybridizing fragment from UW compared to the corresponding fragment in F196 was approximately 8 kbp, which corresponds to the size of the Tn*5luxAB* insert. Neither *Sal*I nor *Bgl*II had restriction sites within the Tn5 insert. When probed with WJP11, hybridization was observed with the positive control (pAS40) and to the same fragments in F196 chromosomal DNA that previously to hybridized to the *csbC* probe (Figure 3.8B). As expected, there was no hybridization to UW DNA. This indicated that F196 was homozygous with respect to the *csbC* mutation, in that all copies of *csbC* had the Tn*5luxAB* insert.

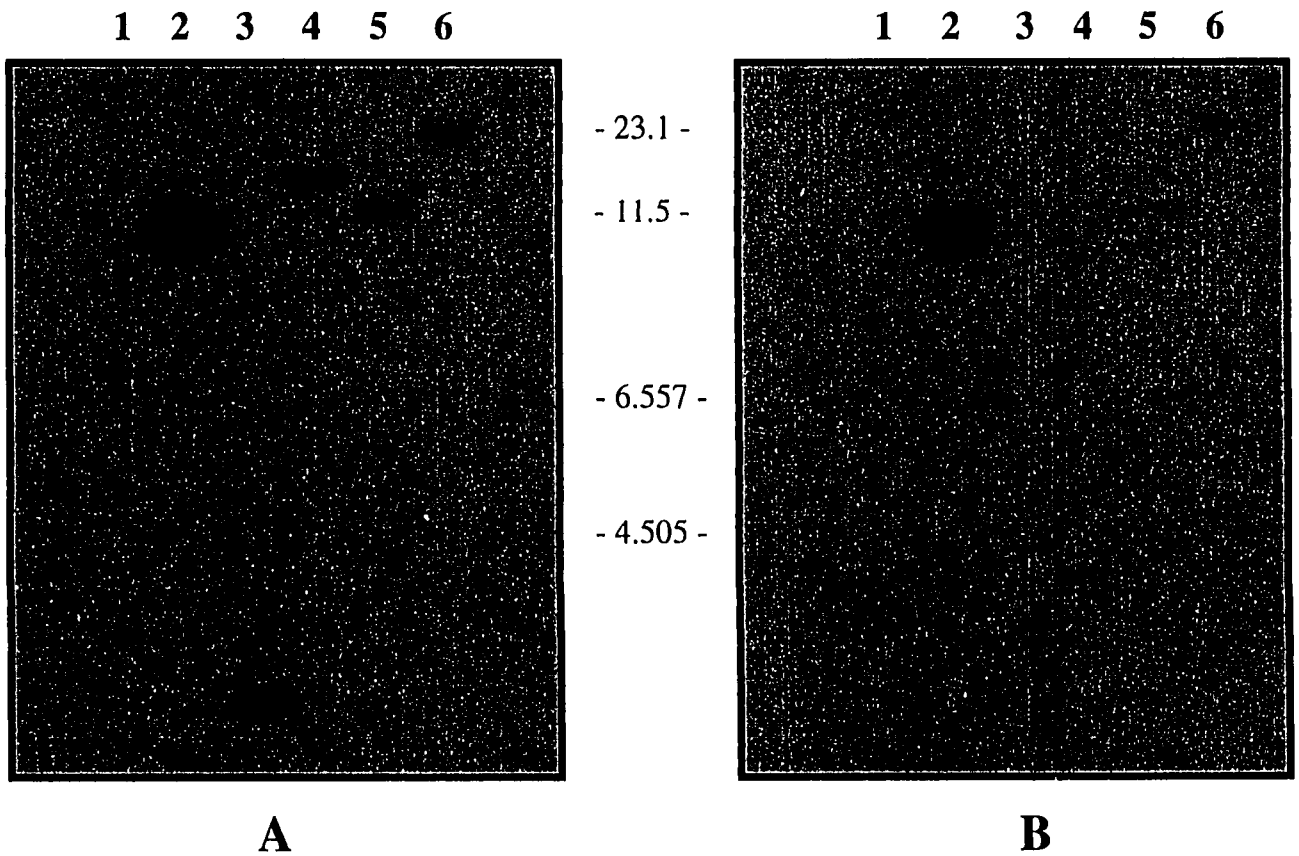


Figure 3.8 Southern analysis of *csbC* in UW and F196. Lane 1: pUC119 *Sal*I digest. Lane 2: pAS40 *Sal*I digest. Lane 3: UW Chromosomal DNA, *Sal*I digest and lane 4: *Bgl*II digest. Lane 5: F196 Chromosomal DNA, *Sal*I digest, lane 6: *Bgl*II digest. A: Blot probed with *csbC* oligonucleotide. B: Same blot probed with the *luxA* oligonucleotide. Size markers (*Hin*dIII and *Pst*I digested λ DNA) are indicated in kbp.

3.6 Gel Shift Assays with the *E. coli* Fur Protein

It has been shown previously that *A. vinelandii* does posses a Fur-like protein (Mehrotra, 1997). In order to further investigate the possibility that *csbC* was regulated by a Fur-like protein, gel shift assays using the *E. coli* Fur protein were done. The DNA target was the 250 bp PCR fragment from pAS50 containing the putative promoters and iron-boxes of *csbC* and *orfA*. These assays were repeated with and without Mn. Mn was used in place of Fe, as Fur's co-repressor, since it is much more stable in an aerobic environment, thus is less readily oxidized.

As shown in Figure 3.9, DNA shift was seen, starting at 200 nM Fur (lane 4), and a second shift is seen starting in lane 7, as expected since there are two iron-boxes within the target DNA. The shift in target DNA mobility was seen only in the presence of the co-repressor; when Mn was absent, protein binding was eliminated (Figure 3.10). The assay was repeated in exactly the same way, using a 250 bp *Eco*RI-*Pvu*II fragment from the *E. coli iucA* (a gene involved in the biosynthesis of aerobactin) promoter, as a positive control, with Mn (Figure 3.11) and without Mn (Figure 3.12). Here, a similar shift is seen, only in the presence of Mn, beginning with 50 nM Fur (lane 4). Assays done with the *csbC* and *iucA* promoter fragments in the absence of Mn and at very high concentrations of Fur (2000 and 5000 nM), resulted in a slight DNA shift likely due to non-specific binding of Fur to the DNA target.

In order to confirm that Fur binds to the sequences which were identified to be the iron-boxes overlapping the *csbC* and *orfA* promoters, 19 bp oligonucleotide pairs WJP28/29, and WJP30/31 were synthesized so that each pair had the same sequence as the two putative iron-boxes shown in Figure 3.5. In addition two other 19 bp oligonuclotide pairs WJP19/34 and WJP32/33 were used as negative controls. These were made from sequence surrounding the putative promoter region. The assay was done

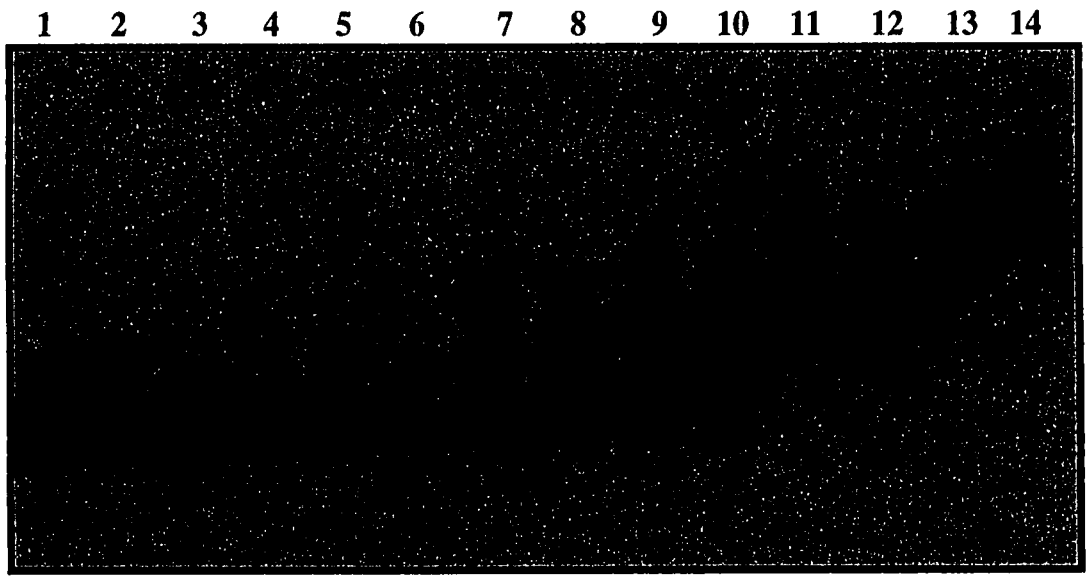


Figure 3.9 Gel shift assay (with Mn) with *E. coli* Fur and 250 bp *A. vinelandii* *csbC* promoter fragment. Lanes 1-14, 0, 0, 25, 50, 75, 100, 200, 300, 400, 500, 700, 1000, 2000, 5000 nM Fur protein

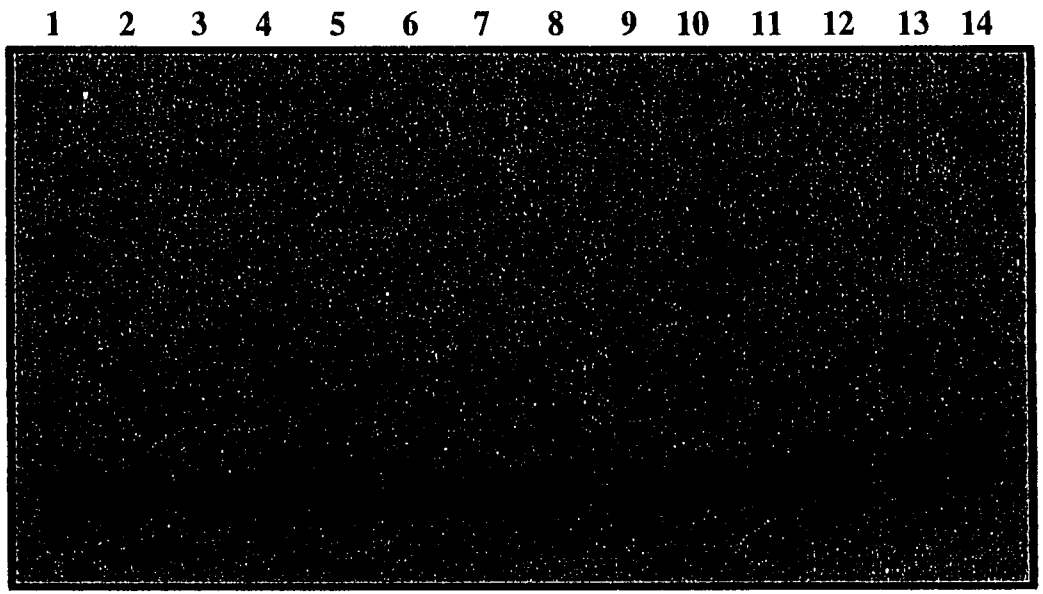


Figure 3.10 Gel shift assay (without Mn) with *E. coli* Fur and 250 bp *A. vinelandii csbC* promoter fragment. Lanes 1-14, 0, 0, 25, 50, 75, 100, 200, 300, 400, 500, 700, 1000, 2000, 5000 nM Fur protein

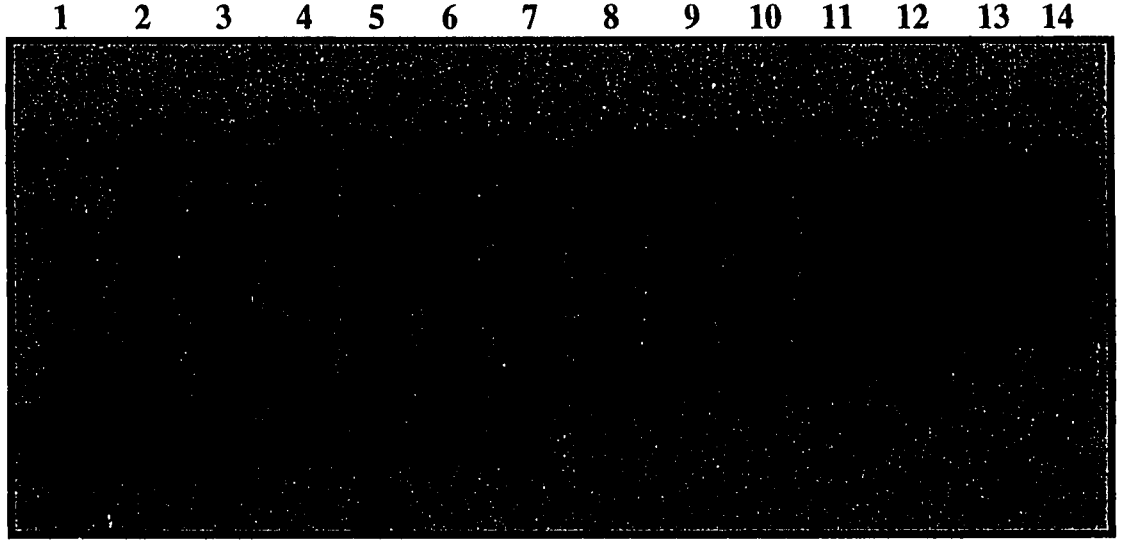


Figure 3.11 Gel shift assay (with Mn) with *E. coli* Fur and 250 bp *E. coli iucA* promoter fragment. Lanes 1-14, 0, 0, 25, 50, 75, 100, 200, 300, 400, 500, 700, 1000, 2000, 5000 nM Fur protein

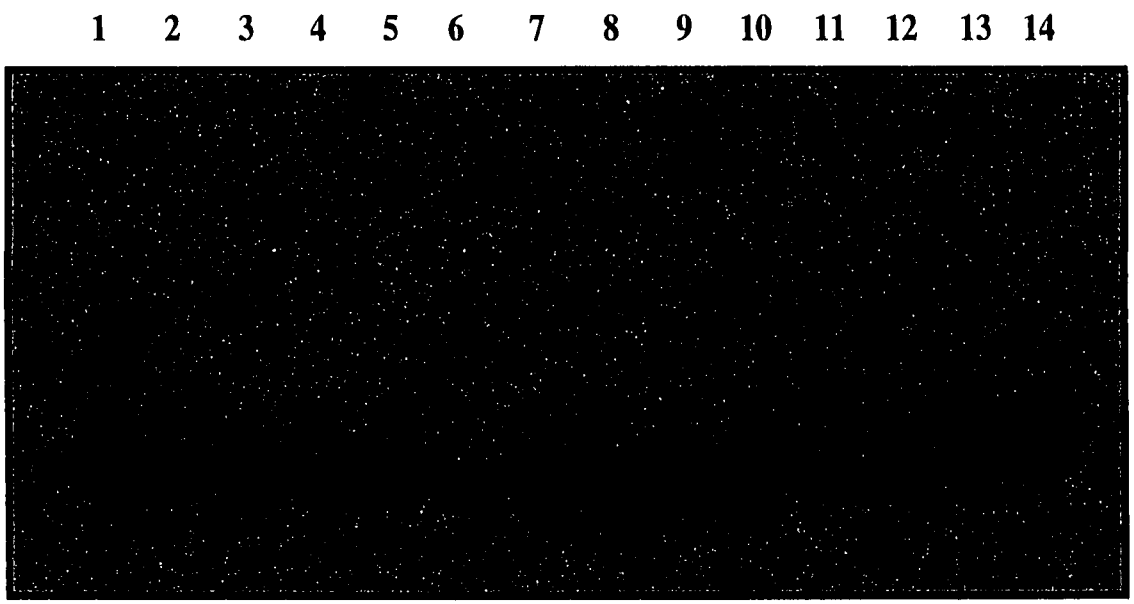


Figure 3.12 Gel shift assay (without Mn) with *E. coli* Fur and 250bp *E. coli iucA* promoter fragment. Lanes 1-14, 0, 0, 25, 50, 75, 100, 200, 300, 400, 500, 700, 1000, 2000, and 5000 nM Fur protein

exactly as before (except that 8% PAG was used). Surprisingly, DNA shift was seen in all four cases, when Mn was present, but no shift was seen in the absence of Mn (data not shown).

This unexpected result indicated that Fur was binding non-specifically to the oligonucleotide targets and, therefore, raised the possibility that the heterologous *E. coli* Fur was also binding non-specifically to the larger *Azotobacter* DNA target used in the initial gel shift experiment (Figure 3.9). In order to confirm that Fur interacts specifically with the 250 bp PCR fragment, a competition binding assay was done. All conditions were exactly the same as in earlier gel shifts, except that the labeled target was mixed with varying amounts of unlabeled 250 bp fragment as a specific competitor or with varying amounts of poly dI-dC as a non-specific competitor, prior to mixing with the binding buffer-Fur mixture. The specific competitor was added in 10- and 50-fold excess of the labeled target and the non-specific competitor was added in 100-, 1000- and 2000-fold excess. As shown in Figure 3.13, it is evident that this protein-DNA interaction was specific, as Fur binding was out-competed by excess specific competitor (lane 6) and to a greater extent when the amount of specific competitor is increased (lane 7). However, Fur still bound to the labeled target DNA even in the presence of a 2000-fold excess of non-homologous DNA (lane 10).

3.7 Methyl Viologen Sensitivity Studies

The possibility that *csbC* is also involved in an O_2-stress response was investigated using plate assays containing the superoxide generating agent methyl viologen to promote O_2-stress conditions. First the minimum level of Fe required to repress *luxAB* on solid medium was determined and was used in the subsequent plates. BBGN was prepared with 0 to 90 μM methyl viologen and F196 was spotted onto the plates. Lux activity was then used as a reporter of *csbC* activity, by placing the plates on

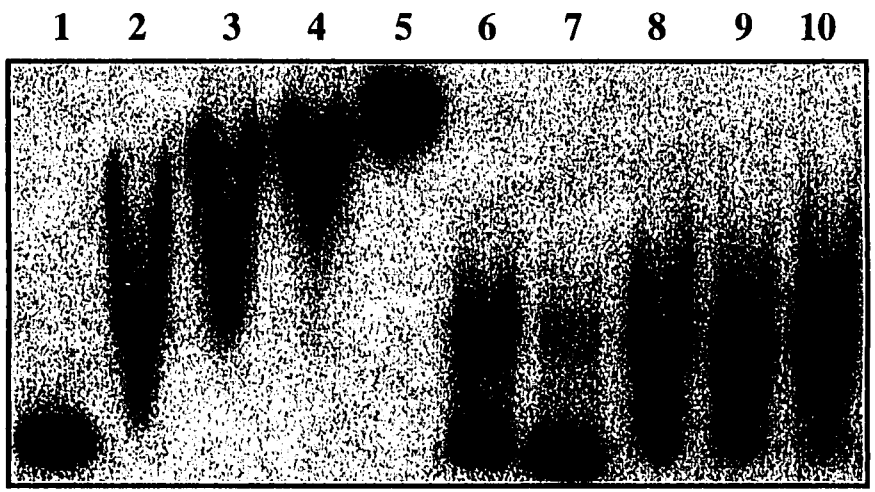


Figure 3.13 Competition gel shift assay with the *E. coli* Fur protein and 250 bp *A. vinelandii csbC* promoter region. Lane1: 0 nM Fur, lane 2: 150 nM Fur, lane 3: 300 nM Fur, lane 4: 500 nM Fur, lane 5: 700 nM Fur, lane 6:150 nM Fur and 10X specific competitor, lane 7: 150 nM Fur and 50X specific competitor, lane 8: 150 nM Fur and 100X non-specific competitor, lane 9: 150 nM Fur and 1000X non-specific competitor, lane 10: 150 nM Fur and 2000X non-specific competitor.

X-ray film in the darkroom. Induction of *luxAB* was observed at 20 μM Fe citrate plus 30, 40, 50 μM methyl viologen while it was repressed in the presence of 20 μM Fe alone. (data not shown)

3.8 Investigating the Expression of *csbC*

3.8.1 Northern Analysis

In order to study the activity of *csbC in vivo*, using a homologous system, Northern hybridization was carried out using RNA extracted from both UW and F196 and a 310 bp *Not*I-*Pst*I fragment from pAS310 as the probe. This way, in addition to studying *csbC* expression, transcript size could also be determined.

Total cellular RNA was extracted from UW and F196 cultures grown under Fe limited (1 μM Fe citrate) and sufficient (50 μM Fe citrate) conditions, after 16.5 and 18 hr growth. *E. coli* controls, also grown under Fe limited (LB + 50 μM EDDHA) and sufficient (LB + 50 μM Fe citrate) conditions, included DH5α (pAS20, pMH15) where a transcript identical to the one seen in F196 was expected and DH5α (pK184, pMH15) which was used as a negative control. As shown in Figure 3.14A, only a degraded transcript was detectable instead of a single band. This transcript was detectable in the Fe-deficient cells and the *csbC* transcript was completely repressed under Fe-sufficient growth conditions. Due to their abundance, it is likely that the 16S and 23S rRNA prevent hybridization of the probe to its target, as seen by the gaps in the smear at approximately 1500 and 2900 bp. It was also evident, especially in wild-type UW, that with increased incubation time, the amount of *csbC* transcript increases, as seen when comparing lanes 8 and 12. As for the controls, transcript was present in Fe-sufficient *E. coli* (pAS20, pMH15) (lane 3) but was more pronounced in Fe-limited cells (lane 4). RNA extracted from Fe-sufficient cells contained some *csbC* transcript, most likely as a consequence of either Fur titration or simply the fact that the *A. vinelandii* promoter was

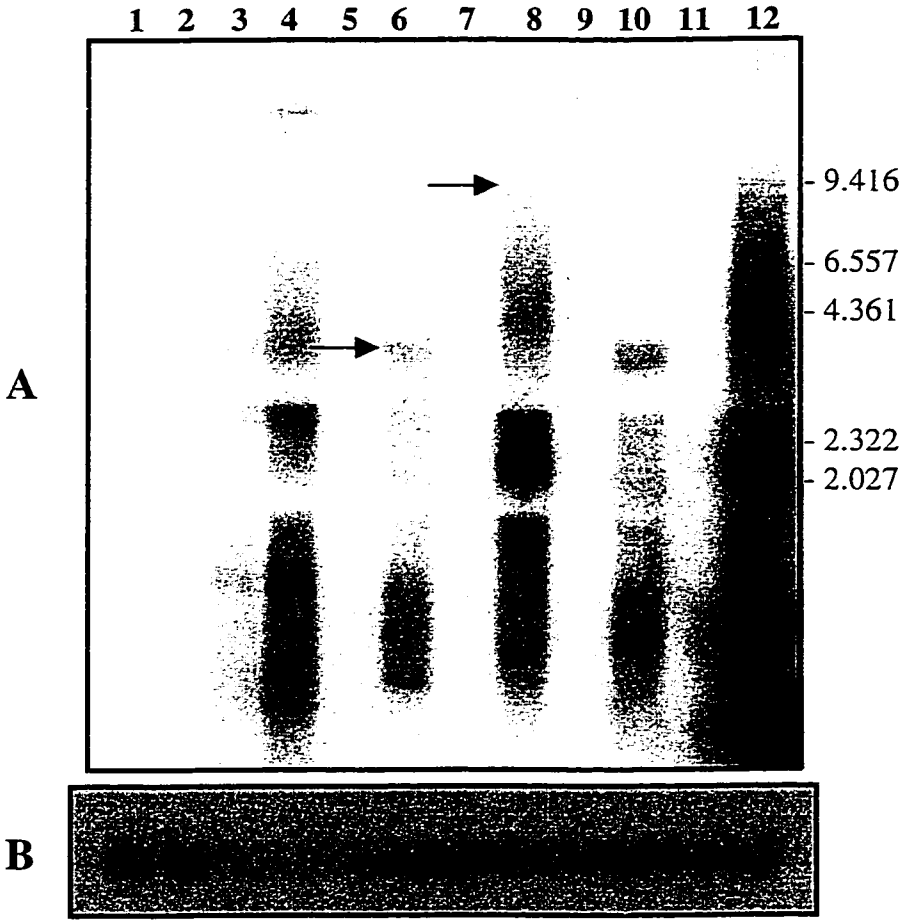


Figure 3.14 Northern analysis of *csbC* in UW and F196. Lane 1: *E. coli* (pK184, pMH15) +Fe, Lane 2: *E. coli* (pK184, pMH15) -Fe, Lane 3: *E. coli* (pAS20, pMH15) +Fe, Lane 4: *E. coli* (pAS20, pMH15) -Fe, Lane 5: F196 +Fe, 16.5 hr, Lane 6: F196 -Fe, 16.5 hr, Lane 7: UW +Fe, 16.5 hr, Lane 8: UW -Fe, 16.5 hr, Lane 9: F196+ Fe, 18 hr, Lane 10: F196 -Fe, 18hr, Lane 11: UW, +Fe, 18 hr, Lane 12: UW-Fe, 18 hr. A: Blot probed with 310 bp *csbC* fragment. Arrows indicate likely transcript size. Size markers (*Hin*dIII digested λ DNA) are indicated in kbp. B: Same blot probed with 16s rRNA oligonucleotide.

not regulated to the same extent in *E. coli*, due to differences in the Fur protein and its specificity for the *Azotobacter* iron-box. However this was consistent with earlier results, where the Lux activity of pAS1 was poorly regulated in *E. coli*. Exact transcript sizes were hard to determine, but the largest transcript was approximately 3.2 kbp in F196 and in pAS40, and approximately 9.5 kbp in UW. This difference in size must be due to the fact that transcription from the *csbC* promoter in F196 likely ends 3' to *luxAB*. Since *luxAB* is approximately 2.4 kbp, and sequence between the putative promoter and start of *luxA* is about 660 bp, thus should generate a truncated transcript of approximately 3060 bp.

As a control for RNA loading, the blot was stripped, and re-probed with a universal oligonucleotide for 16S rRNA (which will hybridize to both *E. coli* and *A. vinelandii* RNA). As seen in Figure 3.14B, bands of approximately even intensity were seen in all lanes, indicating that equal amounts of RNA had been loaded onto each lane of the gel. Thus more or less intense reaction with the *csbC* probe was likely due to increased transcription, not uneven sample loading.

3.8.2 RNA Dot Blot Analysis

Due to the apparently unstable nature of the transcript generated from the *csbC* promoter, it was thought that further study of *csbC* expression could be followed by RNA dot blotting. First the level of Fe required to repress *csbC* was determined. RNA was extracted from UW grown for 16 hr with increasing amounts of Fe citrate added to 0FeBBGN. As shown in Figure 3.15A, *csbC* was repressed when Fe exceeded 7 μM. In repetitions of this experiment, these results were duplicated, but in some cases expression was also seen at 10 μM Fe. Therefore, it can be concluded that *csbC* was repressed by 7-10 μM Fe in the medium.

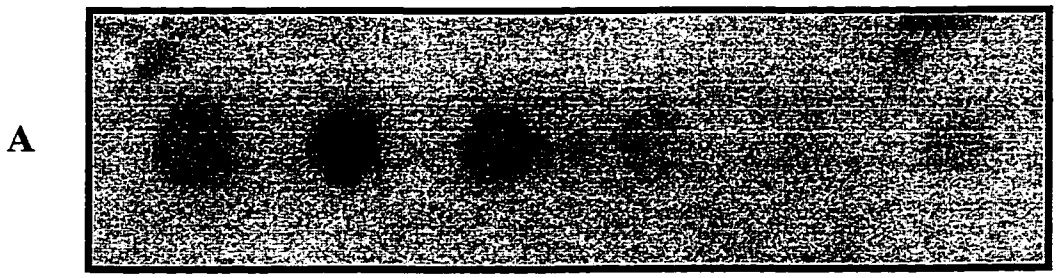


Figure 3.15 RNA dot blot analysis of *csbC* under different Fe levels in *A. vinelandii* strain UW. A: 5 μg RNA extracted from UW cultures grown with 1, 5, 7, 10, 20, 50 μM Fe citrate. B: 5 μg RNA from UWculture grown for 16 hr with 1 μM Fe citrate, and at time 0, 20 μM Fe citrate was added, and RNA extracted after 15, 30, 45 min.

Since *csbC* is repressed when Fe was in sufficient supply (greater than 7 μM), it was expected that the addition of Fe at this level or greater would repress catecholate siderophore biosynthesis. To test this, UW was grown under Fe limited (1 μM Fe citrate) conditions for 16 hr and Fe citrate was added to the culture to a final concentration of 20 μM. RNA was extracted immediately prior to Fe addition and then 15, 30 and 45 min after Fe addition. As shown in Figure 3.15B, *csbC* was repressed within 15 min after addition of Fe, confirming the observation that *csbC* is repressed when Fe is in sufficient supply.

In order to determine if *csbC* is also regulated by O_2-stress, cultures were grown for 22 hr with varying levels of Fe citrate added to 0FeBBGN and under different aeration conditions (where a smaller volume of medium would increase aeration, and cells would be subjected to increased O_2 stress). Results were somewhat inconsistent. In Figure 3.16 two examples of typical results are shown. It does appear that there is some upregulation of *csbC* by O_2 stress, as seen in both cases with the 5 mL culture. In Figure 3.16A it is evident that when comparing the 5 mL culture to the 20 mL culture at 3 μM Fe, there is increased *csbC* transcript under high aeration. However, in Figure 3.16B, *csbC* expression is repressed by a lower level of Fe under conditions of low aeration. It is clear from the graphs that catecholate production (A310) per cell growth is higher under increased aeration, consistent with the results of Cornish and Page, 1998.

3.8.3 S1 Nuclease Mapping of the *csbC* Transcript Start Site.

S1 nuclease mapping was used to confirm that the putative promoter of *csbC*, identified by similarities to the -10 and -35 *E. coli* sigma 70 consensus sequence, was used *in vivo*. Total RNA from UW grown under both Fe sufficient (50 μM Fe citrate) and limited (1 μM Fe citrate) conditions was used, and as controls, total RNA from *E. coli* DH5α (pAS20, pMH15) and DH5α (pK184, pMH15) both grown under Fe limited

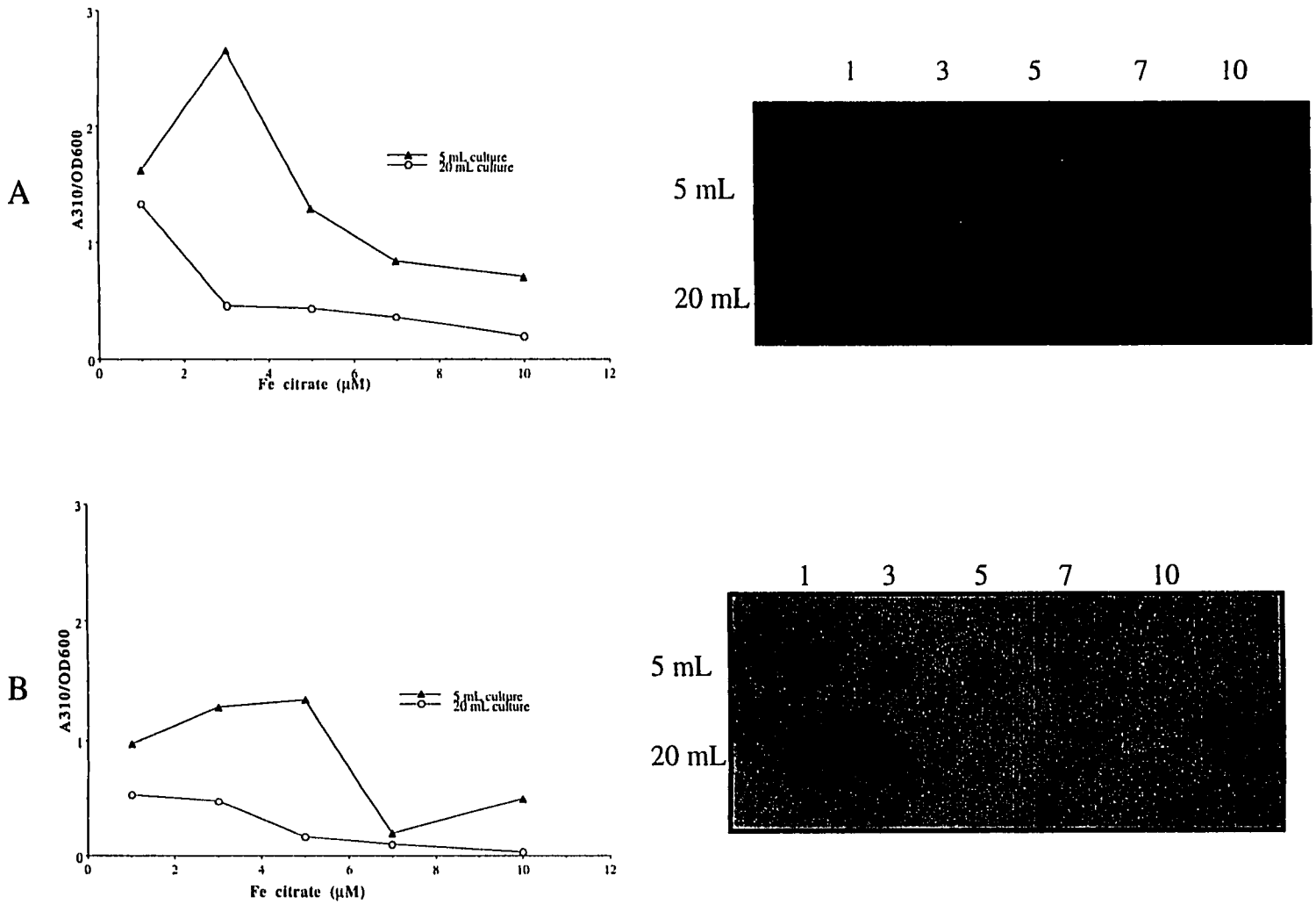


Figure 3.16 RNA dot blot analysis of *csbC* under O_2-stress conditions in UW. A and B: 5 μg RNA extracted from UW cultures grown with 1, 3, 5, 7, 10 μM Fe citrate, in either 5 mL or 20 mL medium. Also shown graphically is an estimate of catecholate production (A310) per growth (OD600) according to the amount of Fe citrate provided in the medium (data generated from the same cultures from which the RNA was extracted).

(50 μM EDDHA) conditions was used. As well, a reaction in which the probe alone (without any RNA added), was carried through all steps as a control. This was done to confirm that any bands observed were not a result of one strand of the probe partially self-annealing (therefore any double stranded region would be protected from S1 nuclease digestion of the probe, and may yield some smaller, labeled product). As shown in Figure 3.17, a small ladder of bands was seen, but it was very hard to deduce which base was the transcript start site. S1 nuclease mapping was repeated seven times with different modifications including varying the amounts of S1 nuclease enzyme, varying the amounts of probe as well as using S1 nuclease from Pharmacia and Sigma. Results from these experiments were inconsistent, where the largest band in the ladder corresponded to either of three bases (as indicated by asterisks in Figure 3.17, which shows a typical result). The results lead to ambiguity as to which base was the transcription initiation point, however, the products of S1 nuclease were within the approximate size that would be predicted as a start site according to the putative promoter sequence.

3.8.4 Primer Extension Analysis of the *csbC* Promoter

As the results from S1 nuclease were difficult to interpret, primer extension analysis was done to clarify the identity of the transcript initiation site. Again, total RNA from UW grown under Fe limited (1 μM Fe citrate) and Fe-sufficient growth conditions, as well as the *E. coli* controls were used. The products obtained after reverse transcription from primer WJP18 were run on a sequencing gel, and as shown in Figure 3.18, one definite band was evident. The start of transcription was an adenine. This result is in agreement with that seen in Figure 3.17, if the top band in the ladder (which corresponds to the longest product seen) is assumed to be the full length protected

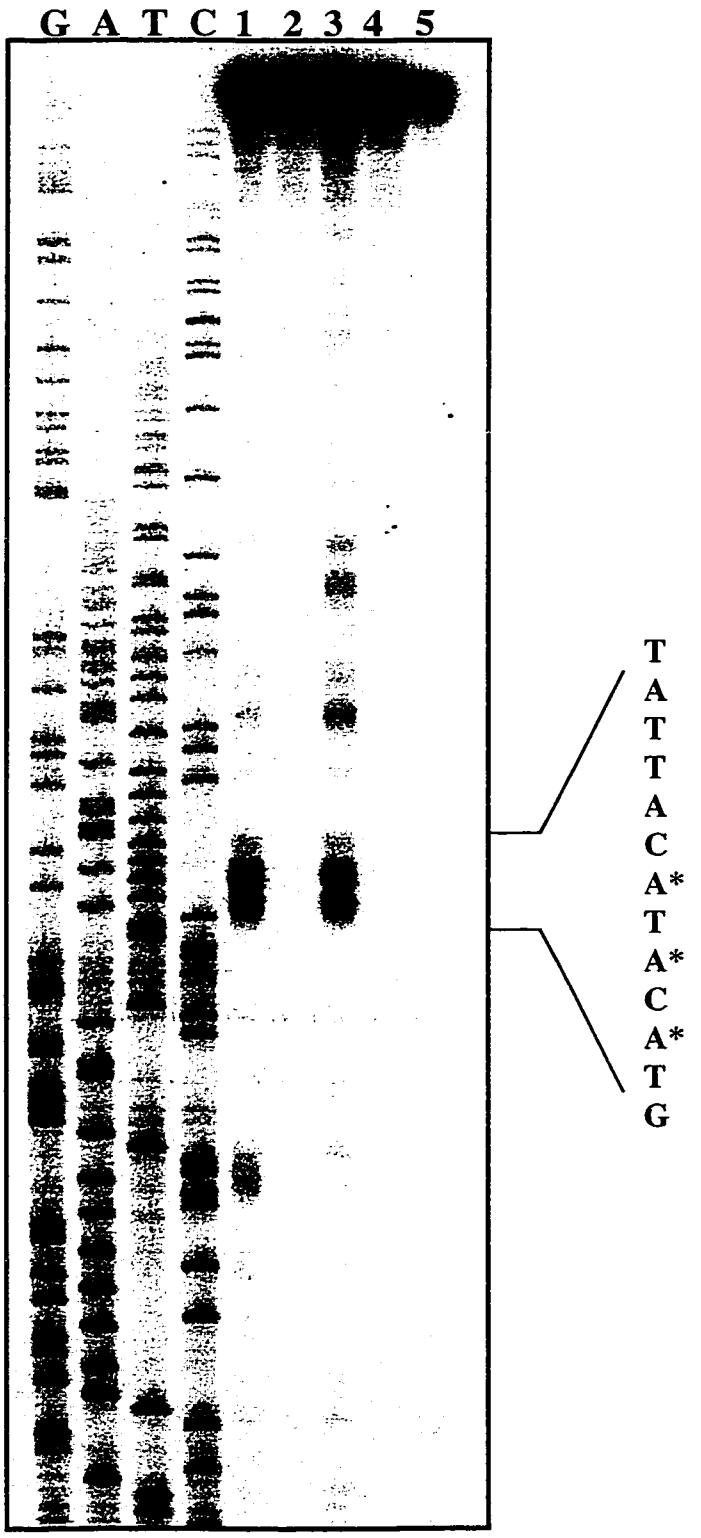


Figure 3.17 S1 Nuclease mapping of *csbC* transcriptional start site. Lane 1: UW RNA from Fe-limited culture. Lane 2: UW RNA from Fe-sufficient culture. Lane 3: *E. coli* (pAS20, pMH15) RNA, Fe-limited. Lane 4: *E. coli* (pK184, pMH15) RNA, Fe-limited. Lane 5: Probe alone. Sequence ladder was generated with primer WJP18. Possible transcript start sites as determined by several S1 experiments are indicated by *.

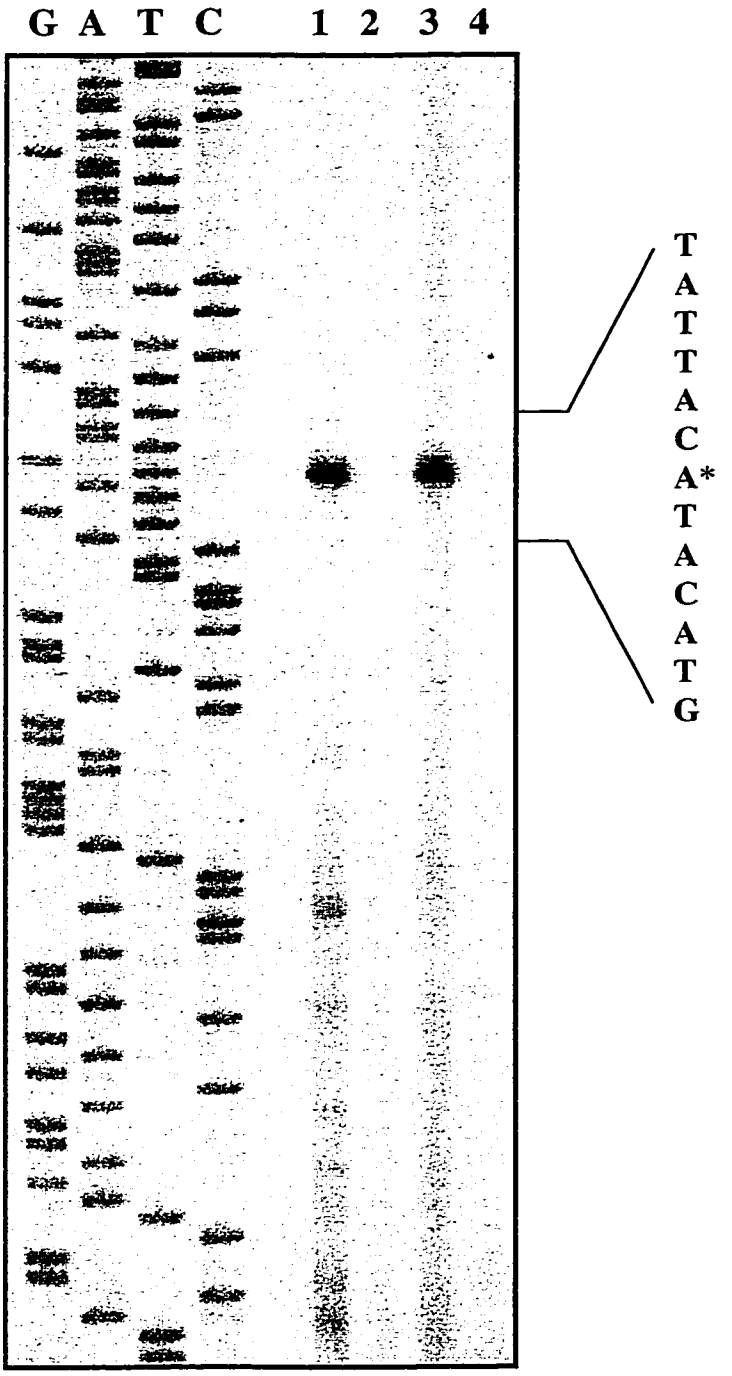


Figure 3.18 Primer extension analysis of the *csbC* promoter. Lane 1: UW RNA from Fe-limited culture. Lane 2: UW RNA from Fe-sufficient culture. Lane 3: *E. coli* (pAS20, pMH15) RNA, Fe-limited. Lane 4: *E. coli* (pK184, pMH15) RNA, Fe-limited. Sequence ladder was generated with primer WJP18. Transcript start site is indicated by *.

transcript. Therefore, based on these results, the transcript start site was an A (nucleotide 214 in Figure 3.5) which was 7 bp downstream of the proposed -10 sequence.

CHAPTER 4. DISCUSSION

The first step to learning more about the nature of the mutation in F196 was to generate a genomic library and isolate the clone containing the Tn*5luxAB* insertion. This generated pAS1 which was easily identified by screening the library for bioluminescent clones. Preliminary analysis of pAS1 included confirmation that the Lux activity was iron-repressible which indicated that the *A. vinelandii* promoter that regulates *luxAB* in F196 was contained within this clone.

It is believed that this promoter is regulated by a Fur-like protein in *A. vinelandii*. There are two reasons for this hypothesis. First, is the fact that from total cell protein, a Fur-like protein was detected in *A. vinelandii* by Western blot analysis using antiserum against *E. coli* and *P. aeurginosa* Fur, which indicates that these proteins share serological homology (Mehrotra, 1997). Second, the basis for cloning *fur* genes from other microorganisms has been by functional complementation of an *E. coli fur* mutant. This emphasizes the fact that Fur proteins of different bacteria are interchangeable which indicates that they share a conserved mechanism of action. Therefore, we predicted that the *E. coli* Fur protein should repress the *A. vinelandii* promoter and it seemed reasonable to look for the ability of this protein to bind to *A. vinelandii* DNA, for the purpose of determining whether or not the *A. vinelandii csbC-orfA* promoter region has functional iron-box(es). Gel shift assays were done using purified *E. coli* Fur, and it was evident that the heterologous Fur protein did bind to the *A. vinelandii* DNA possibly even at both putative iron-boxes as shown by the Mn-dependent binding of Fur to the promoter fragment. Specificity of this binding was confirmed by a competition Fur binding assay. The failure of this assay using oligonucleotides as the DNA target may be indicate that Fur requires some surrounding DNA sequence to confer specificity in binding. It would be ideal if these experiments could be repeated using purified *A. vinelandii* Fur, if it

should become available. In order to confirm that both *csbC* and *orfA* are regulated by Fur, it would be interesting to confirm that the putative iron-boxes are are in fact recognized by Fur. This could be done by DNase I footprinting experiments.

The fact that *luxAB* was only partially repressed (not to the extent seen in F196) in the *E. coli* strains is likely due to repressor dilution, through gene copy number since increasing the level of Fur (by addition of pMH15 carrying the *fur* gene) resulted in better control of this promoter. The Northern results also were consistent with Lux not being completely repressed, due to repressor dilution, as it was evident that there was some transcription from this promoter in the *E. coli* (pAS20, pMH15) grown under Fe sufficient conditions. Similarly, pAS1, which carries at least two iron-boxes, and has a copy number of approximately 15-20, would result in an increased number of Fur target sites. Without a concomitant increase in Fur production, these iron-boxes would be less likely to be repressed. Even in UW, pAS1 was not completely repressed and this is likely due to repressor dilution. It was clear from preliminary analysis that the iron regulated promoter of interest was contained within the pAS1 insert and was Fur regulated.

As proposed by Sevinc and Page, 1992, the observation that DHBA could not restore catecholate siderophore production in F196 could be due to the fact that the genes required for catecholate biosynthesis may be organized in an operon and are therefore affected by insertion of Tn*5luxAB*. In this study, Northern analysis demonstrated that *csbC* is not transcribed alone, but as a large transcript, indicative of an operon. Although the downstream genes have not been identified, it is clear that they are under control of the *csbC* iron regulated promoter. It is therefore possible that not only are *entC, entB* and *entA* homologues encoded in this transcript, but also other genes required in the final steps of the synthesis may be co-transcribed in this operon.

pAS1 was transformed into the different *E. coli ent* mutants to check for the presence of any functional genes involved in catecholate biosynthesis since it is well documented that there is significant functional homology between these biosynthetic

enzymes. It was reasonable to predict that this clone might carry some of the genes (*entA*, *entB* and *entC* equivalents) required for the synthesis of DHBA but less likely that one of the other mutants (*entD*, *entE*, entF or *entG*, which are deficient in the final assembly of enterobactin) would be complemented. None of the *E. coli* transformants showed halos on CAS and thus pAS1 failed to restore enterobactin synthesis. The reason for this became apparent when pAS1 was mapped and determined to carry very little sequence downstream of *csbC*. It is likely that genes required for catecholate siderophore biosynthesis are clustered with *csbC* (as *csbC* is not transcribed alone, as shown by the large transcript detected in Northern analysis) and may complement the *ent* mutants. However in order to confirm this, the region downstream of *csbC* needs to be sequenced so that other genes can be identified. This will require isolation of another clone from the genomic library, using a piece of DNA from the 3' end of *csbC* to probe colony blots. This way, the 3' end of the *csbC* gene could also be sequenced, so as to obtain the complete sequence of this gene.

The mechanism of Tn*5* transposition is conservative, where the transposable element is excised from one location and becomes reinserted at a second location (Berg, 1989). Where the transposon inserts into the target DNA, the Tn*5*-encoded transposase cleaves the DNA target on one strand and 9 bp away on the other strand. Thus, inserts of Tn*5* are bracketed by 9 bp direct repeats. This was the case in the sequence obtained, as when the two pieces of sequence were compiled, taking the sequence immediately upstream of the left border and placing it in front of the sequence obtained immediately downstream of the right border, a 9 bp direct repeat was identified. When the repeat was removed from sequence, this gave 874 bp of sequence upstream of *luxA* and 489 bp of sequence on the other side of the Tn*5luxAB*, downstream of the right border. This gave a total of 1363 bp of sequence, of which both strands of the DNA were sequenced. Comparison of the amino acid sequence to other sequences in the GenBank database revealed that the amino acid sequence shares significant homology to isochorismate

synthase of many bacteria, leaving little doubt as to the identification of the gene disrupted in F196. This fits with what was known about the F196 phenotype, which demonstrated a complete inability to synthesize DHBA or any catecholate siderophore. Identification of isochorismate synthase in *A. vinelandii* indicates that this organism uses a mechanism identical to that used in other bacteria for the conversion of chorismate to isochorismate.

There did not seem to be any deletion of *csbC* sequence in the region surrounding the Tn*5luxAB*. When the two pieces of sequence were entered separately to the GenBank for homology searches, the 874 bp immediately upstream of the left border of the Tn*5luxAB* showed that the amino acid encoded by nucleotides 871 to 873 was identical to amino acid 198 in the *B. subtilus* isochorismate synthase sequence. Taking the 489 bp immediately downstream of the right border showed that nucleotides 3 to 5 encoded an amino acid identical to amino acid 200 of the *B. subtilus* isochorismate synthase. When put together, base 874 of the upstream sequence put together with bases 1 and 2 of the downstream sequence encode for an amino acid identical to amino acid 199 of the *B. subtilus* protein, indicating that there was no stretch of missing sequence in this region. To confirm this, attempts were made to PCR amplify this region of DNA from UW chromosomal DNA, so that the sequence of the wild-type gene could be determined. However, PCR was unsuccessful, and thus it was not possible to confirm the sequence by this method.

In addition to the double stranded sequence obtained, 1185 bp (termed *orfA*) further upstream was obtained by automated sequencing, but in this case only one strand of the sequence was determined. When the 1185 bp of single stranded sequence was compared to sequences in the GenBank, it was revealed that there was homology of approximately 400 nucleotides in the region of nucleotide 1150-1200 to approximately nucleotide 765 (corresponds to the numbering in Appendix 1) to many different antibiotic efflux transport proteins. One such protein that was homologous to the *orfA* sequence

was a chloramphenicol resistance determinant that encodes a putative transmembrane pump in *Streptomyces lividans* (Dittrich *et al.*, 1991). In this paper, the authors found that the protein had 20% homology to an integral membrane protein, FhuB, which is involved with the ferric-hydroxamate uptake system in *E. coli.* The authors were unable to identify any motifs common to these proteins. It is possible that OrfA is involved in siderophore transport and the fact that most of the sequence shares no homology to any known gene may be due to the fact that this region of the protein is involved in recognition of the catecholate siderophores produced by *A. vinelandii.* This result, although preliminary, is very interesting and more work is definitely needed to make conclusive statements as to the function of this upstream gene. The first step would be to sequence the other strand of DNA, in order to obtain more accurate sequence data, which may reveal that other regions of the sequence share homology to proteins of known function, thus giving us a better idea as to the role of *orfA*.

The total 2548 bp of sequence was subjected to open reading frame analysis which revealed two divergently transcribed open reading frames, one which corresponds to *csbC* and the other, upstream corresponding to *orfA*. There appears to be a frame shift mutation in the *orfA* open reading frame. This may be a result of the sequence data being inaccurate, and likely sequencing the other strand would resolve this problem, or alternatively, the region surrounding the frame shift could be PCR amplified and sequenced directly.

There is little information in the literature about *Azotobacter* promoters (no specific σ^{70} consensus sequences for this organism have been identified) as most interest in genetics of this organism has been the study of genes required for nitrogen fixation, which are regulated by σ^{54}. The putative *A. vinelandii* σ^{70} promoters identified have all the characteristics of typical *E. coli* σ^{70} promoters. Therefore the putative promoters were identified by screening for the *E. coli* σ^{70} consensus motifs. In 90% of promoters, the number of bases separating the -10 and -35 sequences is between 16 and 18 (Lewin,

1994), where the putative *csbC* promoter has 18 bp separating the -35 and -10 elements. The -10 sequence has 4 out of 6 bases identical to the σ^{70} consensus, and the -35 sequence has 5 out of 6 bases identical to the σ^{70} consensus sequence. Promoter mapping confirmed that transcription *in vivo* does occur from this promoter. Primer extension analysis identified the transcript start site to be an A. It is common for a start point to be the central base in the sequence CAT, as it is in the case of the *csbC* promoter and as well the start point is a purine (A/G) in 90% of cases. The center of the -10 hexamer is usually 5-9 bp downstream of the transcription start point, and in this case the start site is 9 bases downstream of the center of the -10 sequence.

In the case of the putative *orfA* promoter, both the -10 and –35 sequence had 4 out of 6 bases identical to the σ^{70} consensus sequence. In this case 18 bases separate these elements. Promoter mapping experiments need to be done to characterize the *orfA* promoter. A putative start codon and ribosome binding site were also identified.

The promoter regions were also screened for iron-boxes using the *E. coli* consensus sequence which is normally found to overlap the promoter. Iron-boxes were identified, one of which overlapped the -35 sequence of the *csbC* promoter and had 74% identity (14 out of 19 bases) to the consensus. A putative iron-box was also identified for *orfA*. In this case the iron-box also overlapped the -35 sequence and had 58% identity (11 bases) to the consensus.

The *csbC* promoter region was also screened for sox-boxes, based on the two *E. coli* consensus sequences which have been proposed (Li and Demple, 1996; Fawcett and Wolf, 1994). The differences in the consensus sequences identified may be due to the fact that Fawcett and Wolf used a MalE-SoxS fusion protein, whereas Li and Demple used purified SoxS in their DNase I footprinting studies. The consensus sequence proposed by Fawcett and Wolf is 13 bp longer, but within this sequence is the consensus of Li and Demple. Both sequences identify a GCAY motif, which appears to be the core site for sequence-specific protein-DNA contact by SoxS (Li and Demple, 1996). Two

putative sox-boxes were identified in the *A. vinelandii* sequence, one upstream of the *csbC* promoter which had the greatest homology to the consensus proposed by Fawcett and Wolf, and another, overlapping the –10 region, which showed slightly lower homology to the consensus, but in a position more characteristic of sox-boxes. The GCAY motif is conserved in the putative sox-box overlapping the –10 region, but only the GCA is present in the upstream sox-box. Both sets of authors have identified primary and secondary binding sites for SoxS in the promoter regions of certain genes, but it is unclear what role the secondary sites may play, if any. In the case of *csbC*, the upstream sox-box would likely interfere with *orfA* transcription, while the downstream sox-box would not.

Comparison of this divergent promoter to the *E. coli fepB-entC* divergent promoter revealed strong similarity in the organization of transcriptional initiation signals of these promoters. In *E. coli*, the -35 sequence of the *entC* promoter is separated from the -35 sequence of *fepB* by 31 bp (Ozenberger *et al.*, 1989) and in *A. vinelandii* the -35 sequences of *csbC* and *orfA* are separated by 30 bp. Brickman *et al.* (1990) studied the *fepB* and *entC* promoters by insertion of a DNA fragment carrying both promoters into a vector with divergently oriented reporter gene fusions. Their results suggested some degree of co-operativity between the two promoters and that some minor repressive influence is exerted by Fur occupation of the opposing operator site. Therefore, given the fact that *orfA* may be involved with siderophore transport, and it is known that FepB is a periplasmic protein involved in uptake of enterobactin, it will be interesting to further investigate the function and regulation of *orfA* as well as determining if there are any other genes required for siderophore transport or biosynthesis that may be clustered in this region.

Because the putative *orfA* and *csbC* promoters share so many characteristics common to other bacterial promoters, it is surprising that the promoter probe studies were unsuccessful. Results indicated that there was some β-galactosidase activity, indicating

that both promoters were functional, but the great differences in expression expected when the clones were cultured under high versus low Fe growth conditions were not seen. It is interesting that the *A. vinelandii* promoter was also poorly transcribed when pAS1 was introduced into *E. coli* HB101 (pMH15), where Lux activity was significantly lower than in other *E. coli* hosts. Compounding the problem may be the fact that we were looking at heterologous regulation of an *Azotobacter* promoter in *E. coli*, and as seen in the split plate assays, *luxAB* was not tightly regulated in *E. coli* transformants. Because initial results seemed to raise more questions than answers, and the fact that the value of such results using a heterologous system are likely of limited value, it was thought that efforts would be best focussed on other methods to examine promoter activity. Therefore promoter probe studies were abandoned.

To understand more about the activity of this promoter in *A. vinelandii*, transcriptional analysis was done, including Northern blot analysis, S1 nuclease mapping and primer extension. It is evident from both Northern and promoter mapping experiments that *csbC* is regulated at the level of transcription. In the Northern analysis there is no transcript detectable from UW cultures grown under high-iron conditions. This was confirmed by both the S1 nuclease mapping and primer extension results, where no product was detectable using RNA extracted from UW grown under iron sufficient conditions. This confirms the hypothesis that this gene is strongly regulated in response to iron availability and is consistent with results obtained with promoter mapping of *entC* in *E. coli* where no products of primer extension were detected in RNA from cultures grown under iron sufficient conditions (Brickman *et al.*, 1990). The observation that DHBA is constitutively produced in UW (Page and Huyer, 1984) is likely due to the difficulty in maintaining freely soluble iron in highly aerated medium, and not due to unregulated transcription (Sevinc and Page, 1992). As shown in this study, F196 is unable to produce DHBA due to the transposon insertion in *csbC*. Analysis of the *csbC* promoter activity indicates that the gene is not expressed constitutively, but only under

conditions of iron limitation. Therefore, results of this study agree with the explanation given by Sevinc and Page (1992) as to why DHBA production may appear to be constitutive, but in fact is produced only when *A. vinelandii* is iron limited.

Problems were encountered with Northern analysis. The transcript appeared to be degraded and the probe hybridized to products of varying sizes, resulting in a smear, instead of an intact transcript that was expected to appear as a single band. This made determination of the exact transcript size difficult. These problems are likely due to the fact that this transcript was very large (approximately 9.5 kbp) and sensitive to degradation. It was also possible that an intact transcript was not detectable simply due to the nature of the transcript *in vivo*; perhaps the transcript has a very high turnover rate. In the literature there is a lack of Northern analysis of siderophore biosynthetic operons in other organisms, which may be an indication that the same of problem was experienced by others and not reported or that figures were not considered publishable. We attempted to address this possibility by personal communication via email with another scientist who has worked on the *E. coli* system, but unfortunately we obtained no reply. The fact that a clean transcript was detectable when the Northern was probed for the 16S rRNA indicates that the technique used to isolate RNA did not contribute in some way to RNA degradation.

The fact that S1 nuclease mapping failed to reveal the exact transcriptional start site is likely due to the fact that S1 nuclease, although a single-strand-specific nuclease, can degrade double stranded DNA, RNA or DNA:RNA hybrids exposed to high amounts of this enzyme (Sambrook *et al.*, 1989). As well, S1 nuclease is known to cleave double stranded nucleic acid at nicks or small gaps. The *csbC* promoter region is much more A-T rich than surrounding sequences which would result in weaker base pairing compared to that of G-C pairs. Thus the DNA-RNA hybrid in the region of the transcript start site may have been more susceptible to degradation by S1 nuclease. Primer extension

experiments revealed the transcript start site without any ambiguity and confirmed that the promoter identified was functional *in vivo*.

Northern dot blot results indicated that *csbC* was regulated by the level of iron in the growth medium, such that it was repressed when Fe levels exceed approximately 7-10 μM. This explains the results described in previous studies (Page and Von Tigerstrom, 1988) which demonstrated that catecholate siderophores were detectable when Fe levels were 7 μM or less. This is because transcription of the biosynthetic genes (confirmed at least for *csbC*) is regulated by the level of iron in the growth medium. This gives us some insight as to how differential regulation of the catecholate siderophores and azotobactin might occur. If repression of azotobactin biosynthetic genes also occurs at the level of transcription by a Fur-like protein, it is possible that Fur has a different affinity for the promoters regulating catecholate siderophore biosynthetic genes than for the promoters regulating azotobactin biosynthetic genes.

Repression of *csbC* by addition of Fe to a 16 hr iron limited culture, as seen with the dot blot experiment, was as expected if this gene is regulated by a Fur-like repressor. From what is known about the mechanism of Fur repression, when Fe is in sufficient supply it acts as Fur's co-repressor and binds to the iron-box, thus preventing transcription. Therefore the observation that *csbC* expression is repressed once the cell is supplied with high amounts of iron is indicative of repression by Fur since the cell no longer requires siderophores to supply iron.

The observation that catecholate siderophores are involved in protection against O_2-stress and that catecholate production increases under conditions of increasing aeration (Cornish and Page, 1988) led us to test whether expression of *csbC* would be upregulated as the demand for catecholate siderophores increases. It was thought that due to the identification of putative sox-boxes in the promoter region, that *csbC* might also be under control of a SoxS-like transcriptional activator. Work by Yannone and Burgess (1997; 1998) suggests that *A. vinelandii* might possess a SoxRS-like system.

Initial studies involved using strain F196 and Lux as a reporter of *csbC* activity in plate assays. Here it was seen that *luxAB* was derepressed at 20 μM Fe citrate by addition of methyl viologen.

This result was confirmed (Ottem, 1998) with studies of F196 in liquid medium containing 5 μM Fe citrate which demonstrated increased Lux activity (as measured in a luminometer) with increased amounts of added methyl viologen (0 to 30 μM). However, in liquid culture (Ottem, 1998) or in plate assays, when iron is abundant the varying amounts of methyl viologen did not cause the induction of *luxAB*. This indicates that when Fe levels are high, and presumably Fur is bound to the iron-box, the repressor cannot be displaced by the SoxS activator.

Therefore, further testing of *csbC* upregulatation by O_2-stress would have to be done with iron limited cultures, as was done in the studies by Cornish and Page, (1998). However, since *csbC* is derepressed under such conditions, the observations would have to be made comparing some *csbC* expression to a higher level of expression. Methyl viologen was added as a generator of superoxide and dot blot analysis was done to observe *csbC* expression due to iron limitation compared to expression under iron limitation but with added oxidative stress. Initial results were inconsistent and it was determined that perhaps the volumes of growth medium being used were sufficiently small (7.5 mL/50 mL flask) that the aeration rate was always quite high. Thus *csbC* was being maximally expressed and adding methyl viologen would not affect further *csbC* expression. Therefore, a study was done to compare the expression of *csbC* with varying aeration, with different levels of iron. This revealed that under low aeration, *csbC* was repressed at lower iron levels, whereas *csbC* was still expressed at these higher iron levels when under increased O_2-stress brought on by higher aeration. As well there does seem to be upregulation of the *csbC* transcript under O_2-stress.

Another explanation of sequential control is that azotobactin is turned off by Fe (greater than 3μM). At this otherwise repressive, but relatively low level of Fe,

catecholate transcription may continue to be activated by O_2-stress, via a SoxS-like system. Since catecholates have a role in binding Fe to prevent the Fenton reaction, their continued production in the presence of low levels of soluble Fe has merit. Also, as shown by Cornish and Page (1998), Fe limited cells have very low SOD activity. It is only when Fe levels are much higher (greater than 7-10 μM) that SOD activity is restored (Galibois and Page, 1998), O_2-stress is decreased, and Fur represses *csbC* and associated genes.

The best way to differentiate between the level of *csbC* expression due to iron limitation, versus upregulation due to oxidative stress would be to compare expression of *csbC* with or without SoxS. This would involve generating a SoxS deficient mutant of *A. vinelandii*. *E. coli* SoxRS mutants are available, but a consequence of studying *csbC* expression this way would be the problems of repressor dilution and promoter recognition, already discussed, as well as the added complication of looking at a heterologous SoxS in regulating *csbC*. These factors would likely only complicate interpretation of results, and it would be best to study expression of this gene in a homologous system. A more sensitive assay for RNA transcripts would be to use Reverse Transcriptase PCR which may better indicate differences in *csbC* expression using RNA extracted from cultures grown under conditions of varying aeration and iron. Alternatively, RNA dot blot blots could be repeated, and by exposing the blot to a phosphor screen the amount of radioactivity could be quantified.

More work is needed to determine if the other genes in the *csbC* operon encode proteins required for biosynthesis of catecholate siderophores. This would reveal whether or not *Azotobacter* has a similar organization of siderophore biosynthetic genes as is seen to be the case in other organisms. Also, it would be interesting to identify additional genes in the biosynthetic pathway in order to elucidate the mechanism by which catecholate siderophores are regulated and synthesized in *A. vinelandii*. It would also be interesting to see if Fur and SoxS regulatory elements are present in promoter

regions of other catecholate biosynthesis genes. In order to address the question of differential regulation of azotobactin versus the catecholate siderophores, work needs to continue in the search for and characterization of siderophore biosynthetic genes in *A. vinelandii*.

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APPENDIX 1

Single stranded nucleotide sequence (5' to 3') of *orfA* which follows directly upstream of the sequence reported in Figure 3.5.

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1    GTCGCGGGGCTGGCCCGCCGCCGGGCGAGTCCGGCCGCCGACGTCCAAAG 50
51   CGGCGTCGGCAAGCTCGTGGGTCTCCTGGTCGGGGCGTGGCTGCTAGCGG 100
101  TCCCACCGCTCGTCAGGCCATACCCAGTCGCCGGGCAGCGGCGGTGGGTC 150
151  GTGGTCGCGGCGCCGCGGCCGCCGGTCCCGGTGCGACTTTAGGACCCAGT 200
201  AGAAGACGTCCCGGTACTTTGGCCGCGCGAGGCCGCCGCGGCCTTGTACC 250
251  GACTACCGCCGGCGGTCCGTCTGGCACGCCCGCCGCAACGGCCGGTACTT 300
301  GTCGTGCTTCAAGTAATAGTCGCCGTTCCGCAAGTCCGGCTTCTTGCCGT 350
351  CGTCCGGCTGCCGCTTTGTCCGCGACCGGTGCGGCTGGCCGTCGGTCACC 400
401  GGCACAGCTAGCGGGTCCGCAGGCCGGTCAACGTACCGGGGGTCGCGGTG 450
451  GTCTGGCGGGTGGTCCATGTCGCATGGGTCAACGACTGCGCCCATTAGCT 500
501  CCAAGTCGTCCATCTTGCGCCTCTTCGAGCCCTAGTGGTCCTTCCGCCTC 550
551  TTGACCAACTCGGGGACGTTGTCGCACCGGTCCGCGTGCGGCCCTGCCGA 600
601  GTCGTACCGGTCGCTGCCGAACGGTCGGCCGGGAACGTCCACCCGCCAGT 650
651  CGCCGCCGTTGTCTACCCGGTGCCGCTTCCGCTGGTCGTGGTTGGTCGTG 700
701  TCGGTGGTCGTGCTTCCCGCGGACGGTCGGGCTTCACCAGGCGGTCAAAG 750
751  GTCGCGGTCCCTTAGGGCCGCTACCACCGCTACCTTTTGGGCCAGTACTG 800
801  GAACCGCTACCGACAGGGCGCGACACCGCCGCCGCAGACCAGCTAGTCCT 850
851  AGCGGTACGGCCACCGCTAGCGGCCCCGCCGTGTCTCGGGCCGCTGCCGG 900
901  GCAGGCTCGTCGTCCGAGCCGTTTAGTACGCGGTCCCATGTCCGGTCGCT 950
951  TCGCTCGCGGTCAGGGTGGTCCGAGGTCTCGTCCCGGAACGCCGCTAGCT 1000
1001 TCGCTAGGTCCGCGCCGTGCCACTTGTCTGGCCTGTCGTTCCTGCGTTTA 1050
1051 CATATCGACCGACAATCGGTACGTTGAACTAATGGCACTTTCAACTCTGG 1100
1101 AAACCGGTTTAGGCCCGGCTCGCCGTCCTGGTCGTACTTTAGCTCTTACA 1150
1151 TCCACTTGACGTCCCTCCGCTAGTCCCGCTACTCG
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