Characterization of genetically–programmed responses of *Escherichia coli* to heavy metal stress

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Abstract

Living organisms must monitor and respond to a diverse array of environmental stimuli. These responses, many of which are genetically–programmed, allow the organisms to obtain nutrients as well as cope with toxins. To identify such responses in *Escherichia coli*, a chromosomal *lux*AB gene fusion library was constructed. By screening this library with metals and monitoring for increases in luminescence, two metal-inducible clones were identified. Luminescence of clone LF20110 increases with increasing concentrations of added aluminum, iron, indium, gallium and vanadium, but not added calcium, magnesium or nickel. Luminescence of clone TBT_1 increases with increasing concentrations of added tributyltin, triethyltin, tri–n–propyltin and carbonyl cyanide m–chlorophenyl hydrazone, but not added dibutyltin or tetrabutyltin.

Analysis of clone TBT_1 revealed a *lux*AB fusion to the *uhp*T gene, which encodes a hexose–6–phosphate transport protein. Transcriptional regulation of *uhp*T by glucose–6–phosphate has been well characterized. However, it had not been previously demonstrated that addition of tributyltin could induce transcription of *uhp*T. This effect on *uhp*T is associated with the uncoupling of oxidative phosphorylation.

Isolation and sequencing of the *lux*AB gene fusion from clone LF20110 uncovered a previously uncharacterized gene, which we call *ais* (aluminum and iron stimulated). The *ais* gene has been sequenced, the transcriptional start site has been identified, and the 22 kDa Ais protein has been overexpressed *in vivo*. Mutation of the *bas*S gene abrogates metal–induced transcription of *ais*. However, expression of the BasR–BasS two–component regulatory system, *in trans*, restores the metal–inducible response.

The *E. coli* BasR–BasS regulon is poorly characterized. To identify genes regulated by this two–component system, a Mud*I* (*lac* Ap) gene fusion library was constructed and screened. Gene fusions to *blc*, *glm*US, *iap*, and *ygj*IJK exhibited increased β–galactosidase activity upon overexpression of BasR–BasS, while expression of a *dsd*XA fusion decreased. By searching the *E. coli* genome database for putative BasR binding sequences, four additional genes, *set*C, *yib*D, *yhh*T and *yaf* were identified. Of these nine genes, four have not yet been characterized. The other five are associated with cell membrane functions or sugar metabolism.

Résumé

Les prokaryotes, comme tous les organismes vivants, doivent surveiller et répondre a une diversité de stimuli environnementaux. Leurs réponses, dont la plupart sont génétiquement programmées, permettent aux organismes d'obtenir des nutriments ainsi que de gérer la présence de toxines.

Pour identifier de telles adaptations chez l'organisme *Escherichia coli*, une librairie de fusions géniques *lux*AB chromosomales fut construite. Suivant l'exposition de cette librairie à des metaux specifiques et la mesure d'augmentations significatives de la luminescence chez ces bacteries, deux clones repondant aux metaux ont été identifiés. La luminescence du clone LF20110 augmentait en présence de concentrations croissantes d'aluminum, de fer, d'indium, de gallium et devanadium, mais pas suivant l'exposition au calcium, au magnésium ou au nickel. La luminescence du clone TBT1, augmentait suivant l'exposition à des concentrations croissantes de tributyltin, de triethyltin, de tripropyltin et de cyanure de carbonyl, mais pas après l'exposition au dibutyltin ni au tetrabutyltin.

L'étude du clone TBT_1 révèle une fusion *lux*AB au gène *uhp*T, qui code pour une protéine de transport pour l'hexose–6–phosphate. La régulation de la transcription du gène *uhp*T par le glucose–6–phosphate est bien characterisée. Cependant, ceci est la première démonstration que l'ajout de tributyltin pouvait induire la transcription de *uhp*T. Cet effet du tributyltin est associé au «uncoupling» de la phosphorylation oxadative.

Après l'isolation de la fusion génique *lux*AB du clone LF20110, la séquence fut déterminée, et les résultats suggéraient un gène inconnu que nous avons nommé *ais* (aluminum et iron stimulated). La séquence complète d'*ais* est maintenant connue et

l'origine de transcription a été déterminée. De plus, la protéine Ais de 22 kDa a été surexprimée *in vivo*. La mutation du gène *bas*S abbrège la transcription d'*ais* en réponse au metal, mais l'expression «en trans» du système bi–complexe BasR–BasS rétablit la réponse au métal. Très peu est connu quant au régulon BasR–BasS d'*E. coli*. Afin d'identifier des gènes sous le contrôle de ce système bi–complexe, une librairie de fusion génique Mu*dI* (*lac* Ap) fut construite et analysée. Des fusions géniques aux niveaux de *blc*, *glm*US, *iap*, and *ygf*IJK exhibaient une augmentation d'activité β–galactoside lors de la surexpression de BasR–BasS, tandis que l'expression d'une fusion au niveau de *dsd*XA diminuait. En analysant une base de données d'*E. coli* à la recherche d'éventuels sites de liaison pour BasR, nous avons identifié quatre gènes de plus: *set*C, *yib*D, *yhh*T et *yaf*. D'un total de neuf gènes, quatre ont été identifiés, et les autres sont impliqués dans des fonctions de membrane cellulaire ou dans le métabolisme des sucres.

'Art is I. Science is we.' *Claude Bernard*, French physiologist (1813–1878)

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Some people prefer to live alone. Not I. After five apartments, a frat house, and a year of couch surfing, I can no longer name all of my roommates. However, James Thomson has been an indelible part of my Montréal experience. Jamie, thanks for everything! Mike, Bobby, Sarah, Heide, Vanessa and the boys of ΦΣK kept my life interesting and deserve special mention. Thank you for sharing your space, your food (or at least leftovers), and your bills with me. Sorry about the mess.

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Preface to the Thesis

I have chosen to present this Thesis in manuscript–based format.

The 'Guidelines for Thesis Preparation', established by the Faculty of Graduate Studies and Research, include the following provisions for manuscript–based theses:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis.

I have observed these provisions during the preparation of this Thesis.

The Thesis follows a logical progression. Chapter 1 is a comprehensive review of relevant literature. Chapters 2 through 5, inclusive, are manuscripts describing original research and will be submitted for publication. My peers contributed to this research. Individual contributions are described in the 'Manuscripts and Authorship' section at the beginning of the Thesis. To ensure continuity of the Thesis, Chapters 3, 4, and 5 are prefaced with connecting text. Chapter 6 contains conclusions and a final summary. All text has been formatted properly. Abbreviations used throughout the text are listed at the beginning of the Thesis. References, in alphabetical order, are included at the end of the Thesis.

Contributions to Original Knowledge

1. I discovered that luminescence of the *Escherichia coli ais*::*lux*AB transcriptional fusion strain LF20110 is specifically induced by gallium, indium and vanadium.
2. I determined that metal–induced luminescence of LF20110 is inhibited by added calcium and magnesium.
3. I demonstrated that, in the absence of added metal, changes in pH do not induce luminescence of LF20110.
4. I demonstrated that, upon P1 transduction with *ais*::*lux*AB, recipient strains obtain the metal–inducible luminescence phenotype of LF20110.
5. I have overexpressed the Ais protein, both with and without a six–histidine tag.
6. By generating *bas*S$^-$ and *bas*R$^-$ *bas*S$^-$ mutant strains of *Escherichia coli* and complementing the mutations *in trans*, I demonstrated that the *bas*RS operon is essential for metal–inducible expression of the *ais* gene.
7. I implicated BasS as an iron and aluminum sensing protein.
8. I generated and screened a Mu*dI* (*lac* Ap) transcriptional fusion library and, using β–galactosidase assays, identified five genes regulated by BasR–BasS.
9. Using a computer–assisted strategy, I identified four additional genes which may be regulated by BasR–BasS.
10. I confirmed that triorganotin compounds, but not dibutyltin or tetrabutyltin, induce luminescence of the *Escherichia coli uhp*T::*lux*AB transcriptional fusion strain, TBT_1.
11. I determined that TBT_1 and its parental strain, DH1, are equally sensitive to organotin compounds.
12. I demonstrated that uncouplers of oxidative phorphorylation, such as carbonyl cyanide m–chlorophenyl hydrazone, induce luminescence of TBT_1.
13. Using RNA blotting analysis, I demonstrated that tributyltin induces transcription of the *uhp*T gene in a concentration–dependent manner.

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List of Abbreviations

aa	amino acid
ADP	adenosine 5'–diphosphate
Ap	ampicillin
ATP	adenosine 5'–triphosphate
bp	base pair
CCCP	carbonyl cyanide m–chlorophenyl hydrazone
Cm	chloramphenicol
DBT	dibutyltin
DCCD	N, N–dicyclohexylcarbodiimide
EDTA	ethylenediamine–N,N,N',N'–tetraacetate
EPR	electron paramagnetic resonance
G6P	glucose–6–phosphate
Gm	gentamicin
IRP–1	iron regulatory protein 1
Km	kanamycin
LB	Luria–Bertani
LPS	lipopolysaccharide
NMR	nuclear magnetic resonance
nt	nucleotide
$O_2^{\bullet -}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
ORF	open reading frame
PCP	pentachlorophenol
PCR	polymerase chain reaction
PFU	plaque forming unit
RLU	relative light unit
^{R}	resistance
^{S}	sensitivity
TBT	tributyltin
TEtT	triethyltin
TPT	tri–n–propyltin
Tc	tetracycline
Xgal	5–bromo–4–chloro–3–indolyl–β–D–galactopyranoside

Where the telescope ends, the microscope begins.
Which of the two has the grander view?

Victor Hugo,
Les Misérables

CHAPTER 1:

Introduction

Gene Regulation by Heavy Metals

1.1 HEAVY METAL HOMEOSTASIS

There are ninety naturally occurring elements. Fifty–three are heavy metals. Most are either too rare or too insoluble within the hydrophilic conditions of the biosphere to be readily bioavailable. Yet, because of their abundance, utility, and/or toxicity, several heavy metals are biologically significant. For most prokaryotes, iron, zinc, copper, cobalt and manganese are essential trace elements. Molybdenum, nickel, and tungsten can also be important. Other metals, such as mercury, silver, lead, chromium, cadmium and arsenic are notable for their toxicity.

Metals are important protein cofactors. Metal–containing enzymes are ubiquitous and affect every aspect of cell physiology. For example, iron– (or manganese–) containing ribonucleotide reductases are essential for DNA synthesis (Auling and Follmann, 1994). Zinc and copper oxidases are required for carbon and nitrogen metabolism. In addition, 'blue' copper proteins are important for electron transport reactions, and zinc–binding proteases are commonly produced by pathogenic bacteria (Brown et al., 1994; Hase and Finkelstein, 1993). Cobalt–containing cobalamin (i.e. vitamin B_{12}) is a cofactor for many enzymes, including some involved in glycerol utilization and amino acid metabolism (Ochiai, 1994). Proteins with iron–sulfur clusters or molybdate centers are important for electron transport and oxidation–reduction reactions, especially under anaerobic conditions (Beinert and Kiley, 1999; Hille et al., 1999). This Chapter provides a review of the role of metals, and metalloproteins, in transcriptional regulation. However, the numerous metabolic functions of metalloproteins must not be forgotten.

The impact of a heavy metal is affected by both concentration and reactivity. Concentration determines the number of metal ions that a cell must contend with. For example, if the metal concentration in the bacterial culture medium (e.g. 10^8 cells/ml) is 1 pM, and assuming that the metal ions do not interact with components of the culture medium, then there is an average of six ions/cell, too few to be either useful or dangerous. However, if 1 nM is present, each cell must cope with 6000 metal ions. At this concentration, mercury (Hg^{2+}) can damage a cell, but manganese cannot. Iron and zinc are commonly found at concentrations above 100 nM (e.g. 600 000 or more ions/cell). The consistent and abundant supply of these reactive, but not overly toxic, metals may account for their widespread participation in cellular processes.

Reactivity refers to the capacity of a metal to generate toxic oxygen species, the ability to mimic other metals, and the affinity of a metal for amino acid side chains, such as the imidazole ring of histidine residues or the thiol group of cysteine residues. The formation of reversible metal–thiol bonds is particularly important for proper protein function. Mercury has such a high affinity for thiols that it can displace other metal cofactors, form irreversible thiol bonds, and disrupt protein activity. Silver, chromium, and cadmium are toxic for the same reason. Moreover, the slightly higher toxicity of copper, as compared to zinc, may be due to differences in thiol affinity. Free metal ions can react with molecular oxygen and the thiol groups of glutathione to form reactive oxygen species, such as H_2O_2. Free metals can also catalyze the formation of superoxide radicals from other reactive oxygen species (Touati, 1999). For example, the Fenton Reaction describes the metal (M) induced generation of a hydroxyl radical ($OH^{\bullet}$):

$$M^{3+} + H_2O_2 \rightarrow M^{2+} OH^- + OH^{\bullet}$$

Reactive oxygen species damage DNA, proteins, and membrane lipids, and are a key reason why it is important for the intracellular concentration of free metal ions to remain low. There are additional mechanisms of metal toxicity. Arsenic is chemically similar to phosphorous, and the pentavalent arsenate oxyanion species exerts its deleterious effects by disrupting phosphate metabolism.

Unlike toxic organic compounds, which are subject to anabolism and catabolism, metal ions cannot be destroyed. However, metals can be sequestered by histidine– and cysteine–rich proteins, converted to less reactive species, or exported from the cell. With the notable exception of Hg^{2+}, which can be reduced to the volatile and readily dissipated Hg^{0}, few metals can be effectively detoxified by conversion to less toxic species. As such, sequestration and efflux are the main mechanisms of metal detoxification. A single metal–binding protein can sequester only a few metal ions and it can be expensive for the cell to produce enough protein to sequester even a moderate concentration of metal. A single efflux pump can export many metal ions and is often more efficient. However, if the same ion is repeatedly exported and taken up, efflux becomes less productive. Sequestered ions do not return.

Uptake and efflux mechanisms are classified according to their energy requirements and structure. Chemiosmotic transporters depend on the relative internal and external concentrations of the substrate. Chemiosmotic uptake proteins are active when the external concentration is higher. Chemiosmotic efflux proteins are active when the internal concentration is higher. In proton/cation antiport systems, metal ion efflux is coupled to proton uptake and requires an intact proton gradient. P–type, A–type and ABC transporters are ATP–dependent. In P–type systems, transport and ATPase

activities are controlled by a single protein. In A–type systems, different proteins mediate efflux and ATPase activities. ABC transporters use two integral membrane subunits and two ATPase subunits. These subunits may be discrete proteins, or separate domains of a single protein. ABC–mediated uptake often involves a periplasmic factor while ABC–mediated efflux employs an outer membrane factor.

Heavy metal homeostasis systems are only effective if expressed appropriately. Uptake mechanisms need to be activated when metal concentrations are limiting. If the metal concentration is too high, detoxification mechanisms need to be turned on, and uptake must be repressed. This requires regulation, which generally occurs at the level of transcription. Prokaryotes use many elegant strategies to regulate gene expression in response to heavy metals.

1.2 METAL–DEPENDENT REPRESSOR PROTEINS

A repressor protein regulates gene expression by inhibiting transcription. The repressor protein recognizes a specific, usually palindromic, DNA sequence located within the promoter of a target gene. When the repressor is bound to this sequence, it occludes the –35 and/or –10 promoter elements, preventing transcription initiation by RNA polymerase. The repressor proteins described below may assume either an active conformation, in which affinity for DNA is high, or an inactive conformation, in which DNA binding is abrogated. The shift from an active to an inactive conformation is mediated by the incorporation (or loss) of a metal ion.

1.2.1 Fur

Fur, the ferric uptake regulator, was identified two decades ago in a mutant strain of *E. coli* that demonstrated constitutive siderophore expression (Hantke, 1981). Fur is ubiquitous. Homologs have been described for Gram–negative organisms, including *Acinetobacter*, *Bordetella*, *Campylobacter*, *Erwinia*, *Haemophilus*, *Helicobacter*, *Legionella*, *Neisseria*, *Pseudomonas*, *Salmonella*, *Rhizobium*, *Vibrio*, and *Yersinia*, Gram–positive bacteria, such as *Bacillus* and *Staphylococcus*, as well as *Mycobacteria* and cyanobacteria. Current genome sequencing projects are certain to reveal additional homologs (Bereswill et al., 1998; Berish et al., 1993; Brickman and Armstrong, 1995; Bsat et al., 1998; Carson et al., 1996; Daniel et al., 1999; Ernst et al., 1978; Franza et al., 1999; Ghassemian and Straus, 1996; Heidrich et al., 1996; Hickey and Cianciotto, 1994; Litwin et al., 1996; Prince et al., 1991; Staggs and Perry, 1991; Wong et al., 1999).

Fur directly regulates a diverse array of genes. They are too numerous to describe in detail, but include loci involved with siderophore synthesis, iron transport, oxygen radical detoxification, and virulence factor production (Bearson et al., 1997; Escolar et al., 1999). Fur also controls expression of alternate sigma factors and regulatory proteins, and so indirectly mediates transcription of additional genes. Because of this extensive influence, Fur is known as a global regulatory protein.

Promoters regulated directly by Fur contain the 'Fur box' or 'iron box' consensus sequence. In solution, Fur monomers form homodimers. Dimers assume an active conformation upon incorporation of Fe^{2+}. Recognition of the Fur box and DNA binding is mediated by an N–terminal helix–turn–helix domain. When Fe^{2+} is limiting, Fur binding to DNA is impaired and trancription is derepressed. Studies using electron microscopy

and atomic force microscopy reveal that, when bound to DNA, Fur has a well–ordered, oligomeric structure and increases the rigidity of the DNA (Le Cam et al., 1994). Also, the portion of a promoter bound by protein increases with the Fur:DNA ratio, suggesting that Fur can polymerize when protein concentrations are sufficiently high (Frechon and Le Cam, 1994).

Fe^{2+} is integral to Fur function, but questions about metal incorporation remain. A conserved amino acid motif, $His_6–X_2–Cys–X–Cys$, located near the center of each monomer, is implicated in metal binding, but the role of specific residues in metal coordination has not been resolved. *In vitro*, Fur can be activated by the addition of Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+} Mn^{2+}, or Zn^{2+} but the metals may not be bound in the same way (Althaus et al., 1999; Bagg and Neilands, 1987; Michaud-Soret, et al., 1997; Jaquamet et al., 1998). According to this scheme, one metal binding site is occupied by a Zn^{2+} ion, which serves to stabilize Fur structure. In the inactive Fur conformation, the other metal binding site may contain a second Zn^{2+} ion, or it may be empty. In either case, occupation of this second site (likely, the central metal–binding motif) by a Fe^{2+} ion generates the active Fur conformation. Evidence for this model comes from analysis of the metal content of Fur protein preparations. In the absence of all metal, Fur dimers are unstable. Dimers containing one or two Zn^{2+} ions per monomer are stable. Addition of EDTA, which chelates divalent cations, does not remove all zinc from the protein, but instead leaves Fur containing one Zn^{2+} ion per monomer. Addition of excess iron generates a protein that contains one Zn^{2+} ion and one Fe^{2+} ion, not two Fe^{2+} ions, per monomer (Althaus et al., 1999). When manganese or cobalt are used instead of iron, the Fur preparations contain one Zn^{2+} ion, plus either a Mn^{2+} ion or a Co^{2+} ion (Michaud-Soret et

al., 1997). This suggests that there is a Zn^{2+} binding site that is inaccessible to chelators or other metals. The location of this binding site has not been determined, but X-ray absorption spectroscopy suggests that the Zn^{2+} ion is coordinated tetrahedrally (Jacquamet et al., 1998). Either the central metal-binding motif, or a conserved C-terminal motif, His–X_7–Cys–X_2–Cys, may be involved. The C-terminal motif is intriguing because this region has also been implicated in dimerization (Stojiljkovic and Hantke, 1995). As such, the Zn^{2+} ion may be important for dimer formation.

The interaction of Fur with the Fur box has been closely examined. The consensus Fur box is a 19 nt sequence, $^{5'}$GATAATGATAATCATTATC$^{3'}$, but several longer variants exist, and no single nucleotide appears to be essential (DeLorenzo et al., 1987). This sequence has long been viewed as a palindrome formed by an inverted repeat of the nonamer, $^{5'}$GATAATGAT$^{3'}$. However, the current model suggests that Fur recognizes a hexamer $^{5'}$NATAAT$^{3'}$, that is repeated three times (2 direct, 1 inverted) within the Fur box (Escolar et al., 1998a; 1998b). This model is consistent with DNA footprint analysis, which shows a pattern, repeated three times across the consensus Fur box sequence, of two protected nucleotides followed by four unprotected ones (DeLorenzo et al., 1988). To test this theory, binding of Fur to synthetic promoters containing from one to five repeats of the hexamer was examined. *In vitro*, Fur binding was observed to DNA sequences containing two or more tandem repeats of the hexamer (Escolar et al., 1998a). However, binding to two repeats was quite poor, suggesting that Fur requires a minimum of three repeats to form a stable complex. Fur can bind hexamers repeated in any orientation, but has a higher affinity for the native pattern, direct–direct–inverted, than for three direct repeats or the direct–inverted–direct combination. Changes to the

hexamer, in particular the $^{3'}$T, impair Fur binding. Analysis also revealed that there was one repeat of the footprinting motif (two protected, four unprotected bases) for each hexamer (Escolar et al., 1998a). This indicates that the affinity of Fur for a specific promoter depends on the number and orientation of hexameric repeats. As such, iron concentration may affect different Fur–regulated promoters in different ways. For example, a slight decrease in iron concentrations may trigger derepression of low affinity Fur promoters while more severe iron limitation may be required before high affinity Fur promoters are expressed.

The concentration of Fur has been estimated to be 2500 molecules/cell in *Vibrio cholera* (Watnick et al., 1997) and 5000 molecules/cell in *E. coli* (Zheng et al. 1999). This is many fold higher than the estimate of 20 molecules/cell for the LacI repressor or 300 molecules/cell for the Trp repressor. This may simply reflect the large number of Fur–repressed genes. However, it has been suggested that, in addition to regulating gene expression, Fur could help sequester intracellular iron. Fur defects are accompanied by the accumulation of high levels of free intracellular iron. The combination of free iron and deficient DNA repair is likely responsible for the lethal phenotype of *fur recA* double mutants (Touati et al., 1995).

Several Fur–like proteins have recently emerged. Zur, Irr, and PerR exhibit homology to Fur, bind to similar DNA sequences, and regulate gene expression in response to metal concentration. Zur, the zinc uptake regulator, represses expression of zinc transport operons under zinc–sufficient conditions (Gaballa and Helmann, 1998; Dalet et al., 1999; Patzer and Hantke, 1998; Patzer and Hantke, 2000). Irr, the iron response regulator, represses genes involved in heme biosynthesis (Hamza et al., 1998).

Irr responds to iron, but it acts as a repressor in the absence of Fe^{2+}, unlike Fur, which is active only when Fe^{2+} is present. PerR, the peroxide regulator, mediates expression of genes required for detoxification of reactive oxygen species (Chen et al., 1995; Bsat et al., 1996; Bsat et al., 1998). These genes are expressed under high concentrations of both Fe^{2+} (or Mn^{2+}) and H_2O_2 (or a compound generated by peroxide exposure). However, interaction of the inducers with PerR is not understood. Genome sequencing projects have revealed numerous putative 'Fur homologs'. It is likely that some of these are really Zur–, Irr–, and PerR–homologs.

1.2.2 DtxR/IdeR:

In many Gram–positive organisms, proteins of the DtxR/IdeR family regulate iron uptake and the expression of virulence determinants. The diptheria toxin repressor (DtxR) of *Corynebacterium diptheriae* was the first to be identified (Boyd et al., 1990). However, many homologs have been identified, including DtxR of *Brevibacterium lactofermentum*, the iron–dependent regulatory (IdeR) proteins of *Mycobacterium* spp. and *Rhodococcus* spp., the desferrioxamine synthesis regulator (DesR) of *Streptomyces pilosis* and *Streptomyces lividans*, the *Streptococcus* spp. iron regulator repressor (SirR), and the transport–related operon regulator (TroR) of *Treponema pallidum* (Boland and Meijer, 2000; Doukhan et al., 1995; Dussurget et al., 1996; Gunter-Seeboth and Schupp, 1995; Hill et al., 1998; Oguiza et al., 1995; Posey et al., 1999; Schmitt et al., 1995).

Several crystal structures of DtxR have been solved. They include the DtxR apoenzyme (no bound metal), holoenzyme (metal bound), and DtxR bound to DNA (Pohl et al., 1999b; Qiu et al., 1996). Moreover, a structures for the *M. tuberculosis* IdeR

holoenzyme have been obtained (Feese et al., 2000; Pohl et al., 1999a). These studies reveal structural and functional features of these proteins and illuminate the role of the metal cofactor.

DtxR/IdeR monomers have three domains and each domain is separated by a flexible linker region of two to eight amino acids. In DtxR, domain 1 (residues 1–73) contains a helix–turn–helix motif which is important for DNA recognition and binding. Domain 2 (residues 74–140) is important for dimerization and contributes to the two distinct metal–binding sites. Domain 3 (residues 148–226) is mobile, which may account for its poor resolution in most crystal structures. This domain contributes to the two metal–binding sites and makes additional contacts with domain 1 (Pohl et al., 1999b). Domain 3 is also called the SH3 domain, because of its homology to the SH3 domains of α–spectrin and the tyrosine kinases Fyn and Abl (Qiu et al., 1996). The significance of this homology is unknown. However, the SH3 domains of Fyn and Abl interact with the Src protein. As such, it has been suggested that domain 3 of DtxR may interact with some other (as of yet unknown) proteins.

DtxR/IdeR dimers form in the absence of metal, but metal incorporation is required to generate the active, DNA binding, conformation. Although Fe^{2+} is the physiological relevant ion *in vivo*, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} can be substituted *in vitro*. However, these metals are not bound with equal affinity. Iron activates DtxR at concentrations above 1 μM while zinc is only effective at concentrations above 100 μM. Each monomer binds two metal ions, such that four are bound per dimer. In the holoenzyme, the metal ions exhibit pentavalent coordination. Each metal–binding site is formed by three residues from domain 2 and two residues from domain 3. The residues

that form the metal–binding sites are highly conserved in all homologs. Moreover, mutagenesis of these key residues impairs enzyme function (Tao and Murphy, 1993). In the transition from apoenzyme to holoenzyme, the monomers do not move with respect to one another. In fact, the dimerization domain (domain 2) remains almost completely immobile. Instead, structural changes occur within each monomer. The greatest change occurs within domain 3, which moves to contact domain 2 and complete the metal–binding sites. It is believed that the shifting domain 3 acts as a wedge which also forces domain 1 to move. This rotation of domain 1 relative to domain 2 allows space for DNA binding. Upon binding to DNA, there is additional rotation of domain 1, but it is slight compared to the shift upon metal–binding.

DtxR recognizes the palindromic nucleotide consensus sequence: $^{5'}$TAAGGTTAGNCTAACCTTA$^{3'}$ (Chen et al., 2000; Tao and Murphy, 1993). Two dimers bind to this sequence. Each dimer covers 14 nucleotides such that they overlap across the five central base pairs (Chen et al., 2000). Protein binding is cooperative, but contacts between the two dimers have not been identified. Protein binding distorts the DNA. Although it maintains B–DNA form, it becomes slightly bent and slightly unwound (Chen et al., 2000).

In *Corynebacterium diptheriae*, seven DtxR–regulated loci have been identified. In all cases, binding of the DtxR holoenzyme represses transcription. These loci encode the following iron–regulated protein genes: *irp*1, a membrane lipoprotein and putative siderophore receptor (Schmitt and Holmes, 1994; Schmitt et al., 1997); *irp*2; *irp*3, a putative transcriptional regulator, *irp*4, *irp*5 (Lee et al., 1997); *hmu*O, a heme oxygenase

involved in metal acquisition (Schmitt et al. 1997); as well as the corynephage *tox* gene, which encodes the diptheria toxin (reviewed in Holmes, 2000).

Targets for the IdeR protein of *Mycobacterium tuberculosis* have also been identified (Manabe et al., 1999). A computer-assisted search of the *M. tuberculosis* genome revealed six putative IdeR binding sites, appropriately positioned upstream of ORFs. *In vitro* gel mobility shift assays demonstrated that purified DtxR could bind to five of these sites. The putative IdeR-regulated genes are putative virulence factors, including an alcohol dehydrogenase, a serine protease, a putative transcriptional activator, a 16S rRNA and a homolog to the clinically important 19-kDa antigen.

In *Staphylococcus* spp., SirR regulates expression of the *sit*ABC genes, which encode an ABC-type transporter responsible for iron uptake (Hill et al., 1998). Similarly, TroR of *Treponema pallidum* controls expression of the *tro*ABCD metal transport system (Posey et al., 1999). However, TroR is unusual in that it appears to respond to Mn^{2+} but not Fe^{2+}. *In vitro*, DNA binding by TroR is mediated by Mn^{2+} but not Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, or Fe^{2+} (Posey et al., 1999).

1.2.3 ModE

Molybdate and molybdopterin are common cofactors in nitrogenase enzymes and are essential to nitrogen-fixing bacteria. In *Azotobacter vinelandii*, *Clostridium pasteurianum*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Rhodobacter capsulatus*, high-affinity molybdenum uptake is regulated by ModE (Grunden and Shanmugam, 1997; Hinton and Freyer, 1986; Mouncey et al., 1995; Walkenhorst et al., 1995; Wang et al., 1993). Like Fur and DtxR/IdeR, ModE is a

homodimeric repressor protein that assumes an active, DNA binding, conformation upon metal incorporation. Molybdenum, tungsten, and to a lesser extent, vanadium, can activate ModE. The crystal structure of the 262 amino acid *E. coli* ModE has been solved (Hall et al., 1999). Each monomer has two domains. The N–terminal domain contains a helix–turn–helix motif that mediates DNA binding. The C–terminal domain has two subunits, which are required for metal binding and dimerization, respectively. Each monomer binds one metal oxyanion (i.e. MoO_4^{2-}, WO_4^{2-} or VO_3^-). Tetrahedral coordination of the oxyanion involves four residues that are strictly conserved in all MoeE homologues. One residue (Arg^{128} in *E. coli* ModE) is part of a conserved motif, (Thr/Ser)–Arg–Asn–Gln–X_2–Gly. Mutation of this motif has been shown to eliminate repressor activity. Metal incorporation induces changes in the C–terminal domain which enhance DNA binding by the N–terminal domain (Hall et al., 1999; McNicholas et al., 1998).

The *mod*E gene is divergent to the *mod*ABCD operon, such that the two transcriptional units are oriented, and expressed, in opposite directions. ModABCD encodes an ABC–type uptake system for molybdate, tungstate, vanadate and sulfate oxyanions. Transcription of *mod*E is constitutive (Grunden and Shanmugam, 1997). Expression of *mod*ABCD is regulated by ModE (Grunden et al., 1996; Walkenhorst et al., 1995). Transcription is repressed when molybdenum concentrations are high and derepressed when they are low. Details of ModE binding to the *mod*ABCD promoter remain to be elucidated, but DNaseI protection analysis indicates that the protein protects a 30 bp segment (bp –15 to +15, relative to the transcription start site). ModE may recognize the consensus sequences $^{5'}TA^{C}/_{T}AT^{3'}$ or $^{5'}GTTA^{3'}$, which both occur multiple

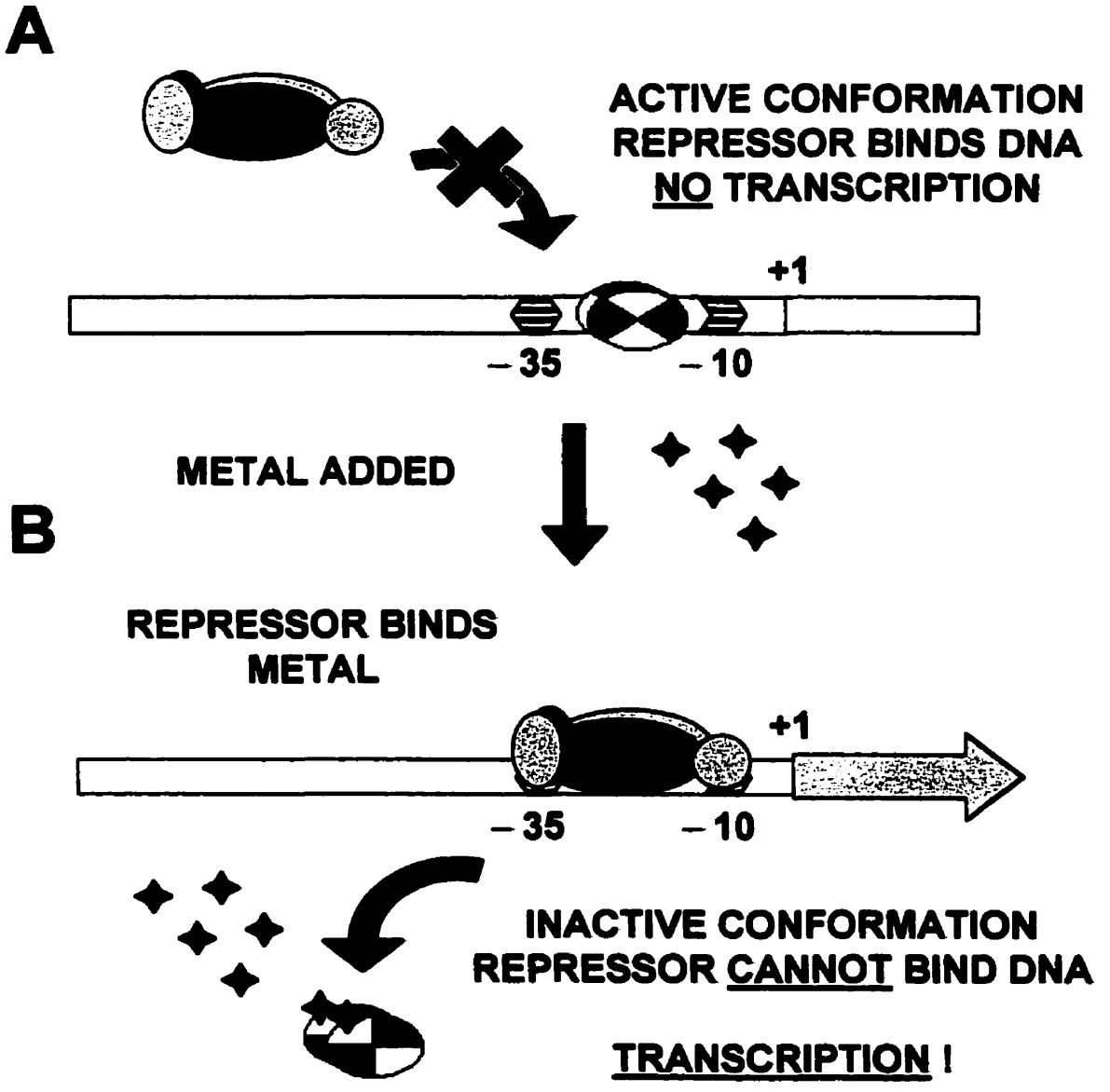
times in the *mod*ABCD promoter region. The $^{5'}TA^{C}/_{T}AT^{3'}$ motif is repeated three times within the DNaseI protected region and is associated with a putative DNA stem–loop structure. ModE binding could stabilize this stem–loop and occlude the −10 promoter element (Grunden and Shanmugam, 1997; Grunden et al., 1999). In *E. coli*, ModE also regulates the *dms*ABC (McNicholas et al., 1998), *moa*ABCDE (McNicholas et al., 1997), and *nar*GHJI (Self et al., 1999) operons, which encode molybdate–containing enzymes.

1.2.4 ArsR/SmtB/CadC/ZiaR

ArsR, SmtB, CadC and ZiaR are representative members of a growing family of metal–responsive repressor proteins that use a simple, yet effective, method to regulate expression of metal resistance genes. In the absence of metal, the apoprotein exhibits DNA binding activity and represses transcription. In the presence of cognate metal, a metal–protein complex forms. This holoenzyme no longer binds DNA. As such, transcription is derepressed, and the metal resistance genes are expressed (Fig. 1.1).

ArsR responds to arsenite (As^{3+}) and antimonite (Sb^{3+}). A variety of plasmid–borne, and chromosomal *ars* operons have been identified (Cai and DuBow, 1996; Cervantes et al., 1994; Diorio et al., 1995). All contain *ars*B, encoding an arsenite/antimonite efflux pump, and *ars*C, encoding an $As^{5+} \rightarrow As^{3+}$ reductase. In addition, some *ars* operons contain *ars*A, whose product converts ArsB from a cation–proton antiporter into an efflux ATPase, and *ars*D, which encodes a second regulatory protein, responsible for establishing the upper limit of *ars* operon expression (Cervantes et al., 1994; Silver and Phung, 1996).

Figure 1.1: Transcriptional regulation by a metal–responsive repressor protein A) In the absence of metal, DNA binding of the active repressor homodimer to the promoter region of a target gene occludes transcription initiation by RNA polymerase B) Incorporation of metal into the repressor homodimer impairs DNA binding such that RNA polymerase is able to initiate transcription.


A
ACTIVE CONFORMATION
REPRESSOR BINDS DNA
NO TRANSCRIPTION
+1
-35
-10
METAL ADDED
B
REPRESSOR BINDS
METAL
+1
-35
-10
INACTIVE CONFORMATION
REPRESSOR CANNOT BIND DNA
TRANSCRIPTION !

SmtB, from *Synechococcus*, responds to zinc (Zn^{2+}), cadmium (Cd^{2+}) and, to a lesser extent, cobalt (Co^{3+}), mercury (Hg^{2+}), and nickel (Ni^{2+}). SmtB regulates expression of *smt*A, which encodes a metal–sequestering metallothionein (Daniels et al., 1998).

CadC homologs are widely distributed. CadC responds to cadmium (Cd^{2+}), zinc (Zn^{2+}), lead (Pb^{2+}) and bismuth (Bi^{2+}) (Endo and Silver, 1995). It regulates expression of the cadmium/zinc efflux P–type ATPase encoded by *cad*A.

ZiaR has only been found in *Synechocystis* (Thelwell et al., 1998). ZiaR responds to zinc (Zn^{2+}) and cadmium (Cd^{2+}) and mediates expression of the *zia*A–encoded zinc efflux P–type ATPase.

ArsR (117 aa), SmtB (122 aa), CadC (122 aa), and ZiaR (132 aa) are of similar size, but amino acid sequence homology is limited to the central regions. This may reflect differences in metal specificity. However, work with ArsR and SmtB indicates that the proteins do have similar structures. A crystal structure of apo–SmtB has been solved (Cook et al., 1998). It shows that the central region contains a helix–turn–helix structure followed by a hairpin–shaped antiparallel β–sheet. The helix–turn–helix mediates DNA recognition and binding. The β–sheet structure is flexible and may affect the stability of DNA binding. This central region is flanked by three α–helices (i.e. two towards the N–terminus and one near the C–terminus). SmtB acts as a dimer and inter–monomer contacts between these helices are important for dimerization. The stoichiometry of metal binding by SmtB has not been resolved and it is not clear if there are one or two metal ions bound per monomer (VanZile et al., 2000). As such, the metal binding site(s) of SmtB has not been clearly defined. However, it has been suggested that metal may bind to the C–terminal dimerization helix. This could disrupt or distort dimer sturcture enough

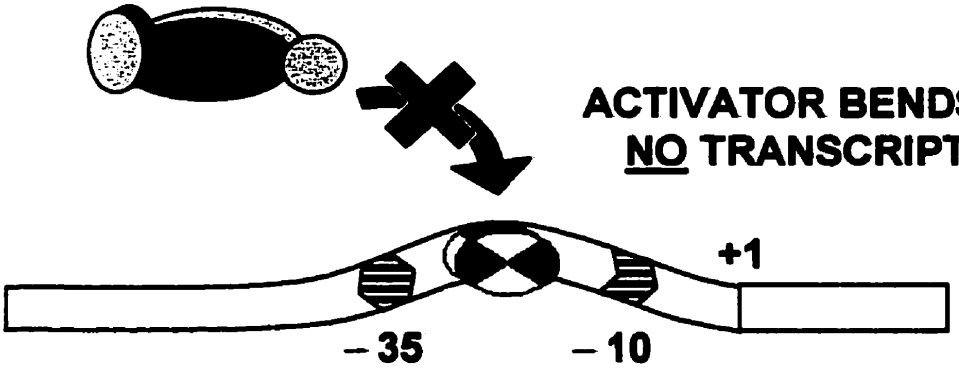
to abrogate DNA binding. Moreover, mutation of SmtB–His^{106}, which is predicted to coordinate the metal ion, results in a constitutively active, metal–insensitive repressor. The implicated residues are not conserved in ArsR or CadC. However, mutation of the equivalent ZiaR–His^{116} residue also generates a metal–insensitive repressor (Thelwell et al., 1998). Alternatively, metal may bind to the helix–turn–helix motif. With ArsR, this appears to be the case (Shi et al., 1996). Arsenite is coordinated by three cysteine residues found within the first helix of the motif. The first two cysteine residues are essential for normal, metal–sensitive repressor activity. These essential residues are conserved in CadC and ZiaR, but only one is found in SmtB. Mutation of these cysteines in ZiaR, but not SmtB, generates a metal–insensitive repressor (Thelwell et al., 1998)..

1.3 METAL–RESPONSIVE ACTIVATOR PROTEINS: MerR/CoaR/CueR/ZntR

MerR, CoaR, CueR and ZntR are similar to the proteins described thus far. They form dimers, recognize characteristic DNA sequences, bind to the promoters of target genes, and sustain conformational changes upon the incorporation of metal ions. However, MerR, CoaR, CueR and ZntR are activator proteins and use a different mechanism to regulate gene expression. Promoters targeted by these activators exhibit unusual spacing of the –10 and –35 promoter elements. In a typical σ^{70} promoter, the spacing is 17 ± 1 bp. The spacing is 19 bp in promoters regulated by MerR or CueR, and is 20 bp in promoters regulated by CoaR or ZntR. The altered spacing disrupts the phasing of the –10 and –35 promoter elements such that they are recognized poorly by

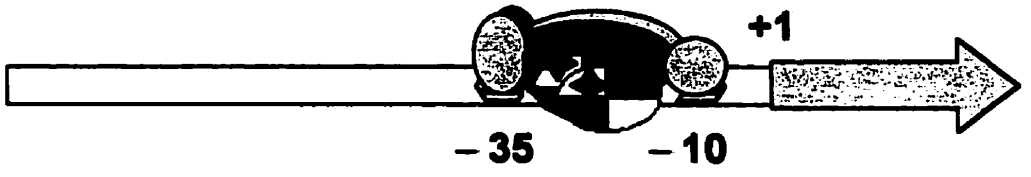
Figure 1.2: Transcriptional regulation by a metal–responsive activator protein A) In the absence of metal, the activator homodimer binds and bends DNA in the promoter region of a target gene, preventing transcription initiation by RNA polymeraese B) Upon metal incorporation, the activator homodimer unbends and unwinds the promoter DNA and activates transcription initiation by RNA polymerase.

A



METAL ADDED

B



ACTIVATOR UNBENDS
and UNWINDS DNA

TRANSCRIPTION !

helix–turn–helix domain near the N–terminus of the protein. Mutation or deletion of this domain abrogates DNA binding and transcriptional activation (Shewchuk et al., 1989; Zeng et al., 1998). Mercury binding is associated with three cysteine residues located in the C–terminal half of MerR. A single Hg^{2+} moiety is coordinated by the two most C–terminal cysteines of one monomer, and a third, more central, cysteine of the other monomer. Substitution of these cysteines (e.g. by alanine) impairs dimerization, but the dimers that do form can bind DNA (Helmann et al., 1990; Shewchuck et al., 1989). Heterodimers, which include the central cysteine on one monomer and the C–terminal cysteines on the other, can bind Hg^{2+} and activate transcription. However, homodimers containing only the central cysteine or only the two C–terminal cysteines are inactive (Helmann et al., 1990). In another Mer construct, all of the residues preceding the conserved, central cysteine were deleted. Despite the deletion, the Mer protein could dimerize and bind Hg^{2+} with wild type affinity (Zeng et al., 1998). The importance of the central cysteine for the asymmetric trigonal coordination of Hg^{2+} has also been confirmed via NMR (Utschig et al., 1995).

Crosslinking studies (Kulkarni and Summers, 1999) indicate that MerR forms protein–protein interactions with RNA polymerase. Interactions occur with both the RNA polymerase core and holoenzymes, and are independent of DNA binding or metal status. A highly conserved and charged region near the middle of MerR may be important for contacts with RNA polymerase (Caslake et al., 1997; Comess et al., 1994). This interaction may help recruit RNA polymerase to the promoter region. In the absence of metal, interactions between RNA polymerase and apo–MerR stabilize the formation of a closed complex such that *mer* operon transcription decreases (Caslake et al., 1997). Basal

The regulatory scheme described for MerR, CoaR, CueR and ZntR is not only used for the activation of metal resistance genes. Homologous activator proteins regulate systems that mediate drug resistance (MtaR), thiostrepton efflux ($TipA_L$), and cell differentiation (BmrR) (Ahmed et al., 1994; Baranoval et al., 1999; Holmes et al., 1993).

1.4 The CopY–CopZ Repressor–Antirepressor System

The *cop*YZAB operon of *Enterococcus hirae* mediates both copper uptake and copper efflux (Odermatt and Solioz, 1995). Although copper is an essential metal, too much can be toxic. The CopY and CopZ proteins provide the proper balance. Together, they mediate a biphasic pattern of gene expression. The *cop*YZAB genes are transcribed when the copper concentration is limiting (<2 μM) or in excess (>0.3 mM), but are repressed at intermediate concentrations. Moreover, this system mediates transport of toxic silver (Ag^+) which has a similar ionic radius to copper (Cu^+).

The CopA and CopB proteins are P–type ATPases that transport Cu^+. However, they work in opposite directions, such that CopA brings extracellular copper into the cell, and CopB expels intracellular copper from the cell (Odermatt et al., 1992; 1994). Although the predominant extracellular form of copper is Cu^{2+}, the current model suggests that, prior to uptake by CopA, a periplasmic reductase converts Cu^{2+} to Cu^+. The putative reductase has not been isolated, but copper reductase activity has been measured *in vivo*. Moreover, expression of the *cop* operon is induced by chelators of Cu^{2+}, which can deplete extracellular Cu^{2+} pools, but not chelators of Cu^+, which cannot enter the

periplasm (Wunderli-Ye and Solioz, 1999). Although CopA and CopB are expressed together, uptake activity dominates when copper is limiting and efflux activity is sufficient to provide resistance when copper is in excess. It is likely that when concentrations are low, free Cu^+ is not available for CopB–mediated efflux. At high concentrations, the rate of free Cu^+ efflux is likely faster than the combined rates of Cu^{2+} reduction and CopA–mediated Cu^+ uptake.

CopY is a metal–dependent transcriptional repressor, 145 amino acids in size. The N–terminus contains a helix–turn–helix domain that mediates DNA binding (Odermatt and Solioz, 1995). The C–terminal features a Cys–X_2–Cys–X_4–Cys–X_2–Cys motif associated with metal binding. A single CopY monomer can bind one Zn^{2+} ion or two Cu^+ ions (Cobine et al., 1999). Zn–CopY, but not Cu–CopY, can recognize and bind a 28 bp DNA sequence that contains an inverted repeat. The *cop* promoter region contains two discrete copies of this sequence, one on each side of the transcriptional start site (Strausak and Solioz, 1997). Zn–CopY binding to either sequence is sufficient to block transcription and repress expression of the *cop*YZAB genes (Wunderli-Ye and Solioz, 1999). Although *in vitro* binding studies indicate that both sites of the wild type promoter become occupied, binding is not cooperative. Displacement of Zn^{2+} ions by two Cu^+ ions abrogates DNA binding. *In vitro*, addition of Ag^+ or Cd^{2+}, but not Ni^{2+}, can also displace purified CopY from the wild type promoter (Strausak and Solioz 1997). Similarily, silver and cadmium, but not zinc or nickel, can induce *in vivo* expression of the *cop* operon (Odermatt et al., 1994; Solioz and Odermatt, 1995).

CopZ, a small, 69 amino acid, protein regulates the metal status of CopY. The N–terminus of CopZ contains a metal–binding motif, Gly–Met–X–Cys–X_2–Cys, which

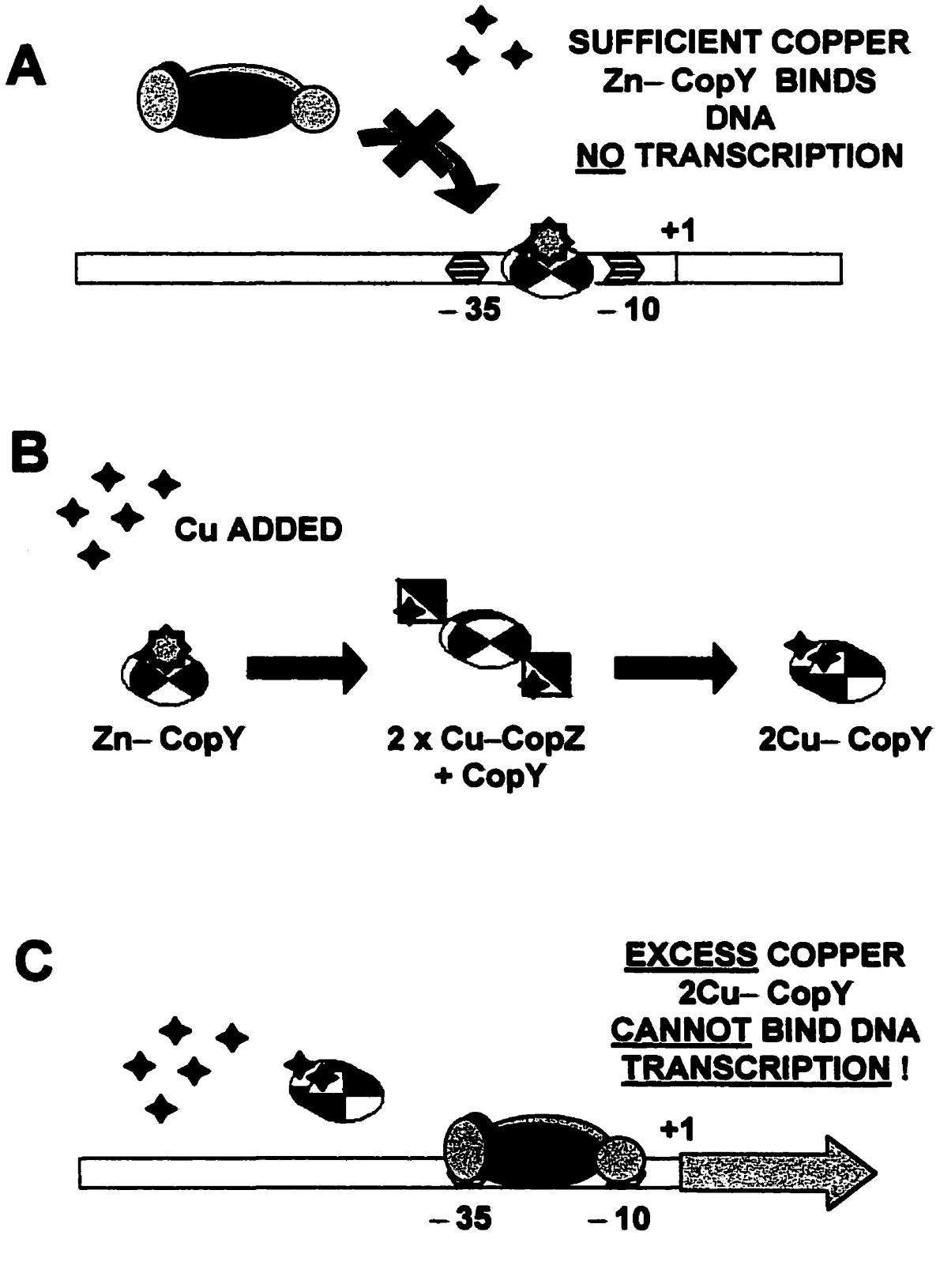
can bind a single Cu^+ ion (Wimmer et al., 1999). When copper concentrations are high, two Cu–CopZ complexes donate their two Cu^+ ions to one CopY moiety, displacing one Zn^{2+} ion (Cobine et al., 1999). Although the stoichiometry of this reaction has been determined, most other details have not. However, it is likely that a positively charged surface of CopZ is important for protein–protein interactions (Wimmer et al., 1999).

These recent findings account for *cop*YZAB repression at intermediate copper concentrations and expression (i.e. derepression) when copper is in excess (Fig. 1.3). Unfortunately, it is unclear why the *cop* operon is transcribed when copper is limiting. One possibility is that, in the absence of copper, apo–CopZ interacts with CopY and impedes DNA binding. This would permit CopA expression and Cu^+ uptake. When copper is no longer limiting, the CopZ–CopY complex may be disrupted by the formation of Cu–CopZ. However, this has not been addressed in the literature.

Although similar procaryotic systems have not been described, CopY and CopZ homologs are found in genome databases. Homologous eucaryotic systems have been reported, including a human copper transport system associated with Menkes' and Wilson's diseases (Rolfs and Hediger, 1999).

Figure 1.3: Transcriptional regulation by the CopY–CopZ repressor pair

A) When copper is sufficient, zinc–containing CopY (Zn–CopY) binds to its target promoter and occludes transcription initiation by RNA polymerase. B) Upon addition of copper, two Cu–CopZ proteins transfer two Cu^{+} ions to CopY, displacing one Zn^{2+} ion. C) The copper–containing CopY homodimer (2Cu–CopY) is unable to bind to its target promoter such that RNA polymerase is able to initiate transcription.

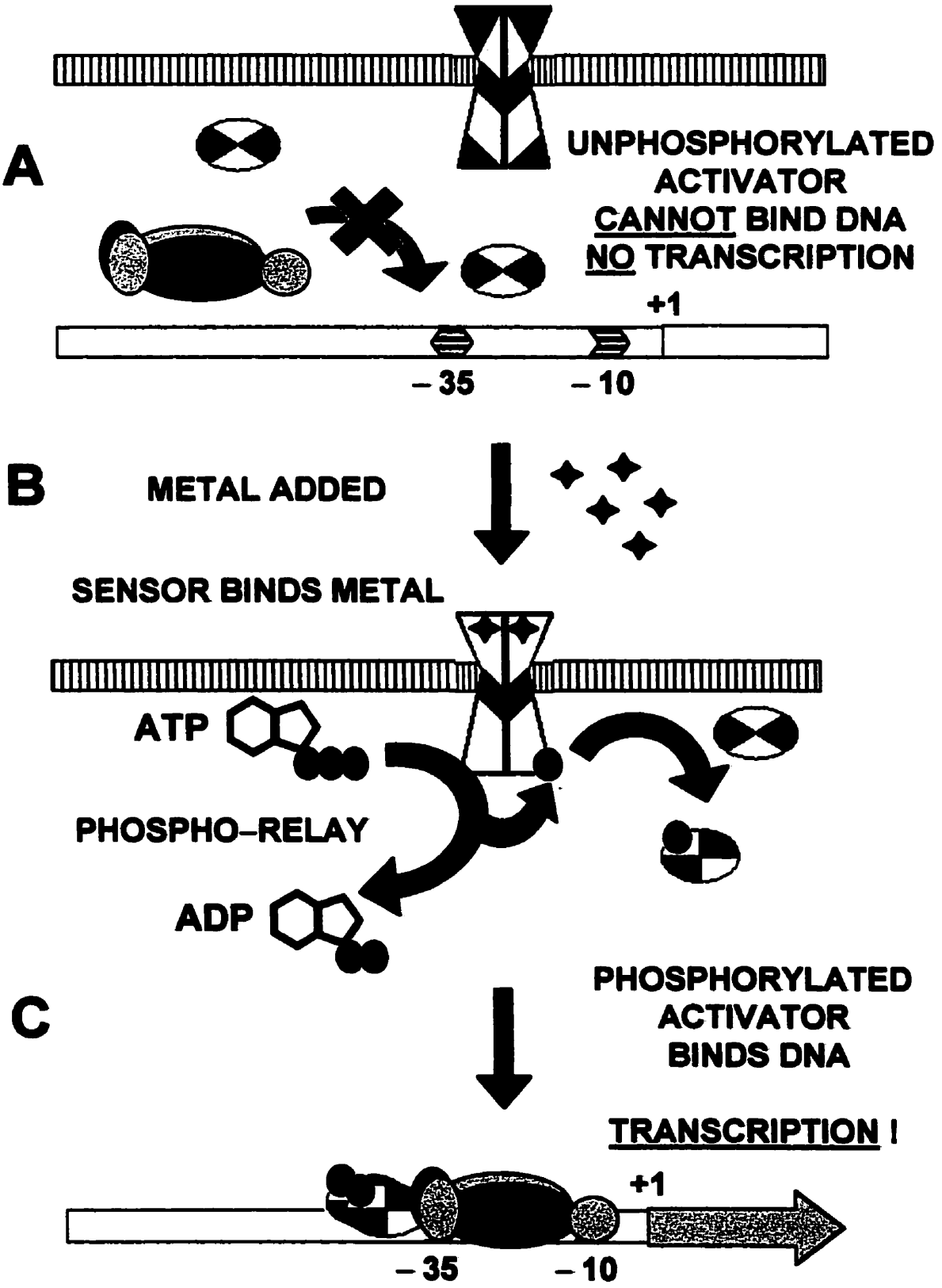

A
SUFFICIENT COPPER
Zn– CopY BINDS
DNA
NO TRANSCRIPTION
+1
–35
–10
B
Cu ADDED
Zn– CopY
2 x Cu–CopZ
+ CopY
2Cu– CopY
C
EXCESS COPPER
2Cu– CopY
CANNOT BIND DNA
TRANSCRIPTION !
+1
–35
–10

1.5 Two–Component Regulatory Systems

Twoy–component regulatory systems are common in procaryotes, but few have been implicated in metal transport. The two–component systems described below are homologous to the EnvZ–OmpR osmosensor system (Dutta et al., 1999; Martinez-Hackert and Stock, 1997). Each includes a transmembrane sensor kinase and a cytoplasmic transcriptional regulator. The periplasmic domain of the sensor kinase is the receptor for a specific ligand (e.g. metal ion). The cytoplasmic domain contains conserved histidine residues and exhibits kinase activity. The transcriptional activator contains a conserved aspartate residue and a DNA binding helix–turn–helix domain. Metal binding to the receptor domain of the sensor induces a conformational change that activates kinase activity and initiates an ATP–dependent phospho–relay cascade. First, a phosphate is transferred from ATP to the conserved histidine residue via an autophosphorylation reaction. Then, the phosphate group is transferred from the histidine residue of the sensor to the conserved aspartate residue on the regulator (Appleby et al., 1996). Addition of the phosphate induces a conformational change in the activator that increases its affinity for DNA. The activator may form homodimers in solution, or DNA binding may assist dimerization. In either case, interactions between the DNA–bound activator proteins and the α–subunit of RNA polymerase enhance transcription from target promoters (Fig. 1.4).

Figure 1.4: Transcriptional regulation by a two–component regulatory system .

A) In the absence of metal, the response regulator is unable to bind DNA or activate transcription of the target gene. B) Addition of metal, and metal binding to the sensor domain of the sensor kinase, initiates a phosphorelay cascade. A phosphate group is removed from ATP, transferred to a conserved histidine residue on the sensor kinase, and finally transferred to a conserved aspartate residue on the response regulator. C) The phosphorylated response regulator binds to the target promoter and mediates transcription of the target gene.


A
UNPHOSPHORYLATED
ACTIVATOR
CANNOT BIND DNA
NO TRANSCRIPTION
+1
–35
–10
B
METAL ADDED
SENSOR BINDS METAL
ATP
PHOSPHO–RELAY
ADP
C
PHOSPHORYLATED
ACTIVATOR
BINDS DNA
TRANSCRIPTION !
+1
–35
–10

1.5.1: Cop/Cut/Pco

A two–component system for copper resistance, involving a copper–sensing membrane protein and a cytoplasmic transcriptional activator, is conserved in *Pseudomonas*, *Xanthomonas*, and *E. coli* spp (Cooksey, 1988; Mills et al., 1993).

In *Pseudomonas*, the system has six components: the sensor, CopS; the regulator, CopR; an inner membrane protein and putative copper efflux pump, CopD; an outer membrane protein, CopB; and two periplasmic proteins, CopA and CopC. The periplasmic proteins can bind and sequester copper. CopA is thought to bind 11 Cu^{2+} ions, while CopC binds a single Cu^{2+} moiety. The genes comprise a single chromosomal operon, *cop*ABCDRS. The chromosomal copper resistance operon, *cop*ABCD, from *Xanthomonas* is similar, but *cop*RS genes have yet to be identified. However, *Pseudomonas* CopR can bind to the *Xanthomonas cop*ABCD promoter (Mills et al., 1994).

The chromosomal *E. coli* copper resistance operon, *cut*ABCDEF, contains two genes not found in the *Pseudomonas* system. CutF is a putative lipoprotein while CutE may be the acetyltransferase that attaches the fatty acid moiety to CutF. The production of this novel lipoprotein may alter the permeability of the cell membrane to copper. The CutE product also contains a putative Cu^{2+} binding motif, but its importance is unknown (Fong et al., 1995; Gupta et al., 1995).

The *pco*ABCDRSE copper resistance operon, from the *E. coli* plasmid pRJ1004, is similar to the *cut* and *cop* systems (Brown et al., 1995). Analysis indicates that *pco*ABCDRS is transcribed as a single unit while *pco*E is transcribed from a separate promoter. Both promoters contain 'copper' boxes, which are DNA sequences containing inverted repeats and recognized by PcoR and CutR. However, *pco*E is more strongly

induced by copper than *pco*ABCDRS. A putative promoter, located upstream of *pco*RS, may also mediate consitutive, low level expression of the regulatory genes (Rouch and Brown, 1997). Although PcoE has not been well characterized, it may be a periplasmic protein capable of sequestering copper.

CopS, CutS, and PcoS are homologous to the EnvZ sensor kinase. Copper sensing is probably mediated by the N–terminal domain. CopR is homologous to the OmpR response regulator. DNA recognition and binding is mediated by a C–terminal helix–turn–helix domain. Transfer of ^{32}P from radiolabeled CopS to CopR has been demonstrated *in vitro* (Mills et al., 1994).

1.5.2 Czc

In *Ralstonia* sp. (formerly *Alcaligenes eutrophus*) strain CH34, the *czc* system mediates resistance to cobalt, zinc and cadmium. The *czc* operon includes at least eight genes, *czc*NICBADRS (Grosse et al., 1999). The functions of CzcN and CzcI are unknown. However, they are predicted to be membrane bound, and periplasmic, respectively. CzcABC comprise a proton–cation antiport system responsible for the energy dependent efflux of Co^{2+}, Zn^{2+} and Cd^{2+} (Nies, 1995). CzcD is membrane bound and provides consitutive, albeit low level, metal efflux (Anton et al., 1999). CzcR is a response regulator and CzcS is its cognate sensor kinase. Primer extention analysis reveals that there are four promoters, P_N, P_I, P_{CBA} and P_{DRS}, which give rise to the *czc*N, *czc*I, *czc*CBA, and *czc*DRS transcripts. RNA blotting experiments suggest that other mRNAs, such as *czc*NICBA, can be generated by transcriptional readthrough (Grosse et al., 1999).

It has been shown that the *czc* system is inducible by Zn^{2+}, Cd^{2+} and, to a lesser extent, Co^{2+}, but the regulatory cascade is not completely understood. CzcD sets the lower limit for induction. In *czc*D deficient strains, basal transcription of *czc*CBA is higher (Anton et al., 1999). Metal efflux by CzcD prevents metal sensing by CzcS. This may involve an interaction between CzcD and CzcS or, more likely, CzcD keeps the local metal concentration below the threshold required for CzcS activation. Metal sensing by CzcS is thought to enhance DNA binding by CzcR, and induce transcription.

CzcR–CzcS do upregulate transcription in response to metals, although the details of their interaction have not been demonstrated. However, the *czc* system is unusual in that CzcR–CzcS are not essential. Even in *czc*RS deleted strains, transcription of *czc*CBA is induced by Zn^{2+} (Grosse et al., 1999). Moreover, purified CzcR does not bind DNA containing P_I, P_{CBA} or P_{DRS} sequences. CzcR only recognizes P_N. The significance of this may not be known until CzcN and CzcI are better characterized.

1.6 Metal–Regulated Extracellular Function σ Factors

Extracellular function (ECF) σ factors are a growing class of proteins that regulate RNA polymerase activity in response to external stimuli. Some are alternative σ factors that interact with the RNA polymerase core enzyme to direct transcription of specific genes. Others are anti–σ factors that antagonize σ factors (such as σ^{70} and σ^{54}) and inhibit transcription of target genes. ECF factors can exist in either an active state, in which they can interact with RNA polymerase, or an inactive state. Stimuli from the cell surface (or periplasm) initiate a signal which is transduced to the cytoplasm (Hughes and Mathee, 1998). The nature of the signal has not been clearly defined, but it involves a cascade of protein–protein interactions and conformational changes which ultimately modify the ability of the ECF factor to promote transcription. As described below, several metal–mediated ECF factors exist. They regulate systems involved with iron transport and metal resistance.

1.6.1 Iron Transport: FecI, PupI

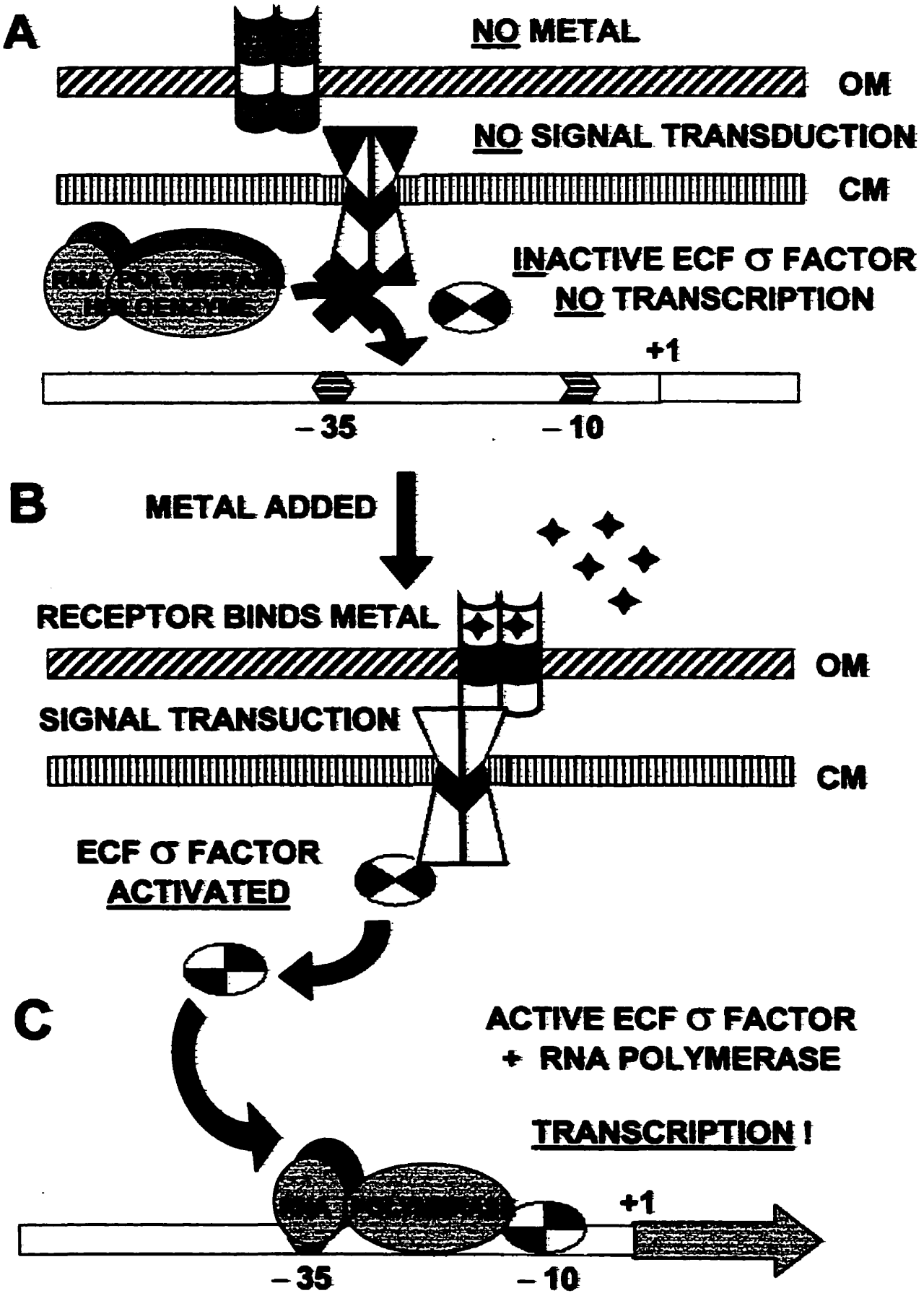
E. coli has at least seven iron transport systems (Braun, 1997). Their transcription is controlled by Fur such that expression (i.e. derepression) can only occur under iron limiting conditions. However, some systems, such as the *fec*IRABCDE operon for ferric citrate transport, are regulated by additional factors. FecA is an outer membrane protein and is the receptor for ferric citrate. FecB shuttles ferric citrate through the periplasm while FecC, FecD and FecE form a complex that transports iron across the cytoplasmic

membrane. FecI is an alternate σ factor that, in its activated form, directs transcription of *fec*ABCDE. FecR is a novel transmembrane regulatory protein required for full activation of FecI. The C–terminal domain of FecR is periplasmic and can interact with FecA. The N–terminal domain is cytoplasmic, and can interact with FecI (Enz et al., 2000; Welz and Braun, 1998).

Under iron replete conditions, transcription from the two *fec* operon promoters, P_{IR} and P_{ABCDE}, is repressed by Fur (Angerer and Braun, 1998). Iron starvation leads to the derepression of both promoters, but the RNA polymerase holoenzyme, containing σ^{70}, only initiates transcription from P_{IR}. Once expressed, the partially active FecI σ factor mediates low level transcription from P_{ABCDE}. This results in expression of FecA, and due to the secondary structure of the transcript, smaller amounts of FecB, FecC, FecD, and FecE.

However, if iron citrate is present, the *fec* operon is strongly induced (Fig. 1.5). Iron citrate binding to the FecA receptor at the outer membrane generates a signal that is transduced, via FecR, to FecI in the cytoplasm. FecI becomes fully activated and interacts with the RNA polymerase core enzyme to direct transcription of P_{ABCDE} (Braun, 1997). It should be emphasized that ferric citrate activates transcription without entering the cell. Several kinds of FecA mutants have been isolated, including those that are: i) deficient for signaling, but not iron transport; ii) deficient for transport, but not signaling, and iii) signal in the absence of ferric citrate. These mutants indicate that the signaling and iron transport functions of FecA can be separated. Several details of signal transduction and iron transport remain to be elucidated. For example, mutation of *ton*B, *exb*B or *exb*D, which encode an energy transducing mechanism required by several iron transport

Figure 1.5: Transcriptional regulation by an extracellular function (ECF) σ factor. A) In the absence of metal, the ECF σ factor in inactive and is unable to direct transcription of the target gene. B) Addition of metal, and metal binding to the outer membrane (OM) receptor, initiates a signal transduction cascade. The signal is transduced from the OM receptor to the cytoplasmic membrane (CM) component, which then activates the ECF σ factor. C) The active ECF σ factor directs transcription of the target gene.


A
NO METAL
OM
NO SIGNAL TRANSDUCTION
CM
INACTIVE ECF σ FACTOR
NO TRANSCRIPTION
+1
–35
–10
B
METAL ADDED
RECEPTOR BINDS METAL
OM
SIGNAL TRANSUCTION
CM
ECF σ FACTOR
ACTIVATED
C
ACTIVE ECF σ FACTOR
+ RNA POLYMERASE
TRANSCRIPTION !
+1
–35
–10

systems, can impair both citrate–mediated iron transport and FecA–mediated induction by ferric citrate (Harle et al., 1995; Moeck and Coulton, 1998). However, TonB is only required for signaling across the outer membrane, not signaling across the cytoplasmic membrane (Braun, 1997). The mechanism by which FecA, TonB and FecR interact is under investigation.

A similar system has been identified in *Pseudomonas putida*. PupB is the outer membrane receptor for the ferric siderophores, pseudobactin BN7 and pseudobactin BN8, and is homologous to FecA (Koster et al., 1993). Homologs to FecI and FecR, called PupI and PupR, respectively, have also been identified. Under iron limiting conditions, binding of ferric BN7 or ferric BN8 to the PupB receptor generates a signal which, once transduced to the cytoplasm, induces transcription of *pup*B. Although the result is similar, the signal is slightly different. Unlike FecI, which promotes transcription, PupI is an anti–σ factor and interferes with transcription. In a *pup*I mutant strain, transcription of *pup*B is actually enhanced, even in the absence of ferric pseudobactin. Under inducing conditions, PupR inhibits PupI activity and transcription can occur (Koster et al., 1994).

Regulation of the ferric pyochellin receptor, FptA, of *Pseudomonas aeroginosa* may involve a similar strategy, although homologs to FecR and FecI have not yet been identified. Transcription of *fpt*A is induced by ferric pyochellin binding to the FptA receptor and requires PchR, a putative transcriptional activator (Heinrichs and Poole, 1996). The mechanism by which PchR and FptA interact is unknown. However, signals transduced from FptA seem to regulate transcription. Distinct signals seem to induce repression of *fpt*A when pyochellin is absent, and activate *fpt*A transcription when ferric pyochellin is bound (Heinrichs and Poole, 1996). An ECF factor could account for this.

1.6.2 Metal Resistance: Cnr and Ncc

The *ncc* system, from *Alcaligenes xylosoxidans*, mediates resistance to nickel cobalt and cadmium (Schmidt and Schlegel, 1994). The related *cnr* system of *Ralstonia* sp. strain CH34 is responsible for cobalt and nickel resistance (Grass et al., 2000). Both systems are metal inducible and mediate resistance via cation–proton antiport. The *cnr*XYHCBA operon is better characterized. The regulatory genes, *cnr*XYH, and the antiport genes, *cnr*CBA, are transcribed independently from the P_{XYH} and P_{CBA} promoters, respectively. CnrH is an ECF σ factor. CnrX and CnrY mediate CnrH activity in response to metals. CnrX monitors periplasmic copper levels while the transmembane CnrY protein transduces signals from the periplasm to the cytoplasm. These roles remain to be confirmed, but translational PhoA fusions indicate that at least the C–terminals of CnrX and CnrY are located in the periplasm. Deletion of *cnr*XYH abrogates metal–inducible transcription of *cnr*CBA. When added in *trans*, *cyn*H increases the basal level of *cnr*CBA transcription, but does not restore metal–inducibility. The basal level of transcription is higher if *cyn*YH is used instead. This suggests that the signal from CnrX and CnrY activates CnrH (Grass et al., 2000). The *ncc* system encodes homologs to all of the *cnr* genes, plus an extra factor, NccN (Koster et al., 1994). The function of NccN is unknown, but it is homologous to CzcN of the *Ralstonia* cobalt, zinc and cadmium resistance system. Although similar, *ncc*XYH and *cnr*XYH cannot complement one another(Grass et al., 2000).

1.7 Regulatory Proteins with Iron–Sulfur Clusters: SoxR and FNR

Iron–sulfur clusters are a heterogeneous group of protein cofactors that perform a variety of structural and functional roles. Their utility stems from their sensitivity to oxidation and reduction. Changes in the redox environment alter the charge, and sometimes the structure, of the clusters. Iron–sulfur clusters are found in proteins associated with anaerobic metabolism including numerous nitrogenase, fumarase and dehydrogenase enzymes. Changes in cofactor structure can precipitate changes in protein conformation and function. Although it is not a prokaryotic protein, the vertebrate iron regulatory protein 1 (IRP–1) is an intriguing example of this. The [4Fe–4S]–containing form of IRP–1 is an aconitase that participates in the tricarboxylic acid cycle. However, oxidation and destruction of the [4Fe–4S] cluster converts IRP–1 into an RNA binding protein that recognizes stem–loop structures called iron regulatory elements (IREs). IRP–1 binding to an IRE stabilizes mRNA structure and enhances translation. Several proteins that transport and sequester iron are post–transcriptionally regulated by this mechanism (Beinert and Kiley, 1996).

In vivo, iron–sulfur clusters are usually coordinated by cysteine residues. *In vitro*, they can also be coordinated by synthetic, cysteine–rich peptides. A single 'maquette' peptide, containing a Cyx–X_2–Cys–X_2–Cys motif, can coordinate a single [4Fe–4S] cluster. However, it requires two peptides containing the smaller Cys–X_2–Cys motif to coordinate the same cluster (Mulholland et al. 1999). In solution, peptide–cluster complexes can form spontaneously. In a cell, a variety of factors, including dithiols and several gene products, are important for generation and maintenance of iron–sulfur

cofactors. Both glutathione and thioredoxin influence the redox state of the cell and facilitate cluster stability. Functions that require iron–sulfur cluster containing proteins are impaired in *E. coli* double mutants that are deficient in both thioredoxin–A and glutathione reductase (Ding et al., 1996). The highly conserved IscS/NifS and IscU/NifU proteins are involved in cluster formation. In *E. coli*, mutation of *isc*S slows cell growth and inhibits functions mediated by [4Fe–4S]–containing enzymes including aconitase, glutamate synthase, fumarase, NADH dehydrogenase, succinate dehydrogenase and FNR (Schwartz et al., 2000). IscS catalyzes the reduction of free cysteine to alanine and sulfide. The sulfide is added to a conserved cysteine residue to form a reactive cysteine persulfide residue. The crystal structure of NifS from *Thermatoga maratima* was recently solved. The conserved cysteine residue is on a flexible, solvent exposed loop, which would allow the sulfide to be readily donated to other proteins involved in cluster biosynthesis, such as IscU/NifU (Kaiser et al., 2000). IscU homodimers provide a scaffold for the assembly of iron–sulfur clusters. Each monomer provides the scaffold for one $[2Fe–2S]^{2+}$ cluster, which can then be combined within the IscU dimer to form one $[4Fe–4S]^{2+}$ cluster (Agar et al., 2000). Once complete, the clusters are transferred to cofactor–deficient apoproteins (Nishio and Nakai, 2000). Another enzyme, SoxR reductase, may mediate the NADPH–dependent reduction of $[2Fe–2S]^{2+}$–SoxR (Kobayashi and Tagawa, 1999). Current efforts are focussed on unravelling the intricacies of iron–sulfur cluster assembly and incorporation.

1.7.1 [2Fe–2S] Centers: SoxR

Genes of the *Escherichia coli* SoxR–SoxS regulon provide defense against damage from superoxide ($O_2^{\cdot-}$). Homologs from *Pseudomonas aeroginosa* are required for virulence, probably because they provide protection against $O_2^{\cdot-}$ generated by their host's defense response (Ha and Jin, 1999). SoxR and SoxS form a regulatory cascade. Both are transcriptional activators. SoxR senses $O_2^{\cdot-}$, but not H_2O_2 or $OH^{\cdot}$, and regulates transcription of SoxS. SoxS, in turn, controls expression of the rest of the genes in the regulon. SoxS is an AraC/MarA–type transcriptional regulator. The small (107 aa) protein contains two helix–turn–helix domains connected by a flexible linker. Each domain binds DNA. SoxS acts as a monomer (Rhee et al., 1998). SoxR is quite different. The functional form is a homodimer (Hidalgo et al., 1995). Each monomer contains one N–terminal helix–turn–helix DNA binding domain. Unlike SoxS, which is fully functional once expressed, SoxR can exist in either an active or an inactive form. Activation is mediated by two [2Fe–2S] clusters. Each SoxR monomer contains one cluster, coordinated by four C–terminal cysteine residues (Gaudu and Weiss, 1996). Upon exposure to oxidative stress, the clusters shift from a reduced state, $[2Fe–2S]^{+}$, to an oxidized state, $[2Fe–2S]^{2+}$. Oxidized, but not reduced, SoxR promotes transcription of SoxS. It has been recently demonstrated that nitrosylation of the [2Fe–2S] clusters (e.g. by NO) also activates SoxR (Ding and Demple, 2000). Both oxidation and nitrosylation are reversible. For example, electron paramagnetic resonance (EPR) spectroscopy can differentiate the paramagnetic $[Fe^{3+}–Fe^{2+}]$ iron in reduced SoxR from the diamagnetic $[Fe^{3+}–Fe^{3+}]$ iron of oxidized SoxR. *In vitro*, experiments using EPR spectroscopy indicate

that purified, oxidized SoxR can be reduced, and then re–oxidized. For example, purified SoxR can mediate *in vitro* transcription of *sox*S under oxidizing conditions, but transcription stops once a reducing environment is established. However, *in vitro* transcription recommences if the oxidizing environment is restored (Ding et al., 1996). This indicates that changes in oxidation state do not destroy SoxR. Similarly, EPR spectroscopy has been used to monitor SoxR in intact cells (Gaudu et al., 1997). Addition of an oxidizing agent (e.g. phenozine methosulfate) decreases the EPR signal of reduced SoxR. However, washing the cells or addition a reducing agent (e.g. sodium dithionite), restores the EPR signal. Control experiments indicate that recovery of the EPR signal does not require *de novo* protein synthesis or represent a change in the concentration of [2Fe–2S] clusters. As such, oxidation/reduction of SoxR is also reversible *in vivo*.

The *sox*R and *sox*S genes are divergently transcribed. SoxR recognizes a sequence between the –10 and –35 elements of the *sox*S promoter. The *sox*S promoter is a poor target for RNA polymerase because the –10 and –35 promoter elements are 19 bp apart, instead of the preferred 17 bp, and are out of phase. Oxidized SoxR, like Hg^{2+}–activated MerR, remodels its target promoter to make it a better substrate for RNA polymerase. DNA binding is independent of the [2Fe–2S] clusters. *In vitro* gel retardation assays demonstrate that oxidized SoxR, reduced SoxR, and apo–SoxR, in which the [2Fe–2S] clusters are absent, bind the *sox*S promoter with similar affinity (Hidalgo and Demple, 1994). However, only oxidized SoxR can stimulate the formation of an open complex by RNA polymerase (Ding et al., 1996). It has also been demonstrated that the amount of *sox*S mRNA is proportional to the amount of oxidized SoxR (Ding and Demple, 1997).

1.7.2 [4Fe–4S] Centers: FNR

The *E. coli fnr* locus was described 25 years ago as a mutation responsible for deficits in fumarate and nitrate reduction (Lambden and Guest, 1976). FNR is now recognized as a global regulator. It mediates expression of approximately 120 *E. coli* genes involved in survival and energy generation under anaerobic conditions. FNR homologs from *Salmonella* spp. (OxrA), *Pseudomonas* spp., *Rhodopseudomonas palustris* (AadR), and *Shewanella putrefaciens* (EtrA) perform similar roles (Cuypers and Zumft, 1993; Dispensa et al., 1992; Lissenden et al., 2000; Saffarini and Nealson, 1993; Strauch et al., 1985). Other homologs, from *Azorhizobium caulinodans*, *Azotobacter vinelandii* (CydR), *Bradyrhizobium japonicum* (FixK), *Paracoccus denitrificans* (NNR) and *Rhizobium* spp. (FixK) mediate nitrogen fixation (Anthamatten et al., 1992; Patschkowski et al., 1996; Saunders et al., 1999; Wu et al., 2000). However, some FNR–like proteins exhibit structural, but not functional homology. A homolog from *Vibrio fischeri* mediates bioluminescence, while regulators from *Actinobacillus pleuropneumoniae* (HlyX), *Bordetella pertussis*, and *Pasteurella haemolytica* (FnrP) are associated with toxin production (Bannan, et al. 1993; MacInnes et al., 1990; Spiro, 1994; Uhlich et al., 2000). The Flp regulators from *Lactobacillus casei* and *Lactococcus lactis* are poorly characterized, but have been implicated in zinc transport (Gostick et al., 1999; Irvine and Guest, 1993). *Pseudomonas stuzeri* is interesting in that it contains at least four FNR homologs (Vollack et al., 1999). One, FnrA, regulates a variety of genes required for anaerobic metabolism. The others, DnrD, DnrE, and DnrS, are functionally similar to FixK and independently regulate several operons involved in denitrification. Structurally, DnrD, DnrE and DnrS are unusual in that they lack several conserved N–terminal

cysteine residues. FnrA is required for DnrS activity, which suggests that these proteins form a regulatory cascade. Hierarchical arrangements of FNR homologs have been described in both *Pseudomonas* and *Rhodopseudomonas* (Arai et al., 1995; Egland and Harwood, 2000).

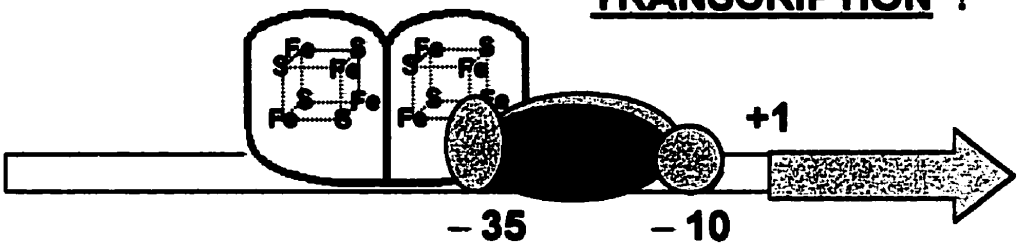
FNR is structurally similar to the CRP–family of regulatory proteins (Shaw et al., 1983). DNA recognition and binding is mediated by a C–terminal helix–turn–helix domain (Sharrocks et al., 1990). Dimerization is mediated by a central, helical domain. However, FNR has an unusual, cysteine–rich N–terminal domain that distinguishes it from CRP. Moreover, wild type FNR contains two [4Fe–4S] clusters per dimer. Under anaerobic conditions, each $[4Fe–4S]^{2+}$ cluster is coordinated tetrahedrally by three N–terminal cysteines plus a fourth, more centrally located cysteine (Green et al., 1993; 1996). Under aerobic conditions, the clusters are unstable. They degrade, irreversibly, to $[3Fe–4S]^{+}$ and then to $[2Fe–2S]^{2+}$ (Khoroshilova et al., 1997). Changes in the clusters trigger alterations in FNR structure. Dimerization is destabilized such that $[2Fe–2S]^{2+}$–containing FNR is predominantly monomeric (Beinert and Kiley, 1999;. Lazazzera et al., 1996). Moreover, the remaining oxidized dimers have seven–fold less affinity for DNA than the active, reduced dimers (Green et al., 1996). In short, oxidative stress inactivates FNR (Fig. 1.6).

FNR is both an activator and a repressor of gene transcription However, the $[4Fe–4S]^{2+}$ cluster is oxygen–labile, so FNR is only functional under anaerobic conditions. Under such conditions, it tends to activate genes required for anaerobic metabolism and repress genes involved with aerobic metabolism. Under aerobic conditions, FNR–activated genes are poorly expressed and FNR–repressed genes become

REDUCED FNR [4Fe-4S]
ACTIVATOR BINDS DNA

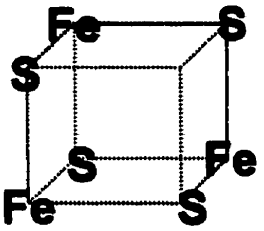
A

TRANSCRIPTION !



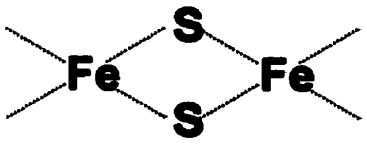
B

OXIDIZING AGENT
ADDED



[4Fe-4S]

IRON-SULFUR
CLUSTER
DESTROYED

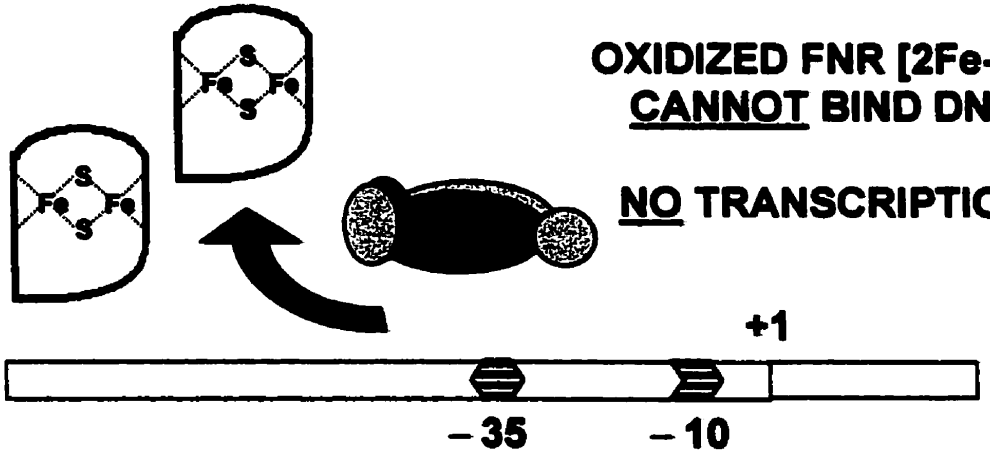


[2Fe-2S]

C

OXIDIZED FNR [2Fe-2S]
CANNOT BIND DNA

NO TRANSCRIPTION



derepressed. FNR recognizes and binds the palindromic DNA sequence $^{5'}$TTGAT X_4 ATCAA$^{3'}$. The regulatory role of FNR is determined by the position of its binding site, relative to the –10 and –35 promoter elements. Promoters activated by FNR fall into two classes. In Class I promoters, the binding site is centered at –61.5. In the more common Class II promoters, the binding site is centered further downstream, at –41.5. Promoters repressed by FNR exhibit more heterogeneity and can have multiple FNR sites. Mutational analyses indicate that activator and repressor functions are mediated by distinct regions of FNR (Williams et al., 1997). For example, mutations in AR1 (activating region 1), a large, solvent exposed surface, impair transcription from both Class I and Class II promoters. Mutations in AR3 (activating region 3), an exposed loop on the opposite surface of FNR, only impair transcription of Class II promoters (Busby and Ebright, 1997). AR1 makes contacts with the α–subunit of RNA polymerase and enhances open complex formation. AR3 makes contacts with σ^{70}. At Class I promoters, FNR is positioned such that only AR1 of the downstream monomer contacts RNA polymerase. At Class II promoters, FNR makes additional contacts with RNA polymerase. AR3 of the downstream dimer interacts with σ^{70} while AR1 of the upstream dimer contacts the α–subunit. FNR mutations that affect repressible promoters converge on a region that overlaps, but is not identical to, AR1. This suggests that there are both favourable and unfavourable FNR–α–subunit contacts. Favourable interactions enhance RNA polymerase recruitment and open complex formation. Unfavourable ones may block recruitment and/or inhibit RNA polymerase activity (Meng et al., 1997).

1.8 SUMMARY:

In prokaryotes, heavy metal homeostasis is mediated by a plethora of systems that sense, import, export and sequester metal ions. These systems are required for the acquisition of essential metals and the detoxification of noxious ones. To be effective, each system must be expressed, or repressed, appropriately. This requires metal–dependent regulation of gene expression. As described in the preceding pages, many elegant mechanisms of transcriptional regulation exist.

In the simplest systems, a single metal–responsive homodimer regulates the transcription of just a few genes, which are expressed only in the presence of the cognate metal. The ArsR, SmtB, CadC and ZiaR repressor proteins, and MerR, CoaR, CueR and ZntR activator proteins use variations of this scheme to mediate metal resistance. It is an efficient strategy because it requires little energy to maintain, and the target genes are only expressed when needed. Also, the few determinants used by these resistance systems may favour their mobility, as demonstrated by the widespread distribution of *mer* and *ars* operons.

The unusual CopY–CopZ system seems convoluted, but the principle difference is that the transcriptional regulator is also regulated. This may be indicative of copper's essential status. Although toxic at high concentrations, overzealous copper efflux can also endanger the cell. Similarly, the two–component regulatory systems that regulate the *cop*, *cut*, *pco*, and *czc* operons are not as complicated as they appear. The metal–sensing and transcriptional regulation activities are divided between proteins, rather than performed by one. The advantage to this is that the two–component systems can monitor and

ferric citrate. However, the presence of ferric citrate is not sufficient for *fec* gene transcription. The cell must also need iron. Fur represses the *fec* genes as long as the cell's iron requirement is satisfied. The *E. coli sod* gene encodes superoxide dismutase, which is important for detoxification of reactive oxygen species. Fur, FNR and SoxS all regulate expression of *sod*. The interacting, and sometimes redundant, regulatory systems provide a set of checks and balances that allow appropriate and efficient gene transcription, and ensure heavy metal homeostasis.

1.9 Rationale and Objectives of the Thesis:

Procaryotes have numerous mechanisms by which they maintain metal homeostasis. Chapter 1 includes examples of genetic systems that are regulated by, or cope with, arsenic, bismuth, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, silver, tellurium, tungsten, vanadium and zinc. However, two common heavy metals are absent from this list: aluminum and tin. Aluminum is the most abundant heavy metal in this planet's crust and has innumerable industrial applications. Yet, almost nothing is known about its effect on prokaryotes. Inorganic tin is one of the least toxic heavy metals. However, organotin compounds are widely employed as antimicrobial agents. Such use has been criticized because of its devastating impact on non-target organisms. Yet again, information about the impact of organotins on the targeted bacteria is severely lacking. The work described in this thesis was

initiated such that the effects of aluminum and organotin on gene expression in prokaryotes could be identified and better understood.

Chapter 2 describes the isolation and preliminary characterization of *ais*, the aluminum and iron stimulated gene from *Escherichia coli*. The *ais* gene was sequenced and Ais protein expressed. Gene expression upon addition of numerous heavy metals, including aluminum, iron, gallium, indium and vanadium, was measured by luminescence from an *ais*::*lux*AB transcriptional fusion and by RNA blotting.

Chapter 3 includes additional details about the transcriptional regulation of *ais*. The effects of divalent cations, pH and the two-component regulatory systems, PhoP–PhoQ and BasR–BasS are described.

Chapter 4 describes the search for BasR–BasS–regulated genes in *Escherichia coli*. Genes were isolated by over-expressing BasR–BasS in a Mu*d*I (*lacZ* Ap) gene fusion library and screening for changes in β–galactosidase activity. Additional genes were identified by searching the *Escherichia coli* genome database for BasR regulatory sequences.

Chapter 5 describes the isolation of the hexose–6–phosphate transport gene, *uhp*T during a search for genetic responses to organotin compounds. Gene expression upon addition of organotin compounds, glucose–6–phosphate, and uncouplers of oxidative phosphorylation, was measured by luminescence from an *uhp*T::*lux*AB transcriptional fusion, and by RNA blotting.

In Chapter 6, the data, and its significance are summarized. Finally, possible directions for future research are discussed.

CHAPTER 2

Isolation and characterization of an aluminum–, iron–, and vanadium–inducible gene in *Escherichia coli*

2.1 Abstract

Upon exposure to toxic metals, living organisms frequently change the expression of certain genes. This enables them to respond to metal-induced effects on cell physiology Aluminum, the third most common crustal element, is not identified as an essential trace element, but is implicated in many toxic processes. The bioavailability and toxicity of aluminum is enhanced under acidic conditions. To identify genes whose expression is inducible by added aluminum, a library of *lux*AB chromosomal gene fusions was constructed in *Escherichia coli*, and luminescence was assayed in the absence and presence of added aluminum at acidic pH. One clone was found that showed increased luminescence in the presence of aluminum, and subsequently by iron, gallium, indium and vanadium, but not nickel or copper. Cloning of the junction of the *lux*AB insertion, and hybridization to the Kohara λ library of the *E. coli* genome, revealed that the aluminum-inducible gene mapped to 51.9 map units. A northern blotting analysis revealed that a transcript of approximately 800 nucleotides was induced in the presence of aluminum or iron. DNA sequencing of 1286 bp surrounding the site of *lux*AB insertion, and expression of this cloned DNA *in vivo*, revealed that it encoded a polypeptide of 200 amino acids (22 kDa) which begins with a GTG codon and whose N–terminus is suggestive of a membrane–localizing signal sequence. This gene was thus designated *ais* (aluminum and iron stimulated). Ais exhibits homology to the PmrG protein from *Salmonella enterica* serovars, the plasmid-encoded AfrS protein from *E.coli* RDEC-1, and the TraG proteins from the *Salmonella enterica* plasmid R64 and *Shigella sonnei* plasmid ColIb–P9. The function of these proteins is currently unknown. However, they are associated with genes involved in lipopolysaccharide modification and pili syntheis and may contribute to bacterial virulence or survival under harsh environmental conditions.

2.2 Introduction

Aluminum is the most abundant crustal metal and the third most abundant crustal element (Martin, 1988). Aluminum is not considered to be an essential trace element, but it does participate in biological processes, often with toxic effects (Ganrot, 1986). Aluminum toxicity is a significant medical and agricultural concern. In mammals, aluminum contributes to a variety of neurological and skeletal pathologies (Macdonald and Martin, 1988) including neuronal cytoskeletal abnormalities (Klatzo et al., 1965), memory impairment (Crapper and Dalton, 1973a; 1973b), and alterations in neurotransmitter metabolism (Crapper and Dalton, 1973a; Crapper et al., 1973). Aluminum is responsible for the dialysis dementia experienced by long term hemodialysis patients (Alfrey et al., 1976) and the incidence of several neurological disorders has been correlated with the concentration of aluminum in drinking water (Nieboer et al., 1995). Aluminum inhibits plant root growth and limits agricultural yields. The solubility, and therefore mobility and bioavailability, of aluminum increases with decreasing pH. As such, plants growing in acidic soils and in regions polluted by acid rain are particularly vulnerable to aluminum toxicity (Delhaize and Ryan, 1995; Delhaize et al., 1999; Taylor, 1988). Aluminum has been shown to affect gene expression in wheat (Richards et al., 1994; Snowden and Gardner, 1993), tobacco (Ezaki et al., 1996) and yeast (Ezaki et al., 1998). The mechanism of toxicity is unknown, but aluminum and related metals, including gallium and indium, can alter the structure of phospholipid membranes and enhance lipid peroxidation (Verstraeten et al., 1997). Moreover, aluminum can alter the function of procaryotic ATPases. In *Escherichia*

hermannii and *Enterobacter cloacae*, this altered activity is associated with multidrug resistance and metal accumulation (Hernandez et al., 1998)

Indium and gallium are not abundant metals and, like aluminum, are not essential trace elements. However, increased use of the radionuclides, ^{111}In and ^{67}Ga, and the semiconductor compounds, gallium arsenide (GaAs) and indium arsenide (InAs), has prompted studies of these elements. Indium can interfere with iron metabolism and, through competitive inhibition, prevent iron binding to siderophores. As such, indium can have bacteriostatic effects on pathogenic enterobacteriaceae, including *Klebsiella pneumoniae* and *E. coli* spp (Rogers et al., 1982). It has also been shown that aluminum, gallium and indium can bind directly to DNA, inhibit gene transcription, and alter the structure of chromatin (Guidon et al., 1993; Walker et al., 1989).

Some bacterial species have strategies for coping with aluminum and other toxic metals. *Pseudomonas fluorescens* produces an extracellular phosphatidylethanol–amine–rich matrix which can immobilize aluminum, gallium, iron and other metals (Appanna and St Pierre, 1996). Additionally, *P. fluorescens* can sequester metals within cytoplasmic inclusion bodies (Appanna and Hamel, 1996). The details of this mechanism have yet to be resolved, but gallium exposure is associated with the induction of at least six cytoplasmic proteins in *Pseudomonas* (al-Aoukaty et al., 1992). A gene which confers aluminum tolerance to *E. coli* was isolated from a strain of *Arthrobacter viscosus* retrieved from acidic soil (Jo et al., 1997). ALU1–P, the protein encoded by this gene, is homologous to ExsB, a putative transcriptional regulator that is highly conserved among procaryotes (Becker et al., 1995).

In an effort to identify aluminum-mediated effects on bacterial cell physiology, and the cellular responses to them, we searched for genetically-programmed responses to aluminum exposure in *Escherichia coli* (Guzzo and DuBow, 1994). A library of approximately 3000 *E. coli* clones, each containing the promoterless *Vibrio harveyi lux*AB genes in a single, random chromosomal location, was previously created (Guzzo and DuBow, 1991). Changes in transcription, as measured by a change in luminescence, were monitored in the absence and presence of aluminum after overnight growth on solid media (adjusted to pH 5.5, such that the metal was readily bioavailable). One aluminum–responsive clone, designated LF20111, was previously shown to contain the *lux*AB genes inserted 97 bp downstream of the *E. coli fli*C translational start site (Guzzo et al. 1991). In addition to aluminum, luminescence of LF20111 was also inducible by copper, iron and nickel. Here, we present the characterization and expression of a second *E. coli* gene whose transcription is induced (as measured by luciferase activity and RNA blotting to the cloned gene) by aluminum in both liquid and solid media. We have designated this novel gene *ais* (for aluminum and iron stimulated) because it is also induced by iron. Subsequently, *ais* was found to be induced by gallium, indium, and vanadium.

2.3 Materials and Methods

2.3.1 Bacterial strains and media

E. coli strain DH1 [F⁻ *recA1 endA1 gyrA96 thi hsdR17* (r_k^-, m_k^+) *supE44 relA1*] (Hanahan, 1983) was used to prepare the library of *lux*AB transcription fusion clones (Guzzo and DuBow, 1991). Strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd-5* ($r_k^-m_k^+$) (F' *proAB lacI*^{q}ZΔ*M15*)] (Gough and Murray, 1983) was used to transform all DNA ligation reactions. Strain BL21(DE3):pLysS [F⁻ *ompT* *hsd*S_B (r_k^-, m_k^+) *gal dcm* (DE3)] [pLysS Cm^r] (Grodberg and Dunn, 1988) was used for protein expression experiments. *E. coli* strains were propagated at 37°C, unless otherwise stated, in Luria–Bertani (LB) broth (10 g NaCl, 10 g tryptone, 5 g yeast extract per L) or on LB plates containing 1.5% agar (Sambrook et al., 1989) and supplemented with antibiotics when indicated. Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada), 40 μg/ml; Cm (Sigma, St. Louis, Missouri), 50 μg/ml; Km (Boehringer Mannheim, Laval, Canada). 50 μg/ml. Tc (Boehringer Mannheim, Laval, Canada), 10 μg/ml in broth, 20 μg/ml in agar. Stock solutions of metal salts were prepared in sterile distilled deionized water at the following concentrations: aluminum chloride (20.1 mg/ml) (Anachemia, Montreal, Canada), ferric chloride (14.5 mg/ml) (BDH, Montreal, Canada), gallium chloride (12.47 mg/ml) (Strem Chemicals, Newburyport, Massachusetts), indium chloride (9.63 mg/ml) (Strem Chemicals, Newburyport, Massachusetts), nickel sulfate (11.15 mg/ml) (Anachemia, Montreal, Canada), vanadium chloride (15.4 mg/ml) (Aldrich Chemical Company Inc., Milwaukee, Wisconsin). The pH of LB was adjusted to 5.5 with 12 M HCl and to 7.0 with

2.3.3 Construction of plasmids

Construction of plasmids pAl-1 and pAG17 are described elsewhere (Guzzo, 1994). Briefly, pAl-1 was generated by ligating *Bam*HI-cleaved chromosomal DNA from strain LF20110 to *Bam*HI-cleaved pUC119 (Vieira and Messing, 1987) and contains the downstream junction of the *ais*::Tn*5*-*lux*AB insertion. Plasmid pAG17 was constructed by ligating *Hind*III-cleaved chromosomal DNA from strain LF20110 to *Hind*III-cleaved pUC119 and contains the upstream junction of the *ais*::Tn*5*-*lux*AB insertion. Plasmids pAG43 and pAG46 were both derived from a 5.0 kb *Hin*dIII–*Bam*HI fragment from phage 379 (Kohara *et al.* 1987) as described previously (Guzzo, 1994). To construct plasmid pAG43, the fragment was ligated to pUC119 hydrolyzed with the same enzymes. To construct plasmid pAG46, the ends of the *Hin*dIII–*Bam*HI fragment were back-filled and then ligated to *Sma*I–cleaved plasmid pKK223–3. For construction of protein expression vectors, plasmid pAG46 was used as a PCR template to amplify the entire coding region of the *ais* gene. The oligonucleotide primers $^{5'}$CCCCGGATCCGCGCTTGTCTATAGGTGG$^{3'}$ (Sheldon Biotechnology Center, Montreal, Canada), which corresponds to bp 375–402 (Fig. 2.5) and contains an *Nde*I site, and $^{5'}$CCCCCCCTCGAGGTAATTAACGAATTCCCC$^{3'}$ (Canadian Life Technologies, Burlington, Canada), which corresponds to the complement of bp 982–999 (Fig. 2.5) and contains an *Xho*I site were used for PCR and products were ligated to the TA vector, pCR2.1 (Invitrogen, Carlsbad, California). Next, a *Nde*I–*Xho*I fragment, containing the *ais* gene, was isolated and ligated to pET29b (Novagen Inc., Madison, Wisconsin) cleaved with *Nde*I and *Xho*I. to construct pDAX29 for the expression of Ais with a C–terminal histidine tag. To construct plasmid pDAX16, for expression of Ais

without a histidine tag, the *Xba*I–*Xho*I fragment of pDAX29 was ligated to pET16b (Novagen Inc., Madison, Wisconsin) cleaved with *Xba*I and *Xho*I.

2.3.4 Luminescence assays

Overnight cultures of *E. coli* strain LF20110, grown in LB broth containing Tc and Ap at 32°C, were diluted 20-fold into fresh LB broth at pH 5.5 or pH 7.0 and grown to mid-logarithmic phase (A_{550} = 0.4). The cultures were then diluted 50-fold in LB broth and grown to an A_{550} of 0.05, at which time the cultures were separated into 50 ml portions. One flask contained no exogenous metal, whereas the appropriate volume of freshly prepared metal salt stock solution was added to the other flasks to reach the appropriate final metal concentration (usually 1, 5, 10 and 20 μg metal/ml). The portions were subsequently incubated at room temperature. At regular intervals, cells were removed and diluted to an A_{550} of 0.05 in LB broth. Luminescence of triplicate 1 mL samples was measured with a Tropix Optocomp I Luminometer (MGM Instruments, Connecticut). Samples were automatically injected with 100 μL of a decyl aldehyde solution (0.5% v/v in LB broth) and light emitted during a ten second period was recorded.

2.3.5 DNA sequencing and mapping with the Kohara λ phage library

Labeling of cloned chromosomal DNA, and mapping using the Kohara λ phage library of the *E. coli* genome (Kohara et al., 1987) was performed as previously described (Guzzo et al., 1991; Guzzo, 1994). A 4.0 kb *Bam*HI–*Eco*RI DNA fragment from plasmid pAl-1 was used as a probe. For sequencing, single-stranded phagemid DNA was prepared from pUC119– or pUC118–based plasmids as described elsewhere (Vieira and Messing,

1987). Dideoxy sequencing reactions, with [γ-^{32}P]dATP (500 Ci/mmol; Du Pont Canada Inc., Mississauga, Canada), were performed with a Sequenase II kit using the universal primer (U.S. Biochemical Corp., Cleveland, Ohio). Determination of the sequence at the junction between IS*50*R and the adjacent chromosomal DNA, cloned into plasmid pAI-1, was performed as previously described (Guzzo, 1994).

2.3.6 Northern blotting analysis

Northern blotting analysis of *ais* mRNA expression was described previously (Guzzo, 1994). To summarize, an overnight culture of strain DH1 was diluted 100-fold into LB broth adjusted to pH 5.5 and grown at 32°C. When the cultures attained an A_{550} of 0.2, they were portioned into separate flasks. One flask contained no added metal. Aluminum chloride, ferric chloride or nickel sulfate was added to the other flasks to achieve a final concentration of 10 μg metal/ml. After 80 minutes of additional incubation, RNA was extracted and purified using the RNaid II kit (Bio 101, Mississauga, Canada). Five μg of RNA were subjected to electrophoresis through a 1% denaturing agarose gel according to Sambrook *et al.* (1989), except that the RNA was prestained by the addition of 100 μg/ml ethidium bromide to the loading buffer, and the running buffer contained 7.4% (v/v) formaldehyde, 40 mM MOPS-NaOH (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After electrophoresis, the gel was soaked in 20X SSC [3 M NaCl, 0.3 M sodium citrate (pH 7.0)] for 20 minutes and then transferred to a Hybond-N membrane (Amersham Ltd., Oakville, Canada) for 16 hours. The filter was baked in a vacuum oven at 80°C for two hours, and then hybridized to an [γ-^{32}P]-labeled probe according to Shackelford and Varmus (1987). The probe was a 700 bp *Bam*HI-*Hin*dIII fragment isolated from plasmid pAG17,

labeled with [γ–^{32}P]dGTP (3000 Ci/mmol, ICN Biomedicals, Mississauga, Canada) according to the procedure of Feinberg and Vogelstein (1983; 1984).

2.3.7 S1 nuclease analysis

S1 nuclease analysis of analysis of *ais* mRNA was described previously (Guzzo, 1994). An oligonucleotide primer $^{5'}$GGTCGCAACGTTCAGCATGACGAA$^{3'}$, corresponding to the complement of bp 598 to 575 (Fig. 2.5) was synthesized (Sheldon Biotechnology Centre, Montreal, Canada), labeled at its 5' end with [γ –^{32}P]ATP (3000 Ci/mmol; Amersham Ltd., Oakville, Canada), and extended using single-stranded DNA isolated from pAG43 as a template (Greene and Struhl, 1989). The double-stranded radioactive products were subsequently hydrolyzed with *Pst*I (Fig. 2.5, bp 39), subjected to electrophoresis through an alkaline agarose gel, and the 560 nucleotide single-stranded S1 nuclease probe was purified (Greene and Struhl, 1989). The probe was hybridized to 10 μg of RNA isolated from strain DH1, grown in the presence of 10 μg/ml of aluminum at pH 7.0, in aqueous hybridization buffer in a total volume of 15 μl at 55 °C (Greene and Struhl, 1989). After twelve hours, 135 μl of S1 nuclease mix, containing 250 units of S1 nuclease (Pharmacia Biotech, Montreal, Canada), was added and the mixture incubated at 37 °C for 60 minutes. The reaction was subsequently stopped, precipitated and suspended in 5 μl of 0.1 M NaOH. An equal volume of formamide loading dye was added and half of the reaction was subjected to electrophoresis through a 5% denaturing polyacrylamide gel (Greene and Struhl, 1989). A dideoxy sequencing reaction was also performed using the synthesized oligonucleotide and single–stranded plasmid pAG43 as a template.

2.3.8 Visualization of plasmid-encoded polypeptides

BL21(DE3):pLysS cells transformed with pET16b, pET29b, pDAX16, or pDAX29 were grown in LB broth at 37°C to an A_{550} of 0.5, at which point 1 ml aliquots were removed. IPTG was then added to a final concentration of 4 mM, and the cultures were returned to the incubator. After two hours, 1 ml of cells was removed. The cells were lysed and boiled in SDS–PAGE loading buffer (Sambrook et al., 1989) and loaded onto a 15% SDS–polyacrylamide gel (Laemmli, 1970). Staining with Coomassie blue was used to visualize polypeptides (Sambrook et al., 1989).

2.3.9 Nucleotide sequence accession number

The DNA sequence presented here was assigned EMBL accession number X83874. However, subsequent DNA sequencing and restriction endonuclease analyses have revealed that nucleotides 996 and 997 (Fig. 2.5), initially reported as TT, are CC.

2.4 Results

2.4.1 Isolation of an aluminum-inducible *lux*AB gene fusion clone

A library of 3000 *E. coli* clones, each containing a single chromosomal copy of the *V. harveyi lux*AB genes in random positions, was previously constructed (Guzzo and DuBow, 1991). The library was screened in the absence and presence of 1 and 10 μg/ml of added aluminum on LB agar plates. Because aluminum solubility, and therefore bioavailability, increases with decreasing pH, the media was adjusted to pH 5.5. One of two

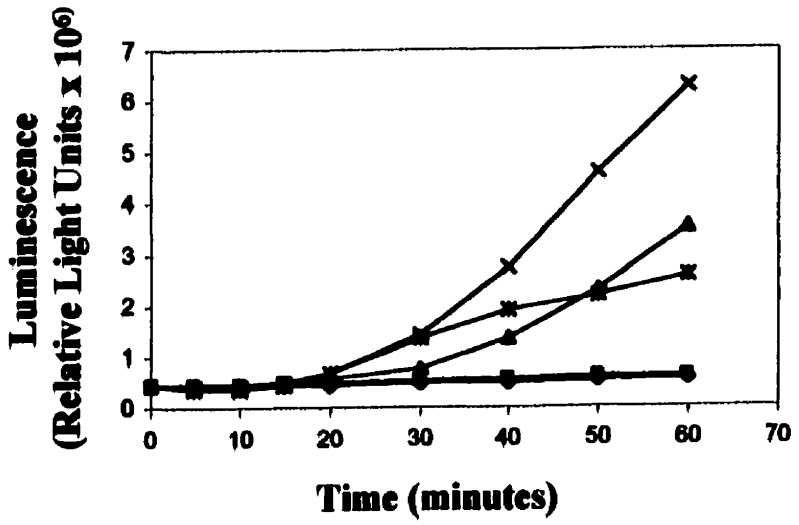
clones (Guzzo et al., 1991; Guzzo, 1994) found to display increased luminescence with increasing concentrations of aluminum was isolated and designated strain LF20110.

Luminescence of strain LF20110 was measured over a 60 minute interval in the absence or presence of 1, 5, 10 and 20 μg/ml aluminum (added as aluminum chloride) in LB broth adjusted to pH 5.5 (Fig. 2.1A) or pH 7.0 (Fig. 2.1B). After a lag period of 15 minutes, luminescence in the aluminum–treated cultures increased. In general, the rate of increase in light emission correlated with the increasing concentrations of added aluminum. Moreover, luminescence, after addition of metal, was affected by pH and was greater at pH 5.5 than at pH 7.0. For example, 60 minutes after addition of 10 μg/ml aluminum, luminescence was three–fold greater at pH 5.5 (Fig. 2.1A) than at pH 7.0 (Fig. 2.1B). Luminescence was subsequently found to be stimulated by the addition of iron (as ferric chloride) (Fig. 2.2). The pattern of light emission, with respect to metal concentration and pH, was similar for both aluminum and iron. A comparable pattern of luminescence was also seen upon addition of vanadium (as vanadium chloride; Fig. 2.3), gallium (as gallium chloride; data not shown) and indium (as indium chloride; Fig. 2.4). Indium was the most potent inducer of luminescence. One hour after addition of 1 μg/ml indium, luminescence increased to $5x10^6$ relative light units at pH 7.0 (Fig. 2.4B) and $1x10^7$ relative light units at pH 5.5 (Fig. 2.4A). In contrast, one hour after addition of 1 μg/ml aluminum, iron, or vanadium, luminescence was less than $1x10^6$ relative light units at both pH 7.0 and pH 5.5. Using the same methods, it was determined that the luminescence of strain LF20110 is not inducible by nickel sulfate or copper sulfate in either solid or liquid media (data not shown).

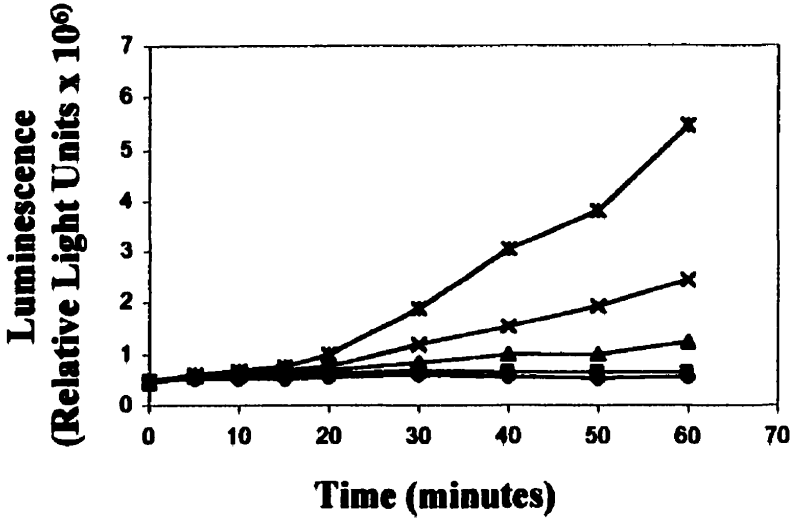
Figure 2.1: Measurement of luminescence from strain LF20110 in the presence of aluminum at pH 5.5 (A) and pH 7.0 (B). Aluminum was added to a final concentration of: 0 (◆), 1 (■), 5 (▲), 10 (×), 20 (∗) μg/ml. Standard error is less than 1%, hence error bars are not shown.

Figure 2.2: Measurement of luminescence from strain LF20110 in the presence of iron at pH 5.5 (A) and pH 7.0 (B). Iron was added to a final concentration of: 0 (◆), 1 (■), 5 (▲), 10 (×), 20 (∗) μg/ml. Standard error is less than 1%, hence error bars are not shown.

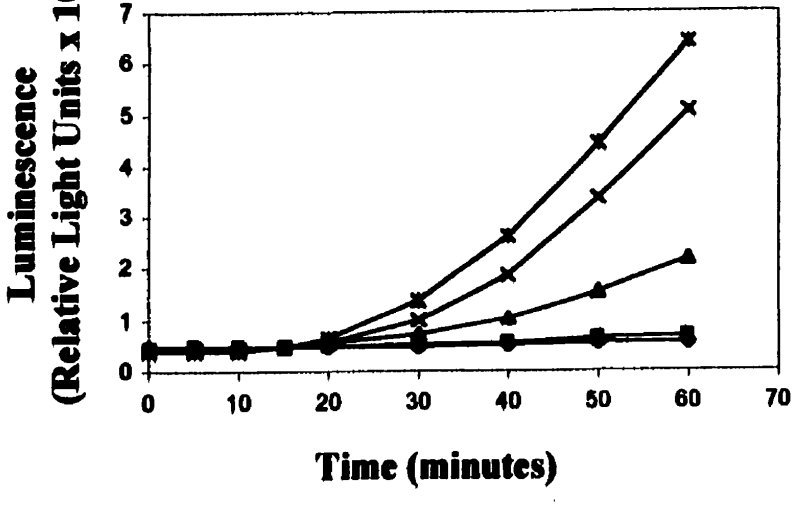
1A


Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (minutes)

1B


Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (minutes)

2A


Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (minutes)

2B

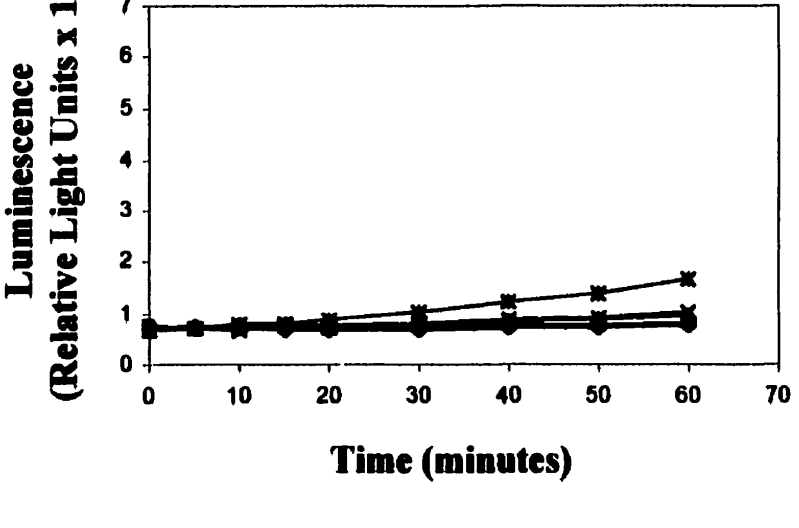
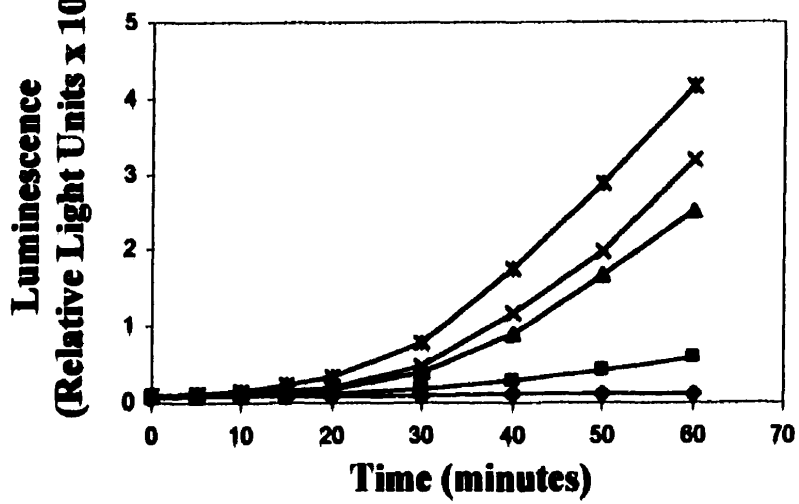

Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (minutes)

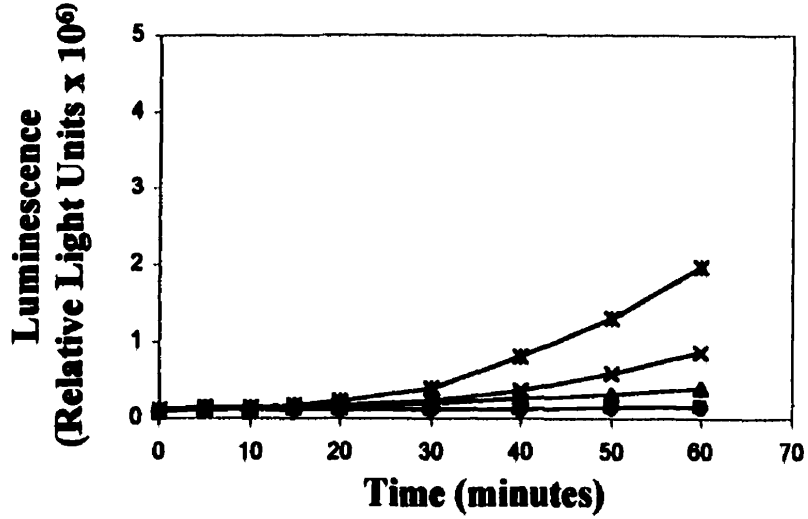
Figure 2.3: Measurement of luminescence from strain LF20110 in the presence of indium at pH 5.5 (A) and pH 7.0 (B). Metal was added to a final concentration of: 0 (◆), 1 (■), 5 (▲), 10 (×), 20 (∗) μg/ml. Standard error is less than 1%, hence error bars are not shown.

Figure 2.4: Measurement of luminescence from strain LF20110 in the presence of vanadium at pH 5.5 (A) and pH 7.0 (B). Metal was added to a final concentration of: 0 (◆), 1 (■), 5 (▲), 10 (×), 20 (∗) μg/ml.Standard error is less than 1%, hence error bars are not shown.

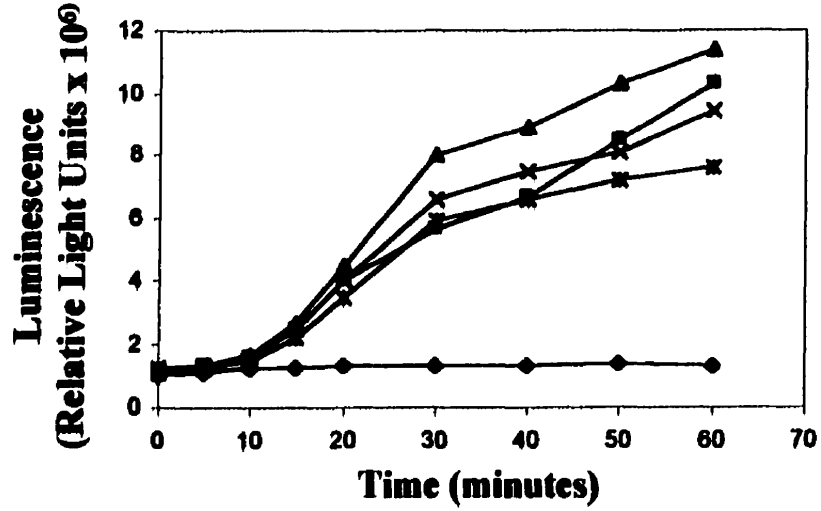
3A


Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
0
10
20
30
40
50
60
70
Time (minutes)

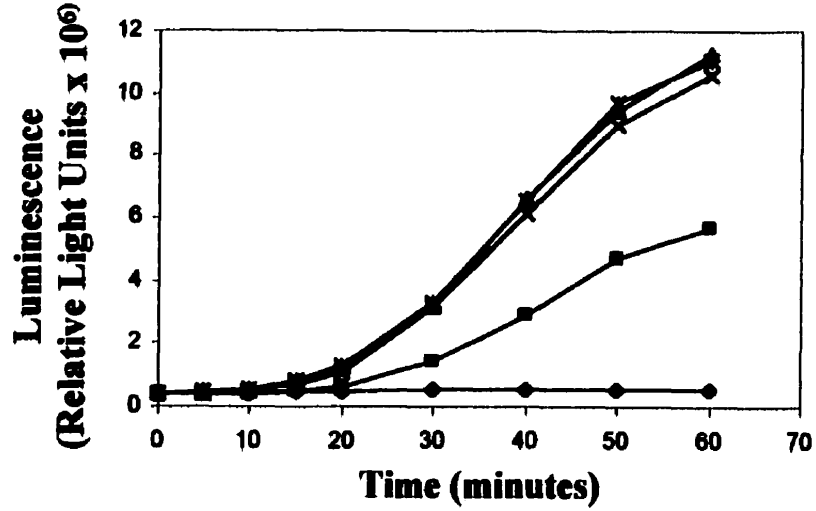
3B


Luminescence
(Relative Light Units x 10⁶)
0
1
2
3
4
5
0
10
20
30
40
50
60
70
Time (minutes)

4A


Luminescence
(Relative Light Units x 10⁶)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
Time (minutes)

4B


Luminescence
(Relative Light Units x 10⁶)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
Time (minutes)

2.4.2 Cloning and sequencing of the aluminum-inducible gene

A Southern blot was performed, and confirmed that the Tn*5-lux*AB element was present at a single chromosomal location in strain LF20110 (data not shown). The right end of the gene fusion was cloned in one step using the *tet* gene in the Tn*5-lux*AB element as a selection marker (Guzzo and DuBow, 1994; Guzzo et al., 1991). The enzyme *Bam*HI cleaves to the left of the *tet* gene, such that digestion of total chromosomal DNA with *Bam*HI, ligation to *Bam*HI–cleaved pUC119 (Vieira and Messing, 1987), and selection of transformants on LB plates containing Tc enabled isolation of plasmids that contain the *tet* gene, IS*50*R and chromosomal DNA downstream of the site of the Tn*5-lux*AB insertion. The resulting plasmid, pAl–1, was shown to contain approximately 4.3 kb of chromosomal DNA, with an *Eco*RI site located 4.0 kb away from the chromosomal *Bam*HI site (Guzzo, 1994). The site of insertion was sequenced using a primer complementary to IS*50*R, as previously described (Guzzo et al., 1991). Initially, comparison of the sequence with the bacterial sequences in the GenBank database (Benson et al., 1999) did not produce a match. The 4.0 kb *Bam*HI–*Eco*RI fragment was labeled and found to hybridize to phage 379 of the Kohara λ library of the *E. coli* chromosome (Kohara et al., 1987) and is transcribed in a right–to–left orientation (Guzzo, 1994). A total of 1286 bp, spanning the site of insertion, was sequenced (Fig. 2.5). It has been subsequently determined that the Tn*5-lux*AB element had inserted after bp 2 363 413 of the complete *E. coli* chromosome (bp 667 of Fig 2.5) (Blattner et al., 1997). The sequenced region contained a nested pair of putative open reading frames (ORFs) of 200 and 94 amino acids which start with GTG and ATG codons, respectively (Fig. 2.5). We decided that the metal–inducible ORF should be called *ais*, for aluminum– and iron– stimulated.

Figure 2.5: DNA sequence of the *ais* region. Shown is the amino acid sequences, in one-letter code, of ORF 22.2/Ais. The potential Shine–Dalgarno (SD) sequences are overlined. The two putative start sites of transcription, P1 and P2, are indicated by boxes. The putative σ^{70} "–10" and "–35" promoter sequences are underlined for P1 and overlined for P2. An inverted repeat embedded in the σ^{70} "–10" and "–35" sites is in boldtype. The potential BasR binding motif, which is similar to the PmrA binding motif described by Wosten and Groisman (1999), is indicated by bold, italicized boxed type. The site of Tn*5*–*lux*AB insertion was determined to be between bp 667 and 668 and is denoted by a vertical arrow. An inverted repeat sequence, which is located downstream of Ais, and is a potential transcription terminator, is indicated by horizontal arrows. The GTG start codon of upstream ORF b2253, and the TAA stop codon for downstream ORF yfaO, are indicated with boxes.

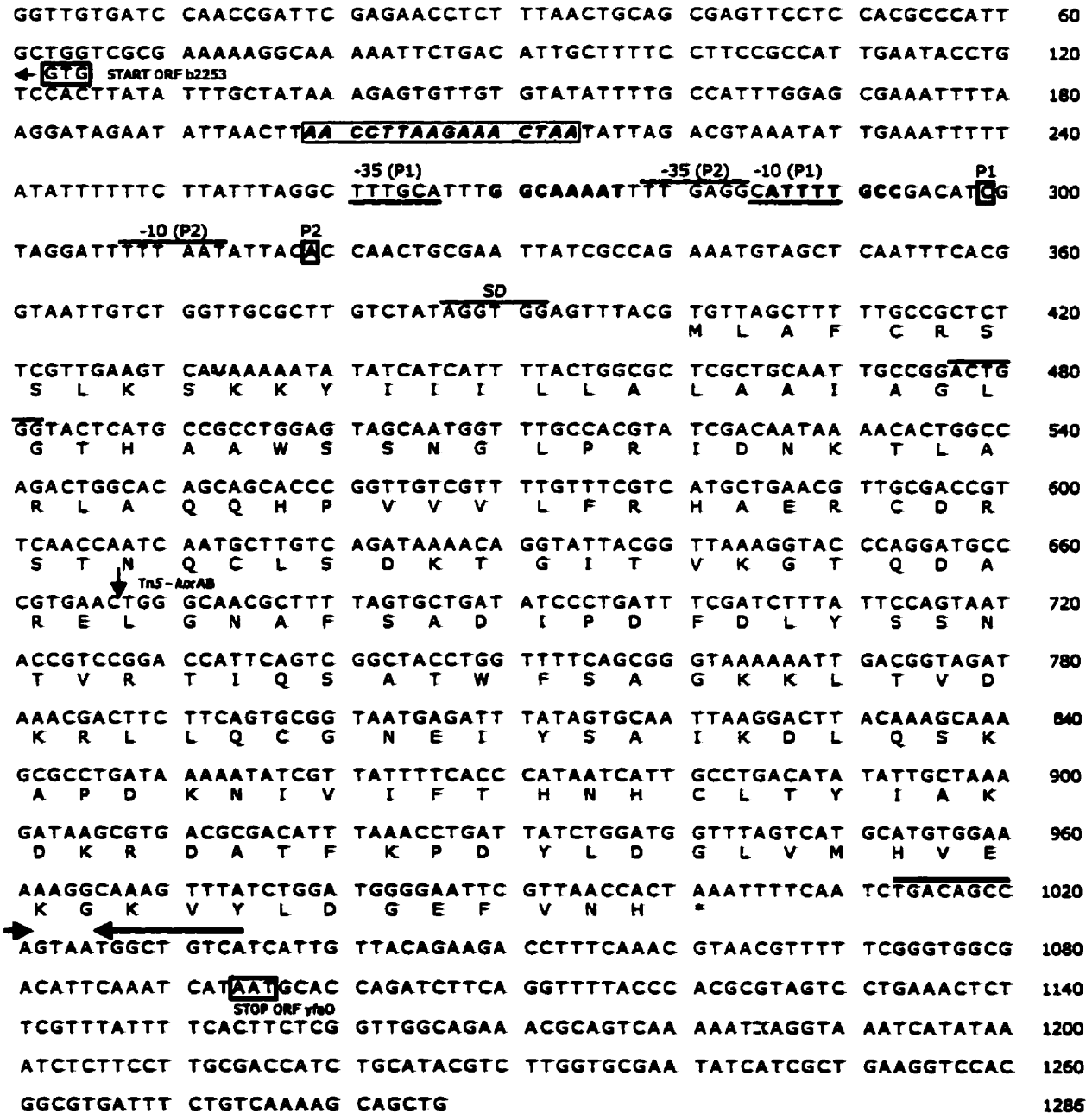
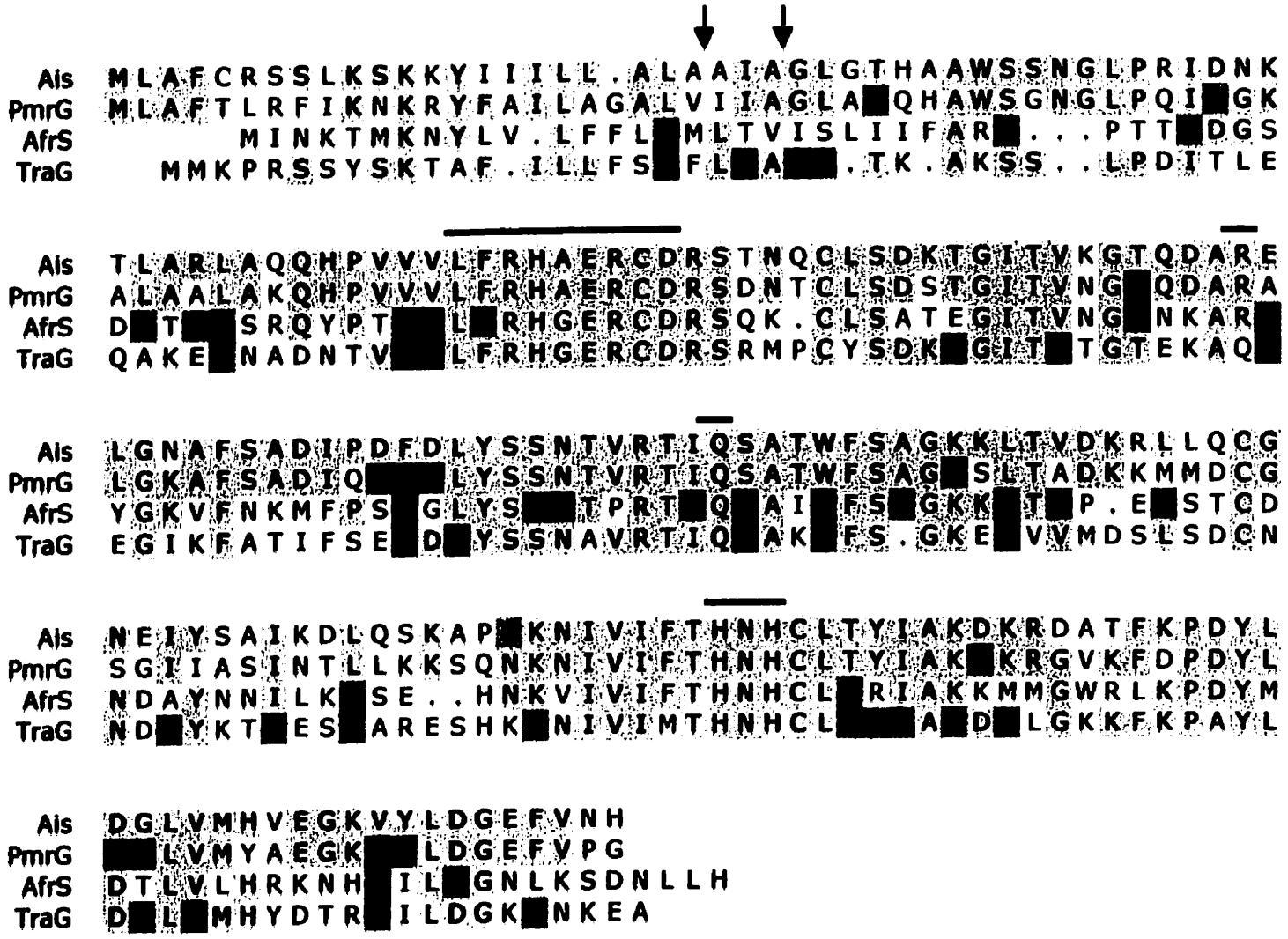

START ORF b2253
-35 (P1)
-35 (P2)
-10 (P1)
P1
-10 (P2)
P2
SD
Tn5-kanAB
STOP ORF yfaO

Figure 2.6: Homology of Ais to PmrG from *Salmonella enterica* serovars, AfrS from the attaching/effacing *E. coli* strain RDEC-1, and TraG from plasmids R64 and pCollb–P9. The amino acid sequences, in one-letter code, are aligned to give maximum homology. Identical residues are lightly shaded. Similar residues are darkly shaded. Overlined regions indicate the putative phosphate binding motif. Arrows mark putative cleavage sites for the Ais signal sequence. Sequence data for PmrG (accession number AF036677), AfrS (accession number L08467), and TraG (accession numbers AB027308.1 for R64 and AB021078.1 for pCollb–P9) were obtained from the GenBank database.


Als
PmrG
AfrS
TraG

2.4.3 Analysis of transcription

To examine the transcription of this region, the left end of the gene fusion was cloned into plasmid pUC119, using *Hin*dIII and selection for Tc^R, resulting in plasmid pAG17 (Guzzo, 1994). When the Tn*5–lux*AB element inserts into the chromosome, a *Bam*HI site (located between the end of IS*50*L and *lux*A) is placed 23 bp downstream of the site of insertion. Hydrolysis of pAG17 with *Hin*dIII and *Bam*HI produced an approximately 700 bp DNA fragment containing sequences upstream from the site of Tn*5–lux*AB insertion. The fragment was labeled and hybridized to RNA isolated from *E. coli* DH1 (no Tn*5–lux*AB insertion) grown in the absence and presence of 10 μg/ml aluminum chloride, ferric chloride and nickel sulfate at pH 5.5. An mRNA of approximately 800 nucleotides in length was detected. The level of *ais* mRNA from cells exposed to aluminum and iron is augmented, whereas the level produced in the presence of nickel is comparable to unexposed cells (Guzzo, 1994). These results also show that increased luminescence is due to an increase in *lux*AB transcription.

An S1 nuclease analysis was performed on RNA from cells exposed to aluminum (Guzzo, 1994). Two protected fragments were seen migrating with nucleotides 299 and 319 (Fig. 2.5), localizing putative transcriptional start sites to a C and an A residue, respectively. The putative promoter starting at bp 299, which produced a more abundant amount of protected fragment, was called P1, while that starting at bp 319 was designated P2 (Fig. 2.5). If the transcript is 800 nucleotides long, then a transcriptional stop site should be located near bp 1119. An inverted repeat sequence, which may form a stem–loop structure, is centered around bp 1122 (Fig. 2.5).

2.4.4 Expression of ORFs

Analysis of the DNA sequence upstream of bp 348, using ATG as a start codon, revealed a potential ORF with a predicted molecular weight of 10.6 kDa spanning bp 488 to 769 (Fig. 2.5). Translation of the DNA sequence using GTG, an alternative start codon, revealed that an ORF with a predicted molecular weight of 22.2 kDa could be encoded from the region spanning bp 400 to 999 (Fig. 2.5). Shine–Dalgarno sequences are present upstream of both ORF 10.6 and ORF 22.2. When the expression of polypeptides from plasmids pET16b and pDAX16 (pET16b containing both ORF 10.6 and ORF 22.2) was monitored *in vivo*, a unique polypeptide, migrating at approximately 22 kDa, was observed in the IPTG–induced pDAX16 culture (Fig. 2.7). This suggests that ORF 22.2 encodes the *ais* gene product. Expression of polypeptides produced *in vivo* from plasmids pDAX29 and pET29b revealed the presence of a unique protein in the IPTG–induced pDAX29 culture (Fig. 2.7). This polypeptide is slightly larger than 22 kDa, consistent with expression of the Ais protein containing a C–terminal six histidine tag.

2.4.5 Homology of Ais to other proteins

A homology search was conducted using the BLASTP algorithm (Fig. 2.6) (Altschul et al., 1997). Ais exhibits 63% amino acid identity, and an additional 12% amino acid similarity, to the *pmr*G (formerly *pag*H) gene product of *Salmonella enterica* serovars, including *S. typhimurium* LT2, *S. typhi* and *S. paratyphimurium* (Gunn et al., 1998), Genome Sequencing Center). The function of PmrG has not been determined, but it is known to be regulated by the PmrA–PmrB two–component regulatory system. Other genes regulated by the PmrA–PmrB system mediate 4–aminoarabinose modification of

Figure 2.7: Expression of the Ais polypeptide. Polypeptides expressed *in vivo* from *E. coli* strains BL21(DE3)pLysS/pET16b (Lanes 1, 5), BL21(DE3)pLysS/pDAX16 (Lanes 2, 6), BL21(DE3)pLysS/pET29b (Lanes 3, 7) and BL21(DE3)pLysS/pDAX29 (Lanes 4, 8) in the absence (Lanes 1–4) or presence (Lanes 5–8) of 4 mM IPTG. Mass of marker polypeptides (in kDa) are shown at the right. The Ais protein and Ais with a N–terminal, six–histidine tag (Ais + 6 His) are indicated with arrows.

lipopolysaccharide and resistance to peptide antibiotics such as polymixin B (Gunn et al., 1998; Roland et al. 1993). Ais exhibits 37% amino acid identity, and an additional 35% amino acid similarity, to AfrS (Genbank accession number L08467) from the attaching/effacing *E. coli* strain RDEC–1 (Wolf and Boedeker, 1990). Recently, it was suggested that AfrS is a novel regulatory protein that, in coordination with AfrR, mediates transcription of *afr*A, the structural subunit of the AF/R1 pilus (Cantey et al., 1999). However, the mechanism of this regulation remains to be elucidated. Ais also exhibits 40% amino acid identity and an additional 22% amino acid similarity to the TraG proteins from plasmid pColIb–P9 of *Shigella sonnei* strain P9 (Genbank accession number AB021078) and plasmid R64 of *Salmonella enterica* serovar Typhimurium (Genbank accession number AB027308). The function of TraG is unknown. Although the N-terminal regions of these proteins show little sequence identity, computer analysis predicts that the N–terminal of each protein contains a signal sequence (Argos and Rao, 1986; Ray et al., 1986).

2.5 Discussion

We have isolated a gene inducible by aluminum, iron, gallium, indium and vanadium, as measured by an increase in luminescence from a luciferase gene fusion and by RNA blotting to the isolated gene. We have designated this gene as *ais* (aluminum and iron stimulated). Induction of transcription, as measured by luminescence, correlates with metal concentration such that the level of light emission increases with increasing concentrations of added metal. Moreover, the luminescence at a given concentration of added metal

increases with decreasing pH (Figs. 2.1–2.4). Exceptions to this trend have been observed at pH ≤ 5.5 in cultures containing 20 μg/ml of added aluminum and 10 or 20 μg/ml added indium. This may be due to impaired cell viability caused by the increased toxicity of the metals at pH 5.5. We have observed that, under acidic conditions, indium and aluminum, added at concentrations as low as 10 μg/ml, can slow cell growth (data not shown). This sensitivity to aluminum and indium is not due to disruption of the *ais* gene by the Tn*5*-*lux*AB insertion. When compared to the parental *E. coli* strain DH1, *E. coli* strain LF20110, which contains the *ais*::Tn*5*-*lux*AB insertion, does not display increased sensitivity to metals (data not shown). Thus, it does not appear that *ais* plays a significant role in detoxification of these metals.

The amount of a mRNA species that hybridizes to a radiolabeled *ais* DNA probe is augmented in aluminum and iron exposed *E. coli* DH1 but not in untreated or nickel exposed cells (Guzzo, 1994). This confirms that the metal–induced changes in *ais* expression occur at the level of transcription.

The Tn*5*-*lux*AB element was shown to be inserted in a previously uncharacterized *E. coli* gene. The DNA sequence spanning the insertion site was determined, and S1 nuclease analysis (Guzzo, 1994) revealed that transcription could start at two positions, termed P1 and P2, corresponding to bp 299 and 319, respectively (Fig. 2.5). Potential σ^{70} "-10" and "-35" recognition sites for these promoters are indicated in Figure 2.5. Analysis of the potential ORFs downstream of bp 348 revealed that there were two possible candidates: ORF 10.6 with an ATG start codon and ORF 22.2 with a GTG start codon. When this DNA region was expressed *in vivo*, a polypeptide migrating at approximately 22 kDa was seen, while no 10.6 kDa polypeptide was observed either *in vivo* (Fig. 2.7) or *in vitro* (data not

and *Salmonella enterica*. Two primer extension products have been obtained for *ais*, P1 corresponds to a C residue (101 bp upstream from the start codon; Fig. 2.5) while P2 corresponds to an A residue (81 bp upstream from the start codon; Fig. 2.5). Only a single primer extension product, starting with a G residue, has been obtained for *pmr*G. Zhou. *et al* (1999) found that NH_4VO_3 induces changes in *E. coli* lipid A that are similar to the lipid A modifications mediated by the PmrA–PmrB pathway in *Salmonella enterica* serovar Typhimurium. They implied that vanadate may induce the BasR–BasS pathway in *E. coli*. Indeed, we have demonstrated that vanadium induces the expression of *ais* (Fig. 2.3). Our data also indicates that BasR–BasS may be required for metal–mediated expression of *ais* (see Chapter 3).

Computer–assisted analysis predicts that Ais and its homologs have N–terminal signal sequences. In Ais, two putative consensus sites, which could direct cleavage either between amino acids 22 and 23 or between amino acids 25 and 26, have been identified (Fig. 2.6) (Argos and Rao, 1986; Ray et al., 1986). This suggests these proteins are membrane bound or periplasmic. The central regions of these proteins are highly conserved. Moreover, this central region features a histidine–containing motif, $LXRH^{G}/_{A}E$ (Fig 2.6.), which resembles the $LXRHG^{E}/_{Q}$ motif present in the active site of the phosphatase domain of the bifunctional eucaryotic enzyme 6–phosphofructo–2–kinase/ fructose– 2,6–bisphosphatase (Hasemann et al., 1996; Yuen et al., 1999). A similar motif is present in cofactor independent phosphoglycerate mutases and acid phosphatases found in both procaryotes and eucaryotes (Hasemann et al., 1996). The active site of the fructose–2,6–bisphosphatase domain features conserved arginine, glutamate and histidine residues. An arginine residue, with spacing identical to that found in the

fructose–2,6–bisphosphatase domain, is conserved in Ais, PmrG and AfrS, and motifs containing glutamine and histidine are conserved in all of the homologs (Fig. 2.6). If Ais is a phosphatase, it may contribute to the modification of lipid A. Indeed, Zhou *et al* (1999) indicate that an enzyme which regulates the level of lipid A 1–pyrophosphate has yet to be identified. Alternatively, Ais may have a regulatory function. Recently, Cantey *et al.* (1999) suggested that AfrS is novel regulatory protein that, in association with AfrR, induces expression of AfrA, the major structural component of the AF/R1 pilus. The mechanism of this regulation has not been determined. However, the activity of many response regulator proteins is dependent on their phosphorylation state. Although described as an AraC homolog, AfrR only exhibits homology to the DNA–binding region of AraC. Information about the regulation of AfrR function, including the role of phosphorylation and/or dimerization, remains to be determined (Cantey et al., 1999).

Although we have demonstrated that *ais* expression is metal–inducible, the role of aluminum is not clear. However, in other systems there are two recurring themes. Gene expression can be mediated by specific, metal–induced regulatory factors, such as ArsR (Diorio et al., 1995) and Fur (Crosa, 1997). Alternatively, gene expression can be an effect of metal–mediated damage to cellular components, such as membrane phospholipids and proteins that require metal–cofactors.

Free metal ions, including iron, vanadium, aluminum, indium and gallium, can participate in Haber–Weiss reactions that generate toxic oxygen radicals (Goldstein et al., 1993; Keller et al., 1989; Martin, 1988). In eucaryotic systems, aluminum treatment induces expression of peroxidase, catalase and superoxide dismutase enzymes that can counter reactive oxygen species (Ding et al., 1999; Ezaki et al., 1996). Aluminum tolerance in

suggests a role in bacterial virulence. For a pathogen to be effective, virulence genes must be expressed when conditions (e.g. sufficient quorum of bacteria, nutritional status, location within the host) are favorable. Although aluminum is not known to be important for bacterial virulence, iron certainly is. Both low and high iron concentrations trigger responses which enhance bacterial survival and pathogenicity (Crosa, 1997; Vasil and Ochsner, 1999). As such, our aluminum–stimulated gene may represent a new iron–regulated genetic cascade in *E. coli*.

We have isolated and expressed a novel gene from *Escherichia coli*. Transcription of *ais* is stimulated by aluminum, iron, indium, gallium and vanadium. Although the function of Ais is unknown, several homologs exist. The homologs share a phosphatase motif and may be involved in bacterial virulence. Experiments to examine the DNA sequences required metal-mediated transcriptional induction of the *ais* gene are currently underway. In this manner, we will to better characterize the *ais* gene, and enhance our understanding of cellular responses to heavy metal stress.

Preface to Chapter 3

In Chapter 2, isolation and preliminary characterization of the *Escherichia coli ais* gene was described. The DNA sequence of the *ais* region was determined, the transcriptional start site identified, and Ais protein was expressed. This study demonstrated that transcription of *ais* is induced by the addition of aluminum, iron, indium, gallium and vanadium. However, the role of pH in *ais* transcription was not fully addressed. Moreover, the homology of Ais and PmrG, as well as the general similarity of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, suggested that a two–component regulatory system might be responsible for metal–induced transcription of *ais*. In Chapter 3, these possibilities are examined.

3.1 Abstract

We previously identified *ais*, an aluminum– and iron–inducible gene from *Escherichia coli*. Although the function of *ais* is unknown, it has several homologs. One of these homologs, *pmr*G from *Salmonella enterica* serovar Typhimurium, is regulated by the PmrA–PmrB and PhoP–PhoQ two–component regulatory systems, pH and magnesium. Using a transcriptional *ais*::*lux*AB reporter gene fusion, we examined the possibility that similar mechanisms regulate *ais*. We found that the BasR–BasS system is required for metal–inducible expression. The *E. coli* PhoP–PhoQ system is not required for metal–inducible expression, but *pho*P and *pho*Q mutations do diminish the magnitude of *ais*::*lux*AB expression. Moreover, pH does not directly induce *ais*::*lux*AB expression, although pH may alter the bioavailablity of aluminum and/or iron and so, indirectly influence transcription. Transcription is not induced by low concentrations of magnesium or calcium. High concentrations of magnesium or calcium have no effect on baseline expression of the *ais*::*lux*AB fusion, but do inhibit aluminum–induced transcription. These results indicate that the *Salmonella* and *E. coli* systems are analogous and indicate that the BasR–BasS mediates cellular responses to metals.

3.2 Introduction

The *ais* gene of *Escherichia coli* was isolated during a search for aluminum-inducible genes (see Chapter 2). Expression of a chromosomal *ais::lux*AB transcriptional reporter gene fusion was found to increase with increasing concentrations of aluminum. It was subsequently found that this pattern of *ais* expression is induced by a variety of metals including aluminum, indium, gallium, vanadium and iron, but not nickel or copper. Ais has several homologs including *pmr*G from *Salmonella enterica* serovar Typhimurium, (Gunn et al, 1998), AfrS from the attaching/effacing *E. coli* strain RDEC-1 (Wolf and Boedeker, 1990), and TraG from the Enterobacteriaceae plasmids pColIb-P9 and R64. Although the function of Ais and its homologs is unknown, they share putative N-terminal signal sequences and a putative phosphatase motif.

Transcription of *pmr*G is mediated by the PmrA-PmrB two-component regulatory system (Wosten and Groisman, 1999). Stimulation of the PmrB sensor kinase results in phosphorylation of the PmrA response regulator. Although it has been suggested that PmrB responds to mildly acidic pH (Bearson et al., 1998), this has been disputed (Gunn et al., 1998; Kox et al., 2000). PmrA increases transcription of several loci, including the *pmr*CAB operon, the *pmr*F operon, *pmr*E, and *pmr*G (Aguirre et al., 2000; Wosten and Groisman, 1999).

In *Salmonella enterica*, the PhoP-PhoQ two-component regulatory system can enhance expression of PmrA-regulated genes. Indeed, several PmrA-regulated genes, including *pmr*G (formerly *pag*H) were initially identified as PhoP-activated genes (Belden and Miller, 1994). The PhoQ sensor protein mediates the phosphorylation state, and

therefore activity of the PhoP response regulator, in response to extracellular magnesium, calcium, and manganese concentrations (Vescovi et al., 1996; Castelli et al., 2000). The current model suggests that phosphorylated PhoP induces expression of *pmr*D (Kox et al., 2000). PmrD then interacts with PmrA to enhance expression of PmrA–regulated genes. The mechanism for this remains to be further elucidated, but it requires a functional PmrA–PmrB system.

The *E. coli* genome includes homologs to all of these genes (Table 3.1). Moreover, the *ais* promoter region includes a sequence which is similar to the DNA binding motif of PmrA (see Chapter 2; Wosten and Groisman, 1999). As such, similar regulatory mechanisms might mediate transcription of the *ais* gene. In this Chapter, the effects of BasR–BasS, PhoP–PhoQ, pH, magnesium and calcium on aluminum–induced expression of a chromosomal *ais*::*lux*AB transcriptional reporter gene fusion are examined.

3.3 Materials and Methods

3.3.1 Bacterial strains and media

The bacterial strains used in this study are listed in Table 3.2. LF20110, the original *ais*::*lux*AB transcriptional fusion clone, was derived from *E. coli* strain DH1 [F⁻ *recA1 endA1 gyrA96 thi hsdR17* (r_k^-, m_k^+) *supE44 relA1*] as previously described (Guzzo and DuBow, 1991; Hanahan, 1983). MG:aisΔS (*bas*S) and MG:aisΔRS (*bas*RS) were derived

Table 3.1: *Escherichia coli* and *Salmonella enterica* homologs

E.coli	*S. enterica*	% Homology (% Identity)	Function
BasR	PmrA	86% (82%)	Response regulator
BasS	PmrB	92% (85%)	Sensor Kinase responds to Al^{3+} Fe^{3+} V^{3+}
*yjd*B	PmrC	87% (78%)	Transport ?
*yjd*E	orf D	89% (86%)	Transport ?
Ais	PmrG	86% (79%)	Phophatase ?
b2253/o390	PbgP1	86% (70%)	Amino transferase
b2254/o322	PmrF/PbgP2	83% (78%)	4 amino arabinose bactoprenol transferase
b2255/o660	PbgP3	89% (79%)	Oxido reductase ?
b2256/o296	PbgP4	78% (69%)	Decarboxylase ?
b2257/o550	PbgE1/PqaB	75%(62%)	Bactoprenol 4 amino arabinose
b2258/o222	PbgE3	72% (62%)	Lipid A transferase
PmrD	PmrD	75% (59%)	Regulator of BasR/PmrA function
Ugd	PmrE/Ugd	94% (88%)	UDP–glucose 6–dehydrogenase
PhoP	PhoP	93% (89%)	Response regulator
PhoQ	PhoQ	88% (81%	Sensor Kinase, responds to [low] Mg^{2+} Ca^{2+} Mn^{2+}

Table 3.2: Bacterial Strains and Plasmids used in these Studies

	Relevant Characteristics	Reference
Bacterial Strains		
LF20110	*E. coli* DH1 *ais*::*lux*AB	Chapter 2
MG1655	*E. coli* K–12 (F^- λ^-)	Bachmann, 1972
MG:ais	MG1655 *ais*::*lux*AB	this study
MG:aisΔS	MG:ais *bas*S::*aac*C1 (Gm^R)	this study
MG:aisΔRS	MG:ais *bas*RS::*aac*C1 (Gm^R)	this study
CSH26		Waldburger 1996
CSH26:ais	CSH26 *ais*::*lux*AB	this study
CSH26ΔQ:ais	CSH26 *ais*::*lux*AB *pho*Q	this study
CSH26ΔPQ:ais	CSH26 *ais*::*lux*AB *pho*PQ	this study
40 R1	*E coli* 40 *ars*R::*lux*AB	Cai, 1996
40 B1	*E coli* 40 *ars*B::*lux*AB	Cai, 1996
DH5α	*supE44* Δ*U169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi–1 relA1*	Woodcock, 1989
Plasmids		
pSU25RS	Ap^R	Nagasawa, 1993
pBAD33	Cm^R	Guzman, 1995
pDAL71	Cm^R	this study
pDAL73	Cm^R Gm^R	this study
pDAL73	Cm^R Gm^R	this study
pK03	Cm^R	Link, 1997
pDK73	Cm^R Gm^R	this study
pDK75	Cm^R Gm^R	this study
pUC7G	Gm^R	Schwizer, 1993

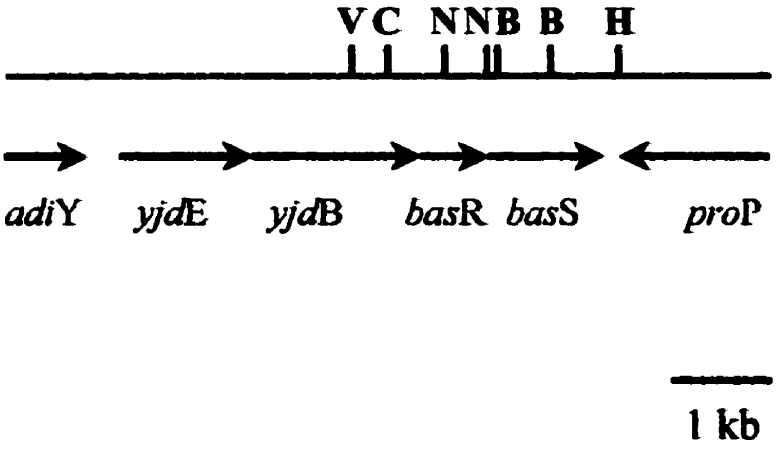
from wild type *E. coli* MG1655 (F^-, λ^-). Strain CSH26 and the mutants CSH26ΔQ. (*pho*Q) and CSH26ΔPQ (*pho*PQ) were kindly provide by Dr. R.T. Sauer 1996 (Waldburger and Sauer, 1996). Derivatives of these strains containing a chromosomal *ais*::*lux*AB transcription gene fusion were generated by transduction with P1 *cml,clr100* using lysates prepared on *E. coli* LF20110 (Miller, 1972). *E. coli* strain DH5α (Woodcock et al., 1989) was used for transforming all DNA ligation reactions and during amplification/isolation of plasmid DNA. *E. coli* strains were propagated at 32°C, unless otherwise stated, in LB broth (10 g NaCl, 10 g tryptone, 5 g yeast extract per litre) or on LB plates containing 1.5% agar (Sambrook et al., 1989) and supplemented with antibiotics as indicated. Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada), 40 μg/ml; Cm (Sigma, St. Louis, Missouri), 50 μg/ml; Gm (Sigma, St. Louis, Missouri) 15 μg/ml., and Tc (Boehringer Mannheim, Laval, Canada) 10 μg/ml in broth, 20 μg/ml in agar. Stock solutions of metal salts were prepared in sterile distilled deionized water at the following concentrations: aluminum chloride (20.1 mg/ml) (Anachemia, Montreal, Canada), magnesium chloride (418.3 mg/ml) (BDH, Montreal, Canada), calcium chloride (182.7 mg/ml) (Anachemia, Montreal, Canada). Where necessary, the pH of LB was adjusted with 12 M HCl or 2 N NaOH.

3.3.2 DNA manipulations

Restriction endonuclease hydrolyses were performed according to the manufacturers' protocol (Gibco-BRL, Burlington, Canada; New England Biolabs, Ltd.,

Figure 3.1 Construction of plasmids used in this study.

Plasmid	Markers	*bas*RS Genotype
pSU25RS	Ap^R	R^+S^+
pDAL71	Cm^R	R^+S^+
pDAL73	Cm^R Gm^R	R^+S^-
pKD73	Cm^R Gm^R Suc^S	R^+S^-
pDAL75	Cm^R Gm^R	R^-S^-
pKD75	Cm^R Gm^R Suc^S	R^-S^-



B = *Bcl*I
C = *Hin*cII
H = *Hpa*I
N = *Nru*I
V = *Eco*RV
G = Gentamicin resistance cassette

volume of freshly prepared metal salt stock solution was added to the other flasks to reach the desired final concentration. Flasks were subsequently incubated at room temperature. At regular intervals, cells were removed and diluted to an A_{550} of 0.05 in LB broth. Luminescence of triplicate 1 mL samples was measured with a Tropix Optocomp I Luminometer (MGM Instruments, Connecticut). Samples were automatically injected with 100 μL of a decyl aldehyde solution (0.5% v/v in LB broth) and light emitted during a ten second period was recorded. When measuring the effect of pH on luminescence, this method was modified such that the second growth step was omitted. Cells were grown to an A_{550} of 0.4 in LB (pH 7) and then directly diluted to an A_{550} of 0.05 in LB of the desired pH.

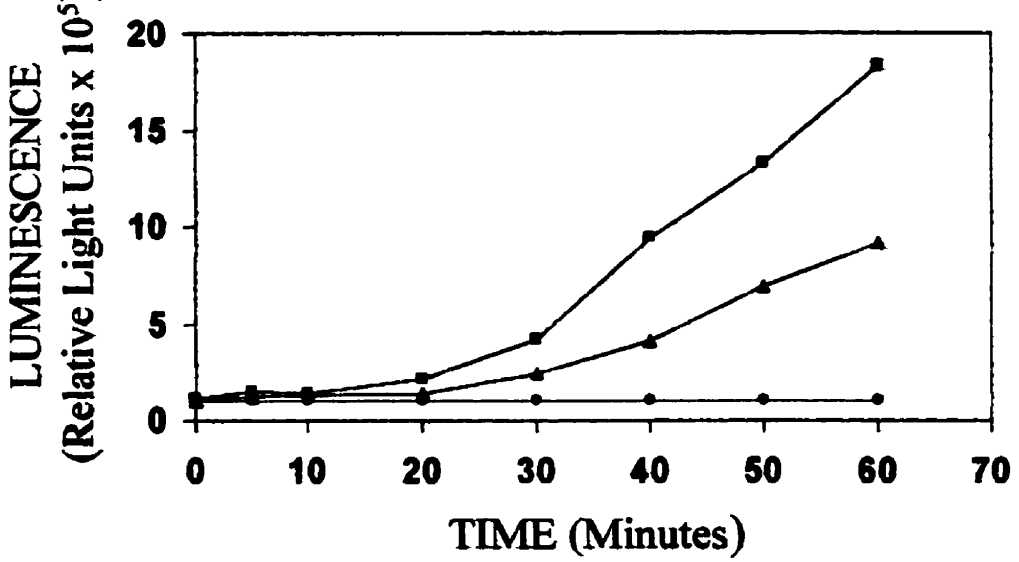
3.4 Results

3.4.1 BasS is required for aluminum–inducible expression of *ais*::*lux*AB

Luminescence of strains MG:ais, MG:aisΔS and MG:aisΔRS was measured upon addition of 0, 10 or 20 μg/ml aluminum (Fig. 3.2). Light emission from MG:ais increased with increasing concentrations of added aluminum (Fig. 3.2A). However, strains MG:aisΔS and MG:aisΔRS showed no increase in luminescence upon addition of exogenous aluminum (Figs. 3.2 B, C). Next, strains MG:aisΔS and MG:aisΔRS were transformed with the *bas*RS–containing pDAL71 or the pBAD33 vector control and luminescence was measured (Fig. 3.3). Upon addition of 0, 10 or 20 μg/ml aluminum, strains transformed with pBAD33 showed no increase in luminescence. However, a

Figure 3.2: Measurement of luminescence from strains (A) MG ais, (B) MG ais Δ*bas*S and (C) MG ais Δ*bas*RS upon addition of 0 μg/ml (●), 10 μg/ml (▲), or 20 μg/ml (■) aluminum. Standard error is less than 1%, hence error bars are not shown.

A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(Relative Light Units x 10^5)
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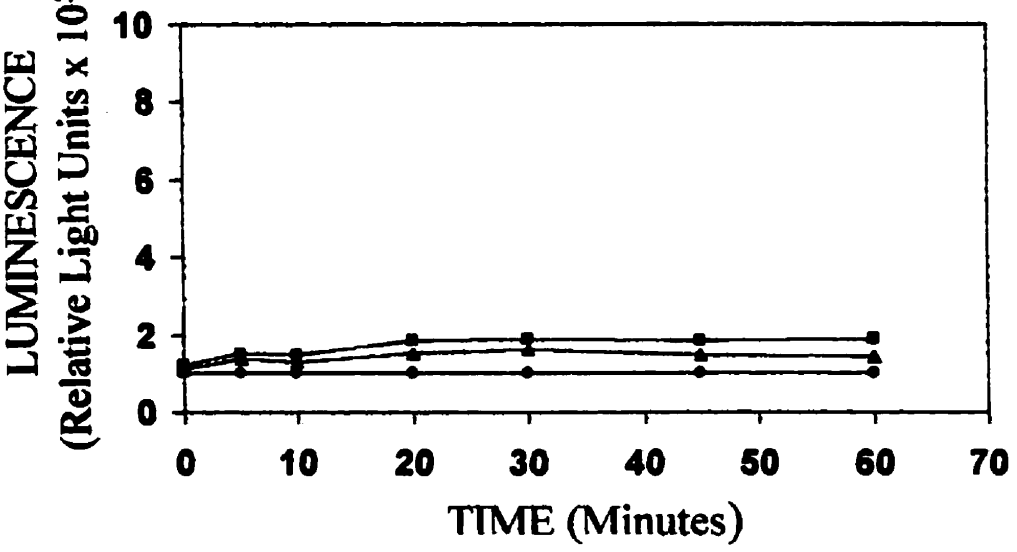
15
10
5
0
0
10
20
30
40
50
60
70
TIME (Minutes)

B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4
2
0
0
10
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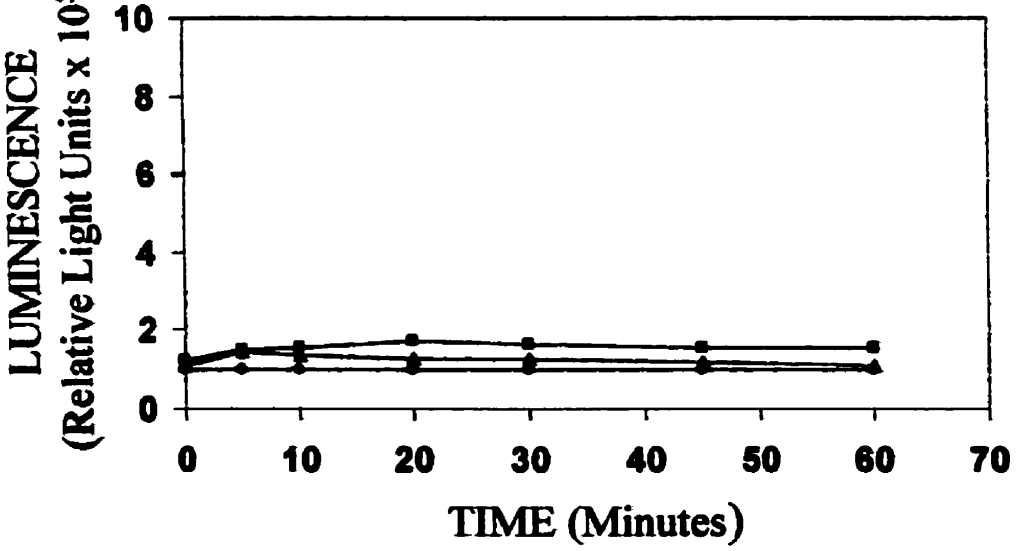
30
40
50
60
70
TIME (Minutes)

C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(Relative Light Units x 10^5)
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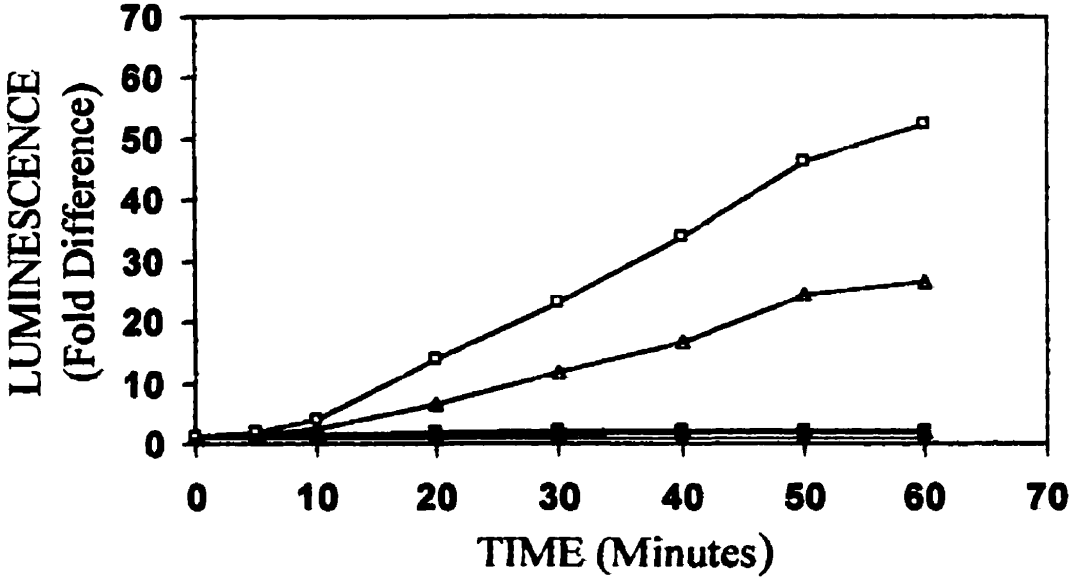
8
6
4
2
0
0
10
20
30
40
50
60
70
TIME (Minutes)

Figure 3.3: (A) Measurement of luminescence from strains MG ais Δ*bas*S:pBAD33 (open shapes) and MG ais Δ*bas*S:pDAL71 (closed shapes) upon addition of 0 μg/ml (circle), 10 μg/ml (triangle), or 20 μg/ml (square) aluminum. (B) Measurement of luminescence produced by strains MG ais Δ*bas*RS:pBAD33 (open shapes) and MG ais Δ*bas*RS:pDAL71 (closed shapes) upon addition of 0 μg/ml (circle), 10 μg/ml (triangle), or 20 μg/ml (square) aluminum. Standard error is less than 1%, hence error bars are not shown.

A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(Fold Difference)
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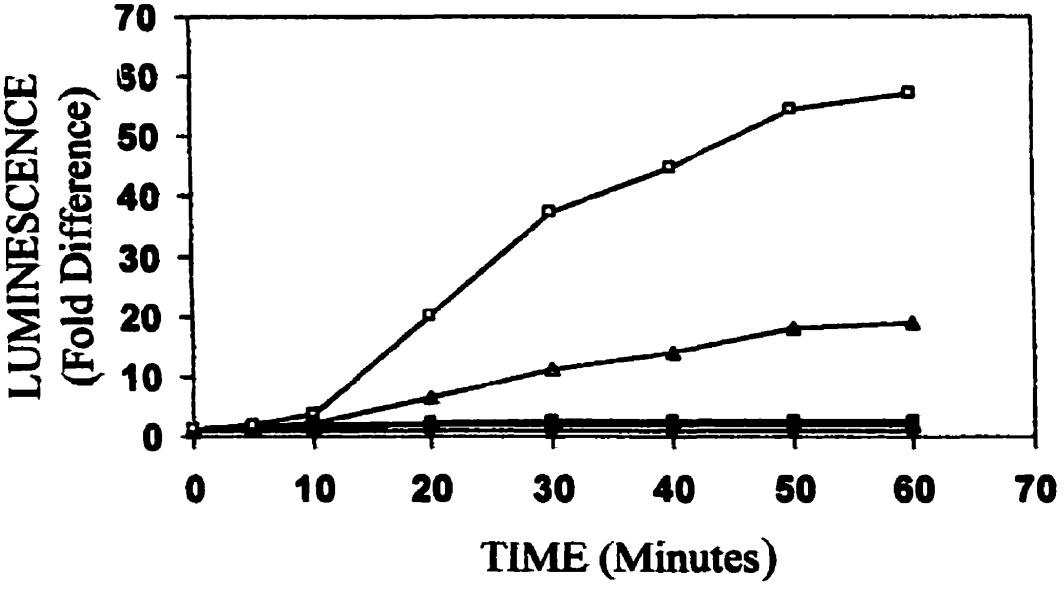
50
40
30
20
10
0
0
10
20
30
40
50
60
70
TIME (Minutes)

B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(Fold Difference)
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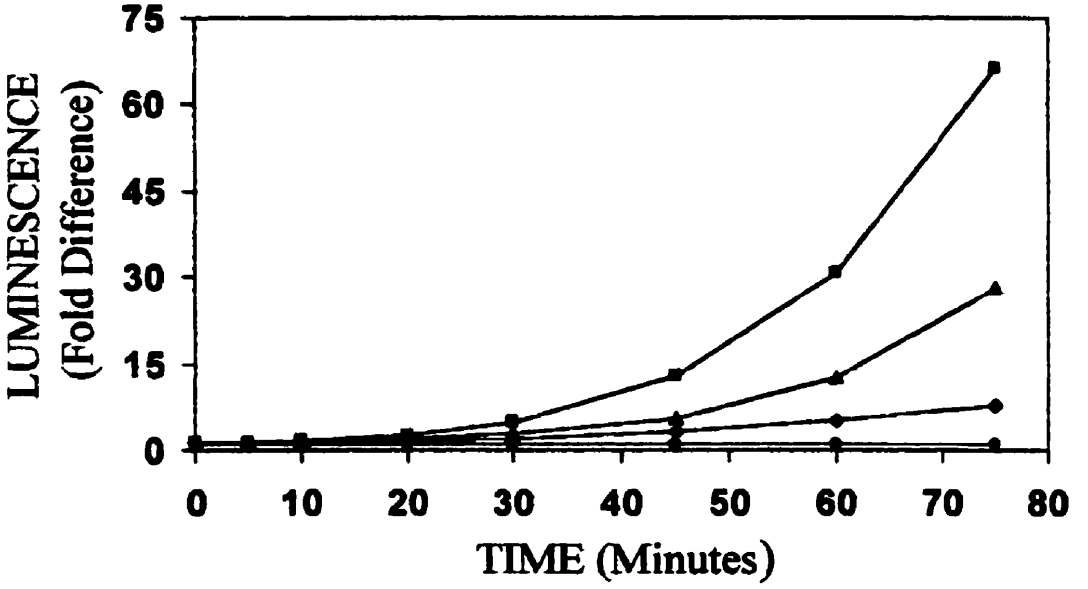
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
TIME (Minutes)

Figure 3.4 Measurement of luminescence from strains (A) CSH26, (B) CSH26 ΔQ and (C) CSH26 ΔPQ upon addition of 0 μg/ml (●), 5 μg/ml (◆), 10 μg/ml (▲), or 20 μg/ml (■) aluminum. Standard error is less than 1%, hence error bars are not shown.

A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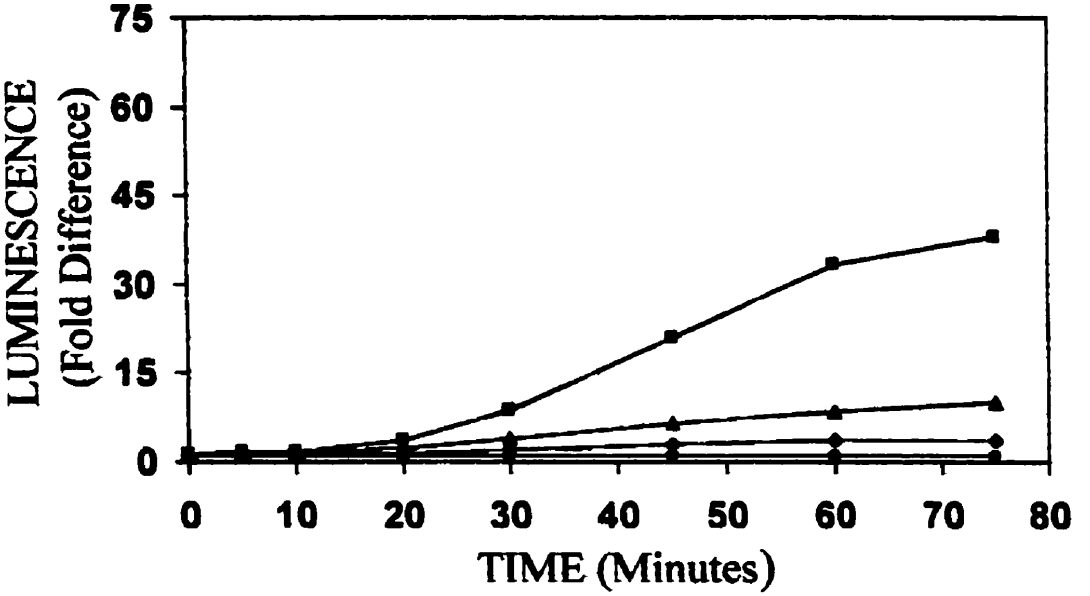
40
50
60
70
80
TIME (Minutes)

B


LUMINESCENCE
(Fold Difference)
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15
0
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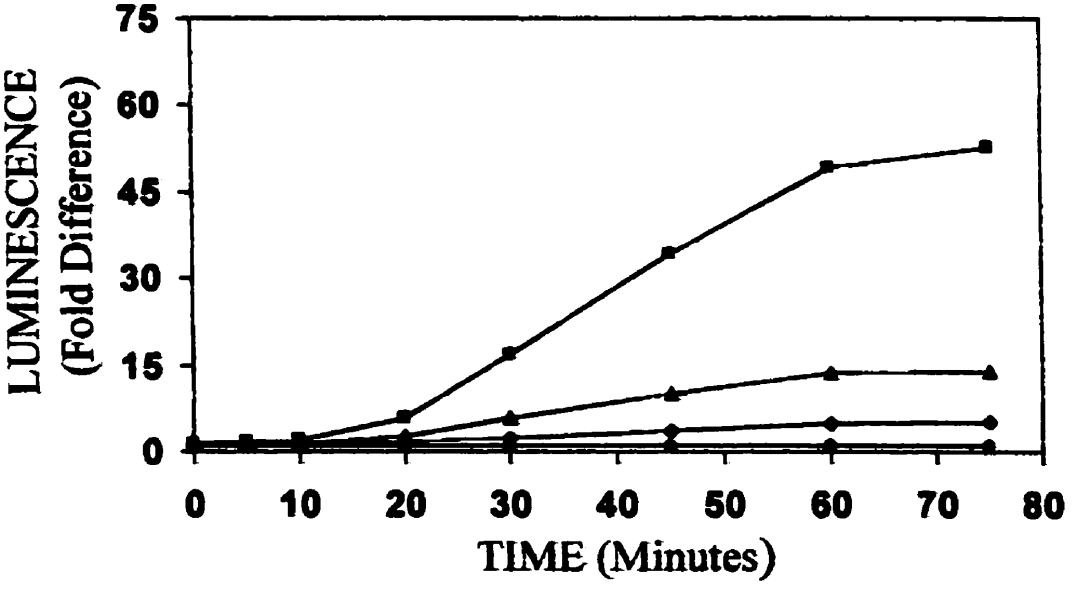
10
20
30
40
50
60
70
80
TIME (Minutes)

C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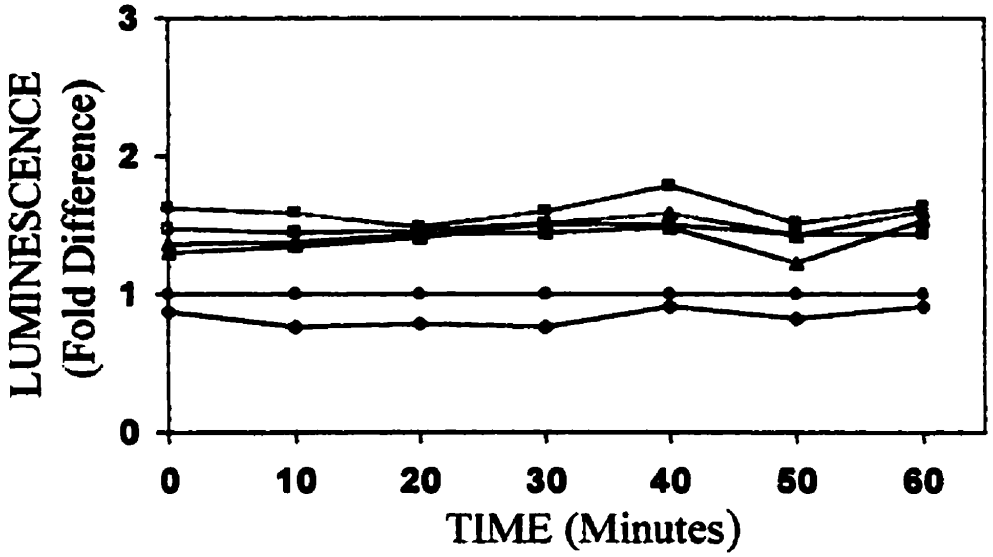
30
15
0
0
10
20
30
40
50
60
70
80
TIME (Minutes)

A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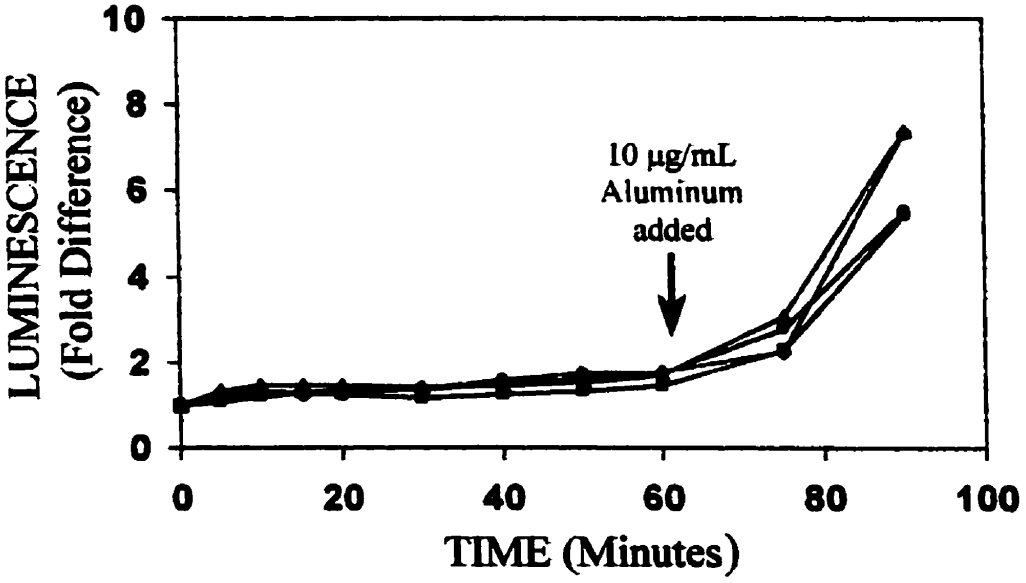
2
1
0
0
10
20
30
40
50
60
TIME (Minutes)

B


LUMINESCENCE
(Fold Difference)
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8
6
4
2
0
10 µg/mL
Aluminum
added
0
20
40
60
80
100
TIME (Minutes)

minutes. Thirty minutes after addition of aluminum, light emission from all four samples increased, but the pH 5.5 and pH 6 samples were emitting much more light than the pH 7 or pH 8 samples (Fig 3.5). This indicates that aluminum induces *ais*::*lux*AB, but extracellular proton concentration does not. However, the solubility, and therefore bioavailability, of aluminum increases as pH decreases. As such, pH may indirectly influence *ais*::*lux*AB expression by increasing the solubility, and/or altering the speciation, of aluminum.

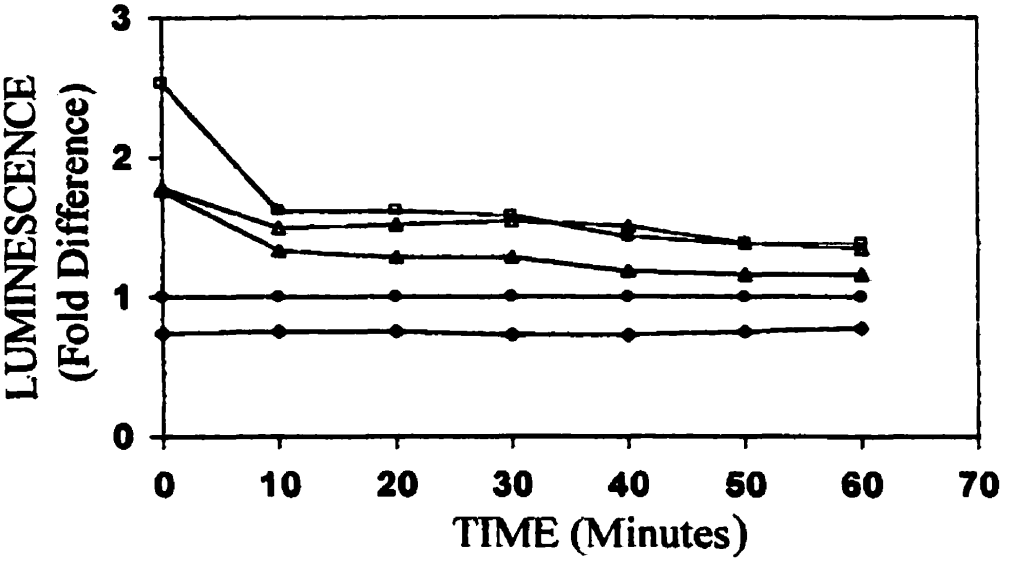
Because the effect of pH on background expression of *ais*::*lux*AB was reproducible, we also examined the effect of pH on expression of two other *E. coli* chromosomal *lux*AB fusions; the constitutively expressed *ars*R::*lux*AB fusion and the arsenic–inducible *ars*B::*lux*AB fusion (Cai and DuBow, 1996). In both cases, basal luminescence was greater under acidic conditions than under neutral or basic conditions (Fig. 3.6). As such, our observations suggest that the luciferase reaction is affected by the extracellular pH.

3.4.4 Effect of divalent cations on expression of *ais*::*lux*AB

In *Salmonella enterica*, PhoP–PhoQ mediates expression of PmrA–PmrB (and therefore PmrA–regulated genes) in response to magnesium, calcium and manganese concentrations. To determine if this effect also occurs in *E. coli*, luminescence of strain MG:ais was measured upon addition of 0, 5, 100, or 250 μg/ml magnesium (Fig. 3.7A). Addition of magnesium had no effect on light emission. In particular, the higher concentrations of magnesium, which lead to decreased PhoP activity, and therefore diminish transcription of downstream genes, did not reduce luminescence below basal levels. Similar

Figure 3.6: Measurement of luminescence from (A) the constitutively luminescent strain, *E. coli* 40 *ars*R::*lux*AB and (B) the arsenic–inducible strain, *E. coli* 40 *ars*B::*lux*AB, at pH 5. (□), pH 6.0 (▲), pH 6.5 (Δ) pH 7.0 (●), and pH 8.0 (◆). Standard error is less than 1%, hence error bars are not shown.

A


LUMINESCENCE
(Fold Difference)
3
2
1
0
0
10
20
30
40
50
60
70
TIME (Minutes)

B

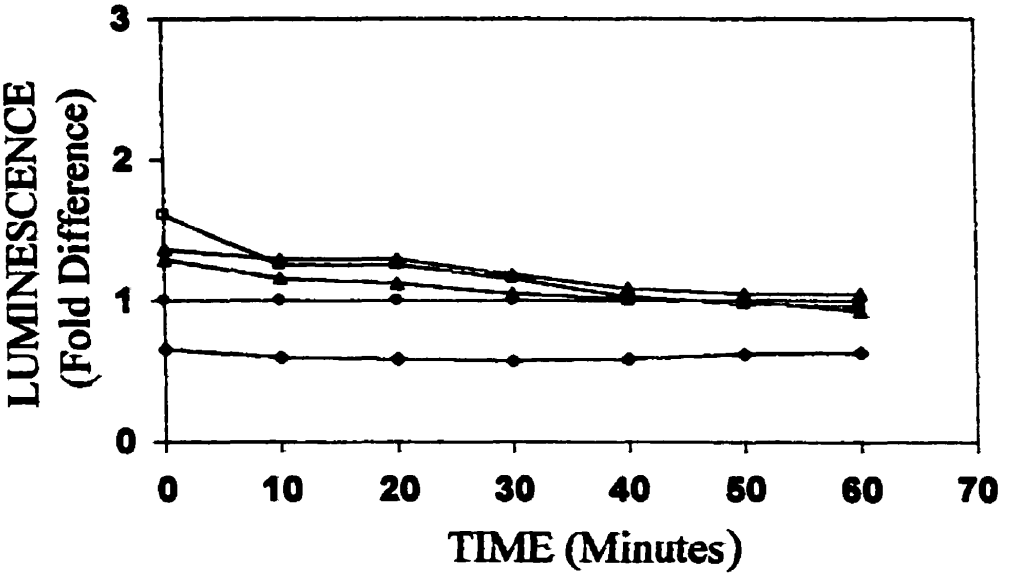
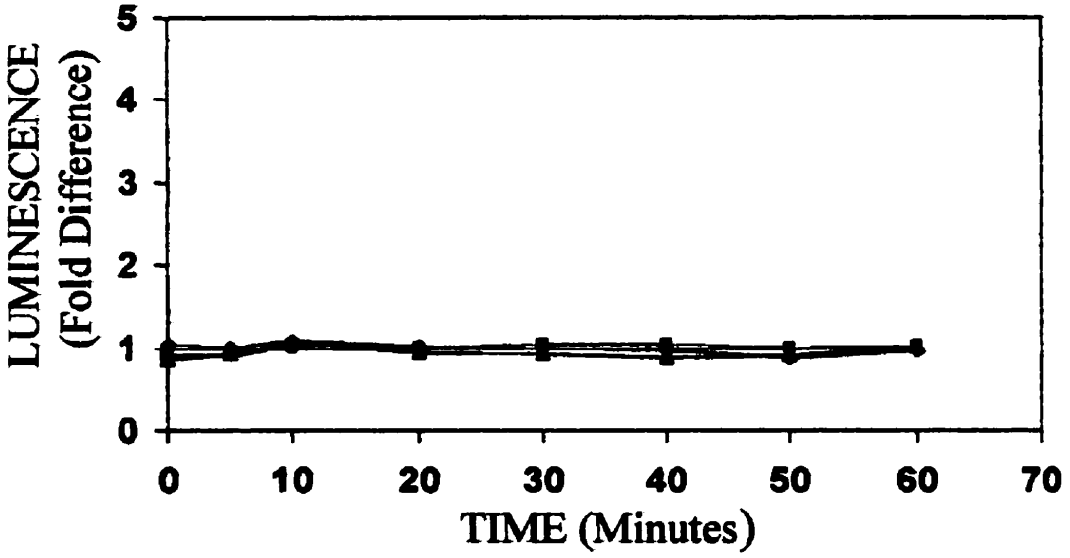

LUMINESCENCE
(Fold Difference)
3
2
1
0
0
10
20
30
40
50
60
70
TIME (Minutes)

Figure 3.7: (A) Measurement of luminescence from strain LF20110 upon the addition of 0 μg/ml (●), 5 μg/ml (◆), 100 μg/ml (▲), or 250 μg/ml (■) magnesium. Quenching of aluminum–induced luminescence by the addition of (B) magnesium or (C) calcium. 10 μg/ml aluminum was added along with 0 μg/ml (●), 10 μg/ml (◆), 100 μg/ml (▲), or 250 μg/ml (■) magnesium or calcium. Control sample to which no metal was added (O). Standard error is less than 1%, hence error bars are not shown.

A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(Fold Difference)
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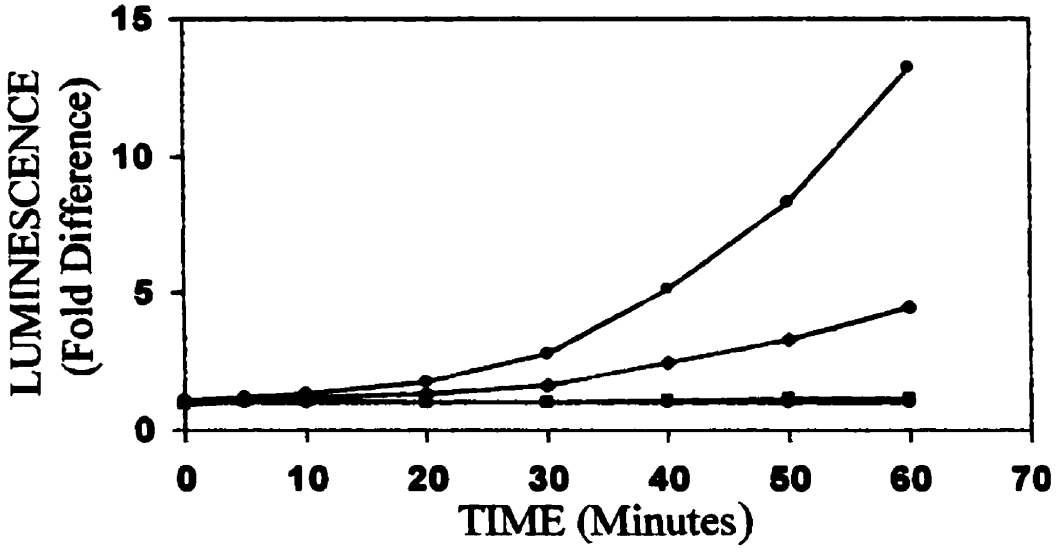
1
2
3
4
5
0
10
20
30
40
50
60
70
TIME (Minutes)

B


LUMINESCENCE
(Fold Difference)
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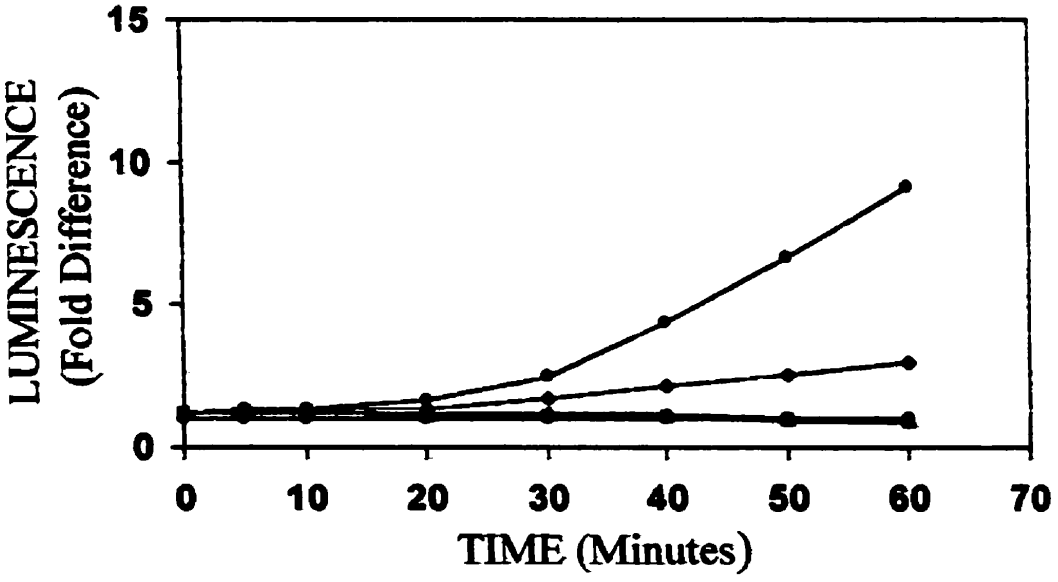
5
10
15
0
10
20
30
40
50
60
70
TIME (Minutes)

C


LUMINESCENCE
(Fold Difference)
0
5
10
15
0
10
20
30
40
50
60
70
TIME (Minutes)

together, AfrR and AfrS do induce *afr*A transcription. Based on homology data, we have suggested that Ais may act as a phosphatase. However, a function remains to be definitively established.

The best studied, and most closely related homolog is PmrG of *Salmonella enterica*. Although its function is also an enigma, transcriptional regulation of *pmr*G has been well established. Transcription of *pmr*G is directly mediated by the PmrA–PmrB two–component system (Wosten and Groisman, 1999). Transcription is initiated upon binding of the PmrA response regulator to a specific site, the PmrA Box, in the *pmr*G promoter region. This binding, and thus *pmr*G transcription, is enhanced by PmrB–mediated phosphorylation of PmrA. PmrB is a sensor kinase, which has been proposed to respond to changes in pH (Bearson et al., 1998).

An additional level of regulation is provided by the PhoP–PhoQ two–component system which acts upstream of PmrA–PmrB. The PhoP response regulator mediates expression of at least 20 genetic loci which are involved in diverse functions including magnesium acquisition and virulence (Bearson et al., 1998; Belden and Miller, 1994; Gunn and Miller, 1996) Its cognate sensor kinase, PhoQ, responds to Mg^{2+}, Ca^{2+} and Mn^{2+}. Under low (μM) but not high (mM) concentrations of these divalent cations, PhoP induces expression of PmrD which acts in concert with PmrA to enhances transcription of PmrA–PmrB regulated genes (Kox et al., 2000).

There is extensive homology between the PhoP–PhoQ/PmrA–PmrB polypeptides of *Salmonella enterica* and the PhoP–PhoQ/BasR–BasS polypeptides of *E. coli* (Table 3.1). Moreover, key nucleotide sequences are conserved in the promoter region of *pmr*G and *ais*

We previously observed that activity of the *ais*::*lux*AB fusion is increased under mildly acidic conditions. Others have noted similar effects on *ugd*::*lacZ*, *pbg*E::*lacZ*, *pbg*P::*lacZ*, *pmr*C::*lacZ* and *pmr*B::*lacZ* fusions (Groisman et al., 1997; Heithoff et al., 1999). However, when LF20110, grown at pH 7, was shifted to pH 5, 5.5, 6, 7 or 8, there was only a slight, albeit consistent, difference in basal luminescence (Fig. 3.5). Moreover, light emission did not increase over time. If aluminum was added to the cultures 60 minutes after the pH shift, there was a dramatic increase in luminescence. This increase was affected by pH, such that light emission increased with decreasing pH. Although it is possible that *ais* behaves differently than other genes in the regulon (to our knowledge, the effect of pH on a *pmr*G reporter gene fusion has not been reported), we believe that the effect of pH is indirect, and BasS does not monitor the extracellular proton concentration. The speciation and solubility of aluminum and iron are affected by pH. It is likely that increased activity of the *ais*::*lux*AB fusion under mildly acidic conditions is due to increased metal bioavailability and/or formation of an aluminum species that more readily interacts with BasS.

We have also examined the effect of pH on activity of a constitutively expressed *ars*R::*lux*AB fusion (Fig. 3.6A) and an arsenic–inducible *ars*B::*lux*AB fusion (Fig. 3.6B). In both cases, there was a slight but consistent increase in luminescence with decreasing pH. As such, the pH–associated change in basal luminescence is likely an artifact of the luciferase reaction. The quantity and oxidation state of the flavin mononucleotide mediates the rate of light production (Eckstein et al., 1993). We suspect that, especially above pH 7, the level of reduced flavin mononucleotide is limited and so luciferase–mediated luminescence is impaired.

Aluminum–inducible luminescnce was maintained in both *pho*Q and *pho*PQ mutant strains (Fig 3.4). This indicates that neither PhoQ nor PhoP is required for aluminum sensing or transcription of the *ais* gene. This is consistent with the finding by Zhou *et al.* (1999) that NH_4VO_3–induced *E. coli* lipid A modifications are unaffected by *pho*Q or *pho*PQ mutations. Although these mutations did not alter the pattern of light emission, they did diminish the magnitude of luminescnce. When compared to CSH26:ais, light emissions from CSH26ΔQ:ais and CSH26ΔPQ:ais were approximately 60% and 75% less, respectively (Fig 3.4). This suggests that PhoP–PhoQ does modulate activity of BasR–BasS in *E. coli*. However, the mechanism of this interaction, and the possible role of *E. coli* PmrD remains to be elucidated.

High concentrations of extracelluar Mg^{2+} and Ca^{2+} lead to diminished PhoP activity and reduced transcription of downstream genes (Vescovi et al., 1996; Castelli et al., 2000). As such, the effect of magnesium and calcium on *ais*::*lux*AB activity was examined. Addition of magnesium (Fig. 3.7A) or calcium (data not shown) in the absence of aluminum had no effect on luminescnce. However, when aluminum was added in combination with magnesium or calcium, quenching of aluminum–inducible light emission was observed (Figs. 3.7B, C). An hour after addition 10 μg/ml aluminum plus 10 μg/ml calcium (equivalent to 25 mM) or magnesium (equivalent to 41 mM), light production was only one third that of the aluminum only control. This is consistent with previous reports that expression of this regulon is inhibited by mM quantities of magnesium and calcium (Groisman et al., 1997). Addition of 100 μg/ml or more magnesium or calcium diminished luminescence to basal levels. This degree of quenching is more dramatic than the effects of

*pho*P and *pho*Q mutations, and suggests that added magnesium and calcium do more than inhibit PhoP activity.

The role of magnesium in PhoP–PhoQ intereaction has recently been dissected (Castelli et al., 2000). PhoQ has a constitutive kinase activiy that mediates phosphorylation and enhances the DNA binding activiy of PhoP. However, PhoQ also possesses a magnesium–dependent phosphatase activity that mediates dephosphorylation of PhoP. Under high (>200 μM) magnesium conditions, PhoQ phosphatase activity dominates such that most PhoP is dephosphorylated and there is little transcription of downstream genes. If the phosphatase activity of PhoQ is gratuitous, it might also dephosphorylate BasR and so diminish transcription of *ais*. Alternatively, magnesium or calcium may directly interact with BasS. BasS may behave in a manner similar to PhoQ such that metal binding (e.g. aluminum, iron or vanadium) shifts the balance between kinase and phosphatase activities. Because these metals enhance transcription of BasR–BasS–dependent genes, they would have to inhibit phosphatase activity and/or augment kinase activity. The former is more likely as aluminum has been shown to alter phosphatase activity and vanadium is a known inhibitor of protein tyrosine phosphatases, acid and alkaline phosphatase, and phophatases involved with gluconeogenesis (Stankiewicz and Gresser, 1988; Stankiewicz et al., 1995). Magnesium and calcium may compete with aluminum, iron and vanadium for BasS binding such that, under high magnesium and calcium conditions, phosphatase activity is not inhibited. Studies on mammalian ATPases have revealed the interaction of calcium and magnesium with aluminum and vanadium. For example, $AlCl_3$ inhibits ATP hydrolysis activity of the Mg^{2+}–dependent Na^+, K^+–ATPase from rat synaptosomes in a dose dependent manner. $AlCl_3$ does not affect Na^+ or K^+ binding or alter the dissociation constant

for ATP. However, it does change the dissociation constant for Mg^{2+}, indicating aluminum disrupts the interaction of Mg^{2+} and the ATPase (Caspers et al., 1994). Vanadium inhibits all E_1E_2-type ATPases, including Na^+, K^+-ATPases and Ca^{2+}, Mg^{2+}-ATPases (Himpens et al., 1991; Stankiewicz et al., 1995). The mechanism for this has not been confirmed, but vanadyl (vanadium(IV)), can behave as a divalent cation (Stankiewicz and Tracey, 1995). The direct interaction of magnesium and calcium with BasS would also explain why the divalent cations inhibit aluminum-induced expression of *ais*::*lux*AB in *pho*P and *pho*Q deleted strains

We have examined the effects of PhoPQ. BasRS, pH, magnesium and calcium, on expression of a chromosomal *ais*::*lux*AB transcriptional fusion gene in *E. coli.* In general, our findings confirm that the *Salmonella* and *E. coli* systems are analogous: BasR-BasS regulate *ais* transcription as PmrA-PmrB regulates *pmr*G. Although not essential for *ais* expression, PhoP-PhoQ can modulate the level of *ais*::*lux*AB activity.

Unlike Groisman *et al.* (1997) we do not find that pH induces expression under either high or low magnesium conditions. However, we do find aluminum-mediated *ais*::*lux*AB activity is enhanced at lower pH. This is most likely due to the increased solubility of aluminum under acidic conditions. In part, the apparent differences may be due to the different techniques used. Groisman *et al* (1997) used *lac*Z fusions and monitored β-galactosidase activity at a single time point, while we used luciferase fusions and followed a time course.

The role of metals in BasR-BasS/Ais expression has not been completely resolved. We believe that BasS senses metals, including iron, aluminum and vanadium. However, at high concentrations, magnesium and calcium seem to interact, or interfere with BasS. Work

with isolated BasR and BasS proteins and construction of additional mutants will allow us to better understand the role of metals in BasR–BasS signalling. In this manner we hope to unravel the mysteries of this enigmatic regulon.

Chapter 4

Identification of genes regulated by the BasR–BasS two–component regulatory system of *Escherichia coli*

Preface to Chapter 4

In Chapter 3, the *Escherichia coli* BasR–BasS two–component regulatory system was examined. It was demonstrated that BasR–BasS mediates expression of the *ais* gene. In Chapter 4, the search for novel members of the BasR–BasS regulon is described.

4.1 Abstract

The *bas*RS (bacterial adaptive response) genes of *Escherichia coli* encode a two–component regulatory system. Although it has been demonstrated that BasR–BasS are required for metal–inducible expression of the *ais* gene, other members of this regulon have not been described. To identify additional BasR–BasS regulated genes, a Mu*dI* (*lac* Ap) transcriptional gene fusion library was constructed. The β–galactosidase activity of more than 7000 clones was monitored in the presence and absence of BasR–BasS, as expressed from the sugar–controlled P_{BAD} promoter. Clones exhibiting altered β–galactosidase activity upon overexpression of BasR–BasS were isolated. For each of these clones, the right end of the Mu*dI* (*lac* Ap) gene fusion was subcloned and sequenced. Gene fusions to *blc*, *iap*, *dsd*XA, *glm*US and *yg*IJK were isolated. Four additional genes, *set*C, *yib*D, *yhh*T and *yaf*B, were identified via a computer–assisted search of the *Escherichia coli* genome database for 'bas box' consensus sequences. In total, nine putative members of the BasR–BasS regulon were identified. Most are associated with sugar metabolism and cell membrane functions.

4.2 Introduction

The *bas*S gene of *Escherichia coli* was isolated during a search for factors that could complement a deficit in the kinase domain of the EnvZ osmosensor (Nagasawa et al., 1993). DNA sequencing of *bas*S revealed the presence of its upstream partner, *bas*R. BasR–BasS were immediately recognized as a two–component regulatory system. BasS is a sensor kinase and EnvZ homolog, while BasR is a transcriptional activator and OmpR homolog. Transfer of ^{32}P from radiolabelled BasS to BasR, a hallmark of response–regulator pairs, has been demonstrated *in vitro* (Nagasawa et al., 1993). Our previous work suggests that BasS senses metals, such as iron and aluminum. We have also demonstrated that metal–inducible transcription of *ais* requires *bas*RS. However, BasR–BasS, and the genes that they regulate, remain largely uncharacterized. The highly homologous PmrA–PmrB system of *Salmonella enterica* serovar Typhimurium has been studied more extensively. The five members of the *pmr*AB regulon were discovered during searches for PhoP–PhoQ regulated genes and loci involved with antimicrobial peptide resistance. Only later was the relationship between these five genes and PmrA–PmrB recognized and confirmed. To date, a focussed search for additional loci has not been attempted.

Herein, we report the identification of novel members of the BasR–BasS regulon in *E. coli*. Two methods were used to find these genes: i) a Mud*I* (*lac*, Ap) gene fusion library was constructed and screened and ii) the complete *Escherichia coli* genome sequence database was analyzed via a computer–assisted search.

4.3 Materials and Methods

4.3.1 Bacterial strains and media

E. coli strain 40 [*Δpro–lac rpsL trp*] was used in construction of the Mud*I* (*lac* Ap) gene fusion library. *E. coli* strain DH5α [*supE44 ΔU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi–1 relA1*] (Woodcock et al., 1989) was used for transformation of all DNA ligation reactions and for amplification and purification of plasmid DNA. *E. coli* strains were propagated at 32°C, unless otherwise stated, in LB broth (10 g NaCl, 10 g tryptone, 5 g yeast extract per litre) or on LB plates containing 1.5% agar (Sambrook et al., 1989). Media were supplemented with antibiotics or sugars as indicated. Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada), 40 μg/ml; Cm (Sigma, St. Louis, Missouri), 50 μg/ml; Km (Sigma, St. Louis, Missouri), 50 μg/ml; and Tc (Boehringer Mannheim, Laval, Canada) 10 μg/ml in broth, 20 μg/ml in agar. Glucose and sucrose, prepared as 20% stock solutions, were used at final concentrations of 0.2%. Xgal (5–bromo–4–chloro–3–indolyl–β–D–galactopyranoside), prepared at 20 mg/mL in N',N'–dimethyl formamide (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin), was used at a final concentration of 40 μg/ml.

4.3.2 DNA manipulations

Chromosomal and plasmid DNA was isolated as previously described (Autexier and DuBow, 1992). Restriction endonuclease hydrolyses were performed according to the manufacturers' protocol (Gibco-BRL, Burlington, Canada; New England Biolabs, Ltd., Mississauga, Canada) using 3 units of enzyme per μg DNA. DNA ligations were performed at 15°C for 18 hours following the manufacturer's protocol (Gibco-BRL, Burlington, Canada). DNA was subjected to electrophoresis in 0.7% TAE agarose gels (Sambrook et al. 1989). Size-selected DNA fragments were purified using the Geneclean II kit (Bio 101, Mississauga, Canada). DNA transformations were performed according to standard methods using $CaCl_2$ or $RbCl_2$ competent cells (Hanahan, 1983). DNA sequencing was performed with the Sequenase II product (Bio101, Mississauga, Canada). Alternatively, DNA sequencing was performed by the Sheldon Biotechnology DNA Sequencing Facility (McGill University, Montreal, Canada), the Core DNA Sequencing Facility (Queens University, Kingston, Canada) or the DNA Sequencing Facility at The Hospital for Sick Children (Toronto, Canada). Forward and reverse M13 primers were used for the DNA sequencing of pUC119–based vectors. The clockwise (#1233) and counterclockwise (#1247) *Bam*HI site primers (New England Biolabs Ltd., Mississauga, Canada), which flank the *Bam*HI site of pBR322, were used for DNA sequencing of pBR322–based vectors.

4.3.3 Plasmids and plasmid construction

Construction of pDAL71, for expression of BasR–BasS, has been described previously (see Chapter 3). Briefly, a *Hin*cII–*Hpa*I fragment of pSU25RS (Nagasawa et al., 1993) containing *bas*RS was ligated to *Sma*I linearized pBAD33 (Guzman et al., 1995).

In pDAL71, the *bas*R gene is proximal to the P_{BAD} promoter. Transcription from the P_{BAD} promoter is induced by arabinose and repressed by glucose. Plasmid pACYC177 (Chang and Cohen, 1978), which has the same p15 origin as pBAD33 and pDAL71, but a different antibiotic resistance profile, was used for vector replacement experiments. Plasmid pBR322 (Bolivar et al., 1977) was used for initial isolation of chromosomal Mu*d*I (*lac* Ap) gene fusions. Some gene fusions were subsequently subcloned into pUC119.

4.3.4 Construction of the Mu*d*I (*lac* Ap) gene fusion library

E. coli 40, transformed with the BasR–BasS expression vector, pDAL71, was used for construction of the Mu*d*I (*lac* Ap) gene fusion library. Generation of Mu lysogens has been described previously (Casadaban and Cohen, 1979). Briefly, recipient cells, grown to early stationary phase in LB broth supplemented with 10 mM $MgSO_4$ and 5 mM $CaCl_2$, were combined with Mu*d*I MAL103 lysate at a multiplicity of infection of 1 pfu/cell. After 20 minutes, cells were diluted 1:10 in fresh LB broth and incubated, with shaking, at 32°C for 30 minutes. Then, cells were plated onto LB agar containing Cm (for maintenance of pDAL71) and Ap (for selection of Mu*d*I (*lac* Ap) lysogens). After 24 hours of incubation at 32°C, transductants were 'mastered', (i.e. individual colonies were picked and plated on a grid, 48 distinct colonies per Petri plate) in triplicate on LB agar containing Cm and Ap. Two of these 'master plates' were required for subsequent experiments and the third was stored at 4°C.

4.3.5 Screening of the MudI (*lac* Ap) gene fusion library

Colonies from a master plate were replica plated on to LB agar containing Cm, Ap, Xgal, and either 0.2% arabinose, 0.2% glucose, or no added sugar. After 24 and 48 hours of incubation, β–galactosidase production was compared. Positive clones, which exhibited differences in β–galactosidase production on the arabinose (i.e. BasR–BasS over–expressed) versus glucose (i.e. BasR–BasS expression repressed) plates were subjected to additional rounds of screening. To begin the second round of screening, each positive clone was streaked (using cells taken from a second master plate) onto LB agar containing Cm and Ap. After incubation, several well isolated single colonies were plated on LB agar containing Cm, Ap, Xgal, and either arabinose, glucose, or no added sugar. Again, β–galactosidase production was compared. Clones that continued to exhibit a positive phenotype (i.e. a consistent difference in β–galactosidase production on arabinose vs. glucose plates) were subjected to a similar, third round of screening. Clones that remained positive after the third round of screening were next tested to ensure that differences in β–galactosidase activity were due to expression of BasR–BasS, and not arabinose or glucose. Colonies were made competent and transformed with pACYC177. Both pACYC177 and pDAL71 have a p15 origin of replication and are incompatible. Transformants were selected on LB agar containing Ap and Km, and then screened on media containing Ap and Cm, or Ap, Km and Cm. Clones exhibiting a Km^R (i.e. transformed with pACYC177) and Cm^S (i.e. loss of pDAL71) phenotype were screened on media containing Ap, Km, Xgal and either arabinose, glucose, or no added sugar. β–galactosidase activity was compared again. Clones that exhibited a difference in β–galactosidase activity, despite the loss of pDAL71, were deemed negative (i.e. arabinose

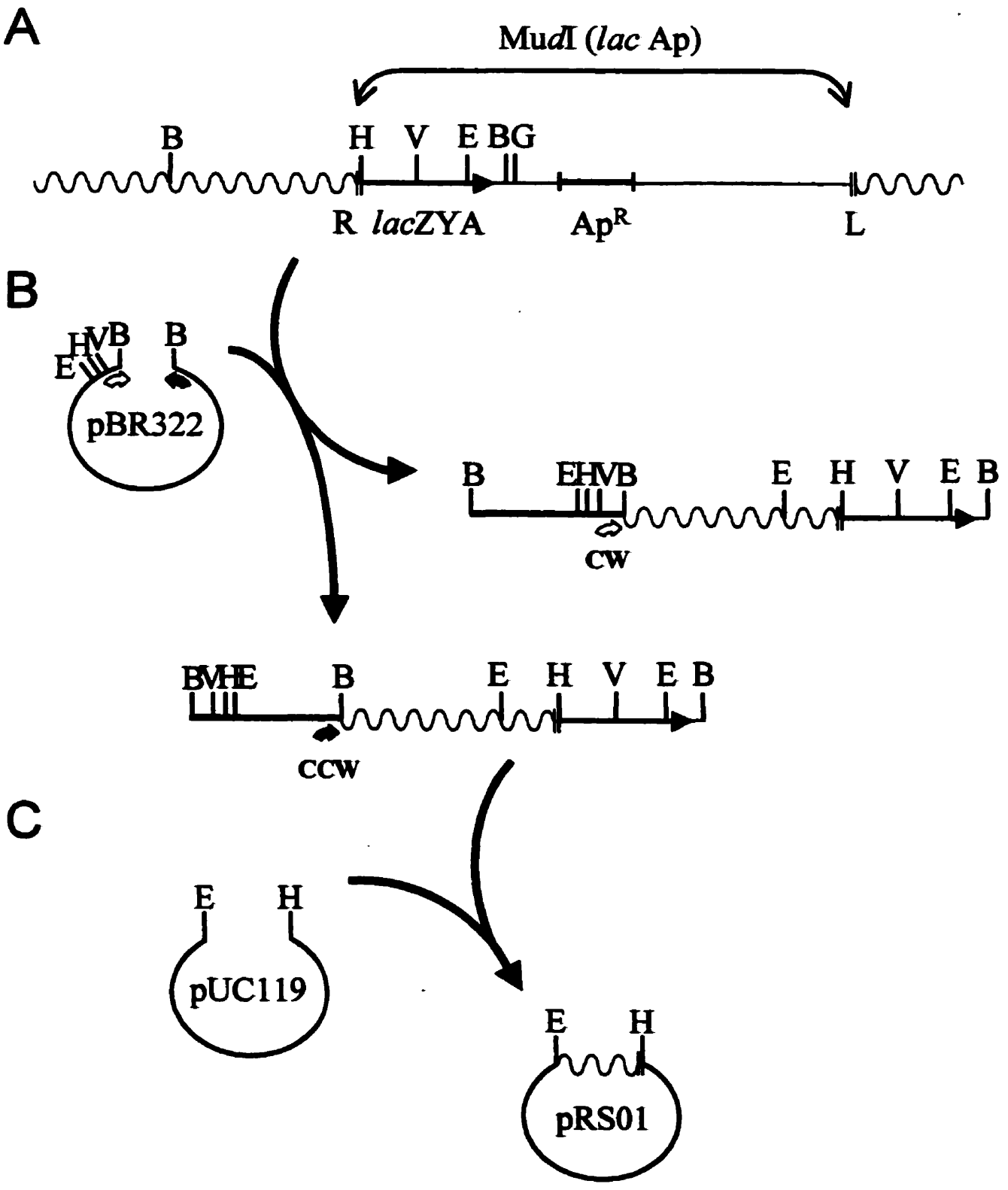
affected gene fusions). Clones that no longer exhibited a difference, such that altered β–galactosidase activity was lost with pDAL71, were deemed positive and retained for further analysis.

4.3.6 Isolation and sequencing of Mud*I* (*lac* Ap) gene fusions

Chromosomal DNA was isolated from positive clones, digested with either *Bam*HI or *Bgl*II and ligated to *Bam*HI–linearized pBR322. Both *Bam*HI and *Bgl*II cleave (approximately) 8 kb from the right end of Mud*I* (*lac* Ap). Although this region does not contain a selectable marker, it does include *lacZ*, which can be used as a differential marker (Diorio et al., 1995). Ligations were transformed into DH5α and plated on LB agar containing Ap and Xgal. Blue colonies were further analyzed. First, plasmid DNA was isolated and digested with a panel of restriction endonucleases. From the restriction map, the orientation of the insert with respect to pBR322, and the approximate size of DNA flanking the right end of Mud*I* (*lac* Ap), could be determined. Because of difficulties associated with sequencing through the right end of Mud*I* (*lac* Ap), the end of the insert DNA distal to the Mud*I* (*lac* Ap) fusion junction was sequenced first. Using the BLASTN algorithm, DNA sequence data was compared to the *E. coli* genome sequence database. If such analysis was insufficient to allow identification of the site of Mud*I* (*lac* Ap) insertion, or if the adjacent chromosomal region was large (> 8 kb), additional subcloning into pUC119, and DNA sequencing was performed.

Figure 4.1: Isolation and DNA sequencing of Mu*d* I (*lac* Ap) gene fusion clones

A) Insertion of Mu*d*I (*lac* Ap) into the *Escherichia coli* chromosome (wiggly line). The right (R) and left (L) ends of Mu*d*I, the *lacZYA* genes, the ampicillin resistance determinant (Ap^R), and restriction endonuclease sites for *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Eco*RV (V), and *Hin*dIII (H) are indicated. B) Subcloning of the Mu*d*I (*lac* Ap) gene fusion by cleavage of chromosomal DNA with *Bam*HI and ligation with *Bam*HI linearized pBR322. The chromosomal fragment, which contains the *lacZYA* genes, the right end of Mu*d*I, and adjacent chromosomal DNA, may be inserted into pBR322 in two orientations. Restriction mapping allows this orientation to be determined and dictates if the 'clockwise' (CW, open arrow) or 'counterclockwise' (CCW, closed arrow) primer is to be used for DNA sequencing. C) Subcloning of large inserts by cleavage of DNA with *Hin*dIII and another restriction endonuclease (in this case, *Eco*RI) and ligation to linearized pUC119.


A
MudI (lac Ap)
B
H V E B G
R lacZYA
Ap^R
L
B
E H V B B
pBR322
B E H V B
E H V E B
CW
B V H E B
E H V E B
CCW
C
E H
pUC119
E H
pRS01

4.3.7 Computer–assisted search for BasR–BasS regulated genes

The BLASTN algorithm was used to search the *E. coli* genome database for DNA sequences matching variations of the 'bas box'. The 'bas box' is the palindromic motif $^{5'}$TAGTTTCTTAAGGT$^{3'}$, found in the *ais* promoter region and potentially recognized by BasR. Sequences returned by the BLASTN algorithm were positioned on the *E. coli* genome and nearby ORFs were identified. An ORF was considered to be a potential member of the *bas*RS regulon if: i) the putative bas box was located appropriately to the gene's –10 and –35 promoter elements, and ii) BasR–BasS regulation was compatible with existing information about the gene.

4.4 Results

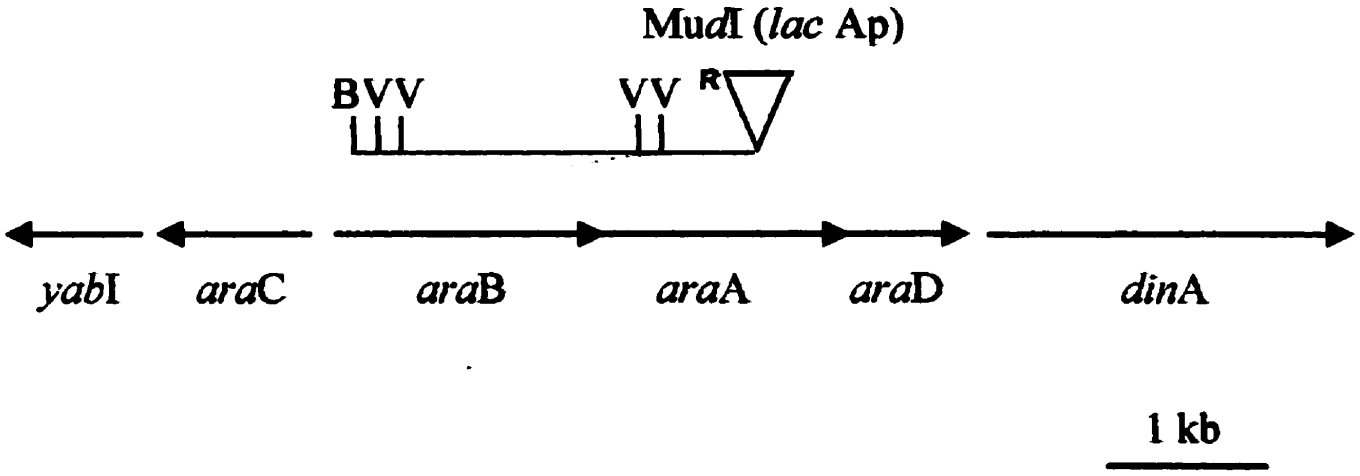
4.4.1 Construction and screening of the Mu*d*I (*lac* Ap) gene fusion library

A library of of more than 7000 Mu*d*I (*lac* Ap) gene fusion clones was constructed and screened. Initially, several hundred clones appeared to exhibit different β–galactosidase phenotypes on arabinose and glucose media. However, most of these proved to be mixed colonies and were eliminated during subsequent rounds of screening. After three rounds of screening, only 16 clones remained. These clones were transformed with pACYC177 such that the BasR–BasS expression vector, pDAL71, was eliminated. Upon screening for β–galactosidase activity, 11 of the 16 clones continued to show differences on arabinose and glucose media. The persistence of altered β–galactosidase activity, despite the absence of pDAL71, indicated that the gene fusion clones were responding directly to arabinose and/or

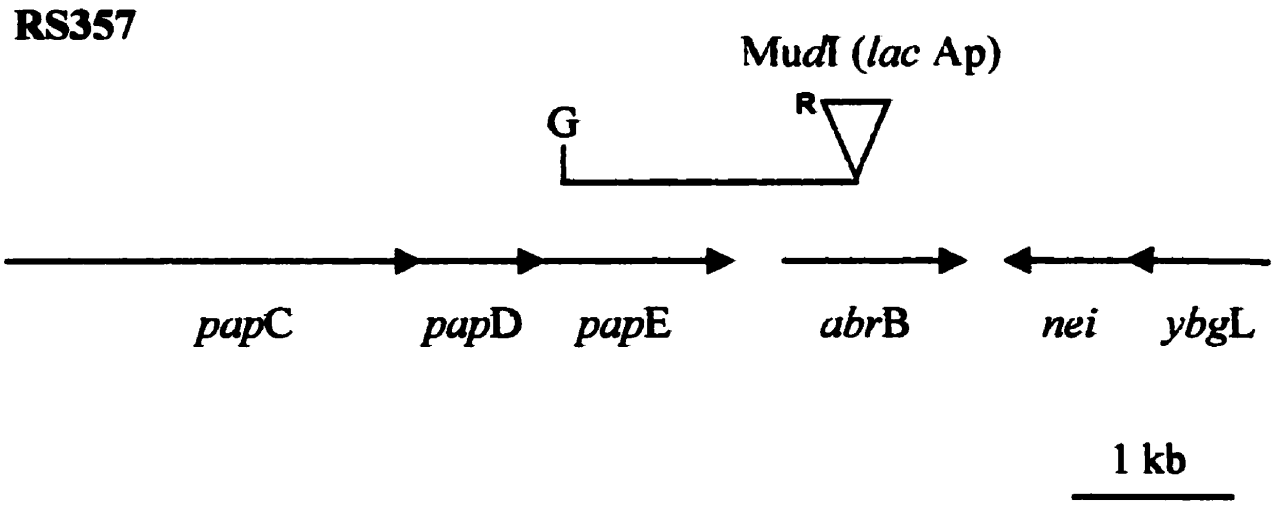
Figure 4.2: Mapping of Mu*d* I (*lac* Ap) gene fusion clones A) RS201, B) RS357, C) RS138, D) RS206, E) RS327, F) RS403, and G) RS411. The site of Mu*d* I (*lac* Ap) insertion, flanking genes, and the restriction map of the adjacent chromosomal region are indicated. The right end (R) of Mu*d* I (*lac* Ap) is denoted. The endonucleases *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Eco*RV (V), and *Hin*dIII (H) were used for restriction mapping.

A

RS201

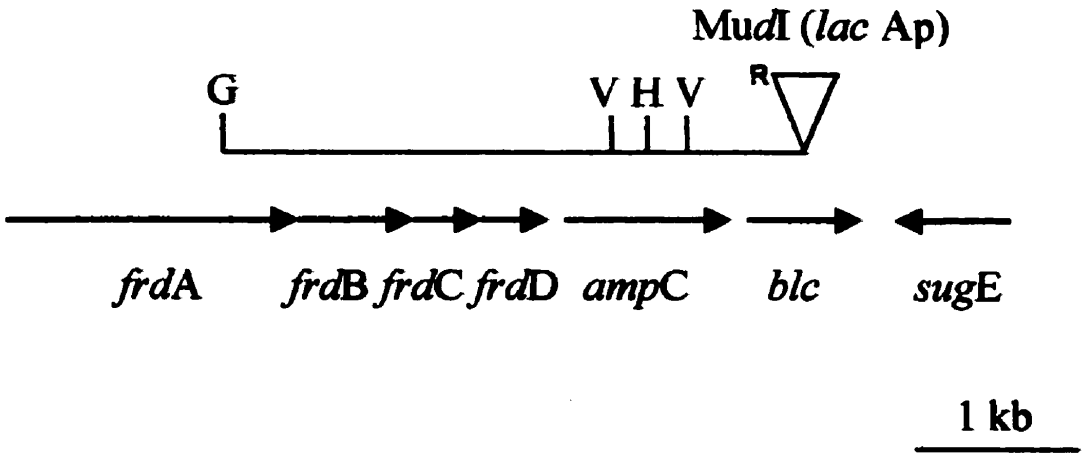


B



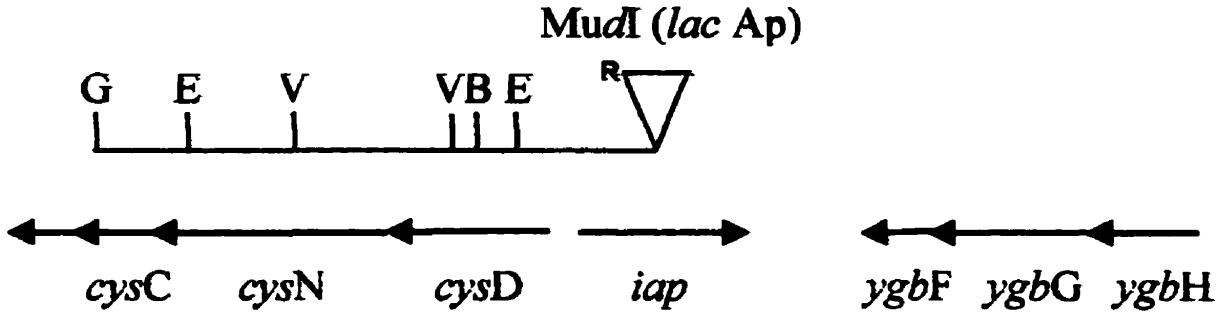
C

RS138



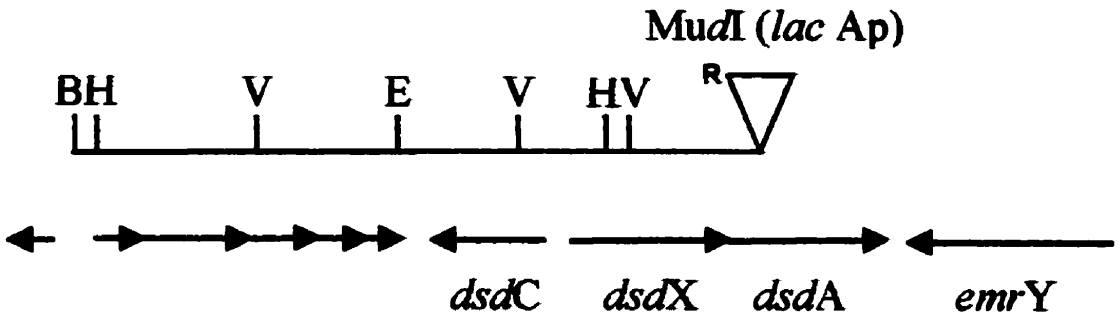
D

RS206



E

RS327



pUC119 linearized with the same enzymes. Initial attempts at sequencing the gene fusion junction, through the right end of Mu*dI* (*lac* Ap), failed. This is likely due to the extensive secondary structure in this region. As such, DNA was sequenced from the plasmid–insert junction and the end of the chromosomal fragment distal to the gene fusion junction was sequenced first (Fig. 4.1C)

The DNA sequencing data was used to query the *E. coli* genome database. A combination of sequence and restriction map data was used to identify the site of Mu*dI* (*lac* Ap) insertion (Fig. 4.2). Two clones, RS201 and RS357, were false positives. The Mu*dI* (*lac* Ap) insertions were localized to the AraC–regulated genes *ara*A (RS201) and *abr*B (RS357) (Figs. 4.2A, B). During replacement of pDAL71 by pACYC177, *ara*C is lost along with *bas*RS. As such, the screening process did not control for overexpression of AraC. The other insertions were localized to *blc* (RS138), *iap* (RS206), *dsd*XA (RS327), *glm*US (RS403) and *ygj*IJK (RS411) (Figs. 4.2C–G). Characterization of these clones is summarized in Table 4.1. The promoter regions were examined for the bas box sequence $^{5'}$TAGTTTCTTAAGGT$^{3'}$, but in no case was such a sequence found.

Table 4.1: Features of the Mud*I* (*lac* Ap) fusion clones.

Clone	Gene Name	Function	Position (Map Units)
RS201	*ara*A	arabinose isomerase	1.4
RS357	*abr*B	putative transcriptional regulator	16.3
RS138	*blc*	bacterial lipocalin	94.3
RS206	*iap*	alkaline phosphatase isozyme conversion	61.2
RS327	*dsd*A *dsd*X	serine dehydratase serine permease	53.6
RS403	*glm*U *glm*S	GlcNAc–1–phosphate uridyltransferase glucosamine synthase	84.3
RS411	*ygj*I *Ygj*J *ygj*K	membrane protein, putative antiporter unknown putative sugar isomerase	69.5

4.4.3 Computer-assisted search for BasR–BasS regulated genes

Dozens of *E. coli* genome sequences exhibited homology to variations of the bas box. However, most sequences were located within the coding region of an ORF, or not suitably positioned within the putative promoter region. Only seven sequences satisfied the search criteria (Table 4.2). Three of these: *ais*, orf b2253 and *ugd*, exhibit homology to PmrA–PmrB regulated genes from *Salmonella enterica* serovar Typhimurium. The remaining four include *set*C, a sugar efflux pump; *yib*D, a putative glycosyl transferase; *yhh*T, a putative transport protein; and *yaf*B, a putative aldose/ketose oxidoreductase.

4.5 Discussion

Using two different strategies we have identified nine putative members of the *bas*RS regulon. They are *blc*, *iap*, *dsd*XA, *glm*US, *set*C, and the uncharacterized *ygf*IJK, *yib*D, *yhh*T, and *yaf* genes. Previous work indicates that genes regulated by *E. coli* BasR–BasS (and/or the homologous *Salmonella enterica* PmrA–PmrB) are responsible for 4–aminoarabinose modifications of lipopolysaccharide. Most of these novel genes are associated with sugar metabolism and/or cell wall functions.

All four genes identified in the computer–assisted search fit this profile. The *set*C gene encodes a sugar efflux pump (Liu et al., 1999a). The pump is not active under standard laboratory conditions, and does not employ glucose, lactose or galactose as substrates (Liu et al., 1999b). However, arabinose utilization has not been examined. Although poorly characterized, *yib*D exhibits homology to glycosyl transferase genes associated with spore

coat and capsular polysaccharide biosynthesis. The *yhh*T gene product is likely a membrane transport protein, while *yaf*B is a putative aldose/ketose oxidoreductase

Isolates from the Mud*I* (*lac* Ap) gene fusion library included *glm*US, which is involved in N–acetylglucosamine and lipopolysaccharide synthesis, and *ygj*K, a putative sugar isomerase (Mengin-Lecreulx and van Heijenoort, 1994). The *blc* gene product, bacterial lipocalin, is an outer membrane protein. It is predicted to be a receptor protein, but its cognate ligand has not been identified (Bishop et al., 1995). The *iap* gene product is a periplasmic protease that cleaves alkaline phosphatase to generate several isozymes (Nakata et al., 1982; Ishino et al., 1987). Finally, *dsd*A encodes a serine dehydratase, and *dsd*X is a serine permease. The *dsd*AX fusion was the only one in which β–galactosidase activity decreased upon BasR–BasS overexpression.

Although the gene fusion clones all exhibited altered β–galactosidase activity upon overexpression of BasR–BasS, their regulation by the two–component system requires further analysis. None of these genes were identified during the computer–assisted search, and inspection of their promoter regions did not reveal bas box sequences. As such, these genes may not be regulated directly by BasR. Instead, their expression may reflect BasR–BasS mediated changes in the cell. For example, NagC regulates expression of *glm*US and other genes required for glucosamine and N–acetylglucosamine metabolism. During BasR–BasS mediated remodeling of lipopolysaccharide, N–acetylglucosamine is required. As such, it is not surprising that members of the NagC regulon are expressed. In fact, *glm*U is essential for lipopolysaccharide synthesis (Plumbridge, 1995).

Mud*I* (*lac* Ap) gene fusions to well characterized members of the *bas*RS regulon, such as *ais*, were not isolated. The possible reason for this is the small library size. *E. coli*

has only 4405 genes, but 7000 Mud*I* (*lac* Ap) insertions do not ensure complete coverage of the genome. Also, essential genes, which would be disrupted by Mud*I* (*lac* Ap) insertion, will be missed. Limitations inherent to the library construction scheme may also be at fault. For example, the host strain, *E. coli* 40, is both *bas*RS$^+$ and *ara*C$^+$. To minimize *bas*RS expression, cells were grown at pH 7 and in the absence of added metal. However, it is possible that residual expression of the host *bas*RS genes prevented identification of some BasR–BasS regulated fusion clones. Efforts were made to achieve tight regulation of plasmid–based *bas*RS expression. When constructing pDAL71, the P_{BAD} promoter and the low copy, P15–based pBAD33 vector were used instead of a high copy number, pUC–based vector or the P_{TRP} promoter. However, BasR–BasS expression from pDAL71 remains leaky. In other experiments, it has been demonstrated that pDAL71 can mediate luminescence of an *ais*::*lux*AB transcriptional gene fusion strain. Light emission is greatest in the presence of arabinose. Glucose diminishes *ais*::*lux*AB activity, but does not reduce luminescence to basal (i.e. no pDAL71) levels. The reason for this is unknown, but the *bas*RS promoter has not been well characterized and the pDAL71 construct may include *bas*RS promoter elements that mediate sugar–independent transcription. Despite these limitations, the isolation of known AraC–regulated genes (even in the *ara*C$^+$ background) indicates that the search strategy was effective.

Nine putative members of the BasR–BasS regulon have been identified. Although this effort marks the first attempt to characterize this regulon, additional work must be done. For example, activity of the Mud*I* (*lac* Ap) fusion clones must be monitored in a Δ*bas*RS background and, to eliminate any peculiarities of *E. coli* 40, in additional Δ*lac* strains. Most of the genes identified during the computer–assisted search are associated with sugar

Chapter 5

Characterization of a Triorganotin–Inducible Gene in *Escherichia coli*

Preface to Chapter 5

In Chapter 2, screening of the Tn*5*–*lux*AB library of *Escherichia coli* with aluminum resulted in isolation of LF20110, the *ais*::*lux*AB transcriptional fusion clone. Screening of the Tn*5*–*lux*AB library with tributyltin resulted in isolation of another *lux*AB transcriptional fusion clone. This is described in Chapter 5.

5.1 Abstract

Organotin compounds, such as tributyltin, are potent biocides. However, because of their toxic and undesirable effects on non–target organisms, widespread use of organotins has been challenged and, in some cases, restricted. Exposure to toxins can result in alterations to cell physiology. Frequently, this involves changes in the expression of specific genes. To identify genes whose expression is inducible by added organotin compounds, a library of *lux*AB chromosomal gene fusions was constructed in *Escherichia coli*, and luminescence was assayed in the absence and presence of added tributyltin. One clone showed increased luminescence upon addition of tributyltin, and subsequently by triethyltin and tri–n–propyltin, but not dibutyltin or tetrabutyltin. Cloning and sequencing of the junction of the *lux*AB insertion revealed that the triorganotin–inducible gene is *uhp*T, which encodes a hexose–6–phosphate transporter. RNA blotting analysis revealed that both glucose–6–phosphate and tributyltin induce transcription of *uhp*T. The role of UhpT in tributyltin metabolism is unclear, but expression of the sugar transporter may result from uncoupling of oxidative phosphorylation.

5.2 Introduction

Organotin compounds are effective biocides with a wide range of applications. Tributyltin is used in antifouling paints and as a preservative for wood and textiles. Dibutyltin is used to stabilize polyvinylchloride products, including plastic piping. Although useful, these compounds are an environmental hazard and devastate target and non–target organisms alike. The deleterious effects of organotins on both procaryotes and eucaryotes have been well documented (Boyer, 1989; Chang et al., 1992; Fent, 1996; Snoeij et al., 1987; White et al., 1999). They are associated with liver damage and thymus atrophy in rats (Boyer, 1989), and neurological damage in rabbits, mice, rats and humans (Chang et al., 1992). Organotin contamination of Stalinon, an antibacterial drug, is credited with the death of more than 100 people (Chang et al., 1992). However, the effects of organotins on aquatic organisms are especially dramatic. These organisms can bioaccumulate organotin to levels thousands of times higher than in the surrounding seawater (Takahashi et al., 1999). Molluscs, such as dogwhelks and mud snails, suffer reproductive problems, including the imposex phenomenon, in which females develop male sexual characteristics (Matthiessen and Sumpter; 1998). These effects have been observed at organotin concentrations as low as 1 ng/mL .

The interactions between organotins and microrganisms have been studied for decades (recently reviewed in White *et al.* 1999). It is known that organotins can interact with lipid bilayers, serve as halide/hydroxide exchangers (Antonenko, 1990), and inhibit ATPase activity. However, much remains to be elucidated about the mechanisms of

organotin toxicity and the effects of sublethal organotin concentrations on cell physiology and gene expression.

In an effort to characterize organotin-mediated effects, we searched for genetically–programmed responses to tributyltin exposure in *Escherichia coli*. A library of approximately 3000 *E. coli* clones, each containing the promoterless *Vibrio harveyi lux*AB genes in a single, random chromosomal location, was previously created (Guzzo and DuBow, 1991). This library was screened in the absence and presence of tributyltin and, by monitoring changes in transcription, as measured by changes in luminescence, three organotin-responsive clones were identified (Briscoe et al., 1995). Here, we present the characterization of one clone, designated TBT_1, which exhibits increased luminescence in the presence of increasing concentrations of tributyltin. TBT_1 also exhibits increased light emission upon addition of triethyltin and tri–n–propyltin, but not dibutyltin or tetrabutyltin. The Tn*5*–*lux*AB insertion has been localized to the *uhp*T gene, which encodes a hexose–6–phosphate transporter (Sonna, 1988). Transcription of *uhp*T, as measured by RNA blotting to the cloned gene, is induced by glucose–6–phosphate and tributyltin. The mechanism of *uhp*T induction remains to be clarified, but is associated with uncoupling of oxidative phosphorylation.

5.3 Materials and Methods

5.3.1 Bacterial strains and media

E. coli strain DH1 [F⁻ *recA1 endA1 gyrA96 thi hsdR17* (r_k^-, m_k^+) *supE44 relA1*] (Hanahan, 1983) was used to prepare the library of *lux*AB transcription fusion clones (Guzzo and DuBow, 1991). Strain DH5α [*endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi1 recA1 gyrA relA1* (Δ*lacIZYA–argF*) *U169 deoR* (ϕ80Δ*lacZM15*)] (Woodcock et al. 1989) was used to transform all DNA ligation reactions. All *E. coli* strains were propagated at 32°C, unless otherwise stated, in LB broth (10 g NaCl, 10 g tryptone and 5 g yeast extract (Difco Laboratories, Detroit, Michigan) per litre) or on LB plates containing 1.5% agar (Sambrook et al., 1989) and supplemented with antibiotics as indicated. Antibiotics were used at the following concentrations: Ap (Ayerst Laboratories, Montreal, Canada) 40 μg/mL, Cm (Sigma, St. Louis, Missouri) 50 μg/mL, and Tc (Boehringer Mannheim, Laval, Canada) 10 μg/mL in broth and 20 μg/mL in agar. Stock solutions of organotin salts were prepared in 70% ethanol at the following concentrations: tributyltin chloride, 5.61 μL/mL; triethyltin chloride, 5.86 μL/mL; tri–n–propyltin chloride, 5.71 μL/mL; dibutyltin chloride 6.52 mg/mL; and tetrabutyltin chloride, 5.0 μL/mL. All organotin compounds were obtained from Aldrich Chemical Company Inc., (Milwaukee, Wisconsin). Stock solutions of glucose–6–phosphate (5.44 mg/mL), carbonyl cyanide m–chlorophenyl hydrazone (CCCP), and KCl were prepared in sterile and deionized water.

5.3.2 DNA manipulations

All restriction endonuclease hydrolysis reactions were performed in the manufacturers' buffers according to the manufacturers' protocol (Gibco–BRL, Burlington, Canada; New England Biolabs, Inc., Mississauga, Canada). DNA ligations were performed at 15°C for 18 hours with T4 DNA ligase following the manufacturer's protocol (Gibco–BRL, Burlington, Canada). DNA was subjected to electrophoresis in 0.7% TAE agarose gels (Sambrook et al., 1989). Size–selected DNA fragments were purified using the Geneclean II kit (Bio 101, Mississauga, Canada). DNA transformations were performed as previously described (Guzzo and DuBow, 1991).

5.3.3 Construction of plasmids

Plasmid pC17 was constructed (in order to obtain the upstream junction of the *uhp*T::Tn*5*–*lux*AB insertion) by ligating *Hin*dIII–cleaved chromosomal DNA from strain TBT_1 to *Hin*dIII–cleaved pUC119, transforming the ligation mixture into *E. coli* DH5α, and selecting for Tc^R clones (Guzzo and DuBow, 1991; Vieira and Messing, 1987). Plasmid pCD159 was constructed by ligating the 5.3 kb *Hin*dIII–*Bam*HI fragment of plasmid pC17 (containing the chromosomal DNA upstream of the fusion junction and 23 bp of IS*50*L) into *Hin*dIII– and *Bam*HI–cleaved pUC119.

5.3.4 Luminescence assays: Solid media

Screening of the *E. coli* Tn*5*–*lux*AB library has been previously described (Guzzo and DuBow, 1991; Briscoe et al. 1995). Briefly, clones (a grid of 48 distinct isolates per Petri plate) were incubated at 37°C on LB agar containing Ap and Tc and then replica plated

onto solid media containing antibiotics and 0, 1 or 10 μg/mL tributyltin. After 20 hours, 100 μL of decyl aldehyde, a substrate for the luciferase reaction, was added to the lids of the plates, and X-ray film (Agfa CURIX RP1 or Kodak XAR-5) was placed over the bases of the inverted plates. After exposure, the film was developed and clones showing a concentration-dependent increase in luminescence were retested. Subsequent light tests were performed in a similar manner, except cell were grown for 24 hours, treated with 50 μL of decyl aldehyde. These light tests were used to monitor TBT_1 upon exposure to 0, 100, 250 and 500 ng/mL of added tributyltin, triethyltin, tri-n-propyltin, dibutyltin and tetrabutyltin, 0, 200, 500 and 1000 ng/mL of added CCCP, and 100, 250, 500, 10 000 and 20 000 ng/mL of added glucose-6-phosphate. ScionImage software (Scion Corporation, Frederick, Maryland) was used for densitometric analysis of films.

5.3.5 Luminescence assays: Liquid media

Overnight cultures of strain TBT_1, grown in LB broth, were diluted 20-fold into fresh LB broth and grown to mid-logarithmic phase (A_{550} of 0.4). The cultures were then diluted 50-fold in LB broth and grown to an A_{550} of 0.05, at which time the cultures were separated into 50 mL portions. Organotin compounds (dissolved in 70% ethanol and added as chloride salts) were added to final concentrations of 0, 10, 100, 250, 500 and 100 ng organotin/mL. Glucose-6-phosphate (dissolved in sterile, deionized water) was added to 5, 10, 25 and 50 μg/mL. Solvent (water or 70% ethanol) was added to a control flask, whereas the appropriate volume of freshly prepared stock solution was added to the other flasks to reach the appropriate final concentrations. The flasks were subsequently incubated at room temperature. At regular intervals, cells were removed and diluted to an A_{550} of 0.05 in LB

broth. Luminescence of triplicate 1 mL samples was measured with a Tropix Optocomp I Luminometer (MGM Instruments, Connecticut). Samples were automatically injected with 100 µL of a decyl aldehyde solution (0.5% v/v in LB broth) and light emitted during a ten second period was recorded.

To assess the effect of halides on luminescence, light emission was measured in the presence of 500 ng/mL tributyltin plus 0, 10. 25, or 50 mM KCl. To assess the possibility that a compound produced upon organotin exposure was responsible for activation of the *uhp*T::*lux*AB fusion, *E. coli* DH1 cells were exposed to 500 ng/ml added tributyltin. After 0, 1, 4, 6 or 24 hours, the culture supernatent fluid was collected, subjected to centifugation at 4000 xg for 10 minutes at 4°C, and filtered through a 0.2 nm Millipore membrane to remove any remaining cells. Finally, TBT_1 cells, grown to mid–logarithmic phase and then diluted to an A_{550} of 0.2, were added to 50 mL of the cell–free supernatent fluid and luminescence was monitored.

5.3.6 RNA dot blotting analysis

An overnight culture of strain DH1 was diluted 20-fold into fresh LB broth and grown to an A_{550} of 0.2 at which time portions were divided into separate flasks. Glucose–6–phosphate was added to a final concentration of 0, 5, 10 or 20 µg/mL and cells were returned to the incubator. After 90 minutes, cells were harvested and total RNA was extracted. Alternatively, tributyltin was added to a final concentration of 0, 100, 250 or 500 ng/mL and cells were harvested after 4 hours of incubation. Five micrograms of total RNA were subjected to RNA dot blotting analysis. RNA was applied to a nitrocellulose membrane (Schleicher and Schuell, Keene, New Hampshire) and fixed by UV crosslinking

(10^5 mJ/cm^2, UV Stratalinker, Stratagene, La Jolla California). The probe, a 400 bp *Eco*57I fragment of *uhp*T isolated from plasmid pCD159, was labeled with [α-^{32}P]dATP (3000 Ci/mmol, ICN Biomedicals, Mississauga, Canada) according to the method of Feinberg and Vogelstein (1983; 1984). Hybridization was performed according to the method of Shackleford and Varmus (1987). Membranes were exposed on a phosphoimager cassette and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, California).

5.3.7 Sensitivity Assay

Overnight cultures of strains DH1 and TBT_1, grown in LB broth, were diluted 20-fold into fresh LB broth and grown to mid-logarithmic phase (A_{550} = 0.4). The cultures were then diluted 10^5 in LB broth and triplicate 100 µL samples were spread on LB agar containing 0, 100, 250, 500, 1000, 5000 or 10 000 ng/mL of added dibutyltin, tributyltin, triethyltin or tri-n-propyltin. After 48 hours of incubation at 32°C, the number of colonies was recorded.

5.4 Results

5.4.1 Isolation of a tributyltin-inducible *lux*AB gene fusion clone, TBT_1

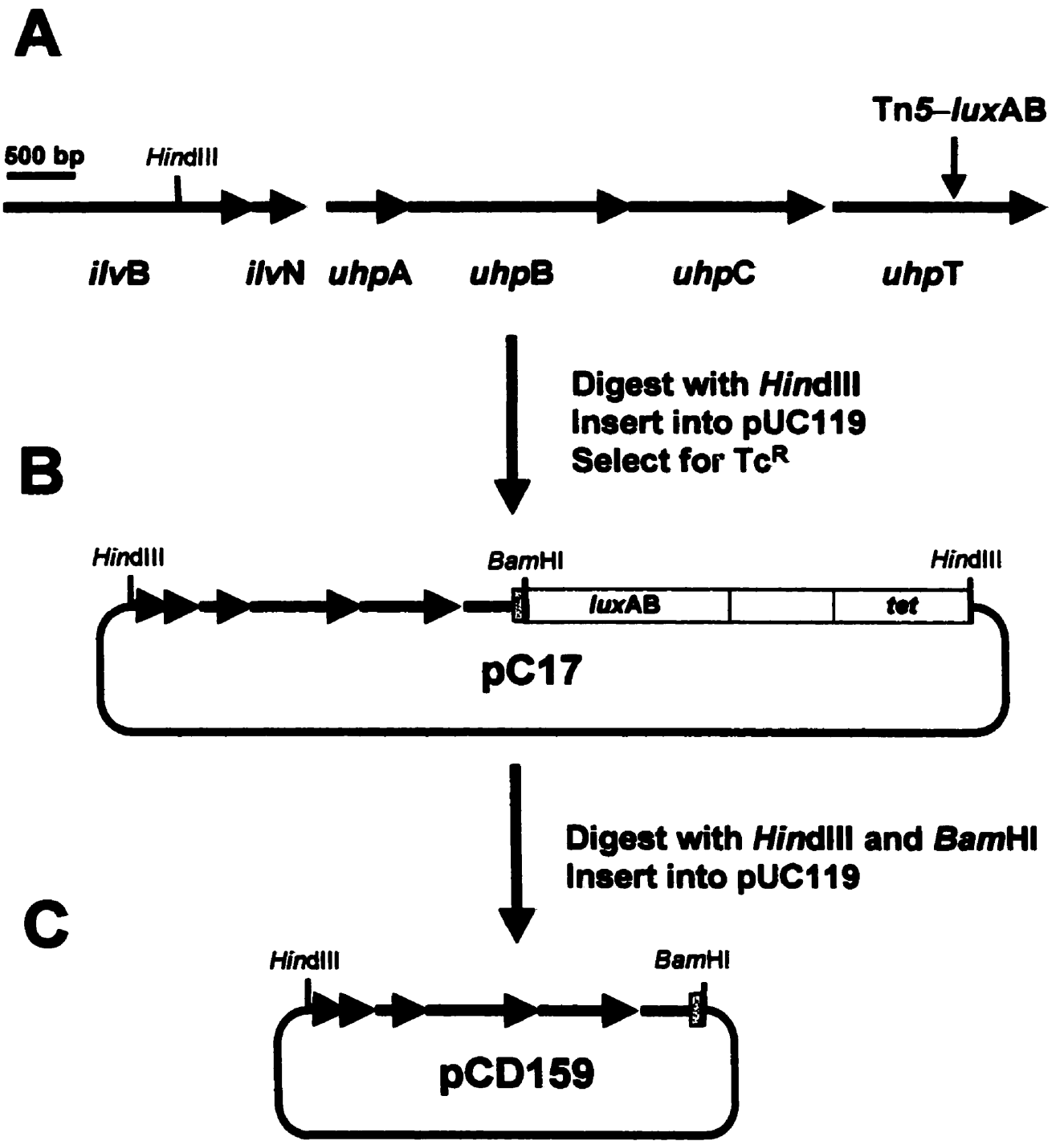
A library of 3000 *E. coli* clones, each containing a single chromosomal copy of the *V. harveyi lux*AB genes in random positions, was previously constructed (Guzzo and DuBow, 1991). The library was screened in the absence and presence of 1 and 10 µg/mL of

added tributyltin on LB agar (Briscoe et al., 1995). One clone, found to display increased luminescence with increasing concentrations of tributyltin, was isolated and designated strain TBT_1.

5.4.2 Cloning and sequencing of the tributyltin–inducible gene

A Southern blot, using the *lux*AB genes as a probe, was performed on genomic TBT_1 DNA cleaved with several restriction endonucleases, and confirmed that the Tn*5*–*lux*AB element was present at a single chromosomal location (data not shown). The left end of the gene fusion was cloned in one step using the *tet* gene in the Tn*5*–*lux*AB element as a selection marker (Guzzo and DuBow, 1991; 1994). The enzyme *Hin*dIII cleaves to the right of the *tet* gene, such that digestion of total chromosomal DNA with *Hin*dIII, ligation to *Hin*dIII–cleaved pUC119 (Vieira and Messing, 1987), and selection of transformants on LB agar containing Tc enabled isolation of plasmids containing the *tet* gene, the promoterless *lux*AB genes, IS*50*L and chromosomal DNA upstream of the site of the Tn*5*–*lux*AB insertion (Fig. 5.1). The resulting plasmid, pC17, contained approximately 5.3 kb of chromosomal DNA. Cleavage of pC17 with *Hin*dIII and *Bam*HI was used to isolate this chromosomal DNA and 23 bp of IS*50*L. This fragment was then ligated into *Hin*dIII– and *Bam*HI–cleaved pUC119 and sequenced using a primer complementary to IS*50*L, as previously described (Guzzo and DuBow, 1991). Using the BLAST algorithm, this sequence was compared to *E. coli* sequences in the GenBank database (Altschul et al., 1997; Benson et al., 1999). It was subsequently determined that the Tn*5*–*lux*AB element had inserted before bp 3 844 000 of the complete *E. coli* chromosome, 794 bp downstream from the translational start of *uhp*T (Blattner et al., 1997).

Figure 5.1: Subcloning of the TBT-inducible gene. A) Insertion of the Tn*5*-*lux*AB transposon into the *uhp*T region of the TBT_1 chromosome. B) Construction of plasmid pC17 by insertion of a *Hin*dIII fragment of the TBT_1 chromosome, containing *tet*, *lux*AB, IS*50*L and upstream DNA, into *Hin*dIII-linearized pUC119. C) Construction of plasmid pCD159 by insertion of a *Bam*HI-*Hin*dIII fragment from pC17, containing IS*50*L and upstream DNA, into *Bam*HI and *Hin*dIII digested pUC119.


A
Tn5–luxAB
500 bp
HindIII
ilvB
ilvN
uhpA
uhpB
uhpC
uhpT
Digest with HindIII
Insert into pUC119
Select for TcR
B
HindIII
BamHI
HindIII
luxAB
tet
pC17
Digest with HindIII and BamHI
Insert into pUC119
C
HindIII
BamHI
pCD159

5.4.3 Luminescence on solid media

Luminescence of strain TBT_L was measured after overnight growth on LB agar containing 0, 50, 100 or 250 ng/mL added tributyltin, triethyltin, tri–n–propyltin, dibutyltin, or tetrabutyltin. A concentration–dependent increase in luminescence was found with tributyltin (Fig. 5.2A), triethyltin (Fig. 5.2B), and tri–n–propyltin (Fig. 5.2C), but not dibutyltin or tetrabutyltin (data not shown). Subsequent tests showed increasing light emission with increasing concentrations of added glucose–6–phosphate (Fig. 5.2D) and CCCP (Fig. 5.2E). Induction of luminescence followed the pattern tributyltin < triethyltin < tri–n–propyltin .≤ CCCP << glucose–6–phosphate.

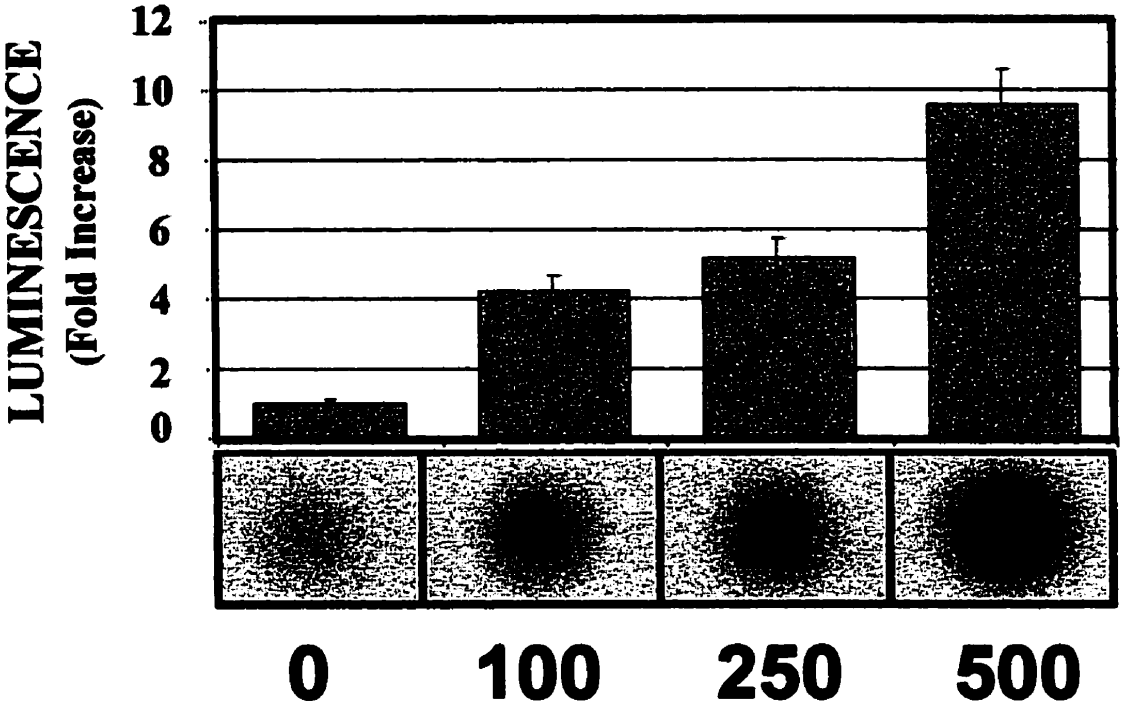
5.4.4 Luminescence in liquid media

Luminescence of strain TBT_L was measured over a six hour period in the absence or presence of 10, 100, 250, 500 and 1000 ng/mL added tributyltin (Fig. 5.3A). In all cultures exposed to tributyltin, light emission was induced after 180 minutes. However, the rate and magnitude of luminescence increased with increasing concentrations of added tributyltin. The initial decrease in light emission in cultures exposed to high (500 or 1000 ng/ml) concentrations of added tributyltin is attributed to the toxicity of the organotin.

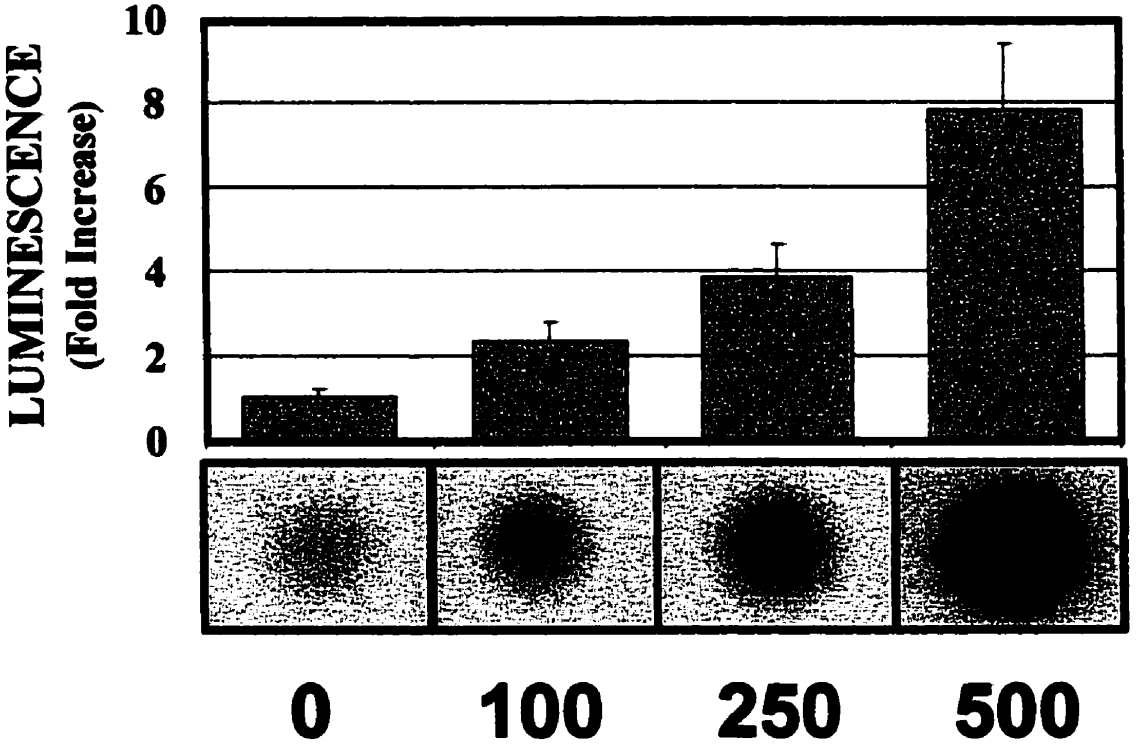
Luminescence of strain TBT_L was also measured over a 90 minute period in LB broth in the absence or presence of 0, 0.1, 0.5, 1, 5 or 10 μg/mL added glucose–6–phosphate (Fig. 5.3B). Light emission was concentration dependent and did not increase in the absence of added glucose–6–phosphate. For concentrations of 5 and 10 μg/mL added glucose–6–phosphate, luminescence was induced within 10 minutes. With 1μg/ml added

Figure 5.2: Luminescence of *E. coli* *uhp*T::*lux*AB fusion strain TBT_1 colonies on solid media upon addition of A) tributyltin, B) triethyltin, C) tri–n–propyltin, D) glucose–6–phosphate, and E) CCCP. Bottom panel: Kodak XAR5 film exposed by luminescent TBT_1 colony. Top panel: graphical representation of luminescence. Concentrations of the added compound and film exposure time are indicated.

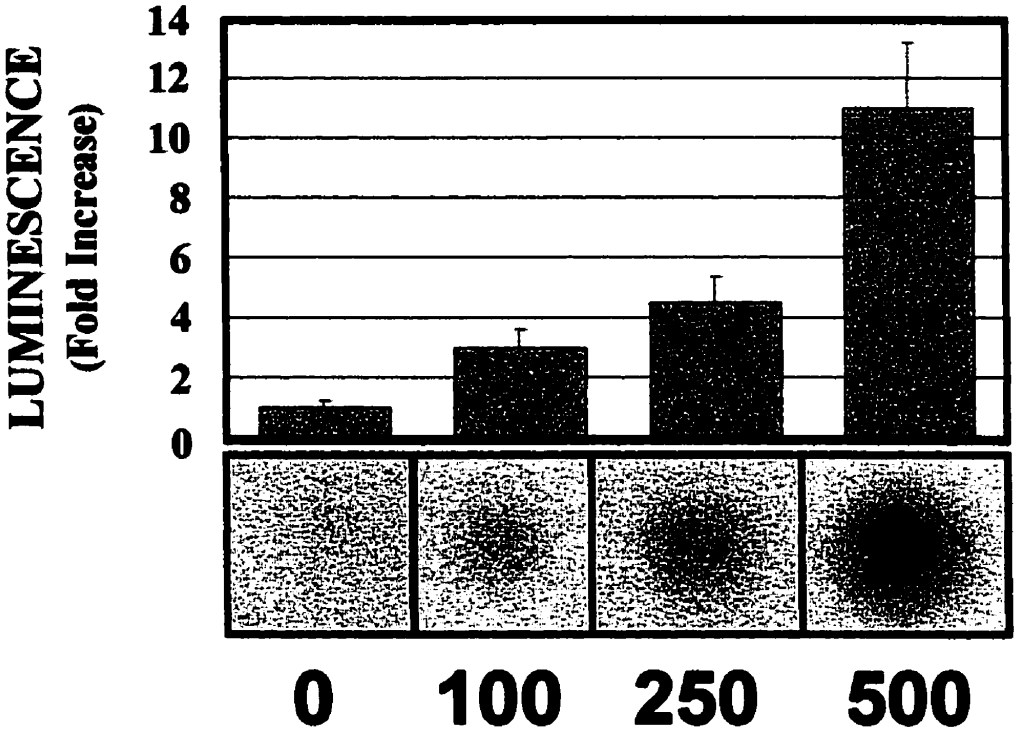
A


LUMINESCENCE
(Fold Increase)
12
10
8
6
4
2
0
0
100
250
500
Added Tributyl tin (ng/mL)
(60 minute exposure)

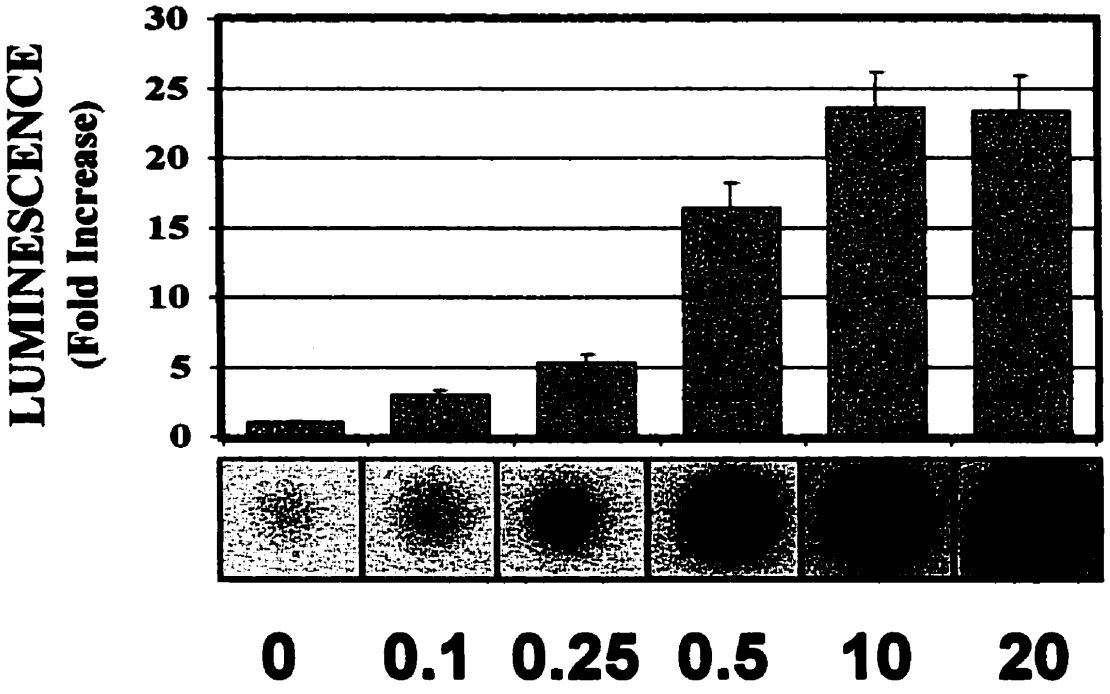
B


LUMINESCENCE
(Fold Increase)
10
8
6
4
2
0
0
100
250
500
Added Triethyl tin (ng/mL)
(60 minute exposure)

C


LUMINESCENCE
(Fold Increase)
14
12
10
8
6
4
2
0
0
100
250
500
Added Tri-n-propyl tin (ng/mL)
(30 minute exposure)

D


LUMINESCENCE
(Fold Increase)
30
25
20
15
10
5
0
0
0.1
0.25
0.5
10
20
Added Glucose-6-phosphate (mg/mL)
(30 minute exposure)

E

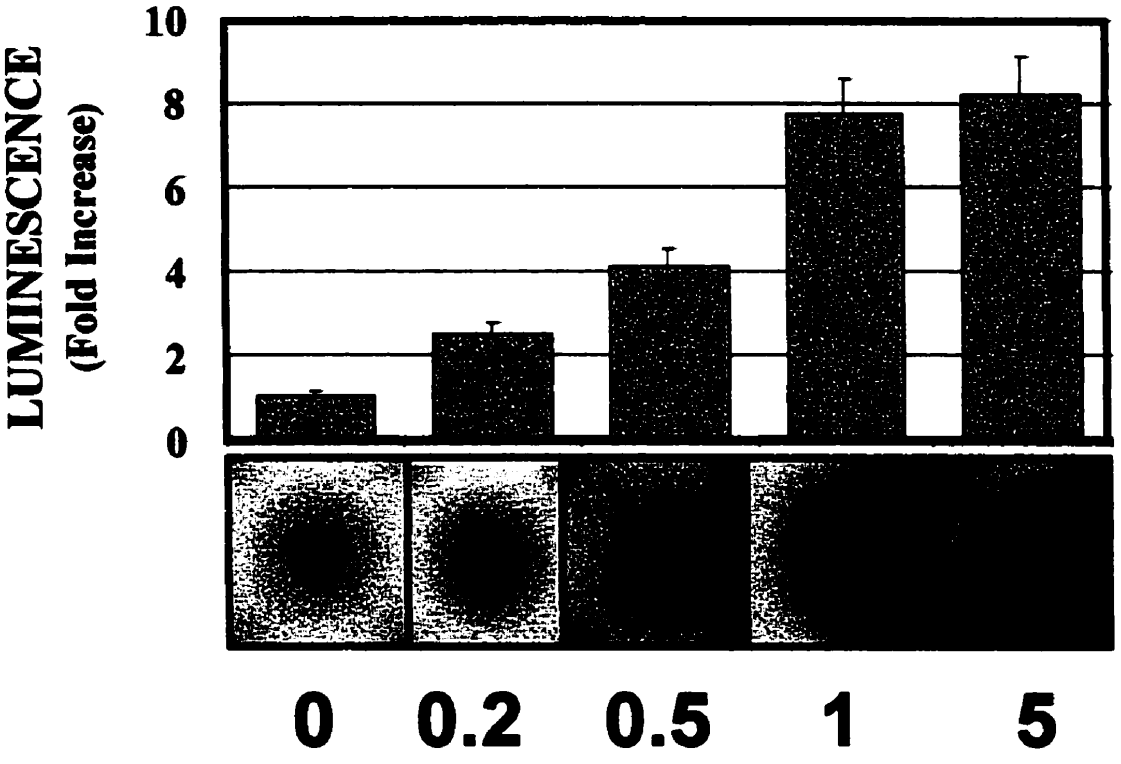
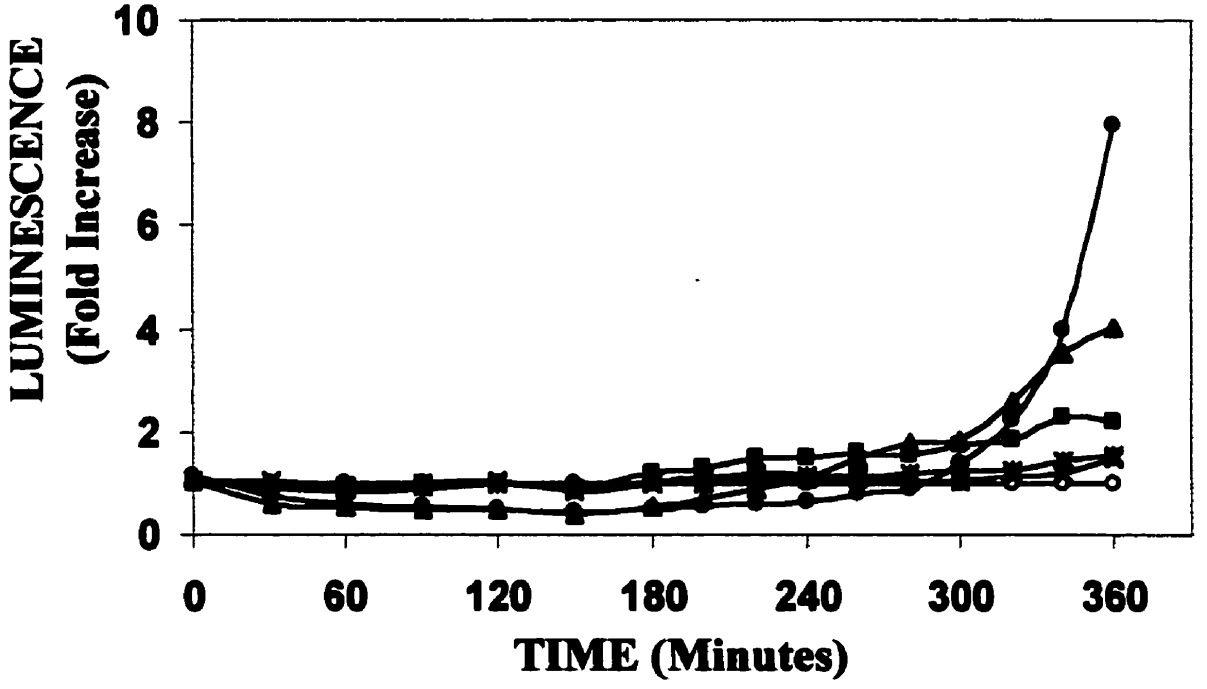

LUMINESCENCE
(Fold Increase)
10
8
6
4
2
0
0
0.2
0.5
1
5
Added CCCP (mg/mL)
(30 minute exposure)

Figure 5.3: Luminescence of *E. coli uhp*T::*lux*AB fusion strain TBT_1 in liquid media upon addition of: A) tributyltin to a final concentration of 0 (O), 10 (×), 100 (∗), 250 (■), 500 (◆), or 1000 (●) ng/mL, or B) glucose–6–phosphate to a final concentration of 0 (O), 0.1 (∗), 0.5 (◆), 1 (●), or 5 (■) or 10 (×) μg/mL. Cells were grown to mid–logarithmic phase (A_{550} of 0.4), diluted in fresh LB broth to an A_{550} of 0.05 and divided into 50 mL aliquots. At time 0, tributyltin or glucose–6–phosphate was added. At each time point, triplicate 1mL samples were removed and assayed for light emission in a Tropix luminometer.

A


LUMINESCENCE
(Fold Increase)
10
8
6
4
2
0
0
60
120
180
240
300
360
TIME (Minutes)

B

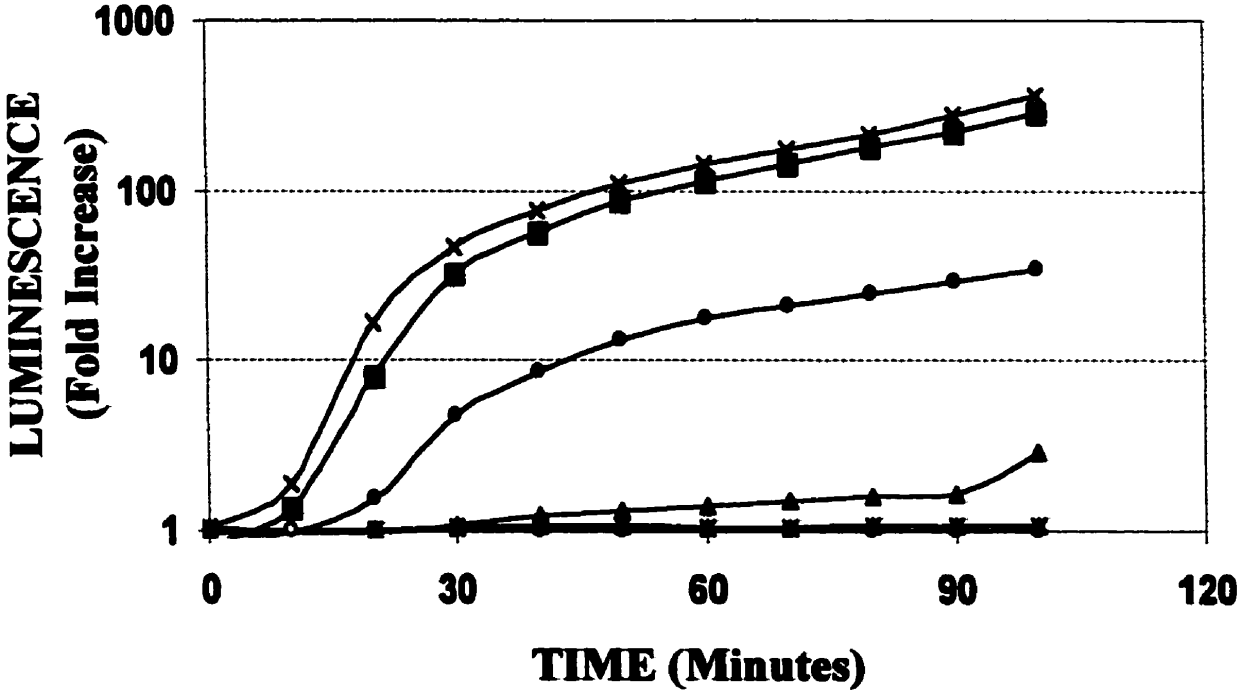
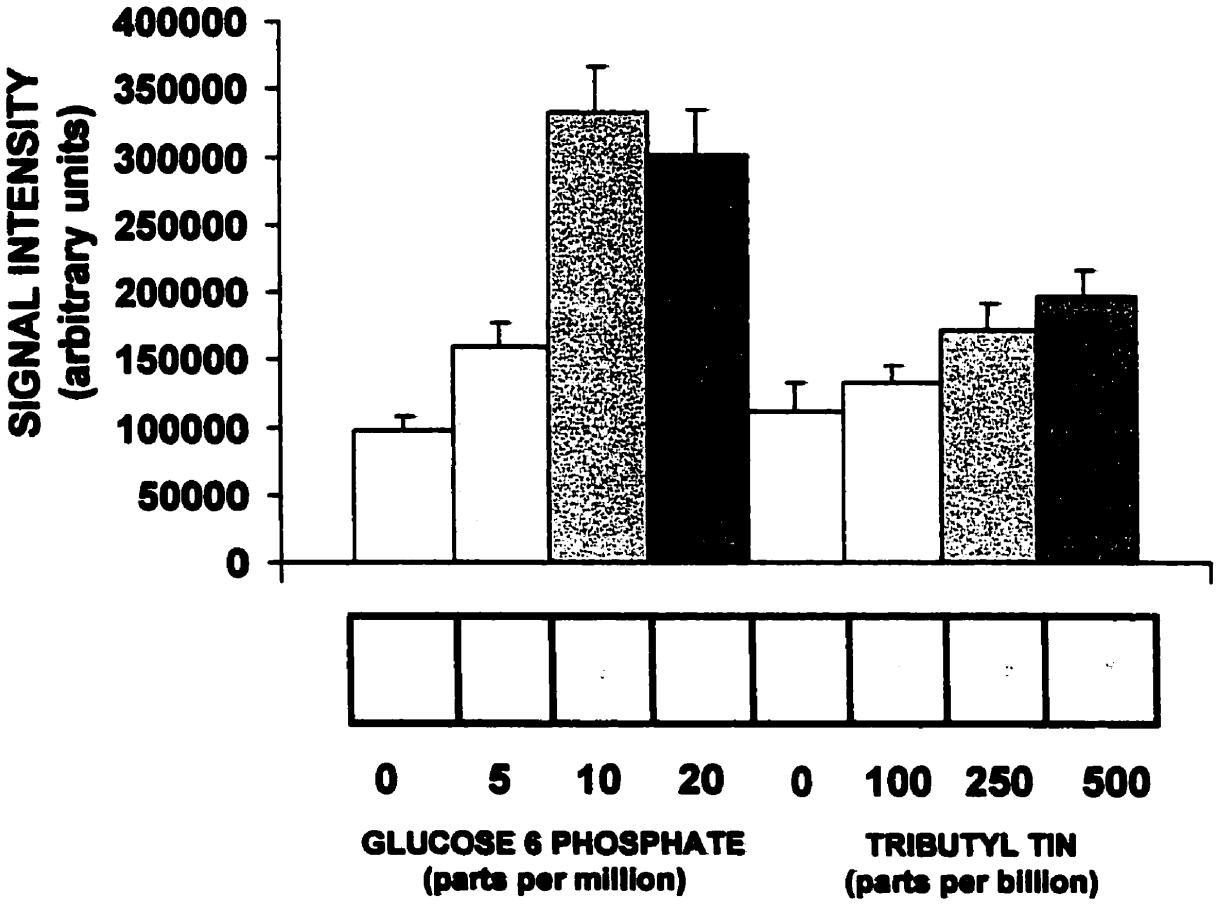

LUMINESCENCE
(Fold Increase)
1000
100
10
1
0
30
60
90
120
TIME (Minutes)

Figure. 5.4: RNA dot blot analysis of *uhp*T transcription from *E. coli* DH1 upon addition of tributyltin or glucose–6–phosphate. Concentrations of added compounds are indicated. Cells were harvested 90 minutes after exposure to glucose–6–phosphate and 4 hours after exposure to tributyltin.


SIGNAL INTENSITY
(arbitrary units)
400000
350000
300000
250000
200000
150000
100000
50000
0
0
5
10
20
0
100
250
500
GLUCOSE 6 PHOSPHATE
(parts per million)
TRIBUTYL TIN
(parts per billion)

5.4.6 Sensitivity to organotins

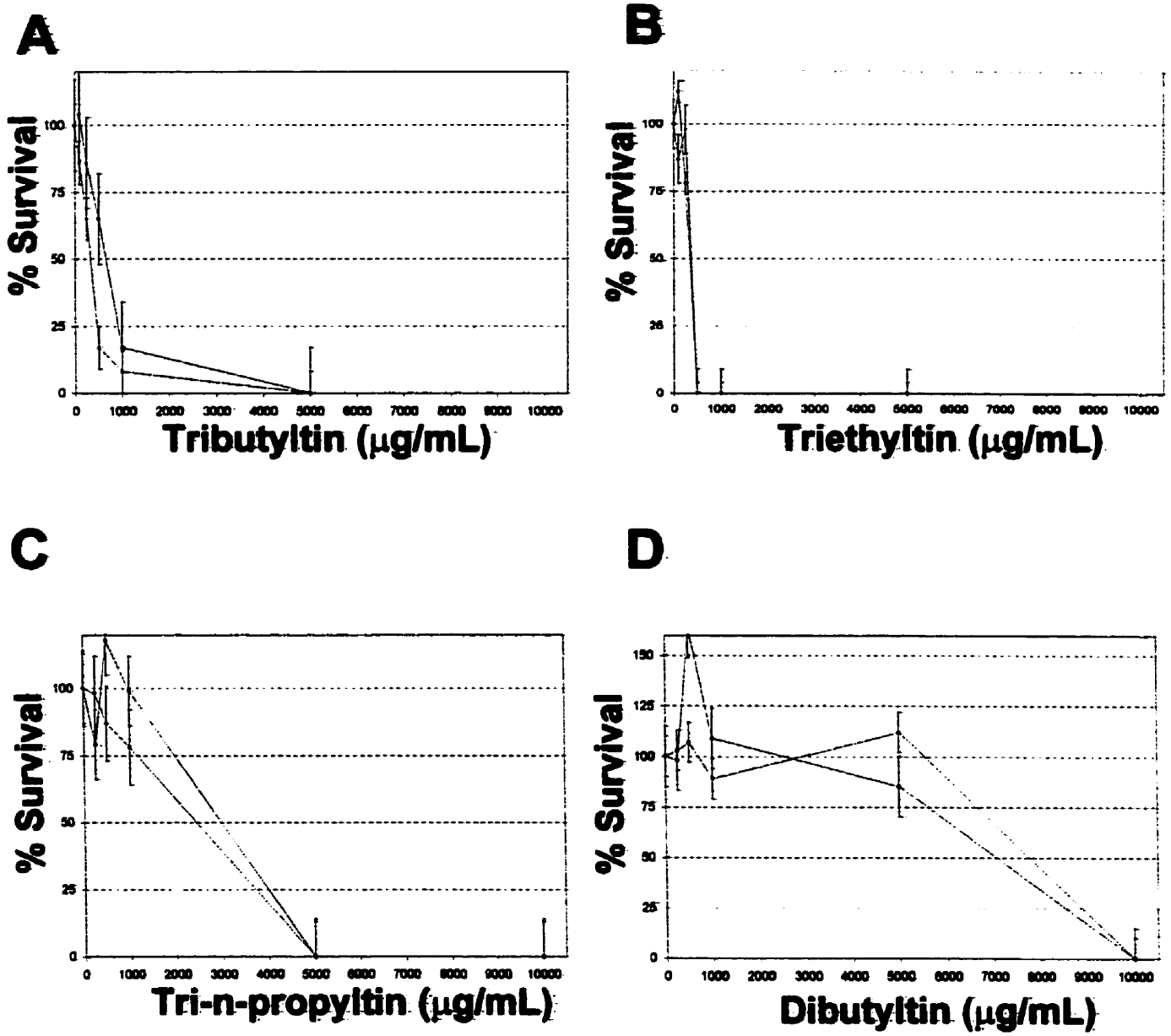
E. coli strains DH1 and TBT_1 were grown on LB agar containing 0, 100, 250, 500, 1000, 5000 and 10000 ng/mL added dibutyltin, tributyltin, triethyltin, and tri–n–propyltin, to determine if the Tn*5*–*lux*AB insertion altered the sensitivity of cells to organotin compounds (Fig. 5.5). The two strains exhibited the same sensitivity profiles, with sensitivity decreasing in the order triethyltin > tri–n–propyltin > tributyltin >> dibutyltin.

5.5 Discussion

We have isolated an *E. coli lux*AB transcriptional gene fusion clone, TBT_1, that is induced by the triorganotin compounds tributyltin, tri–n–propyltin and triethyltin (Figs. 5.2A–C), but not dibutyltin or tetrabutyltin. Cloning and sequencing of the gene fusion revealed that Tn*5*–*lux*AB is inserted into the *uhp*T gene, which encodes a hexose–6–phosphate transporter.

Glucose–6–phophate is the normal inducer of *uhp*T. Briefly, sensing of extracellular glucose–6–phosphate by UhpC and signal transduction via UhpB results in phosphorylation of the transcriptional activator, UhpA (Island and Kadner, 1993; Island et al., 1992). The *uhp*ABC genes are constitutively transcribed as a tricistronic mRNA. Transcription of *uhp*T is inducible and is initiated by binding of UhpA to specific DNA sequences in the *uhp*T promoter region (Merkel et al., 1992). The *uhp*T promoter also contains a binding site for CAP, the catabolite gene activator protein. CAP binding is neither sufficient nor necessary for *uhp*T expression, but it does stimulate transcription (Merkel et al., 1995). Once expressed, UhpT acts as an antiport permease. It catalyzes the uptake of a hexose sugar

Figure: 5.5: Sensitivity of the parental *E. coli* strain DH1 (■) and the *uhp*T::*lux*AB fusion strain TBT_1 (●) to A) tributyltin, B) triethyltin, C) tri–n–propyltin, and D) dibutyltin on solid media. Cells were grown to mid-logarithmic phase (A_{550} of 0.4) then diluted 10^5 in LB broth and plated in triplicate on LB agar containing added organotin at a final concentration of 0, 100, 250, 500, 1000, 5000 or 10000 μg/mL. After 48 hours of incubation at 32 C, colony forming units were counted.


A
% Survival
Tributyltin (µg/mL)
B
% Survival
Triethyltin (µg/mL)
C
% Survival
Tri-n-propyltin (µg/mL)
D
% Survival
Dibutyltin (µg/mL)

phosphate in exchange for the export of an inorganic phosphate (Sonna et al., 1988). This anion exchange does not require a proton gradient. However, uptake of glucose–6–phosphate does require an internal pool of inorganic phosphate.

It is not immediately apparent why triorganotin compounds should induce transcription of *uhp*T. However, several possibilities have been explored: i) UhpBC may recognize triorganotins, or their metabolites; ii) UhpT may influence, directly or indirectly, the resistance of *E. coli* to organotins; iii) *uhp*T transcription may be due to organotin–mediated OH^-/Cl^- exchange; or iv) *uhp*T transcription may be related to organotin–mediated inhibition of ATPase activity and uncoupling of oxidative phosphorylation.

Glucose–6–phosphate induces luminescence from TBT_1 (Figs. 5.2D, 5.3B). In liquid culture, glucose–6–phosphate–mediated light emission is induced within 10 to 15 minutes and, within 60 minutes, results in a 350–fold increase in luminescence. The shape of the curve in Figure 5.3B suggests maximal induction of transcription upon addition of 5 to 10 μg/mL (approximately 8 to 15 μM) or more glucose–6–phosphate. This is consistent with other reports showing maximal induction of *uhp*T transcriptional gene fusions upon addition of micromolar concentrations of exogenous glucose–6–phosphate (Shattuck-Eidens and Kadner, 1981). However, in liquid culture, tributyltin–mediated induction requires several hours and results in a modest 8–fold, increase in luminescence (Fig. 5.3A).

An RNA dot blot, used to examine tributyltin and glucose–6–phosphate induced *uhp*T transcription from *E. coli* DH1, revealed a similar pattern of expression (Fig. 5.4). Glucose–6–phosphate induces *uhp*T transcription more rapidly and to a greater magnitude

than tributyltin. Moreover, 10 and 20 μg/mL added glucose–6–phosphate produced similar, high signals suggesting that the rate of transcription was at a maximum.

The disparate kinetics suggest that triorganotins do not induce transcription in the same manner as glucose–6–phosphate and are not recognized by UhpBC However, it is possible that a metabolite of triorganotin, or some compound generated upon triorganotin–exposure, is recognized by UhpBC. The time required for generation of such inducers would account for the time lag observed in tributyltin–mediated induction. If these inducers exist, they could be present in media conditioned by triorganotin–exposed cells, and their concentration could increase with the duration of exposure. However, when TBT_1 was mixed with such conditioned media, there was no measurable increase in luminescence. As such, there is no evidence that a stable, extracellular inducer recognized by UhpBC is generated upon tributyltin–exposure (data not shown).

Several groups have isolated tributyltin–resistant mutants of *E. coli* (Ito et al., 1983; Sedgwick and Bragg, 1992; Singh and Singh, 1984). Although few of these mutants have been characterized, several classes of mutants have emerged and have enhanced understanding of the effects of organotins. In some mutants, resistance is due to exclusion of organotin. These mutants exhibit slower uptake and lower levels of organotin accumulation than parental strains (Singh and Singh, 1984). Gene(s) responsible for this phenotype have not been identified, but the mucoid appearance of these mutants suggests that some cell membrane component has been modified (Singh and Singh, 1984). Indeed, modification of membrane phospholipid composition is associated with tributyltin resistance in the *Bacillus subtilis* mutant, AG2A (Guffanti et al., 1987).

In other mutants, tributyltin–resistance maps to the *unc* genes, which encode a proton–translocating ATP synthase (Ito et al., 1983). This form of resistance may be indicative of target site modification. Organotins are known to inhibit oxidative phosphorylation in both prokaryotes and eukaryotes. Studies with submitochondrial particles suggest that tributyltin interacts with the F_0 component of ATPase (Matsuno-Yagi and Hatefi, 1993a; 1993b). This is likely to be true for *E. coli*, as soluble (i.e. F_1 only) ATPase activity is not affected by tributyltin, while membrane bound (F_1–F_0) ATPase activity is affected (de Chadarevjan et al., 1979). Many of these *unc* mutants also exhibit resistance to uncouplers of oxidative phosphorylation such as carbonyl cyanide m–chlorophenyl hydrazone (CCCP), N,N'–dicyclohexylcarbodiimide (DCCD), sodium azide and pentachlorophenol (PCP) (Ito et al., 1983). Similarly, some *E. coli* strains, originally isolated as uncoupler–resistant mutants, exhibit resistance to organotins (Ito et al., 1986; Sedgwick and Bragg, 1992).

One well studied tributyltin–resistant mutant, *E. coli ttr–3*, exhibits resistance to tributyltin, CCCP, DCCD, sodium azide and PCP. This strain also has a temperature–sensitive defect in ATP synthesis, but the mutation is not in the *unc* genes. Linkage analysis using phage P1 transduction places the mutation in the 82 map unit region of the *E. coli* chromosome, somewhere between *pyr*E and *dna*A (Nakamura et al., 1989). Although there are several dozen genes between *pyr*E and *dna*A, including many that are uncharacterized, it is interesting to note that the *uhp* operon is in the middle of this region. It is quite plausible that UhpT, a transport protein, could mediate organotin efflux and contribute to organotin resistance. Indeed, organotin resistance in *Altermonas* M–1 is mediated by a putative anion transport protein (White et al., 1999)

However, TBT_1 and the parental DH1 exhibit the same sensitivity to organotin compounds (Fig. 5.5). The sensitivity of these strains to tributyltin were similar to values reported in the literature (e.g. 5 μg/mL is comparable to the 0.001 % (v/v) used by Ito *et al.* (1983) and 10 μM used by Singh and Singh (1984)). Also, the triorganotins are more toxic than the di– or tetra– organotins.

Tributyltin can mediate halide/hydroxyl exchange across lipid bilayers and the *E. coli* cell membrane (Antonenko, 1990; Singh and Bragg, 1979). This inhibits some cell functions, such as uptake of molecules, including proline and glutamine (Singh and Bragg, 1979). To examine the effect of Cl^-/OH^- exchange on *uhp*T transcription, light production by TBT_1 was monitored in the presence of 500 ng/mL tributyltin and 0, 10. 25, 50 mM KCl. Light production decreased with increasing concentrations of KCl (data not shown). The observation that halides reduce light production from TBT_1 is inconsistent with the hypothesis that induction of *uhp*T is due to organotin–mediated halide/hydroxyl exchange.

Tributyltin inhibition of ATPase activity is well established and, as mentioned previously, resistance of *E. coli* to organotin compounds has been associated with the *unc* genes. If the effect of tributyltin on *uhp*T transcription is related to ATPase inhibition, then other uncouplers of oxidative phosphorylation should induce increased luminescence from TBT_1. As such, TBT_1 was exposed to 0, 200, 500, 1000 and 5000 ng/mL CCCP (Fig. 5.2E). CCCP–induced luminescence was greater than that of tributyltin (Fig. 5.2A) and triethyltin (Fig. 2B), and comparable to that induced by tri–n–propyltin (Fig. 5.2C). This suggests that induction of *uhp*T transcription may be related to the uncoupling activity of triorganotin compounds.

Transport of hexose–6–phosphates through the UhpT antiporter is electroneutral, and does not required a proton gradient across the cell membrane. However, our data indicates that uncoupling of oxidative phosphorylation by triorganotins and CCCP does stimulate transcription of *uhp*T. Moreover, there is a least one precedent for our finding. Singh *et al.* (1985) observed increased uptake of sugar phosphates after treatment of *Brochothrix thermosphacta* with CCCP or KCN (Singh et al., 1985).

An intact proton gradient is integral to many aspects of cell metabolism and so, it is difficult to speculate on the precise mechanism of *uhp*T induction. Uncoupling may disturb the interaction between UhpB and UhpC. This might influence phosphatase and/or kinase activity and the phosphorylation state of the activator, UhpA. Alternatively, uncoupling may alter CAP binding. Increased binding of UhpT or UhpT with CAP to the *uhp*T promoter would increase transcription. Future experiments with *uhp*A, *uhp*B, *uhp*C, and *cya* mutants may help to resolve these issues and enhance our understanding of this phenomenon.

Organotin compounds are important to industry and agriculture. Unfortunately, their environmental effects, from enzyme inhibition to imposex, are not fully understood and, despite government legislation, organotin pollution continues to proliferate (Fent, 1996; Matthiessen and Sumpter, 1998). We have found that sub–lethal concentrations of triorganotins, added in ng/mL amounts, can induce transcription of *uhp*T, the hexose–6–phosphate transporter of *E. coli*. Moreover, our experiments suggest that this is related to triorganotin–mediated uncoupling of oxidative phosphorylation.

The artist may be well advised to keep his work to himself till it is completed, because no one can readily help him or advise him with it
But the scientist is wiser not to withhold a single finding or a single conjecture from publicity

Goethe,
Essay on Experimentation

CHAPTER 6

Summary and Future Prospects

6.1 Summary

Procaryotes can monitor and respond to changes in their environment. These reponses, many of which are genetically–programmed, help the organism cope with a diverse array of conditions. For example, genetic cascades regulated by the Fur and FNR proteins mediate responses to iron and oxidative stress. Previously, a chromosomal *lux*AB gene fusion library was constructed in *Escherichia coli*. By screening this library with aluminum, arsenic, selenium and organotin compounds, a variety of novel genes have been identifed.

The current work describes the isolation and characterization of the previously unknown *ais* gene, as well as the identification of a novel pathway for inducing expression of *uhp*T, which encodes a hexose–6–phosphate transport protein.

The *ais* gene was sequenced and the Ais protein was expressed. Although its function has not been demonstrated, Ais contains a motif that is conserved among procaryotic and eucaryotic phosphatases. The Ais protein is also homologous to PmrG from *Salmonella enterica*, AfrS from the pathogenic *E. coli* strain RDEC–1, and TraG, which is conserved among several Enterobacteriaceae plasmids.

The transcriptional start site of the *ais* gene was identified. Transcription was found to be induced by the addition of heavy metals, including aluminum, iron, gallium, indium and vanadium. Magnesium and calcium do not induce transcription of *ais*, but can inhibit aluminum–induced *ais* transcription. In the absence of metal, changes in pH do not induce transcription of *ais*. However, changes in extracellular pH can alter the bioavailability of added metal, and therefore influence transcription.

The PhoP–PhoQ two–component system mediates genetic responses to magnesium and calcium. Although mutation of *pho*P and *pho*Q does not prevent metal–induced luminescence of an *ais*::*lux*AB gene fusion, it does diminish the magnitude of the response. However, mutation of the *bas*S gene abrogates metal–induced expression of *ais*. Moreover, expression of the BasR–BasS two–component regulatory system, *in trans*, restores the metal–inducible response. This indicates that the BasR–BasS system regulates *ais* expression and is involved in *E. coli* responses to heavy metals.

The BasR–BasS two–component system has not been extensively characterized. To identify novel members of this regulon, a Mu*dI* (*lac* Ap) gene fusion library was constructed and screened. Gene fusions to *blc*, *iap*, *glm*US, and *ygf*IJK exhibited increased β–galactosidase activity upon over expression of BasR–BasS, while expression of *dsd*XA decreased. Four additional genes, *set*C, *yib*D, *yhh*T and *yaf*, were identified by searching the *E. coli* genome database for the presence of the putative BasR recognition sequence. Functions for all of these of these genes have not been defined, but most are associated with cell wall functions or sugar metabolism.

The *uhp*T gene encodes a transporter for hexose–6–phosphate. Other labs have demonstrated that, in response to glucose–6–phosphate, UhpA UhpB and UhpC induce transcription of *uhp*T. However, this work indicates that tributyltin, triethyltin, tri–n–propyltin and CCCP can induce luminescence of a *uhp*T::*lux*AB gene fusion. Moreover, RNA blotting analysis confirms that addition of tributyltin increases transcription of *uhp*T. This response may be due to the uncoupling activity of these compounds.

6.2 Future Prospects

The current work can be extended in many exciting directions. The Ais protein remains poorly characterized. Expression and purification of the Ais protein was attempted, but a reliable and high yield method was not developed. Pure Ais protein could be used for the development of antibodies, a valuable tool. Although it has been proposed that Ais is located in the cell membrane (or periplasm), this has not been tested. However, with antibodies, the location of Ais could be determined by analyzing cell fractions via Western blotting, or by examining whole cells with *in situ* hybridization. Also, N–terminal sequencing of the purified protein would identify the extent of the Ais signal sequence. *In vitro* experiments with Ais protein might confirm its role as a phosphatase (although an appropriate substrate would have to be identified). Crystalization of Ais would involve a significant amount of work, but solution of this structure, perhaps using NMR, might confirm the existence of a phosphate binding pocket, and hint at Ais function. More immediately, the function of Ais could be established by comparing the LPS profiles of *E. coli* DH1 and LF20110 (i.e. an *ais* derivative of DH1). Zhou *et al.* (1999) established functions for many of the *pmr* genes by examining LPS changes in a set of *Salmonella typhi* mutants. It would also be interesting to see how aluminum, iron, vanadium and indium affect LPS structure.

BasS has been identified as a metal sensing protein. Mutational analysis of the putative metal sensing domain (e.g. deletion of large or small regions, or alanine scanning) could be used to identify residues required for metal–dependent activities. Purified BasS protein, or BasS–enriched membranes, could be used *in vitro* for kinetic

studies. For example, the effects of Mg^{2+} and Al^{3+} on BasS kinase activity could be monitored. BasR has been identified as a transcriptional regulator. However, the interaction of BasR with the *ais* promoter region remains to be demonstrated. Purification of the BasR protein (again, a project that has been initiated, but has yet to produce satisfactory results) would allow this. Gel mobility shift assays would demonstrate that BasR can bind to the *ais* promoter region. DNA footprinting would further enhance our understanding of how *ais* transcription is regulated. Transcription of *bas*RS has not been characterized. RNA blotting and S1 nuclease analysis should be conducted to identify the size of the *bas*RS transcript, and its transcriptional start site. These analyses would also indicate if *yjd*B, which is located immediately upstream of *bas*R, is cotranscribed with *bas*RS. The *yjd*B gene encodes a putative transport protein. It is found in both *E. coli* and *S. typhi*, but its role is unknown.

Characterization of the BasR–BasS regulon would be another valuable project. An initial attempt is described in Chapter 4. However, the nine genes identified in that study should be examined more extensively. β–galactosidase activity should be monitored in a *bas*R *bas*S background. Moreover, gel mobility shift assays and DNA footprinting analysis would better characterize the interaction of these genes with BasR. Gene fusion libraries, using *lux*AB, Mud*I* (*lac* Ap) or other reporter constructs, have long been used to dissect genetic pathways in procaryotes. However, other systems for monitoring gene expression also exist. Two–dimensional gel analysis, or gene microarray technology, could be used to study the BasR–BasS regulon. Such techniques could also be used to identfy additional effects of aluminum, iron, vanadium, and indium on *E. coli*.

In science one tries to tell people,
in such a way as to be understood by everyone.
But in poetry, it's the exact opposite

Paul Dirac
Quantum Physicist
Nobel Laureate 1933

I could go on making poetry
On my favorite subject, Enterobacteriacea
Yet, poetry is not on my list.
Call me a desperate Bacteriologist
Hoping to convince people:
Microbiology is not only for the smart
But for people who like poetry...
Microbiology is an art.

Teresa Tam
The Body Electric

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Dave, my oldest brother – the one who should have been the scientist but who grew a filmy pony tail instead and who now sells records in an alternative record shop in Seattle (he and his girlfriend, Rain, who only wears black) he's in London, England this Christmas, doing Ecstacy and going to nightclubs.

Douglas Coupland,
Generation X