BINDING SERVICES
Tel +44 (0)29 2087 4949
Fax +44 (0)29 20371921
e-mail bindery@cardiff.ac.uk

Metal-Ion Dependent Transcriptional Regulation in *Escherichia coli*

Saira Khan

A thesis submitted to the School of Biosciences of Cardiff University
for the degree of

DOCTOR OF PHILOSOPHY

School of Biosciences
Cardiff University
May 2004

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

First and foremost, I offer my sincere gratitude and respect to my supervisor Dr Andy Morby who guided me through my PhD with a positive outlook at all times. I am thankful for everything he has taught me and for being so supportive, generous and kind. Also, for having faith in me by giving me the opportunity to work on this project in the first place. This has been a brilliant experience.

All my friends from lab. W315 contributed towards making my PhD a thoroughly enjoyable and memorable experience in an environment full of warmth and laughter. You were there to offer advice and always looked after me. Thanks also to all my friends and family who encouraged me and supported me through my highs and lows.

Special thanks are due to my mother, Tahira Noor, for being such a good example to me. You have been a source of pure light and your love, prudence and support have helped me immensely. Also, my older brother Tabrez, who established the standard for me to follow.

Thanks to my honourable father, Khan Mir, for his clear guidance, wisdom and love. It goes without say that I would not have got here without a father like him and I wish he was here to celebrate this day with me. He always took a deep interest in all aspects of my education and showed me the right path through his own accomplishments. Indeed, he did not have all the advantages that he strived to provide for me.

For all your sacrifices Papa, I dedicate this thesis to you.
This is as much your achievement as it is mine.

ABSTRACT

Escherichia coli possess a variety of metal-ion homeostatic and resistance mechanisms. These assist in tolerance to elevated levels of metal-ions in the surrounding environment. Examples include ZntA, a P-type ATPase that exports Zn(II) and constitutes a major Zn(II) resistance factor in *E. coli*. The transcriptional regulation of ZntA is under the control of ZntR, which responds primarily to Zn(II). ZntR is a dimeric protein belonging to the MerR family of transcriptional regulators that contain highly conserved N-terminal helix-turn-helix motifs and coiled-coil dimerisation motifs. The C-terminal ligand-binding region is less conserved among MerR family members however there are three cysteine residues that are highly conserved within the metal-ion responsive subgroup.

At the time this research project was initiated, the metal-binding residues in ZntR were unknown. A series of mutations were generated in the candidate metal-binding residues, the five histidines and five cysteines, of ZntR. The transcriptional and DNA-binding properties of the corresponding altered proteins were characterised. Competent *E. coli* containing a luciferase reporter plasmid driven by the cloned promoter of *zntA*, P*zntA*, were transformed with plasmids bearing the altered *zntR* derivatives. The transcriptional regulation of P*zntA* by the altered *zntR* derivatives in response to Zn(II), Cd(II) and Pb(II) was monitored. It emerged that four cysteines, three of which are conserved among divalent metal-ion responsive MerR regulators, and three histidines are important for Zn(II) induced transcriptional regulation of *zntA*. The terminal cysteine was found to be important for defining the sensitivity and magnitude of induction of P*zntA* by ZntR.

The global effect of ZntR was studied in an attempt to understand the wider role of ZntR and Zn(II) resistance mechanisms in *E. coli*. An *E. coli* wild-type strain and a strain in which *zntR* had been deactivated were grown in normal LB media and LB media supplemented with Zn(II). RNA was extracted from all the strains and transcription profiles were generated using gene macroarray technology. A previously uncharacterised putative Zn(II) transporter was highlighted. Additionally, a pattern appeared in the *zntR*-deactivated strain of an increase in transcripts which encode proteins that could assist in Zn(II) chelation or transport.

Finally, the function of a series of previously uncharacterised ORFs, designated the *ybh* cluster, was investigated for function by means of bioinformatic analysis. The results suggest that the *ybh* cluster encodes a novel ABC metal-ion importer. Further support came from luciferase assays where luminescence was driven by the cloned promoter of the *ybh* cluster, designated P*ybh,* in response to Zn(II), Cd(II), Co(II) and Ni(II). Co(II) was found to potently decrease transcription from P*ybh*. The other metal-ions also decreased transcription from P*ybh* but at relatively higher concentrations. Minimum inhibitory concentration (MIC) assays and growth studies were carried out with a *ybhR* knockout strain as YbhR is predicted to be one of the integral membrane transporters in this ABC-type importer. The *ybhR* knockout strain demonstrated higher MIC values than the wild-type strain for Co(II) and a definite growth advantage was observed. The *ybh* cluster appears to be the first identified ATP-hydrolysing Co(II) import system in *E. coli*.

CONTENTS

LIST OF FIGURES & TABLES

Figures

Tables

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	Base pairs
BSA	Bovine serum albumin
D_x	Optical attenuance (at *x* nm wave-length)
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5′-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
fig.	Figure
g_{av}	Average acceleration due to gravity
IPTG	Isopropyl-D-thiogalactopyranoside
Kb	Kilo base pairs
kDa	Kilo Daltons
NaCl	Sodium chloride
nt	Nucleotide
ORF	Open reading frame
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris-Acetate EDTA
TBE	Tris-Borate EDTA
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
v/v	Volume / volume
w.t.	Wild-type
w/v	Weight / volume

CHAPTER ONE

INTRODUCTION

1.1 Metal-ions in the environment

Metal-ions are involved in numerous biochemical processes. Whether the role is catalytic, for example in cytochrome oxidase of the respiratory chain where iron is responsible for redox reactions, or structural, such as the zinc-finger motif of eukaryotic transcription factors, most metals are toxic to a cell beyond a certain concentration. A range of metal-ion resistance and homeostatic mechanisms have arisen to enable bacterial cells to survive in environments with variable metal-ion concentrations.

1.2 Overview of metal resistance mechanisms in prokaryotes

The bacterial metal resistance systems in place probably initially evolved to facilitate survival in a highly toxic environment polluted by ongoing volcanic activity (Rouch *et al.*, 1995). Located on the chromosome, plasmids and transposons, resistance determinants exist for a diverse range of biologically required "essential" and biologically redundant "nonessential" metal ions. Essential metals such as iron (Fe(II)/(III)), copper (Cu(I)/(II)) and nickel (Ni(II)) are involved in redox processes whereas magnesium (Mg(II)) and zinc (Zn(II)) serve structural roles, stabilising various enzymes, proteins and DNA through electrostatic forces (Ji and Silver, 1995). Potassium (K(I)) and sodium (Na(I)) are required for regulation of intracellular osmotic pressure. The nonessential metals silver (Ag(I)), arsenic (As(III)/(V)), gold (Au(I)), lead (Pb(II)), mercury (Hg(II)) and in most cases cadmium (Cd(II)) cause toxicity by competing with essential metals for thiol-containing groups and oxygen sites (Ji and Silver, 1995) (a biological role for Cd(II) has been discovered in *Thalassiosira weissflogii* (Lane and Morel, 2000)).

There are no 'general' mechanisms of metal-ion resistance. Some resistance systems are very specific whereas others may protect against a broad range of metal-ion species. Some metal resistance mechanisms involve enzymatic detoxification, converting a highly toxic metal-ion to a less toxic species but this is not always the case. Usually, metals are expelled from the cell cytoplasm by transport systems. Sequestration proteins (chaperones) that can

assist in the localisation of metal-ions are also present in specific cases. Therefore, there is great diversity in resistance mechanisms, the detail of which is dictated by the nature of the metal-ion species concerned and the nature of the environment. Important features of a metal-ion that determine the cognate resistance mechanism are the chemical properties, abundance, biological relevance and most of all, the relative toxicity of the metal-ion species concerned.

1.2.1 ATP-dependent export

Active transport of excess essential or nonessential metal-ions, that can enter cells through normal nutrient import pathways, represents the largest category of metal resistance systems. Microorganisms can export metal-ions from their cytoplasm utilising energy from ATP hydrolysis. The transport proteins belong to the superfamily of P-type ATPases, a ubiquitous group of proteins involved in the transport of charged substrates across biological membranes (Axelsen and Palmgren, 1998). These can be classified into the 'hard' and 'soft' metal-ion ATPases which correspond to transport of group 1a and 2a metals (such as Na(I) and Ca(II)) and d-block metals (such as Cu(I)/(II), Zn(II), Cd(II) and Pb(II)), respectively.

Focusing on just the soft metal-ion transport ATPases, the structure can be described as consisting of a single, multi-domain protein (fig. 1.1) (Rensing *et al.*, 1999). Efflux ATPases are integral membrane proteins with 8 hydrophobic membrane spanning helices (Solioz and Vulpe, 1996). During transport of the substrate, a conserved aspartate residue on the P-type ATPase is phoshphorylated forming a phosphoenzyme intermediate hence the term 'P-type'. Also common to these P-type ATPases is a conserved proline residue in an integral membrane bound domain, which forms part of the ion translocation pathway. There is a conserved ATP-binding domain and it is through ATP hydrolysis that the energy to drive metal-ion translocation is provided. ATP hydrolysis occurs on the cytoplasmic side of the protein allowing a phosphate group to be passed on to the conserved aspartate residue (phospho-enzyme intermediate). This is coupled to the binding of intracellular cations. A

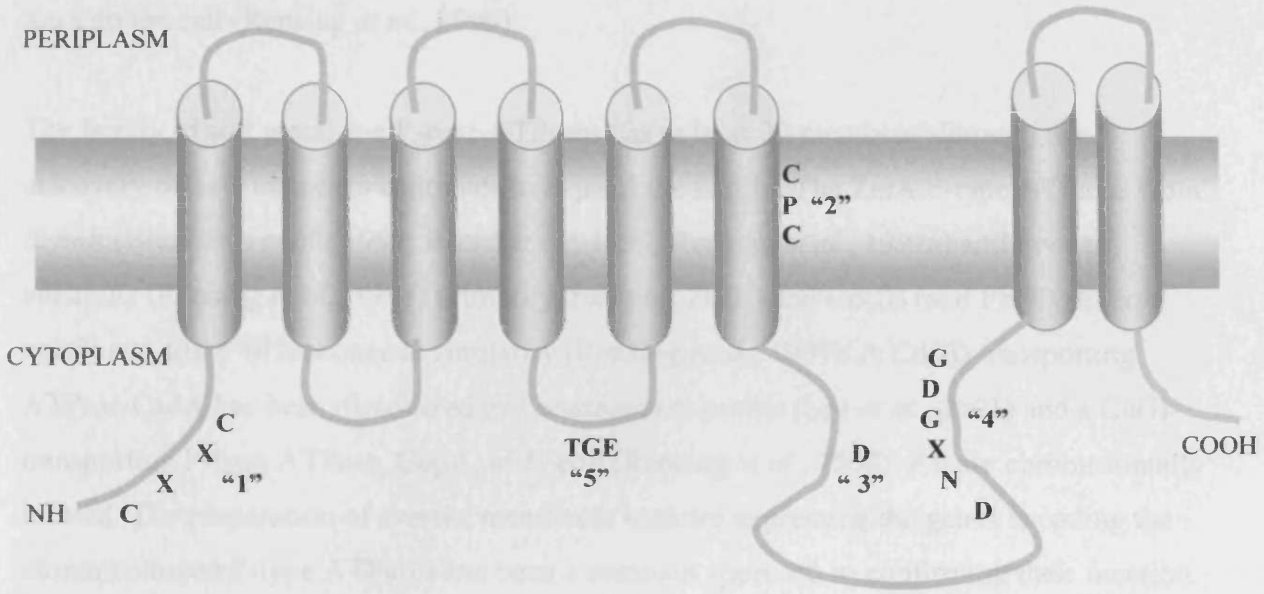


Fig. 1.1 Schematic diagram of a typical soft metal P-type ATPase transporter. Unique and conserved motifs are highlighted in bold text. Motifs "1" and "2" are specific to soft metal-ion P-type ATPases. "1" is the metal-binding motif with cysteines in the sequence CXXC. The number of these motifs varies from one in bacteria to six in some mammalian transporters (Solioz and Vulpe, 1996). "2", located in the sixth transmembrane segment forms the membrane embedded cation translocation pathway containing the CPC (Cys-Pro-Cys) tripeptide. Motifs "3", "4" and "5" are intracellular and are common to all P-type ATPases. Motifs "3" and "4" are part of the aspartyl-kinase domain."3" forms the phosphorylation motif, where the conserved D (Asp) residue is phosphorylated to form the phospho-protein intermediate and "4" is the ATP-binding motif."5" is the phosphatase motif containing the conserved TGE tripeptide which hydrolyses the phospho-intermediate (Rensing *et al.*, 1999).

high-energy intermediate is generated which induces a conformational change causing translocation of the cation across the membrane. The protein, in its low-energy state, binds an extracellular counter-ion. This is associated with dephosphorylation and transport of the ion into the cell (Rensing *et al.*, 1999).

The family of soft metal-ion P-type ATPases has at least 20 members although the discovery of new members continues to expand the family. The ZntA P-type ATPases from *E. coli* (Blencowe *et al.*, 1997, Beard *et al.*, 1997, Rensing *et al.*, 1997a) and *Proteus mirabilis* (Rensing *et al.*, 1998) primarily transport Zn(II) and Cd(II) (and Pb(II) (*E. coli* only)) and share 40% sequence similarity (Rensing *et al.*, 1999). A Cd(II) transporting ATPase CadA has been discovered in *Pseudomonas putida* (Lee *et al.*, 2001) and a Cu(I)-transporting P-type ATPase, CopA, in *E. coli* (Rensing *et al.*, 2000). All are chromosomally located. The preparation of everted membrane vesicles expressing the genes encoding the aforementioned P-type ATPases has been a common approach in confirming their function. The vesicles were found to accumulate radioactive forms of metal-ions, in the presence of ATP. Accumulation was inhibited by micromolar concentrations of vanadate, a potent inhibitor of P-type ATPases (Rensing *et al.*, 1999).

1.2.2 Chemiosmotic export of metal-ions

Other mechanisms of metal-ion expulsion may rely on chemiosmotic gradients. The *czcCBA* system of *Ralstonia metallidurans* located on an indigenous plasmid pMOL30 (Grosse *et al.*, 1999) for example, mediates resistance to Co(II), Zn(II) and Cd(II) and it encodes a cation-proton antiporter driven by a proton gradient (Diels *et al.*, 1995). When these bacteria were grown in media with elevated levels of these metals, an increase in the final pH of the media was observed. The slow build up of alkaline pH indicated the occurrence of proton influx during *czc*-mediated efflux of cations (Diels *et al.*, 1995) (fig 1.2).

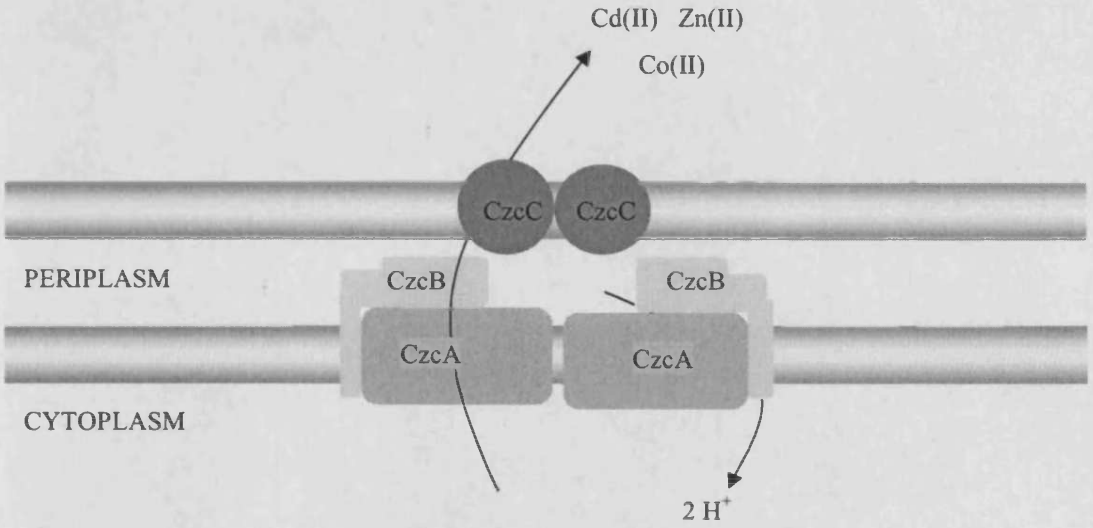


Fig 1.2 A chemiosmotic transporter. The *czcCBA* system of *Ralstonia metallidurans* functions as a dimeric complex, the components of which are embedded in the inner and outer membranes. CzcA is the inner membrane transport protein (Diels *et al.*, 1995) that catalyses the antiport of Cd(II), Zn(II) and Co(II) with H^+. CzcB is an inner membrane anchored periplasmic protein (Rensing *et al.*, 1997b) and CzcC an outer membrane protein (Grosse *et al.*, 1999). Figure adapted from Silver and Phung, 1996.

1.2.3 Extracellular exclusion

In rare cases, microorganisms may protect themselves from toxic metals by blocking their entry. Bacteria generally bind metal-ions non-specifically on their outer membranes or envelopes, which in general are anionic so that metal-ions are readily adsorbed but these barriers are easily saturated (Hoyle and Beveridge, 1983).

Metal-ion specific mechanisms have also been found for example, Cd(II)-binding by *Klebsiella aerogenes* whereby Cd(II) ions are excluded from entry by sulphur excretion resulting in the formation of extracellular cadmium-sulphide precipitates that may aggregate on the cell surface (Aiking *et al.*, 1982).

This type of mechanism has also been discovered in eukaryotes such as *Saccharomyces cerevisiae,* where the absorption of certain metals is reduced by the excretion of large amounts of glutathione, the sulphydryl groups of which bind to heavy metals with great affinity, and the complexes cannot enter the cell membrane (Murata *et al.*, 1985). Increased resistance to Cd(II), Co(II), Zn(II), Ni(II) and Cu(II) has been observed.

Metals can also be excluded by alterations in an organism's cell wall or membrane components in an attempt to protect metal-sensitive sites by reducing the metal-ion permeability. This constitutes another mechanism of extracellular metal exclusion. The outer membranes of many bacteria including *E. coli* contain proteins known as porins which form cylindrical, water-filled channels that allow uptake of selective hydrophilic solutes (reviewed in Jap and Walian, 1996). Mutations in the gene encoding a major porin in *E. coli* B resulted in increased metal tolerance (Lutkenhaus, 1977).

Bacteria that naturally form an extracellular polysaccharide coating demonstrate the ability to 'bioabsorb' metal-ions. The exopolysaccharide coating of these bacteria may provide sites for attachment of metal-ions, as seen in *Klebsiella aerogenes,* and enhances their survival in Cd(II) containing environments (Scott and Palmer, 1990).

1.2.4 Intracellular sequestration

Metal-ions within the cytoplasm may cause toxicity if left to diffuse freely. However, intracellular metallochaperones that bind metal-ions and escort or 'chaperone' them to the required destination can assist in protecting the cellular environment from the metal-ion and shielding the metal-ion from the environment (O'Halloran and Culotta, 2000).

Intracellular sequestration is the accumulation of metals within the cytoplasm to prevent exposure to cellular components. In prokaryotes, there is one prominent example of this form of metal resistance.

Many cyanobacterial strains e.g. *Synechococcus* produce a protein similar to eukaryotic metallothionein (MT) (Olafson *et al.*, 1988). Eukaryotic MT is the most widely studied metallochaperone and is induced following exposure to toxic levels of metal-ions and acts as an effective sponge (Hamer, 1986, Palmiter, 1987). The *Synechococcal* protein, called SmtA, contains a number of cysteine residues that may act as a sink for binding excess Zn(II) and Cd(II).

Prokaryotic intracellular sequestration has also been demonstrated by the cysteine-rich proteins of *Pseudomonas putida*. This strain produces three low molecular weight, cysteine-rich proteins, which may be related to metallothioneins and are involved in binding Cd(II) (Higham *et al.*, 1986, Blindauer *et al.*, 2002).

Although sequestration mechanisms do provide a certain degree of metal-ion tolerance, these mechanisms can be inefficient in providing complete protection as sequestering agents are easily saturated and high levels of expression may be needed to achieve appreciable resistance. In contrast, efflux mechanisms and enzymatic detoxification are efficient, high-turnover systems that can provide effective resistance.

1.2.5 Enzymatic detoxification of a metal to a less toxic form

This type of resistance mechanism is particularly important for highly toxic metals such as Hg(II) or As(III) which can damage outer membrane or periplasmic proteins. Hg(II) resistance is a model example of an enzymatic detoxification system in microorganisms.

The archetypal *mer* operons, located on Tn*501* in the Gram negative bacteria *Pseudomonas aeruginosa* (Stanisich *et al.*, 1977) and Tn*21* from *Shigella flexneri* (de la Cruz and

Grinsted, 1982), provide resistance to Hg(II), which has no known biological function. It is extremely toxic due to its ability to bind strongly to essential thiol groups present in proteins and may result in displacement of native metal-ions (reviewed in Bruins *et al.*, 2000). The *mer* operon has been thoroughly studied and the structure of the *mer* operon from *Pseudomonas aeurginosa* can be seen in fig. 1.3.

The *mer* resistance system combines enzymatic detoxification of Hg(II) to Hg(0), followed by passive diffusion of the volatile Hg(0) out of the cell. The MerT and MerP proteins are collectively responsible for Hg(II) import. MerP is a periplasmic protein, which acts as a Hg(II) scavenger, protecting other proteins in the periplasmic space and the cytoplasmic membrane (Bhriain and Foster, 1986). From MerP-Hg(II), the Hg(II) ions are passed on to MerT, a cytoplasmic membrane protein that facilitates the diffusion of Hg(II) across the membrane (Jackson and Summers, 1982).

Finally, the Hg(II) ions are passed to MerA, a cytoplasmic, NADPH-dependent reductase. MerA is highly specific for Hg(II) and reduces it to the highly volatile and harmless product Hg(0), which escapes from the cell by passive diffusion (Summers and Silver, 1978). Import of Hg(II) followed by reduction to Hg(0) rather than total exclusion has the advantage of protecting both periplasmic and cytoplasmic proteins from damage by Hg(II).

In the absence of Hg(II), transcription of the MerTPAD genes is repressed by the binding of MerR (Lund *et al.*, 1986) to the operator region merO located within the *merTPAD* promoter P_T. Binding of Hg(II) to MerR induces a conformational change in the MerR-Hg(II)-operator complex (O'Halloran *et al.*, 1989) allowing greater accessibility of RNA polymerase to the promoter, inducing transcription of *merTPAD*. This mechanism of switching from a transcriptional repressor to an activator upon binding of the ligand is a typical feature of the MerR family of transcriptional regulators for which MerR is the archetype. The family of MerR regulators will be discussed thoroughly in section 1.5.

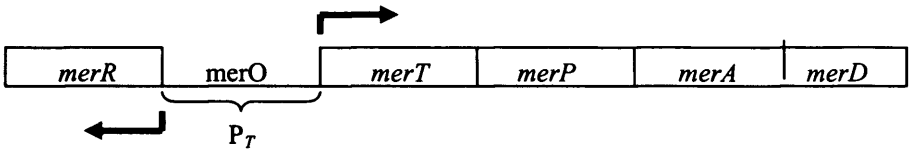


Fig. 1.3 The structure of the *mer* operon on Tn*501* from *Pseudomonas aeruginosa*. *merTPAD* are transcribed as one transcript controlled by a single promoter P_T. The gene for the regulator MerR is transcribed divergently from the MerTPAD genes and its expression is autoregulated from P_T. The operator, merO represents the transcriptional binding site of MerR for regulation of *merTPAD*.

MerD is another regulatory protein that shows similarity to the DNA binding region of MerR and binds weakly to merO (Mukhopadhyay *et al.*, 1991). Experimental data suggest that MerD may compete with MerR for merO however it regulates transcription of the structural genes of the *mer* operon, diminishing their transcription (Nucifora *et al.*, 1989b).

1.3 Role of Zn(II) in prokayotes

Zn(II) occurs exclusively as the divalent cation Zn^{2+}. Its filled d-orbitals render it incapable of undergoing redox changes under biological conditions and it is a strong intracellular Lewis acid (Nies, 1999). Its roles are primarily structural coordinating to the thiol group of cysteine, the imidazole ring of histidine and acidic side-chains of glutamate and aspartate. Zn(II) serves as a cofactor in members of all six classes of enzymes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Vallee and Auld, 1990). Zn(II)

atoms are found in bacterial RNA polymerase and many other enzymes involved in the transcription and replication of nucleic acids (Wu *et al.*, 1992).

Zn(II) is accepted as being necessary for the growth of most living organisms. Its association with cell division was first demonstrated after studies with bacteria (Kung *et al.*, 1976). It is an important component in the zinc finger motif of eukaryotic DNA-binding proteins. This structural motif has been found in some bacterial proteins (Chou *et al.*, 1998).

At elevated concentrations, Zn(II) is known to be a potent inhibitor of the electron transport system of bacteria. In *E. coli*, Zn(II) ions inhibit electron transport at the level of succinate dehydrogenase as well as at the level of the two terminal oxidase complexes, cytochromes *bd* and *bo'* (Beard *et al.*, 1995).

1.3.1 Zn(II) metabolism in *E. coli*

Since prokaryotes lack subcellular compartmentalisation, protection of the cytoplasmic, periplasmic and membrane components from the toxic effects of excess Zn(II) depends largely upon tightly regulated import and export systems, summarised in fig. 1.4. These are particularly important in *E. coli* where the presence of Zn(II) sequestration proteins has not been firmly established (Olafson *et al.*, 1988). Of these transporters, some that primarily transport Zn(II) are not impartial to chemically similar ions such as Cd(II), Co(II) and Pb(II) (Hantke, 2001).

1.3.1.1 Zn(II) import systems

These can be classed into the high affinity, specific import systems ZnuABC (Patzer and Hantke, 1998) and ZupT (Grass *et al.*, 2002), or the broad spectrum, low affinity importers.

Fig. 1.4 An overview of the import and export pathways that operate in *E. coli* under conditions of low, normal or high (toxic) Zn(II) concentrations. Depending on the Zn(II) concentration in the medium, different Zn(II) transporters are synthesised. When Zn(II) is limited, the ZnuABC system is synthesised for high-affinity, specific import of Zn(II) (Patzer and Hantke, 1998). At high concentrations, the potent Zn(II) exporter ZntA, is induced (Blencowe *et al.*, 1997, Beard *et al.*, 1997, Rensing *et al* 1997a). ZupT is also a high affinity importer but it is not as critical as ZnuABC (Grass *et al.*, 2002). Other 'broad range' importers may operate in addition to ZnuABC possibly to assist import and for general homeostasis at normal Zn(II) concentrations. These include the Pit sytem (Beard *et al.*, 2000), MntH (Patzer and Hantke, 2001) and Mg(II) import systems I (CorA) and II (MgtA homologue) (Jasper and Silver, 1997). An additional Zn(II) exporter, ZitB (Grass *et al.*, 2001), may also operate under these conditions.

1.3.1.1.1 The ZnuABC high affinity Zn(II) uptake system

The ZnuABC proteins of *E. coli* belong to the divalent metal-ion ABC transport systems unique to gram-negative and gram-positive bacteria. These transporters possess an ATP-binding cassette (the ABC subunit) and utilise ATP for ligand import (Dintilhac *et al.*, 1997). High-affinity, Zn(II) specific import occurs via proteins encoded by the chromosomal *znuABC* operon (fig. 1.5) under Zn(II)-replete conditions (Patzer and Hantke, 1998).

The primary sequence similarity of ZnuA to the periplasmic binding protein of the manganese (Mn(II)) transport system of *Synechocystis* sp. (Bartsevich and Pakrasi, 1995) and an apparent cleavable leader sequence (Patzer and Hantke, 1998) indicate that it has the potential to bind metal-ions and is located in the periplasm. It has therefore been designated the Zn(II) uptake protein which, after binding Zn(II), directs it to the ZnuB dimer, a hydrophobic dimer possessing similarity to the membrane component of other ABC transport systems. This complex is associated with the ZnuC ATP binding subunits, which hydrolyse ATP in order to import Zn(II) (Patzer and Hantke, 1998).

1.3.1.1.2 High affinity Zn(II) uptake via ZupT

ZupT from *E. coli* is primarily a Zn(II) importer belonging to the eukaryotic ZIP (ZRT, IRT-like protein) family of metal-ion transporters which are predicted to be eight-pass transmembrane proteins (Guerinot, 2000). ZupT transports Zn(II) across the inner membrane into the cytosol (Grass *et al.*, 2002). It was found to confer Zn(II) sensitivity when expressed at high levels although it appears to have a lower affinity for Zn(II) than the ZnuABC system because strains with *zupT* deletions were less inhibited in growth by the addition of high concentrations of EDTA than strains with *znuABC* deletions. ZupT may additionally import Cd(II) and Cu(II) (Grass *et al.*, 2002).

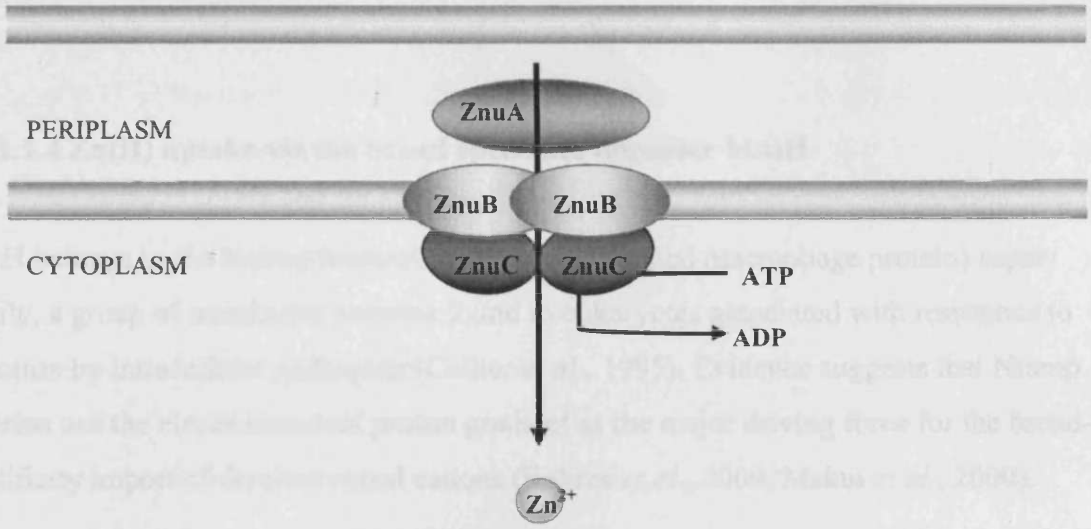


Fig. 1.5 The ZnuABC Zn(II) importing system of *E. coli*. The periplasmic binding protein, ZnuA, acts as a receptor for Zn(II) and presents it to the integral membrane import complex, the ZnuB dimer. This complex is associated with the ZnuC ATP binding subunits, which hydrolyse ATP in order to import Zn(II) (Patzer and Hantke, 1998).

1.3.1.1.3 Import of Zn(II) via the broad spectrum Pit system

In addition to the inducible high-affinity Zn(II) uptake systems in *E. coli*, a constitutive, low-affinity uptake system co-exists. Insertional mutagenesis of *pitA* conferred increased resistance to Zn(II) indicating that co-transport of Zn(II) with phosphate occured via the Pit (phosphate inorganic transport) system (Beard *et al.*, 2000).

Divalent metal cations such as Zn(II) diffuse across into the periplasmic space as a neutral phosphate complex ($Zn^{2+}HPO_4^{2-}$) and then into the cytosol via PitA. As well as Zn(II),

many other metal cations may use this route to enter the periplasm such as Mg(II), Ca(II), Mn(II) and Co(II) (van Veen *et al.*, 1994).

1.3.1.1.4 Zn(II) uptake via the broad spectrum importer MntH

MntH belongs to the Nramp (natural resistance associated macrophage protein) super-family, a group of membrane proteins found in eukaryotes associated with resistance to infection by intracellular pathogens (Cellier *et al.*, 1995). Evidence suggests that Nramp proteins use the electrochemical proton gradient as the major driving force for the broad-specificity import of divalent metal cations (Kehres *et al.*, 2000, Makui *et al.*, 2000).

In *E. coli*, *mntH* encodes a putative membrane protein with high similarity to the Nramp family of transporters. It has been functionally characterised as a proton-dependent divalent cation importer that is able to facilitate the intracellular import of several divalent metal-ions including Zn(II), with substrate preference of Mn(II) > Fe(II) > Cd(II) > Co(II) > Zn(II) > Ni(II) (Makui *et al.*, 2000). MntH is regulated by the external availability of metal ions and by the dual action of the Fe(II) and Mn(II) dependent repressor proteins Fur and MntR (Patzer and Hantke, 2001).

1.3.1.1.5 Zn(II) uptake via broad spectrum Mg(II) import sytems

In *E. coli*, two Mg(II) uptake systems exist that import Zn(II) in addition to Mg(II). System I, a high-affinity, fast non-specific metal transport system, known as CorA was identified as a Mg(II) import protein (Silver, 1969). It is ubiquitously found in gram-negative and gram-positive bacteria and is constitutively expressed (Smith and Maguire, 1995). Evidence suggests it mediates the influx of Mg(II) as well as Ni(II), Co(II) and Zn(II) into the cytoplasm (reviewed in Jasper and Silver, 1997).

System II is a specific Mg(II) transport system that is regulated in response to the Mg(II)

concentration (Jasper and Silver, 1997). System II is analogous to the Mgt system (Park *et al.*, 1976) and has homologues in many other organisms the best characterised of which is MgtA from *Salmonella typhimurium* (Tao *et al.*, 1995). MgtA is a P-type ATPase which catalyses ATP-dependent Mg(II) and Zn(II) uptake. The extremely high similarity (88%) of system (II) (*E. coli*) with MgtA (*Salmonella typhimurium*), suggests that it also transports Zn(II) (Bucheder and Broda, 1974).

1.3.1.2 Putative Zn(II) sequestration proteins

Bacterial metallothioneins (MT) have not been widely reported however, an MT-like protein which can bind Cd(II) has been isolated from *E. coli* (Khazaeli and Mitra, 1981). A Zn(II) binding bacterial MT, containing a Zn(II)-finger motif similar to that found in cyanobacterial SmtA has also been isolated (Blindauer *et al.*, 2002).

In *E. coli*, the membrane associated Zn(II) binding protein ZRAP (Zinc Resistance Associated Protein), formerly YjaI, was characterised and found to be responsible for increased Zn(II) resistance (Noll *et al.*, 1998). It undergoes Zn(II)-induced cleavage releasing the 12 kDa C-terminal fragment into the periplasm where it can bind Zn(II) with significant selectivity Zn(II). Its role may be that of a Zn(II) binding protein that protects the periplasm from Zn(II) (Noll et *al.*, 1998).

The expression of ZRAP is thought to be regulated by a two-component system ZraS/ZraR (formerly HydH/G), inversely orientated to ZRAP on the chromosome at a distance of 237 bp (Leonhartsberger *et al.*, 2001). ZraS has the sequence signature of a sensor kinase, is located in the membrane fraction and specific binding of the activator ZraR to the promoter region of ZRAP and ZraSR has been demonstrated. ZraS forms the sensory part of the system and activates ZraR by phosphorylation. Located in the membrane, ZraS probably senses a change in the external metal-ion concentration via its 13 histidines and cysteine residue (Leonhartsberger *et al.*, 2001).

Though ZRAP probably binds Zn(II) thereby protecting periplasmic proteins from toxic levels, alternatively, its role may be that of a sensor for the Zn(II) concentration in the periplasm which is reported to ZraS by protein-protein interaction and the signal transferred to ZraR. ZraR may in turn induce an unknown protein in addition to ZRAP and the ZraS/R promoter which it autoregulates (Leonhartsberger *et al.*, 2001).

1.3.1.3 Zinc export

There are two characterised Zn(II) export systems in *E. coli.* The first, ZntA, is highly specific and utilises energy from ATP hydrolysis. The second system ZitB, translocates Zn(II) across the inner membrane through chemiosmosis.

1.3.1.3.1 Export of Zn(II) via the highly specific ATPase ZntA

A gene was identified at 77.7 min in the genome of *E. coli* encoding a putative cation transporting P-type ATPase, originally designated *ybhO* (Sofia *et al.*, 1994). The gene was subsequently designated *zntA*, a Zn(II) export protein consisting of 732 amino acids (Beard *et al.*, 1997).

Disruption of *zntA* in *E. coli* resulted in hypersensitivity to Zn(II) and Cd(II) (Beard *et al.*, 1997, Rensing *et al* 1997a) and expression from a heterologous promoter conferred increased tolerance to both these metals (Blencowe *et al.*, 1997). In further experiments, ZntA was shown to catalyse ATP-coupled accumulation of ^{65}Zn(II) and ^{109}Cd(II) in everted membrane vesicles with transport being sensitive to vanadate. The *zntA* disrupted strain did not accumulate these metal-ions and the transport defect was complemented by a plasmid bearing *zntA* (Rensing *et al.*, 1997a).

Furthermore, the amino acid sequence of ZntA contains CPx P-type ATPase features; the presence of an aspartyl kinase domain (conserved aspartate residue), TGE hydrolysis motif,

GDGXND ATP-binding domain, CXXC metal binding motif and CPC cation translocation pathway (Okkeri and Haltia, 1999) (refer to fig. 1.1). The expression of *zntA* is regulated by ZntR belonging to the MerR family of transcriptional regulators (Brocklehurst *et al.*, 1999). This is discussed in more detail in section 1.5.2.

An aspartate residue immediately precedes the CXXC metal binding motif. This domain was cloned and expressed and was shown to increase the overall catalytic rate of metal transfer by increasing the rate of metal-ion binding to the transporter. The solution structure of this motif was determined by NMR (Banci *et al.*, 2002). The conformation of these two cysteines (C59 and C62) and the aspartate (D58) residue in this domain underwent changes during conversion from the apo-ZntA to the Zn(II)-ZntA form. D58 and C59 showed the most significant conformational changes, moving closer to the Zn(II) ion, whereas C62 did not change conformation. In addition to these residues, a water molecule may complete a tetragonal geometry for binding Zn(II), since the Zn(II) is rather solvent exposed. The accessibility is likely to allow for rapid access of other domains of the ATPase to the metal site thus facilitating metal transfer. This structure provided the first example of a Zn(II) ion bound in a protein to 2 cysteines and one aspartate residue (Banci *et al.*, 2002).

ZntA has 30% sequence identity with the products of the human Wilson disease gene and Menkes disease gene both of which are inherited through the X-chromosome and encode defective Cu(I) transporting P-type ATPases. The structural resemblance of these P-type ATPases with ZntA helped elucidate the functional significance of mutations in these disease genes (Okkeri *et al.*, 2002). Mutations in these genes cause characteristic disease phenotypes. In Menkes disease, the defective P-type ATPase fails to transport Cu(I) into the gut and other tissues but not the liver. In Wilson disease the Cu(I) export ATPase of the liver is defective. Consequently, in Menkes disease there is a lack of Cu(I) resulting in lack of important enzyme activities and death occurs in early childhood (Gitschier *et al.*, 1998). In Wilson disease there is an excess of Cu(I) in the liver and brain and symptoms range from acute childhood liver failure to progressive neurological deterioration (Bull and Cox, 1994).

Most of the Wilson disease genotypes consist of amino acid substitutions. ZntA was used as a protein model to explore structure-function relationships within the Wilson disease ATPase by the systematic introduction of substitutions into the primary sequence (Okkeri *et al.*, 2002).

In ZntA, the conserved aspartate D628, is normally phosphorylated as it forms the aspartyl kinase domain. Mutagenesis of this aspartate to a leucine rendered it incapable of undergoing phosphorylation. This led to an understanding of the mechanism of Wilson's disease in patients with the D1267A genotype as D628 in ZntA is homologous to D1267 (Okkeri *et al.*, 2002).

1.3.1.3.2 Chemiosmotic export of Zn(II) via ZitB

The ubiquitous CDF (cation diffusion facilitator) importers and exporters constitute a family of transporters which are essentially membrane integral proteins that catalyse slow efflux of Zn(II), Co(II) and Cd(II), the energy for which is derived from chemiosmosis (Paulsen and Saier, 1997). Members of this family possess common structural characteristics with six transmembrane domains and histidine rich motifs predicted to extend into the cytosol. There are conserved acidic residues present in some of the putative transmembrane domains which may serve as cation binding sites (Paulsen and Saier, 1997).

An additional Zn(II) exporter was identified in *E. coli* called ZitB (formerly YbgR) belonging to the CDF family (Grass *et al.*, 2001). A clear relationship between *zitB* expression and Zn(II) tolerance was found. Although disruption of both *zitB* and *zntA* resulted in hypersensitivity to Zn(II), a strain disrupted only in *zitB* did not exhibit decreased Zn(II) tolerance. However, overexpression of *zitB* on a plasmid led to increased Zn(II) tolerance. These findings suggest that ZitB is an additional Zn(II) transporter in *E. coli*, contributing to Zn(II) homeostasis at non-toxic Zn(II) concentrations while ZntA is required for growth at higher and more toxic concentrations (Grass *et al.*, 2001).

Moreover, it seems that ZitB is an antiporter exchanging Zn(II) for K^+ or H^+ (Lee *et al.*, 2002). This antiport mechanism, where exchange is energised by the transmembrane pH gradient, was first identified in CzcD of *Bacillus subtilis,* a known Zn(II) antiporter also belonging to the CDF family (Guffanti *et al.*, 2002).

1.4 Transcriptional regulation in *E. coli*

The process of transcription is initiated (fig. 1.6) by the binding of RNA polymerase to the promoter region preceding the gene (Epstein and Beckwith, 1968). The RNA polymerase of *E. coli* consists of five subunits; two α subunits, and one each of β, β' and σ. The five-subunit structure, α_2 ββ'σ, has a molecular weight of approximately 465 kDa and is referred to as the holoenzyme (Burgess *et al.*, 1969). The σ subunit (or σ-factor) is crucial for promoter binding specificity and accurate initiation from the required gene (Travers and Burgess, 1969). *E. coli* possess seven different σ-factors for recognition of diverse promoters, the primary one is σ^{70} which controls expression of the majority of housekeeping genes (Helmann and Chamberlin, 1988).

The RNA polymerase holoenzyme binds to the promoter and opens up the double helix, a process called 'promoter melting', exposing bases to create a single-stranded template on the antisense strand. This structure is called the transcription bubble. Ribonucleotides can enter the bubble and base-pair with the antisense strand, of which about 17 bases are exposed. RNA polymerase forms the phosphodiester bonds between the sugars of neighbouring nucleotides forming the backbone of the newly synthesised mRNA in a 5′ to 3′ direction.

After initiation, the σ-factor dissociates leaving behind the "core enzyme", α_2 ββ' which is capable of polymerising mRNA (Chamberlin, 1974). The remaining core enzyme proceeds along the DNA unwinding small sections followed by synthesis of mRNA. As transcription proceeds, the newly synthesised mRNA dissociates from the DNA allowing it to re-form its natural double-helical structure.

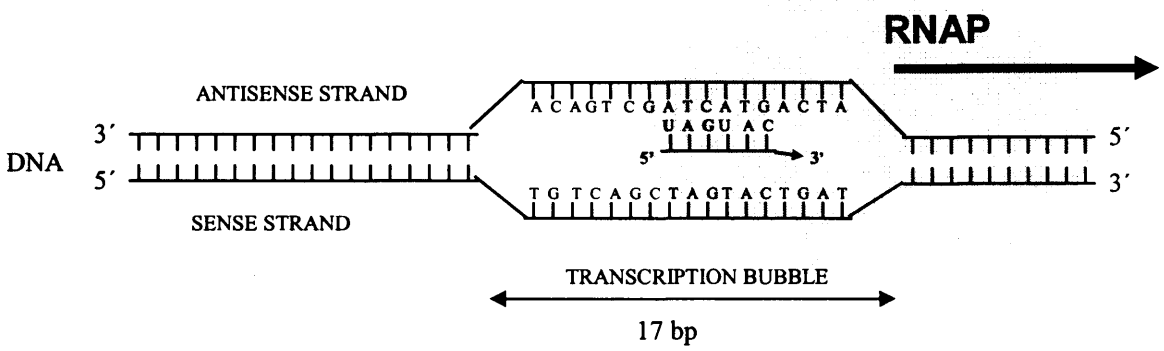


Fig. 1.6 Transcriptional initiation and elongation. RNA polymerase (labelled 'RNAP') binds to the promoter and opens up the helix. A new mRNA transcript is synthesised in a 5′ to 3′ direction. Ribonucleotides can enter the bubble and base pair with the antisense strand of which about 17 bases are exposed. RNA polymerase forms the phophodiester bonds between the sugars of neighbouring nucleotides forming the backbone of the newly synthesised mRNA proceeds along the gene in the direction indicated by the bold arrow, until termination occurs.

RNA synthesis eventually comes to a halt when it reaches a signal that indicates the end of the gene. Secondary structures known as 'hairpins' are formed in the newly synthesised mRNA and cause RNA polymerase to pause synthesis of RNA by blocking its path, leading to eventual dissociation from the structure. The termination signal is composed of a GC-rich dyad symmetry element followed by a run of U residues in the mRNA (Brendel *et al.*, 1986). Termination may sometimes additionally involve a termination factor such as Rho (Roberts, 1969).

Regulation can occur at any of the steps leading to formation of a functional protein. This influences when and how much of a particular protein is made. The main point of control is

transcription. This is greatly influenced by the strength of the promoter and the regulatory proteins that may bind to it.

The activity of σ-factors can be inhibited by anti-sigma factors (Brown and Hughes, 1995). These are proteins that negatively regulate transcription by interacting with a specific sigma factor. This can have inhibitory effects by preventing the σ-factor from recognising and binding to the promoter. In some cases, binding of the anti-sigma factor causes a conformational change in the σ-factor rendering it incapable of binding to the other RNA polymerase subunits (Brown and Hughes, 1995).

1.4.1 *E. coli* promoters

The elements located in promoters which act as the recognition sites for σ^{70}, the major σ-factor, were identified by comparison of promoter sequences from a multitude of *E. coli* genes. The start point of transcription known as +1 is a purine (A or G) over 90% of the time and is commonly the central base in the sequence C A/G T (Hawley and McClure, 1983). There are certain features present in a σ^{70} promoter which enable RNA polymerase to recognise and bind. These are stretches of DNA which are conserved in most promoters and are crucial for RNA polymerase binding: a hexamer, $T_{82}A_{90}T_{52}A_{59}A_{49}T_{89}$ (subscript numbers denote percentage conservation) called the –10 element or Pribnow box, centred about 10 bp upstream of +1; another hexamer, $T_{78}T_{82}G_{68}A_{58}C_{51}A_{55}$ centred approximately 35 bp upstream from the start point of transcription known as the –35 element (Hawley and McClure, 1983).

There is a strong correlation between promoter strength and the degree of sequence identity contained in the –10 and –35 elements compared with the consensus sequences, although no naturally occurring promoter is a perfect match with the predicted consensus sequences (Harley and Reynolds, 1987). Mutational studies on several genes have shown that the strength of transcription increases as the sequence of the promoter approaches the consensus (Youderian *et al.*, 1982, Szoke *et al.*, 1987).

The distance separating the –35 and –10 sites is known as the spacer region. Although the actual sequence of the spacer region is relatively unimportant, the distance is critical in holding the two sites at the appropriate separation for the geometry of RNA polymerase. The optimal spacer region for perfect alignment of the –35 and –10 elements is 17 ± 1bp. In 90% of promoters the spacer length is 17 ± 1bp, however it can be as little as 15 bp or as great as 20 bp in the remaining 10% of σ^{70} promoters (Harley and Reynolds, 1987). The 17 bp spacer length brings the –35 and –10 elements into phase accurately enough to allow them to interact simultaneously with RNA polymerase for transcription initiation. The length of the spacer is critical for determining promoter strength (Harley and Reynolds, 1987).

Other σ-factors can be activated in response to environmental changes and recognise other sequences at –10 and –35, specific for each σ-factor. For example σ^{32} which responds to heat shock specifically recoginises CCCGATNT (–10) and CCCTTGAA (–35) promoters (Helmann and Chamberlin, 1988).

Additionally, A+T rich sequences may be found upstream of the –35 region and are known as UP (upstream promoter elements). UP elements increase the strength of transcription in promoters where they are present (Newlands *et al.*, 1992).

Not all σ factors recognise –10 and –35 elements as in the case of σ^{54}. This σ-factor recognises promoters containing the sequence CTGGCACNNNNNTTGCA and is generally involved in the transcription of genes required in response to nitrogen starvation (Beynon *et al.*, 1983, Hunt and Magasanik, 1985).

1.4.2 Transcriptional regulatory proteins

Transcriptional regulators can repress or activate transcription. Repressors are regulatory proteins that suppress transcription by binding to a specific site (operator) within the

promoter or overlapping it. The operator-repressor complex can inhibit transcription initiation at the promoter by preventing RNA polymerase from contacting the promoter elements. The gene can be transcribed once the repressor has dissociated from its operator, usually in response to some environmental or nutritional stimulus. Small molecules known as effectors can affect the binding affinity of repressors for operator sites. These bind to repressors and occur in two forms known as inducers and co-repressors. Inducers decrease binding of repressors to the DNA whereas co-repressors are essential for the repressor to function.

An example of a repressor protein is Zur in *E. coli.* The *znuABC* system is regulated by Zur which bears 27% sequence similarity to the Fur protein (ferric uptake regulator) in *E. coli* (Patzer and Hantke, 1998). It is highly sensitive and can sense subfemtomolar concentrations of cytosolic Zn(II) (Outten and O'Halloran, 2001). Zur binds to a region of dyad symmetry within the *znuC* promoter in the presence of Zn(II) (Patzer and Hantke, 2000). The binding of Zur to the cognate promoter is inhibited by excess EDTA therefore in the absence of Zn(II), Zur does not compete for DNA binding, allowing RNA polymerase to bind and form an open complex (Outten and O'Halloran, 2001).

Zur, a cytoplasmic protein, is active only in the reduced form (Gilbert, 1990) and functions as a dimer (Patzer and Hantke, 2000). It contains nine cysteine residues, four of which are conserved in *E. coli* Fur. Of these cysteines, C93 and C96 have been shown to bind Zn(II) in Fur and are in fact conserved in all Fur-like proteins except those from *Pseudomonas sp.* (Jacquamet *et al.*, 1998). In Fur, binding of Fe(II) to Fur does not displace the Zn(II) ion; rather, Fe(II) binds at a different site. Zur binds Zn(II) very tightly at this site as in Fur and so its purpose at this site may be structural and not regulatory (Outten *et al.*, 2001). A second Zn(II) binding site in Zur was identified which is similar to the metal sensing site in SmtB from *Synechococcus*. This may act as the sensing site as in Fur where Fe(II) binds at a secondary site (Outten *et al.*, 2001).

The expression of SmtA, the *Synechococcal* metallothionein is regulated by a divergent gene product, SmtB (Morby *et al.*, 1993). This is a transcriptional repressor of *smtA* and

binds to the promoter region, dissociating in the presence of excess Zn(II). SmtB belongs to the ArsR family of transcriptional metalloregulators (Morby et al., 1993). Members of the ArsR family function as dimers and contain a centrally located helix-turn-helix DNA binding motif. This is preceded by the metal binding CVC motif in which the two cysteines are implicated in metal-ion binding. ArsR proteins remain bound to their cognate promoters until the inducer, Zn(II) or Cd(II) in the case of SmtB, binds to the metal binding motif causing a conformational change in the helix-turn-helix motif and subsequent dissociation from the promoter, permitting transcription of *smtA* (Shi et al., 1994).

Activators are regulatory proteins, which enhance binding of RNA polymerase to weaker promoters that don't have an optimal structure compared to the consensus. The activator may enhance transcription for example, by realigning the –10 and –35 elements in the promoter. In the absence of the activator, basal transcription can still occur but the presence of the activator increases the transcription rate because the promoter elements are aligned to the optimal positions for RNA polymerase binding. An example of a metalloregulatory activator is detailed in the following section.

1.5 The MerR family of ubiquitous transcriptional regulators

The regulator of gram-negative mercury resistance operons found on Tn*501* in *Pseudomonas aeruginosa* (Stanisich *et al.*, 1977) and Tn*21* from *Shigella flexneri* (de la Cruz and Grinsted, 1982) is the archetype of the MerR family of transcriptional activators and is called MerR. This transcriptional regulator was shown to activate the *mer* operon in the presence of Hg(II) and repress it in its absence (Lund *et al.*, 1986).

One of the striking features of MerR was the unusually large spacer length of the cognate promoter P_T, at which it acts, suggesting that the promoter structure was important in regulation (Lund and Brown, 1989a). This feature is common to all MerR-regulated promoters (fig. 1.7) where the spacer region is longer than the optimal for a σ^{70} promoter

CoaR	*Pseudomonas denitrificans*	Co(II)	*coaT* – putative export ATPase	AAACC**TTGACA**TTGACACTAATGTTAAGG TTT**AGGCT**GAG	(Rutherford *et al.*, 1999)
BmrR	*Bacillus subtilis*	Rhodamine, TPP	*bmr* - multidrug transporter	CCG**TTGACT**CTCCCCTAGGAGGAGGTC**TT ACAGT**ATAAGGGATAC	(Ahmed *et al.*, 1994)
$TipA_L$	*Streptomyces lividans*	Thiostrepton	Thiostrepton-responsive genes	G**GCTTGCA**CCTCACGTCACGTGAGGAGG**C AGCGT**GGACGGC /GTCA	(Holmes *et al.*, 1993)
SoxR	*E. coli*	Oxidative stress	*soxS*	CGC**TTTACC**TCAAGTTAACTTGAGGAATT **ATACT**CCCCAAC /AGAT	(Amabile-Cuevas and Demple, 1991)

Fig. 1.7 Examples of metal responsive and non-metal responsive MerR type regulators. The sequences of the cognate promoters regulated by the respective MerR proteins are shown with the –10 and –35 elements in bold font. The inverted repeat sequences within the promoters are underlined. The length of the spacer regions between the –10 and –35 elements are 19 bp in most of the promoters with ZntR and CadR regulated promoters as an exception with 20 bp spacers. The transcript start site, where known is indicated by /.

(17 ± 1bp) and are typically 19 or 20 bp, making them poor substrates for RNA polymerase (deHaseth *et al.*, 1998).

The apparent inverted repeats in all of these promoters assist binding of the MerR regulators as dimers. The MerR family of bacterial transcriptional regulators consist of proteins with N-terminal helix-turn-helix DNA binding regions and C-terminal regions which contain coiled-coil dimerisation motifs and the ligand (effector) binding region that is specific to the effector recognised. The effectors are mainly environmental stimuli; heavy metal-ions, antibiotics or oxidative stress.

MerR homologues have been characterised (fig. 1.7) which respond to a range of divalent metal-ions such as Zn(II) (Brocklehurst *et al.*, 1999), Pb(II) (Borremans *et al.*, 2001), Cu(I) (Outten *et al.*, 2000), Co(II) (Rutherford *et al.*, 1999) and Cd(II) (Lee *et al.*, 2001) (ZntR, PbrR, CueR, CoaR and CadR respectively). The MerR family also includes members which are not metal-responsive but respond to oxidative stress (SoxR), thiostrepton ($TipA_L$) and rhodamine (BmrR) (Amabile-Cuevas and Demple, 1991, Holmes *et al.*, 1993, Ahmed *et al.*, 1994).

One of the characteristics of a MerR-type regulator is the high degree of sequence similarity in the N-terminal DNA binding region (fig. 1.8). Similarity in the C-terminal region is considerably less between the different regulators however the metal-ion responsive subgroup are more similar to one another particularly in potential metal-binding sites (fig. 1.8).

1.5.1 The MerR regulatory mechanism

The structure of the promoter P_T (fig. 1.7) at which MerR is bound has highly conserved –10 and –35 σ^{70}-RNA polymerase recognition elements, however the spacer length in P_T is functionally suboptimal at 19 bp. The *mer* promoter structures are both translationally and

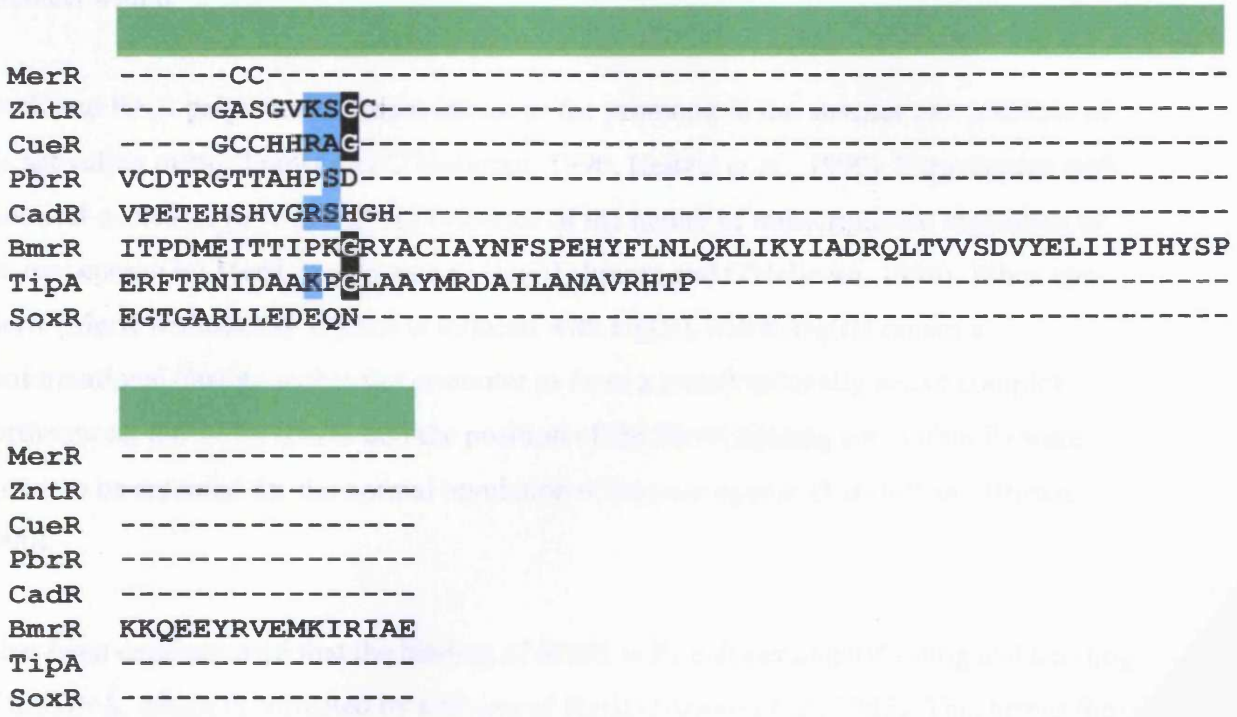


Fig.1.8 Multiple alignment of regulators from the MerR family generated by the CLUSTALW algorithm using BioEdit V 5.0.6. The metal-ion responsive proteins are MerR (*Pseudomonas aeruginosa*), ZntR (*E. coli*), CueR (*E. coli*), CadR (*Pseudomonas aeruginosa*) and PbrR (*Ralstonia metallidurans*). BmrR (*Bacillus subtilis*), $TipA_L$ (*Streptomyces lividans*) and SoxR (*E. coli*) are also shown. Sequence identity is represented by a white font on a black background and similar amino acids are highlighted cyan.

The conserved helix-turn-helix motif within the DNA binding-region was predicted by Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page =/NPSA/npsa_hth.html). The coiled-coil dimerisation motif lies within the region indicated by the yellow boxes. It varies in length depending on the regulator and was predicted using PAIRCOIL (http://www.paircoil.lcd.mit.edu/cgi-bin/paircoil). The ligand-binding region is indicated by green boxes. Red arrows show the conserved cysteines in the metal-ion responsive MerR regulators.

rotationally removed from the optimum structure expected in an *E. coli* promoter that interacts with σ^{70}.

MerR and RNA polymerase remain bound to the promoter in the absence and presence of the activating metal (Frantz and O'Halloran, 1990, Heltzel *et al.*, 1990). Experiments with chemical nucleases provided direct evidence of the nature of transcriptional regulation of the *mer* operon by MerR in response to Hg(II) (Frantz and O'Halloran, 1990). When apo-MerR (MerR without any Hg(II)) is induced with Hg(II), MerR-Hg(II) causes a conformational change within the promoter to form a transcriptionally active complex. Furthermore, the 19 bp spacer and the position of the MerR binding site within P_T were shown to be essential for the normal regulation of the *mer* operon (Parkhill and Brown, 1990).

It has been demonstrated that the binding of MerR to P_T induces underwinding and bending of the DNA, which is corrected by addition of Hg(II) (Ansari *et al.*, 1992). This brings the –10 and –35 elements into phase for transcription by RNA polymerase.

These data support the model of transcriptional induction of the *mer* operon by MerR-Hg(II) in which this complex alters the separation and orientation of the –10 and –35 elements by distorting the promoter P_T, allowing more efficient contact by RNA polymerase. MerR binds Hg(II) in a tricoordinate geometry, with one Hg(II) ion binding per dimer (Shewchuk *et al.*, 1989a). The complete DNA distortion mechanism of Hg(II) induced *merTPAD* transcription is shown in fig. 1.9.

Fig. 1.9 Apo-MerR bends DNA at centre of inverted repeat. RNA polymerase can therefore only contact the –35 element and transcription is repressed. The –10 element is inaccessible due to the length of the spacer and bending of DNA by MerR.

Hg(II) binding to apo-MerR has two effects. Firstly, the bent DNA is straightened to bring both elements towards the plane of RNA polymerase.

Secondly, the DNA is undertwisted so as to realign the –35 and –10 elements, thereby compensating for the structurally supraoptimal spacer length and allowing RNA polymerase to contact both elements simultaneously.

Realignment of the –35 and –10 elements allows transcription of the *mer* operon by MerR. Diagram adapted from Megit (2002).

1.5.2 ZntR: regulator of *zntA* in *E. coli*

A region resembling a MerR type promoter was found upstream of *zntA*. The sequence exhibited strong –10 and –35 consensus elements, a structurally supraoptimal 20 bp spacer and a perfect 11-11 bp inverted repeat. The region was designated P*zntA*, as the promoter for *zntA* (fig. 1.7).

P*zntA* was introduced into a *lux* reporter plasmid called pUCD615 (Brocklehurst *et al.*, 1999). The recombinant plasmid, pUCDP*zntA* was introduced into *E. coli* and assayed for luminescence in response to a range of divalent metal cations. It was concluded that Zn(II) was the primary inducer at P*zntA*, with maximum metal induction occurring at 1.1 mM Zn(II) (Brocklehurst *et al.*, 1999).

The closest known homologue of ZntA is CadA from *Staphylococcus aureus* (Nucifora *et al.*, 1989c). CadA is responsible for Cd(II) efflux and is regulated by a repressor known as CadC which is located immediately upstream of CadA (Endo and Silver, 1995). CadC belongs to the ArsR family of transcriptional regulators (Shi *et al.*, 1994). No CadC or ArsR homologues were found near *zntA*.

Since P*zntA* is very similar in structure to P_T, the *E. coli* database was searched for MerR like proteins using *merR* of Tn*501* as a probe. Two homologues were found, *yhdM* and *o135*, both chromosomal but distantly located to *zntA*. Of the two homologues, *yhdM* had 8.2% more similarity to MerR and 17/22 bp match with the 11-11 bp inverted repeat in P*zntA*, therefore initial efforts were concentrated on *yhdM* as a possible *zntA* regulator (Brocklehurst *et al.*, 1999). *yhdM* was renamed *zntR* and its location was mapped at 74.1 min on the chromosome. Insertional inactivation of *zntR* using a kanamycin cassette resulted in the loss of Zn(II) induced activation of P*zntA* which was restored upon addition of a complementary plasmid copy of *zntR*. Electrophoretic mobility shift assays with purified ZntR resulted in specific binding to P*zntA* confirming ZntR as the cognate regulatory protein for *zntA* (Brocklehurst *et al.*, 1999).

ZntR consists of 141 amino acids and has a predicted molecular weight of approximately 16.2 kDa (Brocklehurst *et al.*, 1999). ZntR binds to P*zntA* as a dimer activating transcription in response to Zn(II), but also to Pb(II), Cd(II) and Hg(II) (Brocklehurst *et al.*, 1999) and bears 34% sequence identity with MerR (Outten *et al.*, 1999).

The sequence of ZntR (fig. 1.10) shows typical MerR-type features with a helix-turn-helix motif located within the N-terminal and a predicted coiled-coil dimerisation domain downstream. ZntR has many potentially metal-binding residues; five histidines and five cysteines, three of which are conserved in MerR. It has been shown that ZntR can bind two Zn(II) ions per monomer (Outten *et al.*, 1999).

In the absence of Zn(II), ZntR can bind to P*zntA* but transcription of *zntA* is induced when Zn(II) levels increase. These parallels with MerR led to the hypothesis that ZntR regulates P*zntA* via a DNA-distortion mechanism similar to MerR regulation of the mercury resistance genes (fig. 1.9) (Outten *et al.*, 1999). To test this hypothesis, a series of modified derivatives of P*zntA* were created (fig. 1.11) (Brocklehurst *et al.*, 1999).

Deletion of 2 nucleotides immediately upstream of the –10 element (P*zntA-2*) brought the length of the spacer down from 20 bp to 18 bp, which is closer to the consensus length of 17 ± 1bp. Constitutive transcription from this construct increased dramatically when assayed in the *lux* reporter system and was probably brought about by the improved alignment of the –10 and –35 elements for RNA polymerase. Addition of Zn(II) to the growth medium led to a decrease in transcription thought to have been caused by a possible over-twisting of the elements. A 1 bp deletion of the nucleotide immediately upstream of the –10 element (P*zntA*-1) had a similar effect. Replacement of the inverted repeat (P*zntA*-IR) with a random sequence abolished Zn(II) induction by ZntR even though the spacer length and base content were conserved, confirming this as the ZntR binding site.

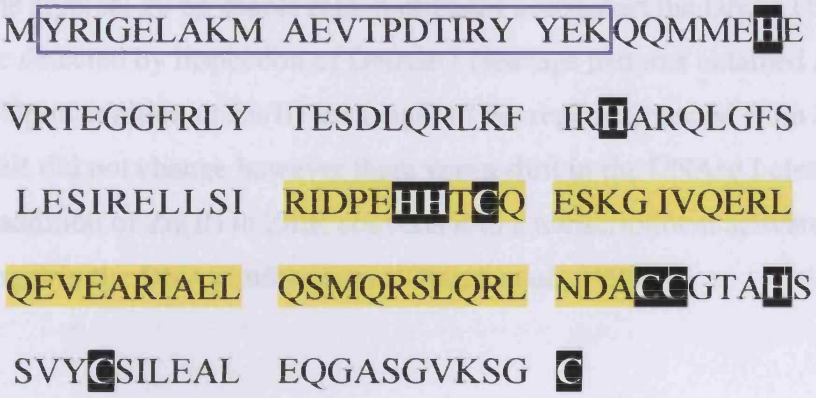


Fig. 1.10 Amino acid sequence of ZntR consisting of 141 amino acids. Within its N-terminal is a helix-turn-helix DNA-binding domain indicated by the blue border. Downstream is the ligand-binding region containing five histidines and five cysteines, all of these are highlighted by a white font on a black background.

The yellow highlighted sequence represents the predicted coiled-coil dimerisation domain, generated using PAIRCOIL (http://www.paircoil.lcd.mit.edu/cgi-bin/paircoil). The location of the helix-turn-helix DNA binding motif was predicted using Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat. pl?page=/ NPSA/npsa _hth.html).

These data help confirm that ZntR regulates *zntA* via a DNA distortion mechanism similar to MerR regulation of P_T (fig. 1.9). Further support came from experiments whereby ZntR was purified to homogeneity and was shown to be a Zn(II) receptor (Outten *et al.*, 1999). Furthermore, DNA footprinting assays with purified ZntR demonstrated that apo-ZntR could bind to the atypical 20 bp spacer region of P*zntA* and distort the DNA. The distortions were detected by inspection of DNAse I cleavage patterns obtained for apo-ZntR at P*zntA*. Upon addition of Zn(II) the length of the region protected from DNAse I cleavage by ZntR did not change however there was a shift in the DNAse I cleavage pattern indicating that addition of Zn(II) to ZntR converts it to a transcriptional activator that introduces changes in the DNA conformation (Outten *et al.*, 1999).

P*zntA* / DERIVATIVE		CONSTITUTIVE TRANSCRIPTION	Zn(II)-INDUCED TRANSCRIPTION
P*zntA*:	**TTGACT**CTGGAGTCGACTCCAGAGTG**TATCCT**	W.T.	W.T.
P*zntA-2*:	**TTGACT**CTGGAGTCGACTCCAGAG****TATCCT**	↑↑↑	↑↑↑
P*zntA-1*:	**TTGACT**CTGGAGTCGACTCCAGAGT***TATCCT**	↑↑↑	↓↓↓
P*zntA-IR*:	**TTGACT**gtggcgcatcgcgtgatcaa**TATCCT**	0	0

Fig. 1.11 A series of deletions of the P*zntA* promoter were created (Brocklehurst *et al.*, 1999). The inverted repeat region is underlined in each case and the –10 and –35 elements are shown by bold font. Deletions are marked by an asterisk (*). The level of constitutive or Zn(II)-induced transcription by ZntR in comparison to P*zntA* from these derivatives is also summarised here. Arrows ↑↑↑ and ↑↑↑ indicate dramatic increases and decreases, respectively. The 0 indicates that there was no transcription.

1.6 Aims

The aims of this study were to elucidate the structure-function relationships in ZntR and to investigate its role on an organismal scale. The characterisation of a novel metal-ion transport system *ybh,* in *E. coli* was the final aim.

The main priorities were to identify residues in ZntR that are essential for metal-ion recognition and binding. ZntR regulates at its cognate promoter as a dimer and the residues involved in dimerisation or promoter binding are also important. Characterisation of the molecular interactions between ZntR and its associated ligands would be approached by the use of site-directed mutagenesis combined with phenotypic assays of regulatory activity.

The possibility that ZntR plays a wider role in cell metabolism other than the regulation of *zntA* will also be investigated with the aid of macroarray technology. *E. coli* (w.t.) and *E. coli* (*zntR* knockout) would be used as control and test strains respectively. These strains would be grown at normal and elevated Zn(II) concentrations and their RNA extracted. Analysis of mRNA transcript profiles may help to elucidate other Zn(II) resistance mechanisms, or Zn(II) induced metabolic changes when the main Zn(II) exporter ZntA is not transcribed due to the absence of its primary regulator, ZntR.

This study concludes with the characterisation of a recently identified gene cluster (*ybh*) on the *E. coli* genome that appears to be involved in metal-ion import. This putative operon is predicted to encode an ABC-transport system along with a novel regulatory protein.

CHAPTER TWO

MATERIALS & METHODS

2.1 Materials

2.1.1 Bacterial strains

Bacterial strains used in this study are listed below in table 2.1.

Table 2.1 The bacterial strains used for general cloning purposes and for assays or other research. For each strain the genotype and reference is shown.

Strain	Genotype	Reference or Source
TG1	*E. coli supE, hsd*Δ5, *thi,* Δ(*lac-proAB*) F′ [*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*Δ15]	(Gibson, 1984)
TG1 (*zntR*::*Km*)	*E. coli supE, hsd*Δ5, *thi,* Δ(*lac-proAB*) F′[*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*Δ15] (*zntR*::*Km*)	(Brocklehurst *et al.*, 1999)
TG2	*E. coli supE, hsd*Δ5, *thi,* Δ(*lac-proAB*) F′[*traD36, proAB*$^+$, *lacI*^{q}, *lacZ*Δ15] Δ(*srl-recA*)306::Tn*10* (*tet*^{r})	(Sambrook *et al.*, 1989)
TOP10	*E. coli* F$^-$ *mcrA* Δ*(mrr-hsdRM mcrBC)* Φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA1 nupG*	Invitrogen

W3110	Wild-type K12 derivative; F⁻ IN(*rrnD-rrnE*) *LAM⁻ rph⁻*	NCIMB Strain No. CNIBM #11296
W3110 (*ybhR::Km*)	Wild-type K12 derivative; F⁻ IN(*rrnD-rrnE*) *LAM⁻ rph⁻* (*ybhR::Km*)	This work

2.1.2 Plasmids

Plasmids used in this study are listed below in table 2.2.

Table 2.2 Plasmids used in the study are shown below along with the reference or source. *Antibiotic markers present in the plasmids are shown by Cm^r, Cb^r, Km^r or Amp^r which respectively confer resistance to Cm, Cb, Km or Amp (refer to section 2.1.3.5).

Plasmid	Relevant characteristic*	Reference or Source
pSU18	Cm^r; cloning and expression vector with *lac* promoter (P_{lac}).	(Bartolome *et al.*, 1991)
pSU18*zntR*	As above (pSU18): *zntR* cloned in *Eco*RI, *Bam*HI sites creating P*lac*::*zntR* fusion.	(Brocklehurst *et al.*, 1999)

pUTS18	Cb^r ; expression vector driven by P*lac*.	(Omori *et al.*, 1994)
pUTS18*zntR*	As above (pUTS18): *zntR* cloned in *Eco*RI, *Bam*HI sites	This work
pUCD615	Km^r, Amp^r ; luciferase assay plasmid. MCS located upstream of promoterless *lux* operon.	(Rogowsky *et al.*, 1987)
pUCDP*zntA*	As above (pUCD615); luciferase operon driven by P*zntA* cloned in *Sau*3A1, *Rsa*1 sites.	(Brocklehurst *et al.*, 1999)
pUCDP*zntA*-1	As above (pUCD615); luciferase operon driven by P*zntA*-1 (1 bp deletion derivative of P*zntA*) cloned in *Sau*3A1, *Rsa*1 sites.	(Brocklehurst *et al.*, 1999)
pUCDP*ybh*	As above (pUCD615); luciferase operon driven by P*ybh* cloned in *Eco*R1, *Bam*H1 sites.	This work
pCR®- Blunt	Km^r ; blunt ended cloning vector for direct cloning of PCR products. Possesses lethal gene *ccdB*.	Invitrogen

pCR®- Blunt-P*ybh*	As above (pCR®- Blunt): P*ybh* cloned in MCS .	This work
pKD4	Km^r ; template plasmid for amplification of kanamycin cassette.	(Datsenko and Wanner, 2000)
pKD46	Cb^r ; Arabinose inducible (P_{araB}) phage λ red recombinase expression plasmid. Temperature sensitive.	(Datsenko and Wanner, 2000)

2.1.3 Chemicals, reagents and laboratory consumables

General laboratory chemicals were obtained from Sigma-Aldrich or Fisher Scientific UK and were of analytical grade or higher. Plasticware and general lab consumables were obtained from Alpha Laboratories, Elkay or Greiner. Other reagent and consumable suppliers are listed below or in the text as required.

0.2 μM sterile filters: Sartorius
Filter papers: Whatman Ltd.
Electrophoresis grade agarose: Bioline
Qiaprep spin miniprep and midiprep kits, Qiaquick PCR purification kit, QIAquick Nucleotide removal Kit, RNeasy mini kit, RNA protect solution, RNase-free DNase, Qiagen 100 columns: Qiagen
Concert gel extraction kit: GibcoBRL
dNTPs: New England Biolabs
10 x tris-borate EDTA (TBE): National Diagnostics

2.1.3.1 Water

Double distilled water was used for preparation of growth media and rinsing of all glassware prior to use. Water used for preparation of buffers and solutions was double distilled followed by chemical purification to 18 MΩ purity using a MilliQ 50 system supplied by Millipore. MilliQ water used for DNA elution from any column-based kits was adjusted to pH 8.0 with the addition of 5 mM tris and autoclaved.

2.1.3.2 Enzymes

Restriction endonucleases, T_4 DNA ligase, Calf intestinal alkaline phosphatase, Klenow DNA polymerase and Vent DNA polymerase supplied with their appropriate buffers were purchased from New England Biolabs. Taq DNA polymerase was obtained from Promega. All enzymes were used according to the manufacturer's instructions.

2.1.3.3 Radiochemicals

[α-^{33}P] dCTP for cDNA incorporation was obtained at a specific activity of 74 TBq.mmol^{-1} and concentration 0.37 MBq.ml^{-1} from Perkin-Elmer Life Science Products.

L-[^{35}S] methionine for maxicell labelling of proteins was also obtained from Perkin-Elmer Life Science Products, at a specific activity of 43.5 TBq.mmol^{-1} and a concentration of 377.4 MBq.ml^{-1}.

[α-^{32}P] dCTP for labelling of 'sticky ended' DNA was obtained from Amersham Pharmacia Biotech, at a specific activity of 220 TBq.mmol^{-1} and a concentration of 370 MBq.ml^{-1}.

2.1.3.4 Growth media

Media were prepared in accordance with the manufacturer's instructions and were either in capsule form (Bio101) or dehydrated form (GibcoBRL). Composition of media is shown in table 2.3.

Media prepared from capsules was used for propagation of plasmids and strains for general cloning procedures. Solid, dehydrated components for preparation of specialised media for specific experimental procedures such as phenotypic characterisation (section 2.2.10) were purchased from Difco Laboratories or Invitrogen.

Table 2.3 Media used for propagation of bacteria during the study. The composition of each medium is shown.

Medium	Composition
LB-Broth (capsule or dehydrated)	10 $g.l^{-1}$ Tryptone 5 $g.l^{-1}$ Yeast extract 10 $g.l^{-1}$ NaCl
LB-Agar (capsule)	10 $g.l^{-1}$ Tryptone 5 $g.l^{-1}$ Yeast extract 10 $g.l^{-1}$ NaCl 15 $g.l^{-1}$ Agar-B
Ψa-Broth	10 $g.l^{-1}$ Tryptone 5 $g.l^{-1}$ Yeast extract 5 $g.l^{-1}$ NaCl

M9 Minimal Medium	2 $g.l^{-1}$ Casamino acids 0.4% (w/v) Glucose 2 mM $MgSO_4$ 100 µM $CaCl_2$ 0.8 $mg.l^{-1}$ Thiamine 1x M9 salts (5x M9 salts composition: 33.9 $g.l^{-1}$ Na_2HPO_4, 15 $g.l^{-1}$ KH_2PO_4, 2.5 $g.l^{-1}$ NaCl, 5 $g.l^{-1}$ NH_4Cl)
Methionine-free Minimal Medium	0.06% (w/v) Methionine-free assay media 0.4% (w/v) Glucose 10 mM $MgSO_4$ 100 µM $CaCl_2$ 0.8 $mg.l^{-1}$ Thiamine 1x M9 salts (5x M9 salts composition: 33.9 $g.l^{-1}$ Na_2HPO_4, 15 $g.l^{-1}$ KH_2PO_4, 2.5 $g.l^{-1}$ NaCl, 5 $g.l^{-1}$ NH_4Cl)
SOC medium	20 $g.l^{-1}$ Bacto-tryptone 5 $g.l^{-1}$ Bacto-yeast extract 0.5 $g.l^{-1}$ NaCl 2.5 mM KCl 2% (v/v) 1 M Glucose

2.1.3.5 Antibiotic selection

Antibiotics were obtained from Melford Laboratories and were used in the following concentrations:

Cb (carbenicillin)	250 $\mu g.ml^{-1}$
Cm (chloramphenicol)	25 $\mu g.ml^{-1}$
Amp (ampicillin)	50 $\mu g.ml^{-1}$
Km (kanamycin)	50 $\mu g.ml^{-1}$
Tc (tetracycline)	15 $\mu g.ml^{-1}$
D-cycloserine	100 $\mu g.ml^{-1}$

All antibiotics were made up as 1000x stock solutions. Cb, Km and D-cycloserine were dissolved in water; Cm and Tc were dissolved in 70% ethanol. Stock solutions were filter sterilised and stored at –20°C.

2.1.3.6 Commonly used solutions

General solutions used in this study are shown in table 2.4 below. Any other solutions used in specific experimental procedures are included in the methods and are shown in the text.

Table 2.4 Commonly used solutions and the composition of each.

Solution	Composition
Iso-saline	0.85% (w/v) NaCl
TAE	40 mM Tris-acetate 1 mM EDTA, pH 8.0

TBE	90 mM Tris-borate 2 mM EDTA, pH 8.3
TE	10 mM Tris-Cl 1 mM EDTA, pH 8.0
DNA gel-loading solution	0.05% (w/v) Bromophenol blue 50% (w/v) Sucrose 10 mM EDTA
RNA gel-loading solution	0.05% (w/v) Bromophenol blue 50% (w/v) Sucrose 25 mM EDTA
Bacterial buffer	0.4 mM $MgSO_4$ 50 mM Na_2HPO_4 20 mM KH_2PO_4 70 mM NaCl
Protein sample application buffer	0.375 M Tris-Cl, pH 8.8 6% (w/v) SDS 30% (v/v) Glycerol 0.05% (w/v) Bromophenol blue
SDS Running buffer	3 $g.l^{-1}$ Tris base 14.4 $g.l^{-1}$ Glycine 1 $g.l^{-1}$ SDS

SSPE	0.18 M NaCl 10 mM NaH_2PO_4, pH 7.7 1 mM EDTA, pH 8.0
Denhardt's Reagent	0.02% (w/v) Ficoll 0.02% (w/v) Polyvinylpyrrolidone 0.02% (w/v) Bovine serum albumin

2.1.3.7 Oligonucleotides

All oligonucleotides were obtained in lyophilised form at a scale of 50 nM from Invitrogen Life Technologies. The oligonucleotides were resuspended in sterile MilliQ to a concentration of 1 $\mu g.\mu l^{-1}$ and stored at –20°C.

2.1.3.7.1 Vector specific oligonucleotides

Vector specific oligonucleotides (table 2.5) were of desalted quality and the annealing temperatures (T_m) were determined using the equation:

$$T_m = 0.41\ (\%\ GC) + 64.9 - (600/N)$$

Where % GC corresponds to the percentage of G or C residues in the oligonucleotide and N is the primer length in bases. In PCR reactions where more than one oligonucleotide was used, the lowest T_m value was used for the annealing step.

Table 2.5 Vector specific oligonucleotides.

Primer name	Sequence (5′ – 3′)
M13 Forward	CGCCAGGGTTTTCCCAGTCACGAC
M13 Reverse	AGCGGATAACAATTTCACACAGGA
P*zntA* For	CCTGCTACTTTGCCGGTCACTTCCTCATCG
P*zntA* Rev	GGAACATGCGCCGTCGCAGCAACAGTCG
P*ybH* For (*Bam*HI)	CGCGGATCCGGTGTAGTTTTCAGGGAGATACTG
P*ybH* Rev (*Eco*RI)	CCGGAATTCGTCATGGCAGGATTATTCATCGC
ybhR K.O. For	ATCACCTTACGTCTAAACATCGCGTACGCCGCTGAGT GTAGGCTGGAGCTGC
ybhR K.O. Rev	CCTGACGTGGCTGAAAACCAAACGTCGGCTGGATTAG ATGAATATCCTCCTTAGTTCC

*ybh*R K.O. For – CHECK	GACCACGACCTGGTATTTTGG
ybhR K.O. Rev – CHECK	CTTCGGCGGTGATGTTTATCG
k1	CAGTCATAGCCGAATAGCCCT
k2	CGGTGCCCTGAATGAACTGC
kt	CGGCCACAGTCGATGAATCC

2.1.3.7.2 Oligonucleotides for site-directed mutagenesis

Oligonucleotides for site-directed mutagenesis (table 2.6) were PAGE purified by the manufacturer. These were designed according to the specifications of Stratagene's QuickChange™ Site-directed Mutagenesis Kit.

Table 2.6 Oligonucleotides used for site-directed mutagenesis. Underlined sequences show the altered bases.

Primer	Sequence (5′ – 3′)
H29A-For	CAGCAGATGATGGAGGCTGAGGTGCGTACTGAAGG
H29A-Rev	CCTTCAGTACGCACCTCAGCCTCCATCATCTGCTG
H53A-For	CCAGCGATTGAAATTTATCCGGGCCGCCAGACAACTAGG
H53A-Rev	CCTAGTTGTCTGGCGGCCCGGATAAATTTCAATCGCTGG
H76A-For	GCATCGATCCTGAGGCCCATACCTGTCAGGAGTCAAAAGC
H76A-Rev	GCCTTTTGACTCCTGACAGGTATGGGCCTCAGGATCGATGC
H77A-For	GCATCGATCCTGAACACGCTACATGTCAGGAGTCAAAAGC
H77A-Rev	GCCTTTTGACTCCTGACATGTAGCGTGTTCAGGATCGATGC
H119A-For	GCCTGTTGTGGGACTGCTGCTAGCCAGTGTTTATTGTTCG
C79A-For	CGATCCTGAACACCATACCGCTCAGGAGTCAAAAGGC

C79A-Rev	GCCTTTTGACTCCTGAGCGGTATGGTGTTCAGGATCG
C79S-For	CGATCCTGAACACCATACCTCACAGGAGTCAAAAGGC
C79S-Rev	GCCTTTTGACTCCTGTGAGGTATGGTGTTCAGGATCG
C114A-For	CGCCTTAACGATGCCGCATGTGGGACTGCTCATAGC
C114A-Rev	GCTATGAGCAGTCCCACATGCGGCATCGTTAAGGCG
C114S-For	CGCCTTAACGATGCCTCATGTGGGACTGCTCATAGC
C114S-Rev	GCTATGAGCAGTCCCACATGAGGCATCGTTAAGGCG
C115A-For	CGCCTTAACGATGCCTGTGCAGGGACTGCTCATAGC
C115A-Rev	GCTATGAGCAGTCCCTGCACAGGCATCGTTAAGGCG
C115S-For	CGCCTTAACGATGCCTGTTCAGGGACTGCTCATAGC

C115S-Rev	GCTATGAGCAGTCCCTGAACAGGCATCGTTAAGGCG
C124A-For	GGGACTGCTCATTAGCAGTGTTTATGCATCGATTCTTGAAC
C124A-Rev	GCTTCAAGAATCGATGCATAAACACTGCTATGAGCAGTCCC
C124S-For	GGGACTGCTCATTAGCAGTGTTTATTCATCGATTCTTGAAC
C124S-Rev	GCTTCAAGAATCGATGAATAAACACTGCTATGAGCAGTCC
C141A-For	GGCGTTAAGAGTGGCGCTTGATTTTTTGCACTGGCAGG
C141A-Rev	CCTGCCAGTGCAAAAAATCAAGCGCCACTCTTAACGCC
C141S-For	GGCGTTAAGAGTGGTTCATGATTTTTTGCACTGGCAGG
C141S-Rev	CCTGCCAGTGCAAAAAATCATGAACCACTCTTAACGCC
C141H-For	GGCGTTAAGAGTGGTCATTGATTTTTTGCACTGGCAGG

C141H-Rev	CCTGCCAGTGCAAAAAATCAATGACCACTCTTAACGCC
C141L-For	GGCGTTAAGAGTGGTCTCTAATTTTTTGCACTGGCAGG
C141L-Rev	CCTGCCAGTGCAAAAAATTAGAGACCACTCTTAACGCC
C141Y-For	GGCGTTAAGAGTGGTTACTAGTTTTTTGCACTGGCAGG
C141Y-Rev	CCTGCCAGTGCAAAAAACTAGTAACCACTCTTAACGCC
C141G-For	GGCGTTAAGAGTGGTGGTTGATTTTTTGCACTGGCAGG
C141G-Rev	CCTGCCAGTGCAAAAAATCAACCACCACTCTTAACGCC
C141D-For	GGCGTTAAGAGTGGTGATTGATTTTTTGCACTGGCAGG
C141D-Rev	CCTGCCAGTGCAAAAAATCAATCACCACTCTTAACGCC

2.2 General methods in molecular biology

2.2.1 Transformation

2.2.1.1.1 Preparation of chemically competent cells

Chemically competent *E. coli* cells were prepared for transformation using the rubidium chloride method (adapted from Hanahan, 1983). The *E. coli* strains to be made competent were streaked on LB-agar plates containing antibiotics where required. The strains were incubated at 37°C, overnight. A single colony was used to inoculate 5 ml Ψa-broth with antibiotics if required at 37°C, 200 rpm, overnight.

This was subcultured into 200 ml (1:40 dilution) prewarmed Ψa-broth and incubated at 37°C with shaking until the optical attenuance at 600 nm (D_{600}) had reached approximately 0.4. The culture was chilled on ice for 5 minutes before harvesting by centrifugation at 6000 g_{av} at 4°C for 5 minutes (Sorvall GSA rotor).

The cell pellet was resuspended in two-fifths original volume of ice cold TfbI (30 mM potassium acetate, 10 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8) and left on ice for 1 hour. The cells were repelleted as before and resuspended in one twenty-fifth original volume of ice-cold TfbII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% (v/v) glycerol, pH 6.5). Following incubation on ice for 4 hours, cells were aliquoted (100 µl) into ice-cold, sterile eppendorfs and snap-frozen in liquid nitrogen before being stored at -80°C until needed.

2.2.1.1.2 Transformation of chemically competent cells

Competent cells were thawed on ice for 10 minutes before the addition of up to 1 µg plasmid DNA. After incubation on ice for 30 minutes, cells were 'heat shocked' at 42°C for 90 seconds and returned to ice for a further 5 minutes.

Expression of selectable markers was achieved by the addition of four volumes of LB-broth with subsequent incubation at 37°C for 1 to 1½ hours in a shaking incubator (200 rpm). The cells were harvested by centrifugation (6000 g_{av}, 4 minutes) and resuspended in half the original volume of LB-broth. The cultures were spread on LB-agar plates containing the appropriate selection and incubated overnight at 37°C.

2.2.1.2.1 Preparation of electro-competent cells

The *E. coli* strains to be made electro-competent were streaked on LB-agar plates containing antibiotics where required and incubated at 30°C overnight (this temperature was chosen for strains such as W3110 containing the temperature sensitive plasmid pKD46, otherwise 37°C). A single colony was used to inoculate 5 ml LB-broth with antibiotics if required at 30°C, 200 rpm, overnight.

The overnight culture was used to inoculate 80 ml fresh LB-broth (1:100 dilution). The media had been prewarmed and contained antibiotics where applicable (in addition, 0.5% arabinose was added for W3110 containing pKD46) and growth was continued as before until the D_{600} was about 0.4. Cells were harvested by centrifugation at 6000 g_{av} (Sorvall GSA rotor) at 4°C for 5 minutes. The pellet was washed in 100 ml ice-cold sterile MilliQ water and this washing step was repeated.

Finally, the cells were washed in 3 ml of ice-cold glycerol (10%) and resuspended in 0.3 ml of ice-cold glycerol (10%). Cells were separated into aliquots of 25 µl in ice-cold, sterile eppendorfs and snap-frozen in liquid nitrogen. Cells were stored at –80°C until needed.

2.2.1.2.2 Transformation of electro-competent cells

Electro-competent cells were thawed on ice for 10 minutes and transferred to a cuvette (with conducting plates) chilled on ice. Up to 1 µg DNA was added and the cuvette inserted

in a micro-pulser (Bio-Rad) with a voltage of 1.8 kV applied for 6.1 milli-seconds. The cells were then transferred to a 30 ml universal tube (Sterilin) and 1 ml of SOC medium was added followed by incubation at 30°C (depending on strain) at 200 rpm for 1½ hours. The cells were harvested by centrifugation (6000 g_{av}, 4 minutes) and resuspended in 500 µl fresh SOC medium. The cultures were spread on LB-agar plates containing the appropriate selection and incubated at 37°C, overnight.

2.2.2 Preparation of DNA

2.2.2.1 Small scale plasmid preparation (miniprep)

A 3 ml culture of plasmid bearing *E. coli* was grown to saturation overnight (37°C, 200 rpm) in LB-broth containing the appropriate antibiotics. DNA extraction was performed using a Qiagen Plasmid miniprep kit as described by the manufacturer. In this procedure, bacteria are lysed under alkaline conditions and the lysate is subsequently neutralised and adjusted to high-salt binding conditions for purification of plasmid DNA on a silica-gel column. For further details, refer to the Qiagen Plasmid miniprep protocol.

2.2.2.2 Large scale plasmid preparation (midiprep)

A 200 ml culture of plasmid bearing *E. coli* was grown to saturation overnight (37°C, 200 rpm) in LB-broth containing the appropriate antibiotics. DNA extraction was performed using Qiagen Plasmid midiprep kits as described by the manufacturer.

2.2.2.3 Genomic DNA purification

Genomic DNA was purified from *E. coli*, TG2 strain according to the US Dept Commerce Molecular Biology protocol. *E. coli* were grown overnight in 5 ml LB-broth with

antibiotics added where necessary. The cells were harvested by centrifugation at 1500 g_{av} for 10 minutes at room temperature and resuspended in 3.5 ml of buffer B1 (50 mM Tris-HCl, 50 mM EDTA, 0.5% (v/v) Tween 20, 0.5% (v/v) Triton X-100, 200 $\mu g.ml^{-1}$ RNaseA). Lysozyme (80 µl of 100.mg ml^{-1} stock solution) and Proteinase K (100µl of 20 $mg.ml^{-1}$ stock solution) were added and the solution incubated at 37°C for 30 minutes. Solution B2 (1.2 ml of 3 M guanidine hydrochloride, 20% (v/v) Tween 20) was added, mixed by inversion several times and incubated at 50°C for a further 30 minutes.

A Qiagen 100 column was equilibrated with 4 ml of QBT buffer (750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100, pH 7.0). The lysate was diluted with an equal volume of QBT buffer and allowed to pass through the column under gravity. The column was washed twice with 7.5 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0) and the genomic DNA eluted with 5 ml of buffer QF (1.25 mM NaCl, 15% (v/v) ethanol, 50 mM Tris-HCl, pH 8.5).

The eluted fraction was purified twice by phenol/chloroform extraction (section 2.2.3.2) followed by precipitation (section 2.2.3.3). The genomic DNA was resuspended in TE buffer and stored at -20°C.

2.2.3 DNA manipulations

2.2.3.1 DNA restriction digests

DNA digestion was performed by incubation for a minimum duration of 1 hour, at the temperature recommended by New England Biolabs. The appropriate buffer for the restriction enzyme, supplied by the manufacturer was used in addition to BSA, when required. DNA that was required for cloning after restriction was purified via gel extraction (section 2.2.3.5).

2.2.3.2 Purification of DNA – phenol / chloroform extraction

One volume of phenol:chloroform was added to the DNA solution and mixed by vortex. The mixture was separated into aqueous and phenol phases by centrifugation for 2 minutes. The upper aqueous phase was removed and retained. One half volume of water was added to the lower phenol layer which was then mixed by vortex. Two layers were formed, the upper of which was removed and added to the previously retained upper phase and precipitated as described below, in section 2.2.3.3

2.2.3.3 Precipitation of DNA

One tenth volume of 3 M sodium acetate pH 4.8, and one volume of ice-cold isopropanol were added to the DNA solution and mixed thoroughly followed by incubation on ice for 5 minutes. The DNA was pelleted by centrifugation at 13000 g_{av}, for 10 minutes at room temperature, washed in 70% ethanol and air-dried. DNA was resuspended in an appropriate volume of sterile MilliQ water, pH 8.0.

2.2.3.4 Purification of PCR products

PCR products were purified to remove unincorporated oligonucleotides, dNTPs, genomic DNA or plasmids and restriction endonucleases using a Qiagen PCR purification kit according to the manufacturer's protocol.

2.2.3.5 Purification of DNA by gel extraction

DNA that had to be isolated from a mixture of DNA molecules of varying sizes was first separated by agarose gel electrophoresis (section 2.2.5.1). Buffer TAE was used in this instance to make up the gel and running buffer; these were autoclaved prior to use.

The gel was mounted on a UV transilluminator (UVP Inc.) for visualisation of DNA bands. The band of interest was cut out with a sterile scalpel and purified using a Concert gel extraction kit.

2.2.3.6 Dephosphorylation of DNA

During cloning procedures, vectors were linearised with restriction endonucleases and the ends dephosphorylated to prevent recircularisation. Dephosphorylation was performed using 0.5 units of Calf intestinal alkaline phosphatase per μg vector DNA, in the buffer provided at 37°C for a minimum duration of 1 hour.

Calf intestinal alkaline phosphatase was heat inactivated at 65°C for 20 minutes in the presence of 20 mM EDTA, pH 8.0. The DNA was purified and precipitated as described in sections 2.2.3.2 and 2.2.3.3.

2.2.3.7 Ligation of DNA molecules

Recombinant plasmids were created by ligating sticky or blunt-ended DNA fragments into dephosphorylated vectors. The ligation reaction was performed using T4 DNA ligase as instructed by New England Biolabs, at 16°C for 1 hour, or 4 hours for blunt-ended inserts. T4 DNA ligase was heat inactivated at 65°C for 10 minutes. Competent *E. coli* were chemically transformed with the reaction mixture to select for recombinant colonies (section 2.2.1.1.2).

2.2.4 RNA purification

Samples of growing *E. coli* cultures were removed in mid-logarithmic phase and harvested by centrifugation. RNA extraction for preparation of cDNA was performed using an

RNeasy mini kit according to the manufacturer's instructions. Treatment with DNase was incorporated in the purification procedure and DNase was removed by washing with buffers containing denaturing agents before the elution step.

The RNA samples were quantified and the purity determined by the absorbance values obtained at 260 nm and 280 nm (A_{260}/A_{280} ratio) using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech). RNA samples were analysed for degradation and remnants of genomic DNA by agarose electrophoreseis (section 2.2.5.2) and stored at –80°C.

2.2.5 Agarose gel electrophoresis of nucleic acids

2.2.5.1 Electrophoresis of DNA samples

Gels containing 1% (unless otherwise stated) agarose were prepared by dissolving solid agarose (Bioline) in TBE and heating to boiling point in a microwave oven. After cooling to approximately 45°C, ethidium bromide was added to a final concentration of 0.5 $\mu g.ml^{-1}$; the molten agarose was poured into a casting tray containing well-forming combs and left undisturbed until it had solidified.

The combs were removed and DNA samples were loaded into wells in volumes up to 12 µl of which 20% (v/v) was DNA gel-loading solution. Electrophoresis was performed at a constant voltage of 200 V, 400 A for 40 minutes in 1 x TBE containing 0.5 $\mu g.ml^{-1}$ ethidium bromide.

DNA bands were visualised and photographed by mounting the gel on a UV illuminating gel camera (MWG-biotech). Estimates of DNA molecular weight were made using a size marker (1 kb ladder obtained from Gibco BRL).

2.2.5.2 Electrophoresis of RNA samples

Electrophoresis of RNA samples was performed by preparing 1% agarose gels as detailed above however, the molten agarose and running buffer were autoclaved prior to use. Also, the casting tray and electrophoresis tank were sterilised by soaking in 3% hydrogen peroxide for at least an hour followed by rinsing with sterile MilliQ before use. RNA samples were loaded into wells in volumes up to 12 µl of which 20% (v/v) was RNA gel-loading solution.

2.2.6 Polymerase chain reaction (PCR)

PCR reaction mixtures were prepared with the following components:

Template DNA	100 – 200 ng (genomic, plasmid or cell culture)
Oligonucleotides (100 ng.µl^{-1} stock)	1 µl
dNTPs (10 mM)	1 µl
DNA Polymerase	0.2 µl
10 x polymerase buffer	5 µl
Mg^{2+}	0 – 4 mM
Water	to 50 µl

Vent polymerase was used for cloning work whereas Taq polymerase was used for PCR screening. The Mg^{2+} sources for Vent polymerase and Taq polymerase were $MgSO_4$ and $MgCl_2$, respectively.

To allow sufficient amplification of DNA, 30 cycles of denaturation, annealing and extension were performed at the following temperatures and durations in a Techne PCR machine:

Denaturation	96°C	30 seconds – 1 minute
Annealing	50 – 68°C	30 seconds – 1 minute
Extension	72°C	1 minute per kb of amplified sequence

Where liquid cell cultures were used for colony PCR screening, a preincubation of 2 minutes at 96°C was allowed before proceeding to amplification. After completion of the 30 cycles of amplification, the reaction mixture was held at 72°C for 10 minutes before cooling to 4°C.

2.2.7 Site-directed mutagenesis

2.2.7.1 QuickChange™ Site-directed mutagenesis kit

Site-directed mutagenesis was performed with a QuickChange™ Site-directed mutagenesis kit obtained from Stratagene and used in accordance with the instruction manual. Oligonucleotides for site-directed mutagenesis of *zntR* were designed according to the specifications of Stratagene's QuickChange™ site-directed mutagenesis kit and were synthesised by Invitrogen Life Technologies (section 2.1.3.7.2).

In this procedure a PCR reaction is performed using a plasmid containing the target gene as a template. This plasmid is denatured and the mutagenic oligonucleotides annealed. The plasmid is amplified by Pfu-turbo polymerase, a high fidelity polymerase. Once the PCR reaction is complete, the sample is cleaned (section 2.2.3.4) and treated with DpnI (section 2.2.3.1) which digests the original methylated template plasmid.

The remaining plasmids were used to transform (section 2.2.1.1.2) TG2 cells for production of individual colonies. Plasmids were purified by miniprep (section 2.2.2.1). The nucleotide sequences of all mutants were confirmed by dideoxy DNA sequencing (section 2.2.8).

2.2.7.2 Site-directed mutagenesis by overlap extension

Alternatively, where the use of the QuickChange™ Mutagenesis kit was unsuccessful for creating the desired mutation, the mutant was constructed by PCR using an M13-reverse oligonucleotide in combination with an oligonucleotide containing the altered bases in its centre.

The resulting PCR product was gel purified with a Concert gel extraction kit and used as a 'primer' in a subsequent PCR reaction with an M13-forward oligonucleotide. The PCR product was purified, cleaved with restriction enzymes and ligated into a similarly digested vector (section 2.2.3.7) and the nucleotide sequences confirmed by dideoxy DNA sequencing (section 2.2.8).

2.2.8 Dideoxy DNA sequencing

DNA samples for sequencing were purified using Qiaprep spin miniprep (section 2.2.2.1) or midiprep (section 2.2.2.2) kits. A 5 µl sample of the purified DNA was quantified using a GeneQuant spectrophotometer. Sequencing reactions were performed using an ABI Prism® BigDye™ Terminator V3.0 Cycle Sequencing Ready Reaction kit (supplied by Perkin-Elmer Life Science Products).

Sequences of samples were resolved by Gareth Lewis, School of Biological Sciences, Cardiff University. Electrophoresis and visualisation of the extension products was accomplished using an ABI Prism® 373 DNA sequencer. The DNA sequencing procedure was based on the original dideoxy-sequencing method elucidated by Sanger *et al.*, 1977.

2.2.9 Methods in protein biochemistry

2.2.9.1 'Maxicell' labelling of proteins

This is a method used for confirming the expression of proteins. It is very sensitive and can be used to detect low amounts of a protein, the gene for which is cloned into an expression vector. A *recA* deficient strain such as TG2 is transformed with the recombinant vector. RecA allows recombinational repair of damaged chromosomal DNA which must not occur in this procedure.

2.2.9.1.1 UV inactivation of cellular genome

TG2 was transformed with the plasmid containing the gene of interest. The resultant strain was maintained on LB plates with antibiotic selection at 37°C. A single colony was used to inoculate 5 ml of M9 medium with antibiotic selection. The culture was incubated at 37°C, 200 rpm, overnight. A 500 µl sample of the culture was diluted 20 fold into the same medium, with selective antibiotics, and incubated at 37°C with agitation until the D_{550} had reached approximately 0.5.

A 3 ml sample of the mid-logarithmic culture was placed in a small petri dish (Nunc™ Brand Products) and evenly distributed over the bottom of the dish. Each petri dish containing cells was placed on a UV transilluminator (UVP, Inc.) and irradiated in the dark for 3 minutes with gentle agitation. This is an important step as it denatures the chromosome (hence the requirement of a *recA* deficient strain). The remaining cellular proteins are sufficient to allow amplification of plasmids.

Still in the dark, the irradiated culture was transferred to a Sterilin universal wrapped in aluminium foil, to exclude any light. Visible light allows induces photolyase to repair damaged chromosomal DNA which must be avoided.

The culture was incubated as before for 1 hour after which D-cycloserine was added to a final concentration of 100 $\mu g.ml^{-1}$ and returned to incubation overnight.

2.2.9.1.2 Radioactive labelling of plasmid encoded proteins

A 1 ml sample of the UV treated culture was transferred to a microfuge tube and the cells were pelleted by centrifugation at 6000 $g_{av,}$ for 2 minutes. The cells were gently resuspended in 1 ml of M9 salts plus 10 mM $MgSO_4$ and washed once more and pelleted. Finally, the cells were resuspended in 500 µl of methionine-free minimal media and D-cycloserine added to a final concentration of 100 $\mu g.ml^{-1}$. Incubation was continued at 37°C, 200 rpm, for 1 hour.

The gene of interest was induced by the addition of 1 mM IPTG (to express genes cloned into *lac* inducible plasmids) and the labelling reaction performed with 0.37 kBq of L-[^{35}S] methionine for 3 hours at 37°C with shaking. The labelling reaction was chased with 10 µl of 'cold' L-methionine, 10 $mg.ml^{-1}$ in order to stop the reaction. Cells containing radioactively labelled protein were collected by centrifugation at 6000 g_{av} for 2 minutes, resuspended in 50 µl of bacterial buffer and used for SDS-PAGE as described in section 2.2.9.3.1.

2.2.9.2 Electrophoretic Mobility Shift Assay (EMSA)

2.2.9.2.1 Preparation of crude cellular extracts

E. coli cells were chemically transformed with control plasmids and recombinant high expression plasmids (section 2.2.1.1.2). The following day, LB medium containing antibiotics was inoculated with resultant *E. coli* colonies and grown at 37°C, 200 rpm, overnight. *E. coli* cultures were diluted 100-fold into 400 ml of pre-warmed fresh LB media

supplemented with 0.2% (v/v) glucose, 0.2 mM $ZnSO_4$, antibiotics and incubated further at 37°C, 200 rpm.

Cultures were induced with 1 mM IPTG when the D_{600} had reached 0.15 and were incubated further until the D_{600} reached 0.5. Samples were spun at 6000 g_{av}, 4°C for 15 minutes. The supernatant was discarded and the pellet resuspended in 20 ml of ice-cold incubation buffer (10 mM Tris-Cl pH 8.0, 100 mM potassium glutamate, 0.1 mM EDTA, 1 mM $CaCl_2$, 5% (v/v) glycerol, 1 mM DTT, 100 $\mu g.ml^{-1}$ BSA). Samples were subjected to French Pressure (3 cycles) in a cold room and centrifuged at 13000 g_{av}, 4°C, 20 minutes. The supernatant was filtered through a 0.2 μM filter and the filtrate collected and its protein concentration determined. This was carried out according to the original method of Bradford, 1976, by comparison to absorbance values obtained for bovine serum albumin standards at a wavelength of 595 nm (A_{595}) in a Thermomax microplate reader (Molecular Devices).

2.2.9.2.2 [α-^{32}P] dCTP labelling of DNA probe

The recombinant plasmid bearing the promoter of interest for binding of specific proteins was digested (section 2.2.3.1) with appropriate restriction enzymes, generating 'sticky' ends. Bands were separated on a 2% agarose gel (section 2.2.5.1) and the band corresponding to the promoter was excised and purified (section 2.2.3.5), and the concentration determined using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech).

A Klenow fill-in reaction was performed using 200 ng of the purified DNA and 0.37 Bq [α-^{32}P] dCTP as instructed by New England Biolabs. Unincorporated dNTPs were removed via a microspin G-25 column (Amersham Pharmacia) and the labelled probe collected.

2.2.9.2.3 Incubation reaction

Proteins (10 μg) present in crude cell extracts were incubated with 20 ng of ^{32}P-labelled probe at 10°C for 1 hour. Herring sperm DNA (100 ng) and MilliQ water were added to a final volume of 20 μl. The whole incubation sample was loaded very gently onto an 8% TBE polycarylamide gel (section 2.2.9.3.2) in a 10°C cold cabinet.

2.2.9.3 Electrophoresis of proteins

2.2.9.3.1 Electrophoresis of proteins via SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) was used to separate proteins on sodium dodecyl sulphate (SDS) denaturing gels. Electrophoresis was performed using the Mini protein II electrophoresis system (purchased from Bio-Rad). All apparatus including the glass plates, spacers and combs were cleaned with ethanol before being assembled.
The separating gel contained acrylamide at a final concentration of 15%. The exact composition of the separating gel is shown below:

1.5 M Tris-HCl, pH 8.8	2.5 ml
10% SDS stock	100 μl
Acrylamide / Bis 29:1 (30% Stock)	5 ml
10% AMPS (ammonium persulphate)	50 μl
TEMED (tetramethylethylene diamine)	5 μl
Distilled water	to 10 ml

The acrylamide solution was purchased from Anachem; AMPS and TEMED from National Diagnostics and SDS from BDH.

The separating gel mixture was loaded into the space between the glass plates, and an inch thick layer of isopropanol applied. When the gel had polymerised, the isopropanol was

washed out with distilled water and the stacking gel mixture poured in its place, containing acrylamide at a final concentration of 4%. The other components and their concentrations are shown below:

0.5 M Tris-HCl, pH 6.8	2.5 ml
10% SDS stock	100 µl
Acrylamide / Bis 29:1 (30% Stock)	1.3 ml
10% AMPS	50 µl
TEMED	10 µl
Distilled water	to 10 ml

The well-forming comb was inserted into the stacking gel mixture and removed after solidification of the stacking gel was complete. Cell samples were heated to 95°C for 5 minutes and allowed to cool.

An equivalent volume of protein sample application buffer was added to the cell sample before loading in the wells at a maximum volume of 20 µl. Prestained (Broad Range) Protein marker (obtained from New England Biolabs) was used to determine sizes of protein bands. Electrophoresis was carried out at a constant voltage of 100 volts for 2 hours in SDS running buffer.

2.2.9.3.2 Electrophoresis of proteins via TBE-PAGE

Electrophoresis was performed using the BioRad Protean® II Cell electrophoresis system (purchased from Bio-Rad). All apparatus including the glass plates, spacers and combs were cleaned with ethanol before being assembled.

The separating gel contained acrylamide at a final concentration of 8%. The exact composition of the separating gel is shown below:

Acrylamide / Bis 37.5:1 (30% Stock)	10 ml
10% AMPS	250 µl
TEMED	5 ml
10 X TBE	5 ml
Distilled water	to 50 ml

TBE polyacrylamide gels were prepared by pouring the above mixture into the space between the glass plates and the well-forming comb inserted. This was done a day prior to use and stored at 10 °C to allow complete polymerisation. Before loading the gel with the incubated samples, the gel was pre-run at 200 V for 1 hour in 1 X TBE, in a 10 °C cold cabinet. After loading the incubated samples (up to 20 µl), the voltage was turned down to 150 V and applied for 4 hours. Gels were removed and visualised as described in section 2.2.9.3.3.

2.2.9.3.3 Visualisation of gels containing radioactively labelled proteins

Gels were transferred to 3MM chromatography paper (Whatman®) and dried at 85°C for 1 hour on a Model 583 gel dryer (Bio-Rad) with vacuum suction (Vacuubrand). Dry gels were wrapped in clear Saran wrap and exposed on Kodak phosphor imaging screens (Bio-Rad) overnight or longer. Images were visualised through a Personal Molecular Imager FX and analysed using Quantity One – 4.1.0 software (Bio-Rad).

2.2.10 General methods of phenotypic characterisation

2.2.10.1 Luciferase assays

Promoter activity was assayed with a luciferase based reporter gene system. Competent *E. coli* cells were transformed (section 2.2.1.1.2) with recombinant luciferase constructs (pUCD615 derivatives). Colonies were used to inoculate 5 ml LB-broth with antibiotic

selection and grown at 37°C, 200 rpm, overnight. Cells were subcultured (1:100 dilution) in fresh LB-broth without antibiotics and grown at 37°C, 200 rpm. Metal-ions were added at a range of concentrations to 200 µl of logarithmically growing cells (D_{600} = 0.2 – 0.3), in triplicate in microtitre plates and sealed with transparent microplate sealers (Greiner).

The cells were incubated at 30°C, 600 rpm for 2 hours in microtitre plate shakers (Camlab). After this incubation period, plates were removed and the D_{600} and luminescence values recorded using a Thermomax microplate reader (Molecular Devices) and a Lumino luminometer (Stratec), respectively.

2.2.10.2 Strain construction

Knockout strains were constructed according to Datsenko and Wanner, 2000, with selection for kanamycin. The kanamycin cassette was amplified by PCR (section 2.2.6) using pKD4 as a template (section 2.1.2). Oligonucleotides were designed which were complementary to the sequences found at the 3′ and 5′ ends of the kanamycin cassette but also contained regions flanking the target gene of inactivation. The completed PCR reaction was cleaned (section 2.2.3.4) and treated with DpnI (section 2.2.3.1) to digest the original methylated template plasmid.

The linear PCR product was used to transform competent *E. coli* W3110 [pKD46] cells by electroporation. The pKD46 plasmid (section 2.1.2) is arabinose inducible and expresses Red recombinase which enhances the rate of homologous recombination occurring between the host strain and the linear DNA. Since pKD46 is temperature sensitive, transformed cells were incubated at 30°C.

The following day, colonies were picked and PCR screened to identify successful insertional constructs. pKD46 was cured by incubating the successful strains at 42°C for 2 hours. The kanamycin cassette was not removed from the strains to allow precise selection of knockout cells in future.

2.2.10.3 Minimum Inhibitory Concentration (MIC) assays on solid media

LB medium was inoculated with *E. coli* strains and grown at 37°C, 200 rpm, overnight. Antibiotics were included in the medium where necessary. *E. coli* cultures were diluted 100-fold in isosaline and streaked onto agar plates containing specific concentrations of metal-ions. The plates were allowed to dry, incubated at 37°C over a period of 72 hours and inspected for growth at regular intervals.

2.2.10.4 MIC assays on metal-ion gradient plates

This procedure was identical to section 2.2.10.3 above, the exception being the preparation of the agar plates. Metal-ion gradient plates were prepared by pouring metal-ion containing molten agar into a tilted 10 cm^2 square petri dish (Bibby Sterilin Ltd.) to create an initial slope and allowed to solidify. The petri dish was transferred to a flat surface and the sloping agar was overlaid with plain molten agar to create a flat plane. *E. coli* cultures were streaked across the plates along the metal-ion gradient and incubated at 37°C for 24, 48 or 72 hours.

2.2.10.5 MIC assays in liquid medium

LB medium (containing antibiotics where necessary) was inoculated with *E. coli* and grown at 37°C, 200 rpm, overnight. The *E. coli* culture was then diluted 100-fold into fresh medium and grown for at least 1 hour until the D_{600} of all sample cultures was identical. The cultures were aliquoted into microtitre plates in 150 µl volumes that contained increasing concentrations of metal-ions in quadruplet, sealed with transparent microplate sealers (Greiner) and incubated at 37°C, 500 rpm, overnight in microtitre plate shakers (Camlab). The MIC values were determined by recording the D_{600} values in a Thermomax microplate reader (Molecular Devices).

2.2.10.6 Growth studies carried out in batch culture

LB medium (containing antibiotics where necessary) was inoculated with *E. coli* and grown at 37°C, 200 rpm, overnight. The *E. coli* culture was diluted 100-fold into prewarmed fresh medium in triplicate conical flasks. Growth was continued and the D_{600} of cell cultures was periodically monitored in a Novaspec II spectrophotometer (Pharmacia).

2.2.11 Gene arrays

The Panorama™ *E. coli* gene arrays (Sigma-Genosys) contain in duplicate, the 4290 PCR-amplified ORFs of *E. coli* K12 representing all of the protein encoding genes within this organism. This allows the study of global gene expression of all ORFs during cellular responses at the transcriptional level.

The procedure involves growing *E. coli* strains under various conditions (for comparison of gene expression), RNA extraction from the control and externally stressed strains and generation of radioactively labelled cDNA from this RNA. This is followed by hybridisation of cDNA to arrays, phosphorimaging and analysis of the expression patterns.

Cultures were grown in triplicate for each strain and for each separate growth condition. RNA extraction was performed when growth was at the mid-exponential phase as detailed in section 2.2.4. cDNA was produced from the purified RNA in a reverse transcription PCR reaction (RT-PCR) where 1 μg of RNA was annealed to 4 μl of *E. coli* specific cDNA labelling primers and the volume adjusted to 15 μl. AMV Reverse transcriptase and *E. coli* specific cDNA labelling primers were purchased from Sigma-Genosys. This mixture was heated to 90°C for 2 minutes and then cooled to 42°C at a rate of 2°C per minute. The mixture was added to the rest of the components shown below and incubated at 42°C for 3 hours:

RNA and annealed primer mixture	15 µl
dATP (10 mM)	1 µl
dGTP (10 mM)	1 µl
dTTP (10 mM)	1 µl
[α-^{33}P] dCTP (10 µCi.µl^{-1})	2 µl
AMV Reverse transcriptase (25 U.µl^{-1})	0.2 µl
5x Reverse transcriptase buffer	6 µl
Water	to 30 µl

Removal of unincorporated nucleotides was carried out by gel filtration through a MicroSpin G-25 Sephadex column (Amersham-Pharmacia), as instructed.

The [α-^{33}P] dCTP labelled cDNA was hybridised to the Panorama™ *E. coli* gene arrays as detailed in the protocol, an outline of which is given here. The cDNA was mixed with 3 ml of hybridisation solution (5x SSPE, 2% (w/v) SDS, 1x Denhardt's Reagent, 100 µg.ml^{-1} herring sperm DNA) and the mixture was added to the macroarrays (these had been prehybridised in glass roller bottles with 5 ml of hybridisation solution at 65°C, for 1 hour). Hybridisation of the cDNA to the arrays was carried out at 65°C, overnight.

The arrays were washed three times with 50 ml of wash solution (0.5x SSPE, 0.2% (w/v) SDS) for 5 minutes at room temperature and three times for 20 minutes at 65°C. The arrays were removed from the roller bottles, partially air dried and wrapped in Saran Wrap.

Arrays were exposed on Kodak phosphor imaging screens (Bio-Rad) overnight or longer depending on [α-^{33}P] incorporation. Images were visualised through a Personal Molecular Imager FX and analysed using Quantity One – 4.1.0 software (Bio-Rad). Arrays were stripped for re-use as instructed by Sigma-Genosys.

CHAPTER THREE

STRUCTURE / FUNCTION ANALYSIS OF ZntR (*E. coli*)

3.1 Summary

The ZntR protein from *E. coli* is a member of the MerR family of transcriptional regulatory proteins and acts as a hyper-sensitive transcriptional switch primarily in response to Zn(II) and Cd(II). The binding of metal-ions to ZntR initiates a mechanism that remodels the cognate promoter, increasing its affinity for RNA polymerase.

The introduction of site-directed mutations in *zntR* has shown that cysteine and histidine residues are important for transcriptional control and have an effect on metal-ion preference, sensitivity and magnitude of induction. Zn(II)-induced transcription was completely abolished in the absence of four of its cysteine resisues, three of which are conserved in the divalent metal-ion responsive MerR family members. A three-dimensional model of the N-terminal region of ZntR is proposed based upon these results and the coordinates of the MerR-family regulator BmrR.

3.2 Introduction

A Zn(II), Cd(II) and Pb(II) specific P-type-ATPase, ZntA, has been identified in *E. coli* which exports ions from the cytosol (Sofia *et al.*, 1994, Beard *et al.*, 1997, Blencowe *et al.*, 1997, Rensing *et al* 1997a). The region upstream of *zntA* contains a σ^{70} promoter, P*zntA*, similar in structure to the mercury resistance (*mer*) promoters of Tn*501* and Tn*21* (Hobman and Brown, 1997, Lund *et al.*, 1986).

The cognate regulatory gene has been designated *zntR* that encodes a polypeptide of 141 amino acids and has been shown to regulate P*zntA* (Brocklehurst *et al.*, 1999). ZntR has a predicted molecular weight of approximately 16.2 kDa (Brocklehurst *et al.*, 1999). The binding constant of ZntR has not been determined.

Each monomer of the encoded polypeptide contains an N-terminal DNA-binding domain, characterised by the presence of a helix-turn-helix (H-T-H) structure, and a C-terminal "signal recognition" domain although the functional protein acts as a dimer. The predicted primary sequence of ZntR shows 34% identity with MerR, containing five cysteine and five histidine residues, and is a member of the MerR-family of regulators which includes SoxR from *E. coli* (the superoxide stress response (Amabile-Cuevas and Demple, 1991)); $TipA_L$ from *Streptomyces lividans* (thiostrepton-regulated gene expression (Holmes *et al.*, 1993)); NolA from *Bradyrhizobium japonicum* (nodulation gene expression (Sadowsky *et al.*, 1991)) and BmrR and BltR from *Bacillus subtilis* (expression of multidrug resistance (Ahmed *et al.*, 1994, Ahmed *et al.*, 1995)) .

In contrast to the majority of the metal-responsive MerR-family members, which are transcribed divergently from the regulated genes, *zntR* is distant on the chromosome (74.1 min) from its associated target gene (*zntA* at 77.7 min) (Brocklehurst *et al.*, 1999).

ZntR mediates transcriptional induction primarily in response to Zn(II) (binding two ions per monomer (Outten *et al.*, 1999)) and Cd(II), however, Hg(II) and Pb(II) induce to a lesser extent (Brocklehurst *et al.*, 1999). ZntR has been shown to induce transcription via a

DNA-distortion (torsional) mechanism (Outten *et al.*, 1999) analogous to that of MerR (Ansari *et al.*, 1992, Ansari *et al.*, 1995) (section 1.5.2) and is very sensitive, responding to femtomolar levels of Zn(II) (Outten and O'Halloran, 2001).

At the time this research was initiated, the residues important for ligand-binding in ZntR had not been identified. ZntR contains many histidine and cysteine residues and these are the candidate residues for binding metal-ions. In this study, the transcriptional regulation of P*zntA* by derivatives of ZntR which contain single directed alterations in the primary amino acid sequence are analysed. It is shown that single amino acid alterations can dramatically change the metal-ion specificity and dynamic range of the P*zntA*/ZntR regulatory complex.

3.3 Results

3.3.1 Creation of *zntR* mutants

The medium-copy number plasmid pSU18*zntR* (section 2.1.2) was used as a template for the creation of *zntR* mutant derivatives H29A, H53A, H76A, H77A, C79A, C79S, C114A, C114S, C115A, C115S, C124A, C124S, C141A, C141S, C141H, C141L, C141Y, C141G and C141D using the QuickChange™ Site-directed Mutagenesis kit (section 2.2.7.1). The H119A derivative was created by the overlap extension method (section 2.2.7.2). Oligonucleotides for site-directed mutagenesis were designed bearing base alterations (section 2.1.3.7.2). Resultant plasmids were used to transform (section 2.2.1.1.2) TG2 cells (section 2.1.1) and nucleotide sequences of all *zntR* mutants were confirmed by dideoxy DNA sequencing (section 2.8).

3.3.2 Transcriptional activity of ZntR derivatives at P*zntA-1*

All of the ZntR mutant derivatives were assayed for their ability to elicit transcription from the P*zntA-1* derivative of P*zntA*. The spacer region of P*zntA-1* has been shortened from the normal 20 bp in P*zntA* w.t. to 19 bp. The critical feature of this promoter is that ZntR w.t. induces transcription in the absence of Zn(II) i.e., interaction of ZntR w.t. with P*zntA-1* alone can be measured. The structural integrity of each of the ZntR derivatives was measured by their capacity to induce transcription at P*zntA-1*.

Competent *E. coli* TG1(*zntR*::*Km*) cells (section 2.1.1) were prepared (section 2.2.1.1.1) and transformed (section 2.2.1.1.2) with pUCDP*zntA-1*. Resultant colonies were used to prepare a batch of chemically competent TG1(*zntR*::*Km*) [pUCDP*zntA-1*] cells (section 2.2.1.1.1).

The control plasmid pSU18, pSU18-*zntR* and all the mutant derivatives were used to transform (section 2.2.1.1.2) TG1(*zntR*::*Km*) [pUCDP*zntA-1*] cells and luciferase assays

were performed as detailed in section 2.2.10.1 without the addition of any metal-ions or IPTG.

The results of the luciferase assays are shown in fig. 3.1. The sensitivity of P*zntA-1* can be seen by the difference in luminescence values obtained in response to the negative and positive control plasmids pSU18 and pSU18-*zntR* which were close to 0 and 40000 RLU, respectively

Although some of the mutant derivatives appeared to be much better at activating P*zntA-1* than pSU18-*zntR,* such as H119A, C79A, C114A, C141S, C141H, C141Y, C141G and C141D all of which produced RLU values of over 50000, the actual values obtained are not as important as the scale of difference between the pSU18 control sample and ZntR w.t. which clearly represents non-activation and activation of P*zntA-1*, respectively. So any mutant derivative that produced values nearer to ZntR w.t. than the pSU18 control sample was regarded as activating P*zntA-1*. All of the ZntR mutant derivatives were active at P*zntA-1* in the absence of Zn(II) except C124A and C124S.

These results are summarised along with the results in the section 3.3.3 and are shown in table 3.1. This table is presented at the end of section 3.3.3.2.

3.3.3 Transcriptional activity at P*zntA* w.t.

All of the ZntR mutant derivatives were assayed for their ability to elicit transcription from the cognate w.t. promoter, P*zntA*. Competent *E. coli* TG1(*zntR*::*Km*) cells (section 2.1.1) were transformed (section 2.2.1.1.2) with pUCDP*zntA* (w.t.). Resultant colonies were used to prepare a batch of chemically competent TG1(*zntR*::*Km*) [pUCDP*zntA*] cells (section 2.2.1.1.1).

The control plasmids pSU18 and pSU18-*zntR,* and all the mutant derivatives were used to transform TG1(*zntR*::*Km*) [pUCDP*zntA*] cells and luciferase assays were performed in

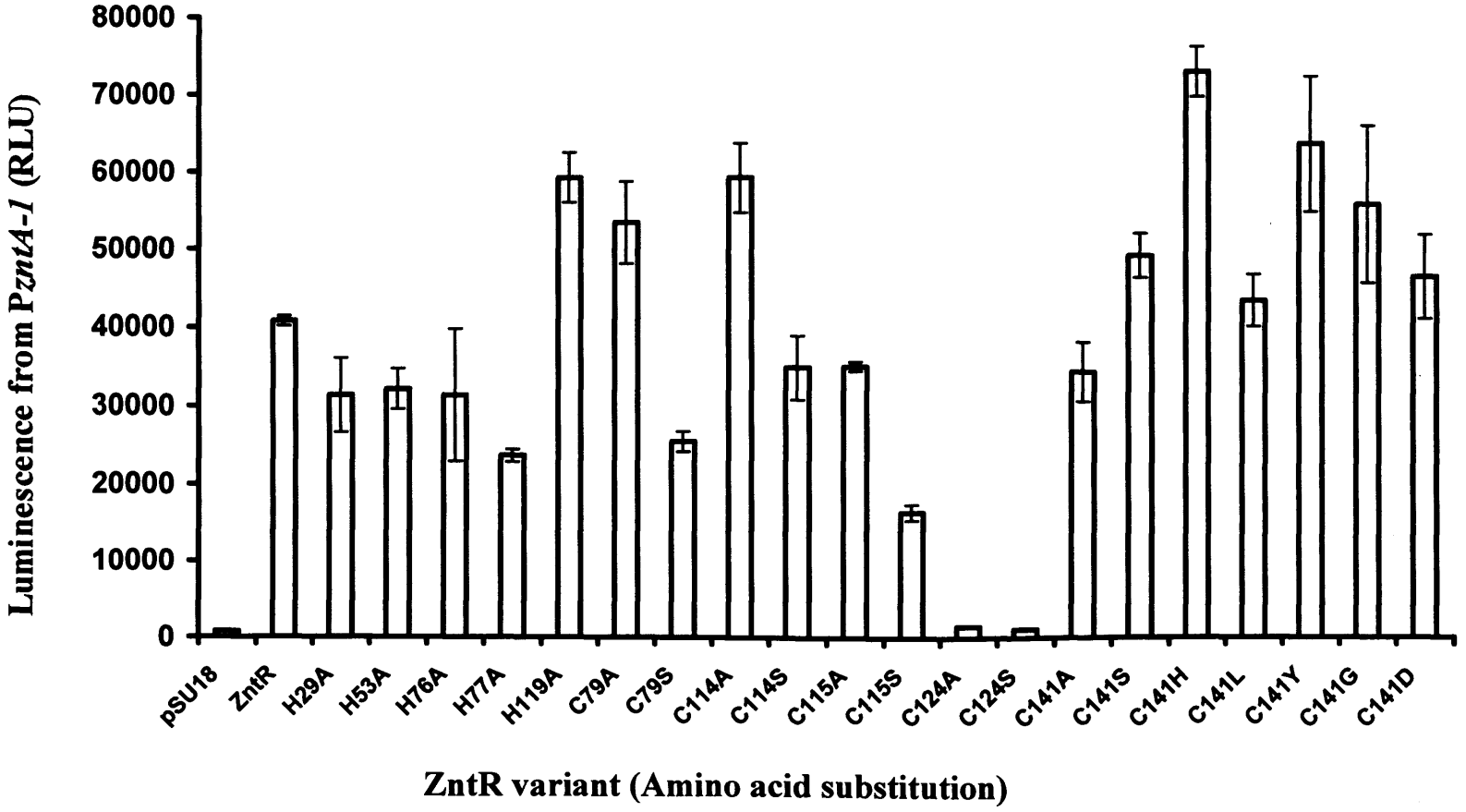


Fig 3.1 . Effect of histidine and cysteine substitutions in ZntR on transcription of P*zntA-1*.

Luminescence was measured in *E. coli* TG1(*zntR*::*Km*) [pUCDP*zntA-1*] with the control pSU18, ZntR w.t. or its derivatives expressed *in trans*. The data, presented as the average raw luminescence values (Relative Light Units (RLU)) (open columns) and the standard error (vertical bars), are derived from a triplicate data set in each case.

triplicate as detailed in section 2.2.10.1. Transcription of *zntR* and the mutant derivatives cloned in pSU18 was under the control of a *lac*-inducible promoter, therefore 1 mM IPTG was used to induce transcription of these genes. A range of metal-ion concentrations were added to the cultures in the microtitre plates: 0 – 1.2 mM Zn(II), 50 μM Cd(II), 0.5 mM Pb(II).

The figures presented in this section show luminescence measurements that have been adjusted for final D_{600} values to take into account any effects of toxicity from the metal-ions and so that an inaccurate representation of luminescence values is not given due to the cumulative effects of basal transcription that may be caused by differences in cell numbers rather than transcriptional activity. Variation in the assay has been normalised by presenting the results as a percentage value based on the maximal level of induction derived from P*zntA*/ZntR + Zn(II) for any given assay set. The assays were performed a minimum of five times for each ZntR derivative and a representative induction profile is displayed for each one.

3.3.3.1 Transcriptional activity of ZntR-histidine derivatives

The transcriptional profiles of the ZntR-histidine derivatives H29A, H53A, H76A, H77A and H119A in response to 0 – 1.2 mM Zn(II) along with the controls pSU18 and ZntR w.t are shown in fig. 3.2. ZntR w.t. elicits transcription from P*zntA* steadily from 0 – 1.1 mM Zn(II) at which there is maximal induction. The derivatives H29A, H53A and H119A do not significantly induce transcription at P*zntA* producing profiles similar to that of pSU18 at this promoter. The alterations H76A and H77A diminished Zn(II) induction to some extent but each shows a similar profile to that for ZntR w.t.

Fig 3.3 shows the transcriptional response of P*zntA* to ZntR and all the mutant derivatives after the addition of 50 μM Cd(II) and 0.5 mM Pb(II). H29A responded to both Cd(II) and Pb(II) to a greater degree than w. t., exhibiting a 6-fold increase in transcriptional response to Cd(II). Both H53A and H119A responded at almost w.t. levels of Cd(II) and Pb(II).

.

Fig 3.2 Effect of histidine substitutions in ZntR on Zn(II) induced transcription of P*zntA* .

Luminescence was measured in *E. coli* TG1(*zntR::Km*) [pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Zn(II) ranging from 0 - 1.2 mM (open columns).

The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. at 1.1 mM Zn(II) (7500 RLU). The standard error (vertical bars) in each case are derived from a triplicate data set.

Fig. 3.3 Effect of histidine and cysteine substitutions in ZntR on Cd(II) and Pb(II) induced transcription of P*zntA* .

Luminescence was measured in *E. coli* TG1(*zntR::Km*)[pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Cd(II) at 50 μM (A (100% ZntR w.t. activity or less) and B (greater than 100% ZntR w.t. activity)) or Pb(II) at 0.5 mM (C (100% ZntR w.t. activity or less) and D (greater than 100% ZntR w.t. activity)) (open columns).

The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. at 50 μM Cd(II) (6200 RLU) or 0.5 mM Pb(II) (4300 RLU). The standard error (vertical bars) in each case are derived from a triplicate data set. Note that the value axes are of different ranges.

H76A induced P*zntA* to a greater extent than ZntR w.t. in the presence of Cd(II) and Pb(II) however the response of H77A was slightly decreased as compared to ZntR w.t.

3.3.3.2 Transcriptional activity of ZntR-cysteine derivatives

Replacement of the first four cysteine residues of ZntR, C79, C114, C115 or C124 resulted in a complete loss of activity with Zn(II) as the inducer whether replaced with alanine or serine (fig 3.4) and ZntR modified at positions C79, C114 and C124 also showed no activity with Cd(II) or Pb(II) (figs 3.3 A-C). Surprisingly, both C115S and C115A derivatives of ZntR mediated induction at P*zntA* in response to Cd(II) and Pb(II). C115S induced at wild-type levels to Cd(II) and Pb(II) but C115A respectively increased transcriptional levels by 2.5- and 3-fold in comparison to ZntR w.t., despite showing no activity with Zn(II).

The C-terminal residue of ZntR is a cysteine (C141) residue and this was altered to alanine, serine, histidine, leucine, tyrosine, glutamate and aspartate. The induction profiles are shown in fig. 3.5.

Initial experiments with C141A and C141S derivatives demonstrated that replacement of this cysteine had very different effects on function. The C141A derivative showed dramatic differences in its Zn(II) induction profile with respect to ZntR w.t. The C141A derivative increased maximal induction of ZntR in response to Zn(II) by 2.5-fold but also changed the sensitivity of the regulator/promoter complex with maximal induction now occurring at 0.1 mM Zn(II) whereas ZntR w.t. and most of the other active derivatives showed maximal induction in the 0.9 – 1.2 mM range. C141A was little altered with respect to Cd(II) (Fig 3.3 B) but 3-fold more responsive to Pb(II) (fig 3.3 D). In contrast, C141S retained the usual transcriptional profile in response to Zn(II) but was 3-fold more responsive at the maximal induction (1.1 mM Zn(II)). This derivative was massively more responsive to Cd(II) and Pb(II), 33-fold and 16-fold, respectively (figs. 3.3 B and D).

Fig 3.4 Effect of ZntR-cysteine substitutions in ZntR on Zn(II) induced transcription of P*zntA* .

Luminescence was measured in *E. coli* TG1(*zntR*::*Km*) [pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Zn(II) ranging from 0 - 1.2 mM (open columns).

The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. at 1.1 mM Zn(II) (7500 RLU). The standard error (vertical bars) in each case are derived from a triplicate data set.

Fig. 3.5 Effect of ZntR-cysteine 141 substitutions in ZntR on Zn(II) induced transcription of P*zntA* .

Luminescence was measured in *E. coli* TG1(*zntR*::*Km*) [pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Zn(II) ranging from 0 - 1.2 mM (open columns).

The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. at 1.1 mM Zn(II) (7500 RLU). The standard error (vertical bars) in each case are derived from a triplicate data set.

Residue C141 was also replaced with histidine, leucine, tyrosine, glycine and aspartate; the latter four derivatives showed a slightly diminished response to Zn(II) with ZntR (C141G) being most responsive to Cd(II) (4-fold) and exhibiting altered profiles and sensitivities (fig. 3.3 A, B, C). ZntR (C141H) showed an increased maximal induction (3-fold) with Zn(II) and an alteration in sensitivity with maximal induction occurring at 0.1 mM similar to that seen in C141A. Induction was slightly increased with Cd(II) but little changed with Pb(II) for C141H.

A summary of the results for sections 3.3.2 and 3.3.3 is shown in table 3.1.

Table 3. 1 Summary table of effect of amino acid substitutions on ZntR transcriptional activity from P*zntA-1* and P*zntA*.

The cysteines that are conserved in MerR (*Pseudomonas aeruginosa*) are marked with an asterisk (*). A tick, ✓, indicates that transcription occurred from the corresponding promoter and — indicates a lack of transcription. The fold-induction (columns headed with F.I.) values are shown for transcription from P*zntA* in response to Zn(II), Cd(II) and Pb(II) as compared to ZntR (w.t.) which is assigned a value of 1 in each case.

3.3.4 Maxicell labelling of ZntR and derivatives

Maxicell labelling of ZntR and its mutant derivatives was carried out to confirm stable expression of these proteins (currently, no antibodies against ZntR are available for Western blotting). Maxicells were performed using *zntR* and its mutant derivatives cloned in pSU18 however the nature of the pSU18 plasmid (medium copy no.) is such that effective expression of the cloned genes did not occur and no corresponding bands were obtained after maxicell labelling.

Therefore, the *zntR* gene and its mutant derivatives had to be extracted from pSU18 and re-cloned into the high expression plasmid pUTS18 (section 2.1.2). Firstly, the recombinant plasmids pSU18-*zntR* and the pSU18- mutant derivatives were digested with *Eco*RI and *Bam*HI (section 2.2.3.1) and the resulting DNA fragments were separated on 1% agarose gels (section 2.2.5.1). The bands corresponding to *zntR*/derivatives were extracted (section 2.2.3.5) and religated (section 2.2.3.7) into pUTS18 that had been *Eco*RI/*Bam*HI digested (section 2.2.3.1) and dephosphorylated (section 2.2.3.6). The ligation reaction mixture was used to transform (section 2.2.1.1.2) competent *E. coli* TG2 cells and resultant colonies were picked and the plasmids were extracted (section 2.2.2.1). These were screened for inserts by digestion with *Eco*RI/*Bam*HI and visualised by agarose gel electrophoresis.

Recombinant pUTS18 plasmids were used for maxicell labelling of the insert as detailed in section 2.2.9.1, 2.2.9.3.1 and 2.2.9.3.3. Fig 3.6 shows the results of the maxicell labelling of ZntR and its mutant derivatives. The first lane was the result of maxicell labelling with an empty control plasmid, labelled pUTS18. In this sample, the Cb resistance marker gene, present on the plasmid was expressed and this appeared to be the only protein labelled. All of the other lanes showed 2 bands, the top band corresponding to the Cb resistance protein, and the lower band to the ZntR/mutant derivative.

It can be concluded that all mutants are fully expressed. The C141H sample seems to be less well expressed however on other occasions the intensity of this sample was equivalent to the other samples but for simplicity the best representative gel has been shown. Also,

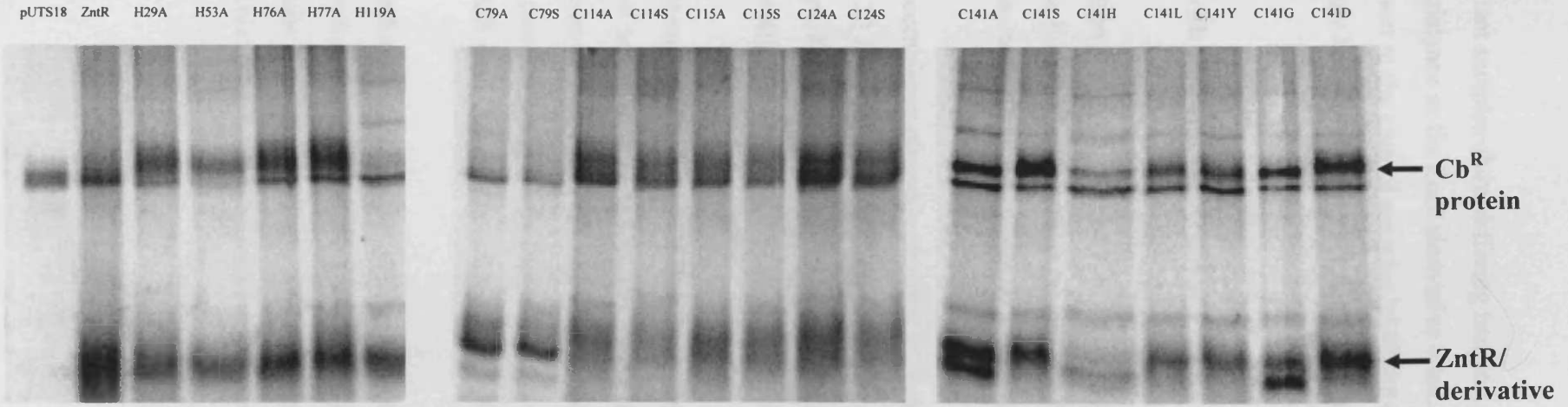


Fig 3. 6 Bands obtained from maxicell labelling of pUTS18, ZntR w.t. and its mutant derivatives. The band marked Cb^R, representing the carbenicillin resistance protein (approximately 30 kDa) is visible in all lanes and the lower band corresponds to ZntR (16.2 kDa) or its amino acid substituted variant, which is expressed and can be seen in all lanes except pUTS18.

some of the mutant samples show differing banding patterns with the appearance of double bands for Cb resistance or the ZntR/derivative band. Since the bands are in the correct places with respect to the control samples (pUTS18 and ZntR w.t.), the appearance of double bands may be caused by proteolyic activity and hence degradation products.

3.3.5 EMSAs with ZntR and derivatives

EMSAs were attempted with ZntR and its mutant derivatives to derive information about the impact of the histidine and cysteine residues on the structure/function of ZntR by analysing the interaction of the mutants with the cognate promoter P*zntA*.

The pUTS18 recombinant plasmids prepared in section 3.3.4 were used to transform (section 2.2.1.1.2) *E. coli* TG1(*zntR*::*Km*) cells. The DNA for the probe bearing P*zntA* was extracted from pUKP*zntA* (Brocklehurst *et al.*, 1999) as described therein. EMSAs were carried out as detailed in section 2.2.9.2, 2.2.9.3.2 and 2.2.9.3.3.

EMSAs were attempted with all of the ZntR derivatives but due to time constraints and the non-reproducible nature of the EMSAs they had to be restricted to pUTS18, ZntR w.t. and two of the cysteine derivatives. C124A/S were chosen because of the results obtained in the P*zntA-1* assays (section 3.3.2) where these were the only derivatives that did not activate P*zntA-1*. C115A/S were chosen as representatives of mutants that activate P*zntA-1*.

EMSAs were perfomed using soluble cell extracts (section 2.2.9.2.1) and the results of the EMSAs are shown in fig. 3.7. The lanes marked pUTS18 do not show a retarded band and only the free probe is visible. ZntR w.t. retards the probe albeit very faintly. This retarded band is also visible in the C115A/S lanes but is absent from the C124A/S lanes.

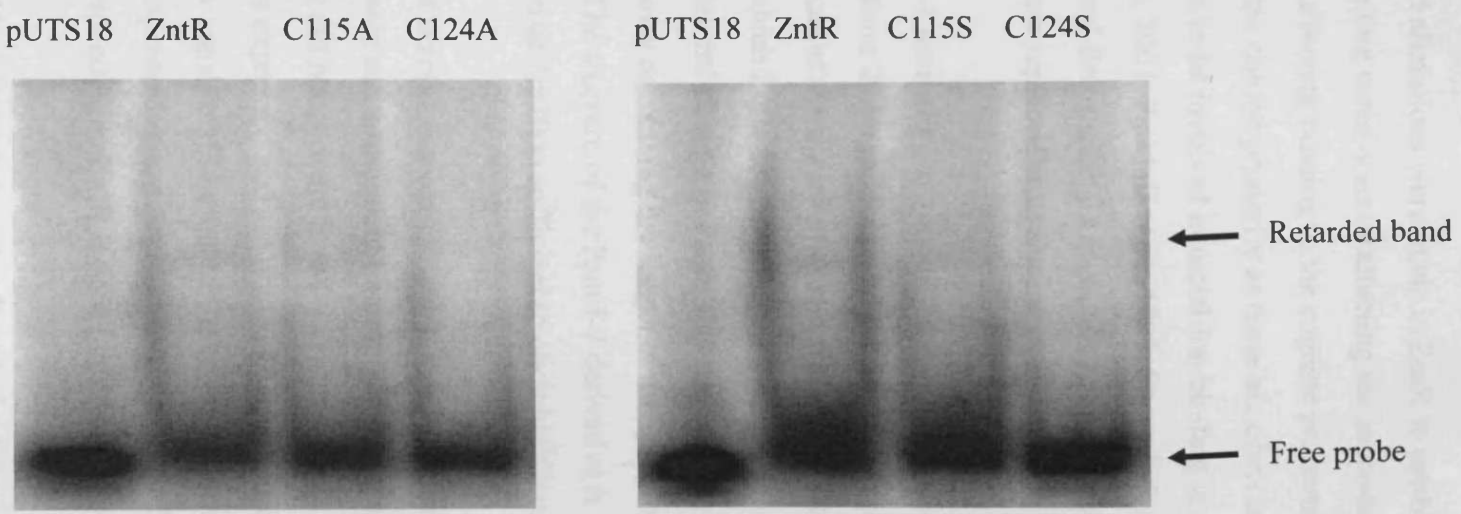


Fig 3. 7 Gel shift pattern obtained from EMSAs of P*zntA* by ZntR and its derivatives C115A, C124A, C115S and C124S, using pUTS18 as a control. The free probe is visible in all the lanes but retardation of the probe is seen only with ZntR, C115A and C115S.

3.4 Discussion

Amino-acid alterations were made in ZntR to establish the role of candidate residues in directly binding metal-ions or affecting the secondary, tertiary or quaternary structure and ultimately affecting binding to the cognate promoter P*zntA*. The cysteine and histidine residues were chosen primarily as these are often involved in metal-ion binding and have been shown to be involved in metal-ion binding in a number of ZntR homologues (Kidd and Brown, 2003, Parkhill *et al.*, 1993, Shewchuk *et al.*, 1989b, Shewchuk *et al.*, 1989c, Stoyanov and Brown, 2003). Histidine residues were replaced with alanine and the cysteine residues were replaced with alanine or serine.

The P*zntA-1* derivative is a modified P*zntA* promoter in which the spacer region has been shortened from 20 bp to 19 bp and this makes the promoter P*zntA-1* much more sensitive to ZntR because of the nature of the proposed DNA distortion mechanism (sections 1.5.1 and 1.5.2) by which ZntR activates P*zntA*. In this mechanism, binding of Zn(II) to ZntR induces a conformational change in P*zntA* that makes it more optimal for transcription by σ^{70} RNA polymerase by decreasing the length of the spacer region and realigning the –10 and –35 elements. The stucture of the P*zntA-1* derivative is such that binding of ZntR alone, without the addition of Zn(II) is sufficient to elicit high levels of transcription.

The mutant derivatives were tested at P*zntA-1* where activation would indicate expression of the mutants and subsequent binding. Of all the mutants, only the C124A and C124S derivatives did not activate P*zntA-1*. This indicates that all of the mutants that did activate P*zntA-1* are expressed and are not toxic, do not affect the dimerisation of ZntR and are structurally capable of binding to the cognate promoter. The C124A/S derivatives may not be stably expressed or are structurally incapable of binding P*zntA-1*, although a protein band corresponding to ZntR is seen in maxicell analysis of these mutants.

Of the histidine mutants tested at P*zntA,* the diminished profiles seen with Zn(II) with H29A, H53A and H119A suggest that these histidines either have a direct Zn(II) binding

role in ZntR or take part in the definition of structure e.g. dimerisation or torsional restructuring of P*zntA*.

Both H53A and H119A responded at almost w.t. levels to Cd(II) and Pb(II) and H29A induced transcription in response to these metal-ions more effectively than ZntR w.t. exhibiting a 6-fold increase in transcriptional response to Cd(II). These data suggest that these histidines have a significant effect on the selectivity and dynamic range of ZntR.

Although slight deviations from ZntR w.t. were seen with the profiles obtained from H76A and H77A with Zn(II), the overall profile was similar to w.t. and therefore no Zn(II)-binding or structural importance can be assigned to these histidines from the results. The much improved response of H76A to Cd(II) and Pb(II) in comparison to ZntR w.t. suggests that this histidine may play a role in determining substrate preference. Mutagenesis of the histidines found in this region in MerR and CueR may shed more light on this aspect of regulatory function.

The roles that H29, H53, H76 and H119 may have in determining substrate preference are of interest. The recently characterised MerR-like regulator ZccR from *Bordetella pertussis* which responds to Zn(II), Cd(II) and Co(II) (Kidd and Brown, 2003) possesses six histidine residues (fig. 3.8) and mutagenesis of these to asparagine had some effect on metal induction by reducing the response to Zn(II), Cd(II) and Co(II) in comparison to ZccR w.t. at the cognate promoter of a predicted export ATPase (Kidd and Brown, 2003). The most pronounced effects were seen with the H90N, H140N and H142N mutants, which resulted in a much diminished response to Zn(II), Co(II) and Cd(II). Interestingly, mutagenesis of H73 in ZccR produced similar results to those seen with H77A in ZntR, where induction of the cognate promoter of ZccR diminished slightly in response to Zn(II), Cd(II) and Co(II).

Mutagenesis of the first four cysteines in ZntR at positions C79, C114, C115 and C124 resulted in a complete loss of activity with Zn(II) as the inducer. In addition, mutants of C79, C114 and C124 showed no activity with Cd(II) or Pb(II) and surprisingly both C115A and C115S derivatives mediated induction at P*zntA* in response to Cd(II) and Pb(II)

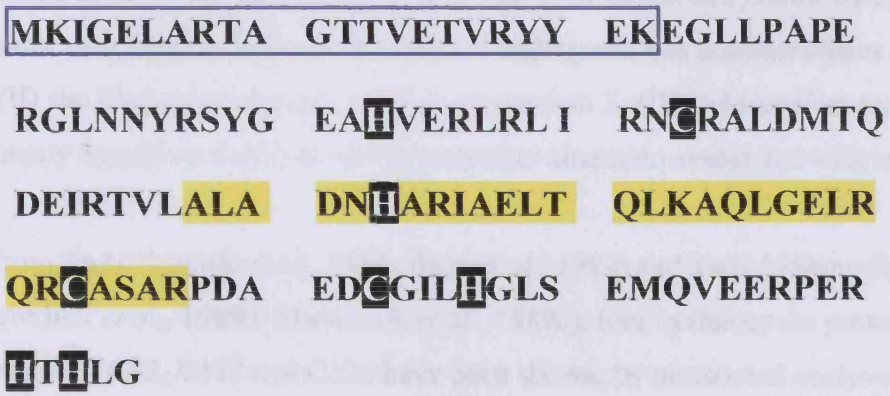


Fig. 3.8 Amino acid sequence of ZccR consisting of 125 amino acids. Within its N-terminal region is a helix-turn-helix DNA-binding domain indicated by the blue border. Downstream is the ligand-binding region containing five histidines and three cysteines, all of these are highlighted by a white font on a black background.

The yellow highlighted sequence represents the predicted coiled-coil dimerisation domain, generated using PAIRCOIL (http://www.paircoil. lcd.mit.edu/cgi-bin/paircoil). The location of the helix-turn-helix DNA binding motif was predicted using Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat. pl?page=/ NPSA/npsa _hth.html).

despite showing no activity with Zn(II). Indeed C115A induces the promoter 2.5- and 3-fold greater than w.t. with Cd(II) and Pb(II), respectively. It is unclear why this derivative should respond so well to Cd(II) and Pb(II) given that it does not respond well to Zn(II). The position of C115 within ZntR may be such that replacement of cysteine by the smaller alanine residue may help to create an alternative binding site that accommodates Cd(II) and Pb(II). Cd(II) can bind more strongly to thiol groups than Zn(II) and therefore may not require as many ligands as Zn(II) to enable structural alteration associated with induction.

In MerR, from Tn*21* (Livrelli *et al.*, 1993, Ross *et al.*, 1989) and Tn*501* (Shewchuk *et al.*, 1989a, Shewchuk *et al.*, 1989b, Shewchuk *et al.*, 1989c), four cysteines are present. Three of these cysteines, C82, C117 and C126 have been shown, by mutational analysis to serve as ligands for Hg(II) binding. These three cysteine residues are conserved with respect to other MerR family members (fig 1.8) and correspond to residues C79, C114 and C124 respectively in ZntR. In CueR (*E. coli*), C112 and C120 correspond to C117 and C126 in MerR, and C114 and C124 in ZntR. Site-directed mutagenesis of C112 and C120 in CueR (Stoyanov and Brown, 2003) eliminated the response to Cu(I), Ag(I) and Au(I).

Also, site directed mutagenesis of three cysteines in ZccR which are analogous to the conserved MerR cysteines showed that these were required for full induction by Zn(II), Cd(II) and Co(II) (Kidd and Brown, 2003). It is therefore unsurprising that C79, C114 and C124 in ZntR are essential for the full transcriptional response of ZntR. Additionally, C115 in ZntR may be required for a tetragonal arrangement to accommodate Zn(II) but not Cd(II) or Pb(II). The complete loss of function in these cysteine mutants suggests that the conserved cysteine residues play a central role in the mechanism of induction and are likely to act as ligands for metal-ions.

The C-terminal residue of ZntR (position 141) is a cysteine, and shows no sequence conservation with other MerR-family members (fig. 1.8). It is of interest that the most diverse region in the MerR family (fig. 1.8) is at the C-terminal region where many MerR-like proteins have putative metal-binding ligands such as thiols and imidazoles. It is perhaps in this C-terminal region where some measure of specificity is directed.

Initial experimentation demonstrated that replacement of C141 in ZntR with either an alanine or serine residue had very different effects on function and this led to further extensive mutagenesis of this cysteine. It appears that the C-terminal C141 in ZntR may control access of metal-ions to the binding sites in the protein as mutagenesis had profoundly different effects depending on the nature of the mutation and of the metal.

Maxicell labelling of ZntR mutant derivatives was carried out to confirm expression of the mutants. Although this had already been achieved by performing luminescence assays at P*zntA-1*, maxicell labelling was necessary for ascertaining expression of non-activating derivatives at this promoter namely, C124A and C124S and also for confirming the presence of the mutant proteins in the system to be used for EMSAs. All of the mutant proteins were found to be fully expressed. EMSAs were carried out with all of the mutant derivatives however results were highly non-reproducable given the time constraints of this study so efforts were focused upon C124A and C124S. C115A and C115S were also used as a comparison for C124.

EMSA's were carried out with C124 derivatives to test the binding to P*zntA*. C115 derivatives were used for EMSA's to rule out arguments that binding of mutants to P*zntA-1* may not necessarily prove binding to P*zntA* w.t. The results of the EMSA's were in accordance to the predicted hypothesis that activation of P*zntA-1* represents binding of ZntR or its derivatives to P*zntA* w.t. since ZntR w.t. and C115A/S did retard the P*zntA* probe whereas C124A/S did not. It appears therefore that C124 is an important residue for structural stability and perhaps ligand binding.

BmrR is a member of the MerR family and activates transcription of the multi-drug transporter gene *bmr* in response to cellular invasion by certain drugs in *Bacillus subtilis* (Ahmed *et al.*, 1994). The crystal structure of BmrR bound to its cognate promoter and its ligand was elucidated recently (Heldwein and Brennan, 2001). The ligand is coordinated between the 2 monomers (fig 3.9 B). It is held in place by residues on the ligand-binding domain of one monomer and residues from the DNA binding domain of the other monomer. The linker helices within BmrR form a coiled-coil holding the monomers

Fig 3.9 (A) A structural model for the first 95 amino acids of ZntR was created using SWISS-MODEL (Guex *et al.*, 1999) (http://swissmodel.expasy.org/SWISS-MODEL.html) with BmrR coordinates as the template. Histidine residues are shown in blue and spacefill, cysteine 79 is shown in yellow and spacefill. The H-T-H motif within the N-terminal DNA-binding region (grey) is highlighted red. The coiled-coil dimerisation structure is shown in magenta.

(B) A dimer of BmrR (Heldwein and Brennan, 2001) complexed with DNA (cyan/spacefill) is shown. The H-T-H motifs within the N-terminal DNA-binding regions of BmrR are highlighted red.

together to form the dimer, and have been predicted to be present in all MerR family proteins (Heldwein and Brennan, 2001). It is unsurprising, given the chemical similarity of Zn(II), Cd(II) and Pb(II), that simplistic interpretations of the structure–function relationships within ZntR are not easily defined, although the 3-dimensional structure of BmrR, the only published structure of a MerR family protein to date (Heldwein and Brennan, 2001), allowed the creation of a model of the first 95 amino acids of ZntR (fig. 3.9 A) which positions three of the histidine (H53, H76 and H77) and one of the cysteine residues (C79) in regions expected to interact with metal-ions e.g. at the proposed dimer interface.

From the data of the histidines in ZntR, H76 and H77 do not play a critical role in Zn(II) responsiveness. A coiled–coil structure is present in BmrR (fig. 3.9 B) (Heldwein and Brennan, 2001) and a similar structure is thought to link the two domains of ZntR (fig. 3.9 A) and other MerR-like regulators (Caguiat *et al.*, 1999). The predicted position of H76 at the proximal end of the coiled–coil structure places this residue in the centre of the proposed dimer-interface region known to be important for substrate binding in BmrR (Heldwein and Brennan, 2001)

The model of ZntR (fig. 3.9 A) predicts that H29 lies close to the DNA-binding H-T-H region of ZntR and is distant from a proposed binding site at the dimer interface although our data shows that this residue has a significant effect on the selectivity and dynamic range of ZntR. It is tempting to suggest that since alterations in these residues appear to affect the substrate range of ZntR that they act as ligands for one of the two Zn(II)-ions predicted to bind each ZntR monomer, perhaps acting in a structural role rather than a primary driver of DNA distortion. Irrespective of the detailed function of these residues, these results show that all 5 histidine residues are important for ZntR function.

In the ZntR model, C79, C114 and C115 are located at the ends of the proposed linker region and C124 is within the ligand binding region (fig 1.10). It may be plausible to allocate the roles of C114, C115 and C124 as Zn(II) binders on one monomer and C79 on the other in order to convert ZntR into its active state.

It is not clear if C124 is important for binding Zn(II) as a switch for activating ZntR or if it binds Zn(II) to structurally stabilise ZntR or simply to provide disulphide bonds with another cysteine for maintenance of the protein structure. In any case, if C124 is important structurally, the possibility that some of the histidines or other candidate metal-ion binding residues within ZntR such as aspartate or glutamate may be involved in binding Zn(II) for a regulatory effect cannot be ruled out.

Recently, the x-ray crystal structure of ZntR was solved (Changela *et al.*, 2003). The comparison of the solved structure with the results from this study are compared in section 6.1.

Concluding remarks

An interesting aspect of this research is the mutability of the specificity, sensitivity and dynamic range of ZntR, with 7 of the 10 derivatives showing substantial differences from w.t. This mutability of ZntR is in sharp contrast to that seen during mutational analysis of MerR (Comess *et al.*, 1994, Parkhill *et al.*, 1993, Parkhill *et al.*, 1998, Ross *et al.*, 1989) which appears to be rather more refractory to functional alteration (Caguiat *et al.*, 1999). Perhaps the differences in the strength of interaction with biological molecules between Zn(II) and Hg(II) has allowed MerR to become more selective and less open to change than seen here for ZntR.

Three of the cysteines required for optimium ZntR function are known to be conserved in MerR, namely C79, C114 and C124. These correspond to MerR residues C82, C117 and C126 where they have already been shown to coordinate Hg(II) in a tridentate orientation sandwiching a Hg(II)-ion between 2 monomers of the MerR dimer (Shewchuk *et al.*, 1989a, Shewchuk *et al.*, 1989b, Shewchuk *et al.*, 1989c). In ZntR, the involvement of an additional cyteine, C115, for coordinating the primary ligand Zn(II) ties in with the assumption that Zn(II) is co-ordinated tetrahedrally within ZntR. ZntR is known to bind 2

Zn(II) ions per monomer (Outten *et al.*, 1999). It is not known if any Zn(II) ions are structural and how many are required for switching apo-ZntR to the activating form. It is likely that these cysteines coordinate Zn(II) between 2 monomers as is the case for the ligands of both MerR and BmrR.

The data show that both cysteine and histidine residues are important in the function of ZntR and that alteration of the C-terminal amino acid can have a profound effect on the properties of this regulator.

Further study of this family of transcriptional regulators will yield important information regarding the evolution of specificity and mechanism, particularly with respect to metal-ion dependent transcription in prokaryotes.

CHAPTER FOUR

TRANSCRIPTIONAL PROFILING IN *E. coli* (w.t.) AND *E .coli* (Δ*zntR*) IN RESPONSE TO Zn(II)

4.1 Summary

Whole-genome transcriptional profiles were generated using RNA isolated from TG1 (w.t.) and TG1 (*zntR*::*Km*) at an elevated Zn(II) concentration to investigate the effect of *zntR* in *E. coli* on an organismal scale.

Significant alterations in expression patterns between the two strains were observed. Zn(II) appeared to affect strain TG1 (*zntR*::*Km*) more dramatically than TG1 (w.t.). Trends observed included interference with other metal-ion utilising systems in TG1 (*zntR*::*Km*), enhanced chemotaxis and other proposed mechanisms of coping with elevated Zn(II), such as chelation. A potential Zn(II) exporter was also highlighted.

4.2 Introduction

The advent of array technology has allowed the expression patterns of all 4290 (Blattner *et al.*, 1997) genes in *E. coli* to be studied simultaneously. Expression profiles generated by cDNA array technology can present a holistic view of transcriptional regulation in response to stressful environmental stimuli. This has been demonstrated in *E. coli* for a range of stimuli such as Fe (McHugh *et al.*, 2003), Zn(II)/Ni(II)/Co(II)/Cd(II) in adapted strains (Brocklehurst and Morby, 2000), hydrogen peroxide (Zheng *et al.*, 2001) and osmotic stress (Weber and Jung, 2002).

The main Zn(II) resistance protein in *E. coli* is ZntA (detailed in section 1.3.1.3.1). The transcriptional activation of this Zn(II) exporting ATPase is dependent on ZntR (detailed in section 1.5.2). It may be possible to glean a great deal of information pertaining to the metabolism and toxicology of Zn(II) by array analysis of a *zntR* deficient strain of *E. coli* in response to Zn(II) with comparison to the transcript profile of the wild-type strain.

This methodology was used for this study to identify novel Zn(II) transport/binding mechanisms in *E. coli*. These may be accentuated when one of the prominent Zn(II) resistance systems, ZntR-ZntA system is absent. Also, the role of ZntR and Zn(II) in general cell metabolism can be reaffirmed or novel pathways elucidated. The identification of other promoters activated directly by ZntR is another possibility.

4.3 Results

4.3.1 Growth curves of TG1 and TG1 (*zntR*::*Km*) strains

LB medium was prepared from a single batch which was aliquoted into separate flasks to keep conditions as standard as possible. The growth of strain TG1 (*zntR*::*Km*) was maintained with kanamycin until inoculation of this strain into fresh LB medium from an overnight culture was required for the growth curve. Growth studies of TG1 and TG1 (*zntR*::*Km*) strains (section 2.1.1) were performed as detailed in section 2.2.10.6, in triplicate aliquots of 250 ml. Growth was followed over a 16 hour period with D_{600} measurements at hourly intervals in standard LB medium and LB medium supplemented with Zn(II) at 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM concentrations.

The growth curves of both strains in standard LB medium and LB medium supplemented with 0.3 mM Zn(II) are shown in fig. 4.1. Growth of the TG1 (*zntR*::*Km*) strain in LB media with either 0.2 mM, 0.4 mM or 0.5 mM Zn(II) was not suitable for extracting mRNA and therefore the growth curves are not shown.

4.3.2 RNA extraction from TG1 and TG1 (*zntR*::*Km*) strains

LB medium was prepared from a single batch which was aliquoted into separate flasks to keep conditions as standard as possible. TG1 and TG1 (*zntR*::*Km*) strains were grown overnight in standard LB medium at 37°C, 200 rpm. These were used to inoculate 100 ml (1:100 dilution) of standard LB medium and LB media supplemented with 0.3 mM Zn(II), in triplicate, and returned to the previous growth conditions.

Cultures were grown to D_{600} of 0.5 ± 0.005 and RNA was extracted and the concentration determined as detailed in section 2.2.4. RNA purification typically yielded concentrations of total RNA of approximately 1 $\mu g\ \mu l^{-1}$. RNA samples prepared from identically treated triplicate cultures for each of the strains were subjected to electrophoresis (section 2.2.5.2)

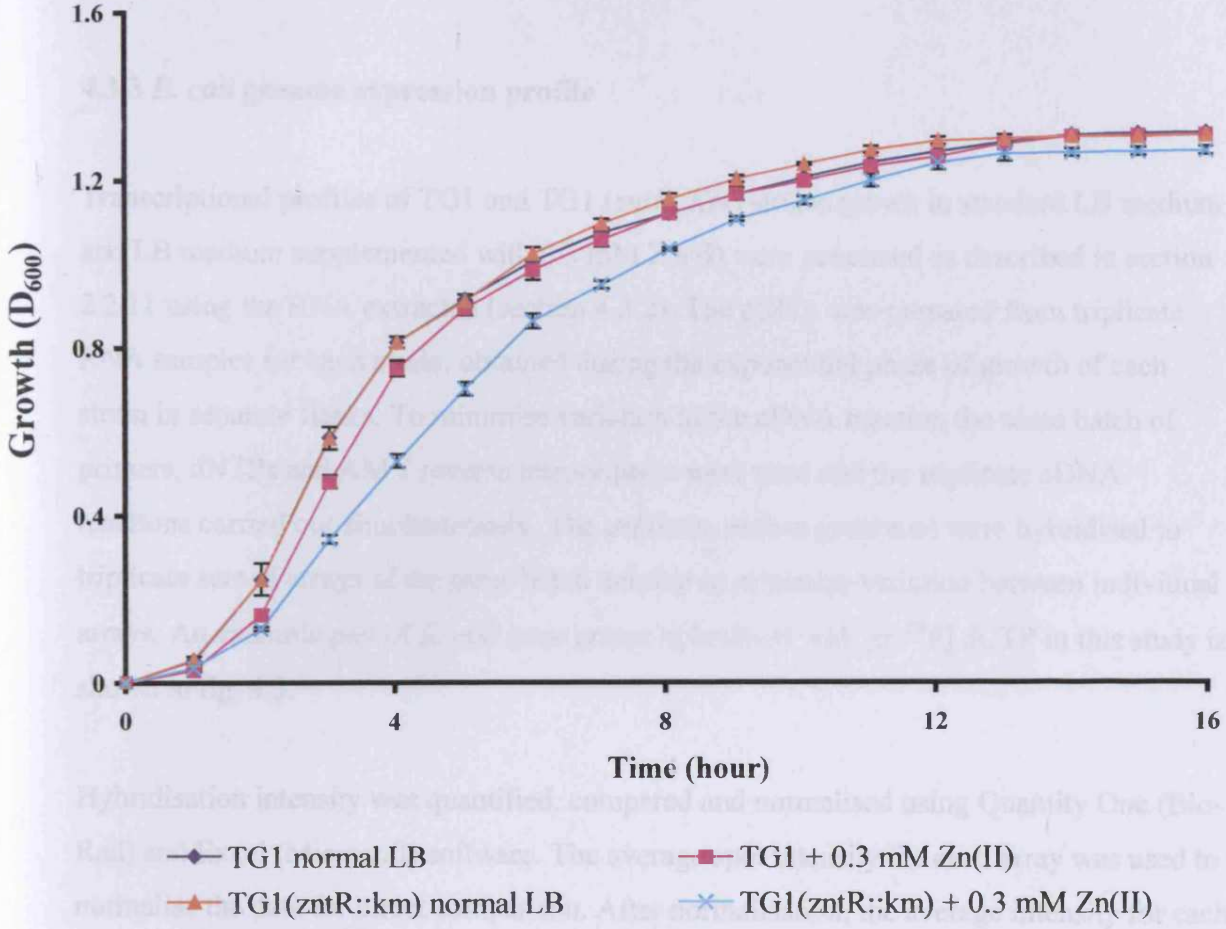


Fig. 4.1 Growth curves for TG1 and TG1 (*zntR*::*Km*) in standard LB medium and LB medium supplemented with 0.3 mM Zn(II).

The graphs show D_{600} against time after initial inoculation. Assays were performed in triplicate and the standard error bars are shown (I).

to check for degradation and genomic DNA contamination. The RNA gel obtained is shown in fig. 4.2, where 1 μg of RNA was loaded into each lane.

4.3.3 *E. coli* genome expression profile

Transcriptional profiles of TG1 and TG1 (*zntR*::*Km*) strains grown in standard LB medium and LB medium supplemented with 0.3 mM Zn(II) were generated as described in section 2.2.11 using the RNA extracted (section 4.3.2). The cDNA was prepared from triplicate RNA samples for each strain, obtained during the exponential phase of growth of each strain in separate flasks. To minimise variation in the cDNA reaction the same batch of primers, dNTPs and AMV reverse transcriptase were used and the triplicate cDNA reactions carried out simultaneously. The triplicate probes generated were hybridised to triplicate sets of arrays of the same batch number to minimise variation between individual arrays. An example pair of *E. coli* gene arrays hybridised with [α-^{33}P] dCTP in this study is shown in fig. 4.3.

Hybridisation intensity was quantified, compared and normalised using Quantity One (Bio-Rad) and Excel (Microsoft) software. The average spot intensity for each array was used to normalise the data for direct comparison. After normalisation, the average intensity for each ORF was compared and the fold increase/decrease calculated. The transcriptional profile of each strain grown in LB medium supplemented with 0.3 mM Zn(II) was compared to that of the same strain grown in standard LB.

4.3.4 Transcriptional analysis of TG1 and TG1 (*zntR*::*Km*) strains

Gene expression was considered to be significantly changed when the fold-change was ± 1.5-fold, this is below the threshold set by the manufacturer Sigma-Genosys who recommended ± 2-fold. Significance analysis was performed using the statistical program SAM (Tusher *et al.*, 2001). This statistical method identifies genes with statistically

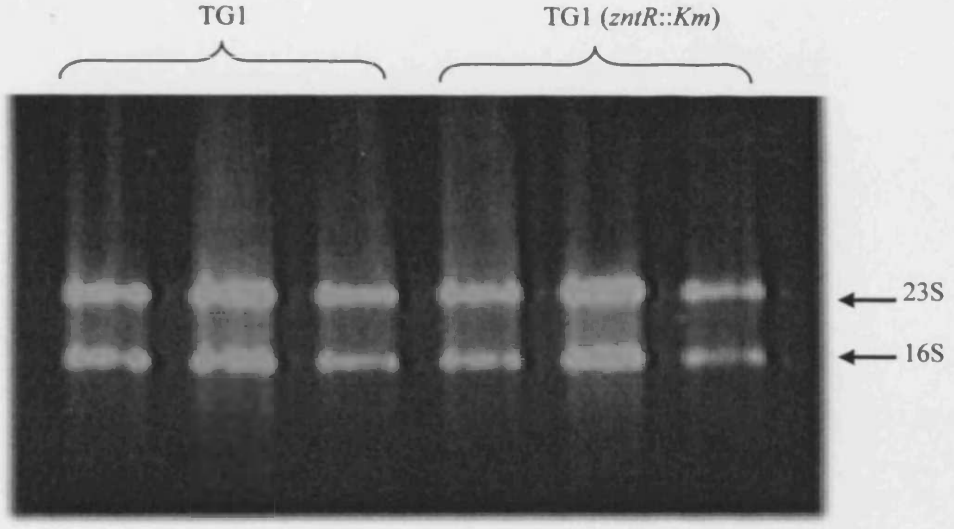


Fig 4.2 A typical RNA extraction from triplicate TG1 and TG1 (*zntR*::*Km*) RNA samples electrophoresed on a 1% agarose gel. The two bands visible in each lane represent 23S and 16S ribosomal RNA (rRNA). No genomic DNA or mRNA degradation is apparent in the samples.

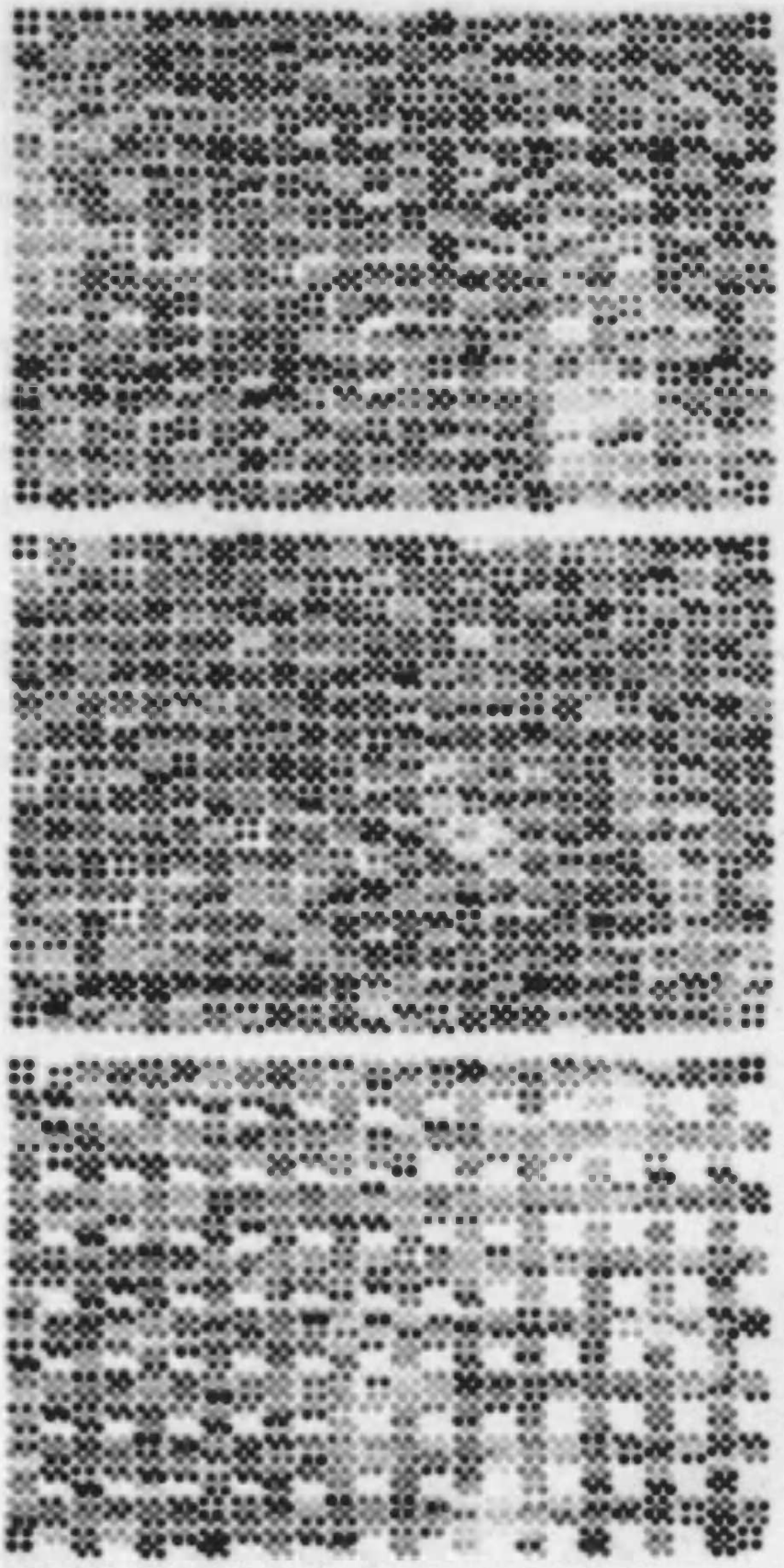


Fig 4.3 Panorama *E. coli* gene arrays hybridised with [α-^{33}P] labelled cDNA probes.

significant changes in expression by assimilating a set of gene specific t-tests. Each gene is assigned a score based on change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM then estimates the percentage of genes identified by chance, referred to as the false discovery rate (FDR). For further details refer to Tusher *et al,* 2001.

The significantly altered genes in strain TG1 (w.t.) are shown in table 4.1. Alterations are based on comparison of TG1 grown in medium supplemented with 0.3 mM Zn(II) against TG1 grown in normal LB medium. These results represent the background profile for comparing strain TG1 (*zntR*::*Km*), for which an analogous table of results is shown (table 4.2).

Transcripts that were altered in both strains are shown separately, in table 4.3. For brevity, genes of unknown function have been omitted from the tables in this chapter. The full set of results can be found in appendix I, tables 1, 2 and 3.

Table 4. 1 Genes of known function for which transcript levels were altered in strain TG1 (w.t.) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (w.t.) to transcript levels for untreated TG1 (w.t.). Down-regulated genes are indicated by a minus sign (-) in the 'Fold change' column. Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array _ Info.xls. Genes are ordered according to function.

Gene	Gene ID	Function	Fold Change
nrdA	b2234	2'-Deoxyribonucleotide metabolism	6.66
slyA	b1642	Adaptations, atypical conditions	4.08
cysM	b2421	Adaptations, atypical conditions	2.94
glnB	b2553	Amino acid biosynthesis: Glutamine	5.06
hisL	b2018	Amino acid biosynthesis: Histidine	4.09
hisG	b2019	Amino acid biosynthesis: Histidine	3.71
hisC	b2021	Amino acid biosynthesis: Histidine	2.73
hisH	b2023	Amino acid biosynthesis: Histidine	3.79
ivbL	b3672	Amino acid biosynthesis: Isoleucine, Valine	4.32
leuC	b0072	Amino acid biosynthesis: Leucine	-2.05
leuB	b0073	Amino acid biosynthesis: Leucine	-1.79
wrbA	b1004	Amino acid biosynthesis: Tryptophan	2.69
pheS	b1714	Aminoacyl tRNA synthetases, tRNA modification	4.58
aspS	b1866	Aminoacyl tRNA synthetases, tRNA modification	4.69
entF	b0586	Biosynthesis of cofactors, carriers: Enterochelin	7.24
entA	b0596	Biosynthesis of cofactors, carriers: Enterochelin	3.96
hemL	b0154	Biosynthesis of cofactors, carriers: Heme, porphyrin	3.17
lipA	b0628	Biosynthesis of cofactors, carriers: Lipoate	4.82
lipB	b0630	Biosynthesis of cofactors, carriers: Lipoate	2.78
ygiC	b3843	Biosynthesis of cofactors, carriers: Menaquinone	2.58
molR_3	b2117	Biosynthesis of cofactors, carriers: Molybdopterin	5.48
nadC	b0109	Biosynthesis of cofactors, carriers: Pyridine nucleotide	3.56
pdxB	b2320	Biosynthesis of cofactors, carriers: Pyridoxine	-2.63
thiD	b2103	Biosynthesis of cofactors, carriers: Thiamin	3.76
mlrA	b2127	Biosynthesis of extracellular matrix and curli: regulator (MerR)	3.98
yefB	b2052	Biosynthesis of extracellular polysaccharide colanic acid	2.43
wcaF	b2054	Biosynthesis of extracellular polysaccharide colanic acid	2.83
wcaE	b2055	Biosynthesis of extracellular polysaccharide colanic acid	2.90
wcaB	b2058	Biosynthesis of extracellular polysaccharide colanic acid	3.22
ppdA	b2826	Biosynthesis of macromolecules: fimbria, pili	4.15
chpA	b2782	Cell growth regulation	3.47
chpS	b4224	Cell growth regulation	4.87
mukF	b0922	Cell killing	3.63
hlyE	b1182	Cell killing	3.94
surE	b2744	Cell protection	2.68
mobB	b3856	Central intermediary metabolism: Nucleotide interconversions	3.57
phnN	b4094	Central intermediary metabolism: Phosphorus compounds	3.30
phnM	b4095	Central intermediary metabolism: Phosphorus compounds	4.17
phnK	b4097	Central intermediary metabolism: Phosphorus compounds	4.03
amtB	b0451	Central intermediary metabolism: Pool, multipurpose conversions	2.99
citF	b0615	Central intermediary metabolism: Pool, multipurpose conversions	10.57
ugd	b2028	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.62
yefC	b2051	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	3.26
cysD	b2752	Central intermediary metabolism: Sulfur metabolism	9.89
cysI	b2763	Central intermediary metabolism: Sulfur metabolism	5.42
yabH	b0055	Chaperone	7.11
yhaP	b3111	Degradation of amino acids: L-serine	4.16
yafB	b0207	Degradation of carbohydrates	2.75
abgA	b1338	Degradation of carbohydrates	3.00
yeeZ	b2016	Degradation of carbohydrates	2.70
yieL	b3719	Degradation of carbohydrates (putative)	3.31
paaZ	b1387	Degradation of phenyl acetic acid	4.09
paaY	b1400	Degradation of phenyl acetic acid	3.26
clpA	b0882	Degradation of proteins, peptides, glycopeptides	3.56
pepT	b1127	Degradation of proteins, peptides, glycopeptides	2.88
rne	b1084	Degradation of RNA	-2.26
ymeH	b1524	Degradation of small molecules (putative): Amino acids	3.13

ansA	b1767	Degradation of small molecules: Amino acids	3.16
dsdC	b2364	Degradation of small molecules: Amino acids	6.58
mhpC	b0349	Degradation of small molecules: Carbon compounds	3.90
rbsR	b3753	Degradation of small molecules: Carbon compounds	-2.80
fadD	b1805	Degradation of small molecules: Fatty acids	3.49
atoA	b2222	Degradation of small molecules: Fatty acids	3.74
atoB	b2223	Degradation of small molecules: Fatty acids	3.10
b2224 (o394)	b2224	Degradation of small molecules: Fatty acids	3.19
fadA	b3845	Degradation of small molecules: Fatty acids	2.50
sodC	b1646	Detoxification	-2.66
xerD	b2894	DNA - replication, repair, restriction/modification	-2.38
cho	b1741	DNA repair	3.64
nuoI	b2281	Energy metabolism, carbon: Aerobic respiration	-1.86
ppc	b3956	Energy metabolism, carbon: Fermentation	-1.72
ydcW	b1444	Enzyme (putative): aldehyde dehydrogenase	2.78
yfaW	b2247	Enzyme (putative): dehydratase	4.50
ygbO	b2745	Enzyme (putative): dehydrogenase	2.68
yhjY	b3548	Enzyme (putative): lipase	3.55
yfiC	b2575	Enzyme (putative): methyltransferase	2.97
yohF	b2137	Enzyme (putative): oxidoreductase	2.74
yffG	b2468	Enzyme (putative): oxidoreductase	5.73
ynbD	b1411	Enzyme (putative): phosphatase	5.00
fabD	b1092	Fatty acid and phosphatidic acid biosynthesis	-2.58
cstA	b0598	Global regulatory functions	3.06
era	b2566	Global regulatory functions	2.38
arcA	b4401	Global regulatory functions	-2.32
pgsA	b1912	Macromolecule synthesis, modification: Phospholipids	2.96
plsC	b3018	Macromolecule synthesis, modification: Phospholipids	2.77
pssR	b3763	Macromolecule synthesis, modification: Phospholipids	2.87
ompA	b0957	Outer membrane constituents	-2.26
yegA	b2064	Outer membrane constituents: lipopolysaccharide biosynthesis	7.32
yedS	b1964	Outer membrane constituents: pore protein	3.56
ompG	b1319	Outer membrane constituents: pore protein	3.20
yjhH	b4298	Phage-related functions and prophages	3.24
yjhO	b4305	Phage-related functions and prophages	3.29
ybcT	b0556	Phage-related functions and prophages	8.21
wzxC	b2046	Protein, peptide secretion	6.30
yaqF	b0296	Ribosomal protein (putative)	2.78
rpmI	b1717	Ribosomal proteins - synthesis, modification	4.75
rplV	b3315	Ribosomal proteins - synthesis, modification	-2.40
nimJ	b1066	Ribosomes - maturation and modification	2.99
cspF	b1558	Stress adaptation	4.56
gltK	b0653	Transport of small molecules: Amino acids, amines	5.74
glvG	b3681	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.53
kefB	b3350	Transport of small molecules: K(I) / H(I) antiporter	3.07
zntA (o732)	b3469	Transport of small molecules: Zn(II), Cd(II), Pb(II)	4.23
zur	b4046	Transport of Zn(II): high-affinity Zn(II) import, repressor	3.08
ydgF	b1600	Transport protein: multidrug	6.19
ycjN	b1310	Transporter (putative), ABC-type	2.31
ycjV	b1318	Transporter (putative): sugars	4.45
b1601 (o344)	b1601	Transporter (putative)	4.89
ydjE	b1769	Transporter (putative)	3.81
yeaN	b1791	Transporter (putative)	3.70
yicM	b3662	Transporter (putative)	4.21
yjeH	b4141	Transporter (putative)	4.99
ynjC	b1755	Transporter (putative), ABC-type	2.99
yejB	b2178	Transporter (putative), ABC-type: Oligopeptides	3.87
afuC	b0262	Transporter (putative): Ferric transport	2.71
yheD	b3323	Transporter (putative): protein export	4.75
yi22_3	b1996	Transposon-related functions	6.68
yi21_4	b2861	Transposon-related functions	3.27

Table 4.2 Genes of known function for which transcript levels were altered in strain TG1 (*zntR ::Km*) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (*zntR ::Km*) strain to transcript levels for untreated TG1 (*zntR ::Km*) strain. Down-regulated genes are indicated by a minus sign (-) in the 'Fold change' column. Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array_Info.xls. Genes are ordered according to function.

Gene	Gene ID	Function	Fold Change
argF	b0273	Amino acid biosynthesis: Arginine	2.65
glnD	b0167	Amino acid biosynthesis: Glutamine	4.52
glnG	b3868	Amino acid biosynthesis: Glutamine	2.54
ilvL	b3766	Amino acid biosynthesis: Isoleucine, Valine	3.59
leuC	b0072	Amino acid biosynthesis: Leucine	2.27
thrB	b0003	Amino acid biosynthesis: Threonine	2.92
trpC	b1262	Amino acid biosynthesis: Tryptophan	-2.25
yjjT	b4371	Aminoacyl tRNA methylase, tRNA modification	3.52
spoU	b3651	Aminoacyl tRNA methyltrasferase, tRNA modification	2.62
tgt	b0406	Aminoacyl tRNA synthetases, tRNA modification	4.64
truB	b3166	Aminoacyl tRNA synthetases, tRNA modification	2.69
trmA	b3965	Aminoacyl tRNA synthetases, tRNA modification	5.61
kdtB	b3634	Biosynthesis of cofactors, carriers: Coenzyme A	3.77
yglG	b3058	Biosynthesis of cofactors, carriers: Folic acid	4.06
moaB	b0782	Biosynthesis of cofactors, carriers: Molybdopterin	3.13
grxB	b1064	Biosynthesis of cofactors, carriers: Thioredoxin, glutaredoxin, glutathione	3.22
yddC	b1494	Biosynthesis of cofactors, carriers: Ubiquinone	2.75
wcaK	b2045	Biosynthesis of extracellular polysaccharide colanic acid	2.83
rcsC	b2218	Cell division: capsule biosynthesis	5.72
xerC	b3811	Cell division: chromosomal segregation	2.92
fic	b3361	Cell division: filamentation, induced in stationary phase	2.57
talA	b2464	Central intermediary metabolism: Non-oxidative branch, pentose pathway	3.70
psiF	b0384	Central intermediary metabolism: Phosphorus compounds	2.47
phnP	b4092	Central intermediary metabolism: Phosphorus compounds	2.53
caiF	b0034	Central intermediary metabolism: Pool, multipurpose conversions	6.83
gltD	b3213	Central intermediary metabolism: Pool, multipurpose conversions	2.98
gadA	b3517	Central intermediary metabolism: Pool, multipurpose conversions	5.80
glpK	b3926	Central intermediary metabolism: Pool, multipurpose conversions	-2.35
rfe	b3784	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.78
rffD	b3787	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	3.45
rffH	b3789	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.31
agaR	b3131	Central intermediary metabolism:Amino sugar conversions	2.64
cbpA	b1000	Chaperones	3.09
mopB	b4142	Chaperones	-1.54
yhaQ	b3112	Degradation of amino acids: L-serine	3.58
maa	b0459	Degradation of carbohydrates	2.70
mcrB	b4346	Degradation of DNA: methylation dependent endonuclease	8.55
mcrC	b4345	Degradation of DNA: methylation dependent endonuclease	2.90
mhpR	b0346	Degradation of fatty acids	3.75
ygeV	b2869	Degradation of nucleotide triphosphates (putative)	3.01
hycI	b2717	Degradation of proteins, peptides, glycopeptides	4.46
sohB	b1272	Degradation of proteins, peptides, glycopeptides	4.40
tdcB	b3117	Degradation of small molecules: Amino acids	-2.11
xylR	b3569	Degradation of small molecules: Carbon compounds	3.41
yhdP	b3245	Degradaton of proteins, peptides, glycopeptides	-3.24
pqiA	b0950	Detoxification: paraquat inducible protein	4.66
pqiB	b0951	Detoxification: paraquat inducible protein	3.48
dnaT	b4362	DNA replication: assembly of primosome	2.99
ampC	b4150	Drug/analog sensitivity	5.11
hyaD	b0975	Energy metabolism, carbon: Aerobic respiration	2.55
hyfB	b2482	Energy metabolism, carbon: Anaerobic respiration	2.49
hyfF	b2486	Energy metabolism, carbon: Anaerobic respiration	2.36
glpE	b3425	Energy metabolism, carbon: Anaerobic respiration	2.39
dmsC	b0896	Energy metabolism, carbon: Anaerobic respiration- dimethyl sulphoxide	3.13
frdC	b4152	Energy metabolism, carbon: Anaerobic respiration- fumarate reductase	2.48
narJ	b1226	Energy metabolism, carbon: Anaerobic respiration- nitrate reductase	2.52
dsbE	b2195	Energy metabolism, carbon: Electron transport	5.09

aldH	b1300	Energy metabolism, carbon: Fermentation- aldehyde dehydrogenase	2.37
gapC 1	b1417	Energy metabolism, carbon: Glycolysis	2.62
ybjR	b0867	Enzyme (putative): amidase	2.57
yfbE	b2253	Enzyme (putative): aminotransferase	3.34
mae B	b2463	Enzyme (putative): bifunctional oxidoreductase/phosphotransacetylase	-2.36
ydjN	b1629	Enzyme (putative): oxidoreductase	4.57
ybhA	b0766	Enzyme (putative): phosphatase	3.90
hydH	b4003	Enzyme: kinase, Zn(II) sensor	7.99
cdsA	b0175	Fatty acid and phosphatidic acid biosynthesis	2.68
cfa	b1661	Fatty acid and phosphatidic acid biosynthesis	2.40
aas	b2836	Fatty acid and phosphatidic acid biosynthesis	2.29
suhB	b2533	Global regulatory functions	3.89
dps	b0812	Global regulatory functions, iron storage	3.34
rfaK	b3623	Macromolecule metabolism: Lipopolysaccharide	2.44
pgpB	b1278	Macromolecule synthesis, modification: Phospholipids	2.43
glgB	b3432	Macromolecule synthesis, modification: Polysaccharides - (cytoplasmic)	2.48
bolA	b0435	Murein sacculus, peptidoglycan	3.15
mrcA	b3396	Murein sacculus, peptidoglycan	2.94
rhlB	b3780	Not classified	-2.89
ybeK	b0651	Not classified	-2.55
fhuA	b0150	Outer membrane constituents: ferric uptake	2.90
flu	b2000	Outer membrane constituents: fluffing protein	3.14
wzzB	b2027	Outer membrane constituents: modification of polyliposaccharide chains	3.29
ompN	b1377	Outer membrane constituents: pore protein, non-specific	3.00
phoE	b0241	Outer membrane constituents: pore protein; phosphate uptake	3.56
ykfA	b0253	Phage-related functions and prophages	3.21
ybaN	b0468	Phage-related functions and prophages	5.09
b1145 (f224)	b1145	Phage-related functions and prophages	3.27
yjhL	b4302	Phage-related functions and prophages	3.33
lit	b1139	Phage-related functions and prophages	5.26
ybgP	b0717	Pilin chaperone, periplasmic (putative)	2.88
ybcG	b0534	Pilin-like protein (putative)	4.59
ydeS	b1504	Pilin-like protein (putative)	8.43
emrR	b2684	Plasmid-related functions: repressor of multidrug resistance pump	2.70
msyB	b1051	Protein, peptide secretion	6.49
secF	b0409	Protein, peptide secretion	-2.27
yaaD	b0028	Proteins - translation and modification	3.79
prfA	b1211	Proteins - translation and modification	2.68
infC	b1718	Proteins - translation and modification	4.68
hrsA	b0731	Proteins - translation and modification	-1.95
purL	b2557	Purine ribonucleotide biosynthesis	2.91
rplL	b3986	Ribosomal proteins - synthesis, modification	3.70
rplB	b3317	Ribosomal proteins - synthesis, modification	-2.90
baeS	b2078	RNA synthesis, modification, DNA transcription	3.14
nusG	b3982	RNA synthesis, modification, DNA transcription	5.02
basR	b4113	RNA synthesis, modification, DNA transcription	3.39
cspH	b0989	Stress adaptation - cold shock	4.12
cspI	b1552	Stress adaptation - cold shock	3.75
treA	b1197	Stress adaptation - osmotic shock	2.94
pspA	b1304	Stress adaptation - osmotic shock: *psp* operon	4.49
pspB	b1305	Stress adaptation - osmotic shock: *psp* operon	2.69
pspE	b1308	Stress adaptation - osmotic shock: *psp* operon	2.82
yeqD	b2069	Stress adaptation (putative)	2.81
yggB	b2924	Stress adaptation (putative) - osmotic shock	3.24
flgM	b1071	Surface structures: biosynthesis of flagella	2.41
flgI	b1080	Surface structures: biosynthesis of flagella	4.21
fliE	b1937	Surface structures: biosynthesis of flagella	2.85
fimC	b4316	Surface structures: biosynthesis of pili	2.97
cueR	b0487	Transport of Cu(II) : Transcriptional regulator of Cu(II)-exporting ATPase	2.65
sapB	b1293	Transport of proteins, peptides	2.94
yecC	b1918	Transport of small molecules (putative): Amino acids, amines	3.08
yaaJ	b0007	Transport of small molecules: Amino acids, amines	3.50
tauC	b0367	Transport of small molecules: Amino acids, amines	4.27
gltJ	b0654	Transport of small molecules: Amino acids, amines	4.77
potH	b0856	Transport of small molecules: Amino acids, amines	3.90
livF	b3454	Transport of small molecules: Amino acids, amines	2.88
livM	b3456	Transport of small molecules: Amino acids, amines	2.59
crr	b2417	Transport of small molecules: Carbohydrates, organic acids, alcohols	5.60
cmtA	b2933	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.70
exuT	b3093	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.82
ugpE	b3451	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.71
ugpA	b3452	Transport of small molecules: Carbohydrates, organic acids, alcohols	3.46

fecC	b4289	Transport of small molecules: Fe(III)	4.71
fecB	b4290	Transport of small molecules: Fe(III)	2.81
tsx	b0411	Transport of small molecules: Nucleosides, purines, pyrimidines	-1.89
pstA	b3726	Transport of small molecules: phosphate (phosphatase)	3.12
pstS	b3728	Transport of small molecules: phosphate (phosphate binding protein)	4.63
znuC	b1858	Transport of Zn(II): high-affinity Zn(II) import	3.59
znuB	b1859	Transport of Zn(II): high-affinity Zn(II) import	2.59
yraP	b3150	Transporter (putative)	4.90
yfdC	b2347	Transporter (putative)	2.78
yhfC	b3364	Transporter (putative)	3.81
yjgA	b4234	Transporter (putative): ABC-type	2.89
ybiT	b0820	Transporter (putative): ABC-type, ATP-binding component	3.36
yadG	b0127	Transporter (putative): ABC-type, ATP-binding component	3.48
allP	b0511	Transporter (putative): allantoin transport	2.90
yjdL	b4130	Transporter (putative): di-/ tripeptides	3.09
ddpC	b1485	Transporter (putative): dipeptide transport, ABC-type	4.40
afuB	b0263	Transporter (putative): membrane component of ferric transport system	2.53
ybbM	b0491	Transporter (putative): metal resistance	4.33
yrbG	b3196	Transporter (putative): Na(I)/Ca(I) antiporter	-3.35
yjcQ	b4081	Transporter (putative): Pit family	3.88
gspE	b3326	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	2.56
gspL	b3333	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	3.38
gspM	b3334	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	4.00
yicK	b3659	Transporter (putative): sugars	2.71
yjiO	b4337	Transporter: multidrug	2.46
tehB	b1430	Transporter: tellurite resistance	3.66
b0299 (o288)	b0299	Transposon-related functions	3.88
tra5_2	b0541	Transposon-related functions	3.67
tra5_3	b1026	Transposon-related functions	3.67
tra5_4	b2089	Transposon-related functions	2.54

Table 4.3 Genes of known function for which transcript levels were commonly altered in both of the strains, TG1 (w.t.) and TG1 (*zntR*::*Km*) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (w.t.) and TG1 (*zntR*::*Km*) strain to transcript levels for untreated TG1 (w.t.) and TG1 (*zntR*::*Km*) strains, respectively. Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array_Info.xls. Genes are ordered according to function.

Gene	Gene ID	Function	Fold Change TG1	Fold change TG1 (*zntR*::*Km*)
aroM	b0390	Amino acid biosynthesis: Chorismate	2.50	3.12
cysK	b2414	Amino acid biosynthesis: Cysteine	8.34	4.79
metG	b2114	Aminoacyl tRNA synthetases, tRNA modification	3.36	3.5
chpB	b4225	Cell killing	4.06	4.08
cysQ	b4214	Central intermediary metabolism: Sulfur metabolism	4.08	3.12
ebgA	b3076	Degradation of small molecules: Carbon compounds	3.85	3.55
yncA	b1448	Drug/analog sensitivity	3.90	4.0
yneI	b1525	Enzyme (putative): aldehyde dehydrogenase	2.98	2.34
ydcP	b1435	Enzyme (putative): collagenase	3.45	3.53
yeiA	b2147	Enzyme (putative): dehydrogenase	6.15	3.78
yggC	b2928	Enzyme (putative): kinase	2.83	3.15
ydiJ	b1687	Enzyme (putative): oxidase	4.24	3.22
nifJ	b1378	Enzyme (putative): oxidoreductase	3.32	3.56
yidC	b3705	Transport of proteins across inner membrane	4.59	3.38
intE	b1140	Phage-related functions and prophages: integrase	4.26	19.21
wzxC	b2046	Protein, peptide secretion	6.30	7.25
rpsV	b1480	Ribosomal proteins - synthesis, modification	3.34	4.57
spy	b1743	Spheroblast formation	5.73	3.64
yeeE	b2013	Transport of small molecules: Amino acids, amines	6.65	4.45
xylF	b3566	Transport of small molecules: Carbohydrates, organic acids	5.46	4.55
yehY	b2130	Transporter (putative): glycine, betaine, choline	7.08	3.66
zupT	b3040	Transport of Zn(II): low-affinity Zn(II) import	4.56	4.47

4.4 Discussion

Transcriptional profiles of *E. coli* strains TG1 (w.t.) and TG1 (*zntR*::*Km*) grown in standard LB media (control experiment) and LB media supplemented with Zn(II) (test experiment) were generated. ZntR is required for transcription of the primary Zn(II) exporter *zntA*, in *E. coli*, therefore strain TG1 (*zntR*::*Km*) where *zntR* is absent provides a useful system for identifying other mechanisms that may assist in providing Zn(II) tolerance.

The TG1 (*zntR*::*Km*) results were compared to the TG1 (w.t.) strain to elucidate novel Zn(II) transporters as well as understanding other mechanisms that may be present for enabling bacterial cells to cope with heavy metal stress. Also, changes to the general cell metabolism in the absence of optimum Zn(II) export were noticed.

Results were presented (tables 4.1, 4.2, 4.3) in which the TG1 strain (table 4.1) was used to show the background genes, or the 'normal' mechanisms of Zn(II) tolerance in a fully functioning ZntR-ZntA system. Table 4.3 showed genes induced by Zn(II) in both strains, and table 4.2 showed genes only induced in TG1 (*zntR*::*Km*), i.e. in the absence of ZntR-ZntA.

The Zn(II) concentration of 0.3 mM was chosen because the growth of TG1 (*zntR*::*Km*) at 0.3 mM Zn(II) trailed smoothly behind its growth in normal LB medium (refer to fig. 4.1). TG1 (*zntR*::*Km*) did not grow very well above 0.3 mM Zn(II) and higher concentrations were unsuitable for RNA extraction since growth of TG1 (w.t.) entered stationary phase at which point growth of TG1 (*zntR*::*Km*) remained in early exponential phase making the samples incomparable.

Thus the MIC of strain TG1 (*zntR*::*Km*) was established at 0.4 mM Zn(II). This unfortunate dependency of the strain on ZntR at low concentrations (maximum induction of *zntA* by ZntR occurs at 1.1 mM Zn (II)) meant that the efficiency for highlighting novel transporters may have been somewhat compromised as the TG1 (w.t.) strain demonstrates an MIC of 2 mM Zn(II) (Rensing *et al.*, 1997a). Nevertheless, the growth curves of TG1 (*zntR*::*Km*) (fig.

4.1) reaffirmed the importance of ZntR as being crucial for Zn(II) tolerance. The relatively low number of down-regulated genes for each strain (17 out of 387 for TG1 and 14 out of 400 for TG1 *zntR*::*Km*) suggests that apart from the applied Zn(II) stress, some growth effects may have caused a proportionally increased number of up-regulated genes.

Also, it appears that for some operons, only part of the operon is up-regulated. This is considered as a general up-regulation of the whole operon as differences in hybridisation can occur despite careful methodology. This is because each transcript is different in terms of its hybridisation strength.

Recognised Zn(II) transporters

Reassuringly, *zntA* was induced in TG1 (w.t.) but not in TG1 (*zntR*::*Km*). The transcript of ZntR was not induced in either strain. These results confirmed the *zntR* knockout status of TG1 (*zntR*::*Km*) and that activation of *zntA* by ZntR occurred in TG1 (w.t.). Although visible induction of *zntR* in TG1 (w.t.) would have been desirable, the copy number of *zntR* may have been undetectable at 0.3 mM Zn(II) and may be an indication of the sensitivity of the *zntA* promoter to ZntR. These results also indicate that ZntR may be the sole transcriptional regulator of *zntA*.

In TG1 (w.t.), it was noteworthy that *zur* (detailed in section 1.4.2), which encodes the transcriptional repressor of the *znuABC* highly Zn(II) sensitive import system (detailed in section 1.3.1.1.1) was induced, as would be expected in a normally functioning *E. coli* system at elevated Zn(II) levels, since this system should be repressed even in the presence of subfemtomolar concentrations of Zn(II) (Outten and O'Halloran, 2001). Curiously, *zur* was not induced in TG1 (*zntR*::*Km*) although an increase in transcription of *znuB* and *znuC*, which form the membrane transporting and ATP-hydrolysis components of this ABC transport system respectively, was observed.

The induction of *znuB* and *znuC* in TG1 (*zntR*::*Km*) was highly unexpected since it would seem logical that in this strain when Zn(II) resistance is already compromised, the further

uptake of Zn(II) would be strongly inhibited. One possibility is that these proteins may be utilised to export Zn(II) under some circumstances. Although ABC transporters have generally been accepted as being strictly unidirectional, there are exceptions. For example, amino acid transporters of this family in *Rhizobium leguminosarum* and *Salmonella typhimurium* have been found which are bidirectional (Hosie *et al.*, 2001). The lack of *znuA* induction may further support this theory. ZnuA is the periplasmic binding protein of this ABC-type import system and if it had been induced under these conditions, the effect of export by ZnuB and ZnuC might be cancelled as ZnuA would be delivering Zn(II) back to these other components of the import system, having a cyclic effect. As for the induction of *zur* in TG1 (*zntR*::*Km*) being affected, a detailed study of the promoter region of *zur* may reveal more information about its own regulation. It is most likely however, that *znuB* and *znuC* were induced for the purpose of binding Zn(II).

The gene encoding ZupT (section 1.3.1.1.2), a low-affinity Zn(II) importer (Grass *et al.*, 2002) was induced in both strains and Zn(II) import via ZupT is thought to occur via an electrochemical gradient. These results were unexpected and in fact a down-regulation would be expected.

The inductions of other known Zn(II) transporters (detailed in sections 1.3.1.1 and 1.3.1.3) were checked in the array profiles but *zntA*, *zur*, *znuB*, *znuC* and *zupT* were the only transcripts detected.

Transcripts detected in strain TG1 (*zntR*::*Km*)

The full list of transcripts up-regulated and down-regulated with a 1.5 fold induction cut-off, in TG1 (*zntR*::*Km*) grown in LB medium supplemented with 0.3 mM Zn(II) in comparison to TG1 (*zntR*::*Km*) grown in normal LB medium is shown in appendix I, table 2. It contains a total of 400 genes. 386 of these were up-regulated of which 101 genes were also induced in TG1 (23 of known function 78 unknown) (appendix I, table 3), and 285

genes were unique to TG1 (*zntR*::*Km*) (146 of known function 139 unknown). Only 14 genes were down-regulated.

The transcription of *hydH* (also known as *zraS*) (refer to section 1.3.1.2), encoding a Zn(II) sensor protein (Leonhartsberger *et al.*, 2001), was detected in TG1 (*zntR*::*Km*). This protein, together with HydG (ZraR), is thought to activate *ZRAP* (*yjaI*), a Zn(II) resistance protein (Noll *et al.*, 1998). An increase in sensitivity of TG1 (*zntR*::*Km*) is indicated by the transcription of *hydH* in TG1 (*zntR*::*Km*). *hydH* may be inducible in TG1 (*zntR*::*Km*) at a lower Zn(II) concentration compared to TG1 (w.t.) (hence the lack of induction in this strain) because of an increased demand for Zn(II) tolerance in the absence of a functioning ZntR-ZntA system.

Zn(II) has long been known to inhibit the aerobic respiratory chain in bacteria (Anraku *et al.*, 1975). In *E. coli*, excessive cytoplasmic Zn(II) can interfere with electron transport by inhibiting the ubiquinol oxidase activity of cytochrome b_{562}-O, the terminal oxidase in aerobically grown *E. coli* (Beard *et al.*, 1995). More precisely, Zn(II) binds to a histidine residue within this complex and inhibits the oxidase activity non-competitively (Kita *et al.*, 1984).

Table 4.2 showed evidence of a push towards anaerobic respiration and perhaps reflects a Zn(II)-induced inhibition of aerobic respiration. Six genes involved in anaerobic respiration were switched on (*dmsC, narJ, hyfB, hyfF, glpE, frdC*) in addition to an 8-fold induction of *aldH*, encoding aldehyde dehydrogenase, a key enzyme in fermentation.

The genes *pqiA* and *pqiB*, normally induced in the presence of paraquat, a superoxide radical generating agent, were upregulated. These genes are controlled by the SoxRS regulon (Koh and Roe, 1995) which also induces many other genes required to cope with superoxide stress (Tsaneva and Weiss, 1990). *pqiA* and *pqiB* may in this case have been switched on directly in response to radicals generated from interference of the electron transport chain. Alternatively, these may have been induced simply to help pool excess

Zn(II) since a closer look at the amino acid content of PqiA revealed 16 cysteine residues indicative of metal-binding properties.

cueR was upregulated in TG1 (*zntR*::*Km*). CueR, a Cu(I) responsive MerR type regulator controls the transcription of *cueO*, encoding a Cu(I) efflux oxidase (Grass and Rensing, 2001), and the Cu(I) export gene CopA, a P-type ATPase (Rensing *et al.*, 2000). In this way excess Cu(I), a redox-active metal species is expelled from the cell. Transcription of *cueO* and *copA* by CopR is controlled in much the same way as *zntA* is controlled by ZntR in response to Zn(II) (Outten *et al.*, 2000). CopR responds to Cu(I), Au(I) and Ag(I) but not Zn(II) (Stoyanov *et al.*, 2001, Stoyanov and Brown, 2003) and in fact demonstrates a zeptomolar sensitivity to free Cu(I) (Changela *et al.*, 2003). Therefore, since CopR does not bind Zn(II) the question remains as to why *copR* transcripts were elevated in this strain at 0.3 mM Zn(II). One answer may be that excess Zn(II) in the cell is displacing Cu(II) from its native binding sites in proteins which utilise Cu(II), due to high similarity in their coordination chemistry as previously demonstrated (Korbashi *et al.*, 1989). If this was happening here, then there would be an increased demand on the cell to keep more Cu(II) inside, in an attempt to counter the effects of excess Zn(II). This may explain why *cueR* transcripts were increased in TG1 (*zntR*::*Km*), because CueR represses the Cu(I) exporter, CopA. A NADH-linked cupric reductase was found in *E. coli* that reduces Cu(II) to Cu(I) *in vivo*, and much of the intracellular copper is assumed to be present as Cu(I) (Rapisarda *et al.*, 1999) thereby activating CueR. CueR is required to switch on *copA* and *cueO*, in order for the expulsion of excess Cu(I). Alternatively, the effects of Zn(II) on the regulation of *cueR* itself could be studied to identify a link.

The induction of several Fe transport related genes was apparent, namely *fecC, fecB, fhuA, afuB*. This is not surprising considering its importance in many major biological processes where it is incorporated into proteins involved in aerobic and anaerobic respiration, gene regulation and DNA biosynthesis (Harrison and Hoare, 1980) and these processes would probably be in demand in stressful conditions such as heavy-metal toxicity. Also, an excessive increase in one metal-ion species can cause stress to systems utilising other

metal-ions. Excessive Zn(II) may be disrupting Fe utilising proteins and therefore there may be an increased demand for Fe import and storage.

FhuA is an Fe transporting protein located in the outer membrane of *E. coli* (Braun *et al.*, 2002). It actively imports ferrichrome, a complex of Fe(III) (ferric form) bound to a siderophore. Siderophores are low molecular mass, high affinity ferric chelators that are extracellularly secreted to solubilise the highly insoluble Fe(III) species in response to Fe restriction.

The *fecABCDE* system of *E. coli* determines another Fe(III)-siderophore transporter, the citrate dependent Fe(III) uptake system (Staudenmaier *et al.*, 1989). The system is transcribed from *fecA* to *fecE*: *fecA* encodes an outer membrane receptor protein, which transports Fe(III)-dicitrate to FecB, a periplasmic protein. Fe(III)-dicitrate is passed on to FecC and FecD, two integral inner membrane proteins and finally translocated into the cytoplasm utilising energy from ATP-hydrolysis via the membrane associated protein FecE. However, only *fecB* and *fecC* were found to be up-regulated in the arrays.

afuB is a putative membrane component of another ferric transport system, however no literature is currently available on this.

Though the primary role of Dps in *E. coli* is to protect DNA from redox stress it can also act as an Fe storage protein (Zhao *et al.*, 2002) and therefore its transcript may have been generated to store incoming Fe for future use.

McrB and McrC form an endonuclease, which recognises modified cytosines on methylated DNA (Panne *et al.*, 1998). These may have been induced due to the effects of Zn(II) on DNA structure, which can be altered because of interaction between Zn(II) ions and phosphate groups.

Other effects of higher than normal Zn(II) levels suggest an attempt at increasing the rate of cell division reflected by *dnaT* (DNA replication), *xerC*, *fic* and *rcsC*. Zn(II) appears to be

involved in *E. coli* cell division and has the potential to play a role in the global orchestration of the fundamental cellular process. Higher levels of Zn(II) have been shown to stimulate *E. coli* cell division (Blencowe, 2002). This data is supported by up-regulation of transcripts such as *dnaT*, *xerC*, *fic* and *rcsC*. *rcsC* is part of a two-component system, *rcsC-rcsB* encoding sensor and regulator respectively, of *ftsA* and *ftsZ*, which are directly involved in cell division (Carballes *et al.*, 1999).

Other observations of the TG1 (*zntR*::*Km*) strain in response to Zn(II) also showed up-regulation of transcripts involved in biosynthesis of surface structures and flagella, enhancing chemotaxis of bacteria away from the Zn(II) in the growth medium. Genes from the *gsp* operon, a transporter of proteins required for pili biosynthesis were also up-regulated, however, this role has been assigned putatively.

An increase in the level of phosphate uptake transcripts was observed. Intracellular phosphate may be used to chelate excess Zn(II). *pstA* and *pstS* encode products involved in phosphate transport and *phoE*, encodes an outer membrane porin selective for cations such as phosphate (Samartzidou and Delcour, 1998). They are part of a single phosphate uptake system, the *pho* regulon (Makino *et al.*, 1989). The *pho* regulon is inducible by phosphate starvation. In addition, a putative transporter *yjcQ*, was induced that may belong to the Pit family.

Excess Zn(II) may affect the osmotic balance of the cell as several 'osmotic shock' induced genes were up-regulated. These were *treA*, *yggB* and *pspA/B/E* (part of the *psp* stress induced operon (Brissette *et al.*, 1991)). It is also plausible that these stress-responsive proteins which respond to changes in protein structure, are required to assist directly against the effects of Zn(II) on intracellular protein structure.

Genes of interest that are rich in cysteines and histidines were found to be up-regulated. Firstly of interest, *tehB* (3 cysteines, 3 histidines) is involved in tellurite (TeO_3^{2-}) resistance and *gutS* (5 cysteines, 7 histidines) involved in selenium and tellurite export may be induced to help in Zn(II) export/binding. It is unlikely that tellurite was present in the media

used as tellurium (Te), ranked 75th in abundance of all elements in the earth's crust, is extremely toxic due to its strong oxidising ability (Taylor, 1999). The cysteine residues in *tehB* have been found to be essential for tellurite binding (Turner *et al.*, 1995). *gutS* induction in response to various metals including Zn(II) has been tested but it was unresponsive (Guzzo and Dubow, 2000), however no experimental details or data were shown in the publication and the experiments with Zn(II) may have consisted of basic phenotypic screens and further study of *gutS* may characterise it as a general, broad-range metal transporter.

Several genes encoding proteins that have been characterised but do not appear to be involved in metal-ion export such as EmrR were up-regulated. EmrR regulates a multidrug transporter and it contains 4 cysteines and 7 histidines. It is a cytoplasmic protein that may have been induced to chelate Zn(II). AllP, involved in allantoin transport contains 5 cysteines and 6 histidines. Additionally, *ybhA*, a putative phosphatase was up-regulated and the encoded protein is extremely histidine rich. It contains 14 histidines and 3 cysteines.

ybbM, is predicted to encode a putative metal transporter (Serres *et al.*, 2004) with 4 cysteines and 4 histidines was up-regulated. BLAST searches were performed but this protein has not been assigned to any known metal transport family as yet. Phenotypic studies of this gene would enable assignment of the precise role of *ybbM*, although it could possibly encode an additional Zn(II) transporter.

Biosynthesis of the extracellular polysaccharide, colanic acid, was apparent due to induction of *wcaK* and *wzxC* part of the colanic acid synthesis genes cluster, as found in TG1 also. Extracellular polysaccharides may be synthesised to help chelate Zn(II) as demonstrated in *Bacillus firmus* which removed Zn(II), Pb(II) and Cu(II) ions from aqueous solution by producing extracellular polysaccharide (Salehizadeh and Shojaosadati, 2003). The adsorption of heavy metals can be brought about by interactions between metal-cations and the negative charge of acidic functional groups on polysaccharides which have an extensive chelation capacity due to their position and size.

cspH and *cspI* are cold shock proteins that may be induced during transient removal from the incubator during the experiment. Although this was also seen in TG1 (w.t.) with different cold shock proteins being induced, this may be due to non-uniform sensitivity within the gene array.

Transcripts detected in TG1 (w.t.)

The full list of transcripts up-regulated and down-regulated with a 1.5 fold induction cut-off, in TG1 grown in LB medium supplemented with 0.3 mM Zn(II) in comparison to TG1 grown in normal LB medium is shown in appendix I, table 1. It contains a total of 387 genes. 370 genes were up-regulated of which 101 genes were also induced in TG1 (*zntR*::*Km*), (23 of known function 78 unknown) (appendix I, table 3) and 269 genes were unique to TG1 (104 of known function 165 unknown). Only 17 genes were down-regulated.

The histidine biosynthesis operon was induced in TG1 but not in TG1 (*zntR*::*Km*). This operon was investigated for ZntR binding sites but none were apparent. *slyA* encodes an adaptation protein produced in response to various cellular stresses (Heroven and Dersch, 2002) and was up-regulated. This protein normally induces *hlyE,* a hemolytic protein involved in pathogenesis (Fernandez *et al.*, 1998). This may represent an example of induction of one gene, *slyA* which is being induced because of heavy-metal stress and thereby inducing *hlyE* as a biproduct.

Other noteworthy up-regulations included *cho* (nucloetide excision repair), *kefB* (maintaining osmotic balance), *cspF* (cold-shock response), fatty acid degradation genes and many genes of the colanic acid biosynthesis cluster; *wcaG, wcaF, wcaE wcaB, wzxC* (induced in TG1 (*zntR*::*Km*) also) and the regulator of this cluster, *djlA* (Genevaux *et al.*, 2001).

Concluding remarks

The results obtained from these arrays further support and consolidate the importance of *zntR* in Zn(II) resistance in *E. coli*. Strain TG1 (*zntR*::*Km*) has a Zn(II) MIC of 0.4 mM in comparison to 2 mM for a normally functioning *E. coli* TG1 (w.t.) strain.

The arrays did help to highlight other mechanisms that a bacterium can employ to deal with an environment polluted with heavy metal cations for example chelation by extracellular polysaccharides and intracellular chelation by virtue of phosphate. The effects of Zn(II) on the general metabolism of the cell were also highlighted.

Some of the results showed that predicted alterations are not always observed as in the case of *zupT* and *znuB* and *znuC* inductions. However, a potential Zn(II) exporter *ybbM*, was identified.

The reliability of these results is affected by several factors. Macroarray results are influenced by array production, RNA extraction, probe-labelling, hybridisation conditions and image analysis. Furthermore, non-specific and cross-hybridisation of probes may be inevitable due to sequence similarity among gene-family members.

Ideally, the results need to be validated further using specific RNA probing with techniques such as Northern blots or real time PCR. Proteomic studies are also invaluable in confirming actual expression of up-regulated or down-regulated genes in stress responses.

CHAPTER FIVE

PHENOTYPIC CHARACTERISATION OF A NOVEL ABC-TYPE METAL-ION IMPORTER

5.1 Summary

The transcriptional abundance of b0795, encoding a member of a predicted ABC transporter complex was significantly reduced in *E. coli* adapted to grow in elevated levels of Zn(II), Cd(II), Co(II) or Ni(II) when compared to growth in normal media. These results, derived from macroarray analysis, and bioinformatic analysis of the operon containing this ORF indicated that this region encodes an ABC-type metal-ion importer.

The promoter region of the genes encoding this putative ABC complex was introduced into a reporter plasmid and luciferase assays were performed in response to various metal-ions. Additionally, a gene encoding one of the predicted integral membrane transport proteins was inactivated, and solid and liquid MIC assays were performed as well as growth studies in response to Zn(II), Cd(II), Co(II) and Ni(II). Collectively, the results led to an early phenotypic characterisation of this complex as a novel metal-ion importer that primarily imports Co(II) and Ni(II) but its transcription may to some extent be affected by Co(II), Ni(II), Zn(II) and Cd(II).

5.2 Introduction

E. coli were adapted to grow in media containing substantially elevated levels of Zn(II), Cd(II), Co(II) and Ni(II) and RNA was extracted for macroarray analysis (Brocklehurst and Morby, 2000). The adapted strains demonstrated MICs for Zn(II), Cd(II), Co(II) and Ni(II) of 9 mM, 4 mM, 23 mM and 19 mM, respectively. These were significantly higher than the MICs of the non-adapted, wild-type strain which demonstrated MICs for Zn(II), Cd(II), Co(II) and Ni(II) of 2.2 mM, 1.2 mM, 1.7 mM and 4 mM, respectively (Brocklehurst and Morby, 2000). A common trend amongst all four adapted strains was the significant decrease in the transcript abundance of b0795, an unassigned ORF.

Hypothetical functions of b0795 and the ORFs flanking it were collected from the GenProtEC database (Serres *et al.*, 2004) and suggested that b0795 and the other ORFs, *ybhF* (b0794), *ybhS* (b0793) and *ybhR* (b0792) are components of an ABC-type importer, designated here as the *ybh* cluster. An ORF preceding b0795 called *ybiH* (b0796), is predicted to encode a regulatory protein, perhaps regulating the *ybh* cluster.

The focus of the work in this chapter was to investigate the function of the *ybh* cluster and to test the hypothesis that these genes together encode a Zn(II)/Cd(II)/Co(II)/Ni(II) import system as suggested by the macroarray results of metal-ion adapted strains (Brocklehurst and Morby, 2000) and bioinformatic analysis presented below.

The promoter of this region, P*ybh,* was introduced into a luciferase reporter plasmid (pUCD615) and was subsequently assayed for activity in the presence of various metal-ions to identify which of the metals affected transcription of the *ybh* cluster and whether transcription was repressed or activated.

A knockout strain of one of the potential integral membrane channels of the complex, YbhR, was constructed and used for MIC assays and growth curves with the metal-ions. Data suggest that the *ybh* cluster encodes a Co(II) and Ni(II) importer which may also be transcriptionally responsive to Zn(II) and Cd(II).

5.3 Results

5.3.1 Bioinformatic analysis of the *ybh* cluster

The ORF b0795 is preceded by a predicted regulatory gene, *ybiH*, and the downstream genes *ybhF*, *ybhS* and *ybhR*, referred to here as the *ybh* gene cluster are located on the reverse strand in the genome at the locus 820765-829878 bp. A schematic of the ORFs in the *ybh* cluster is presented (fig. 5.1). The protein sequences of the members of the *ybh* cluster including all relevant features are shown in fig. 5.2.

A transmembrane region consisting of 21 amino acids is predicted to lie within the immediate N-terminal region of b0795 (fig. 5.2). Furthermore, a Kyte-Doolittle hydropathy plot (Kyte and Doolittle, 1982) showed that downstream from the predicted transmembrane region, the remaining protein is largely hydrophilic in nature. Additionally, a signal peptide consensus sequence was present at the N-terminal region, although it was predicted to be uncleavable (Nielsen *et al.*, 1997). These predictions indicate that b0795 is likely to be located in the periplasm but anchored to the inner membrane. b0795 bears strong similarity to EmrA, HlyD and AcrA (appendix II, fig. 1) all of which are inner-membrane anchored periplasmic proteins from *E. coli*. All of these proteins are involved in the transport of various, specific substrates: EmrA is involved in resistance to nalidixic acid and other toxic compounds (Lomovskaya and Lewis, 1992); HlyD and AcrA are membrane fusion proteins that respectively transfer hemolysin and antibiotics to an outer membrane protein TolC, for secretion (Gentschev *et al.*, 2002, Jellen-Ritter and Kern, 2001). Additionally, b0795 shows similarity to CzcB, a component of the Cd(II), Zn(II) and Co(II) efflux pump from *Ralstonia metallidurans* (appendix II, fig. 1).

Bioinformatic sequence analysis of *ybhF*, *ybhS* and *ybhR* point to an assignment of the *ybh* cluster as an ABC transporter, and more specifically, a binding protein-dependent import system. These are the most ubiquitous ABC transporters in prokaryotes and the most widely studied amongst these are the histidine and maltose importers in *E. coli* (reviewed

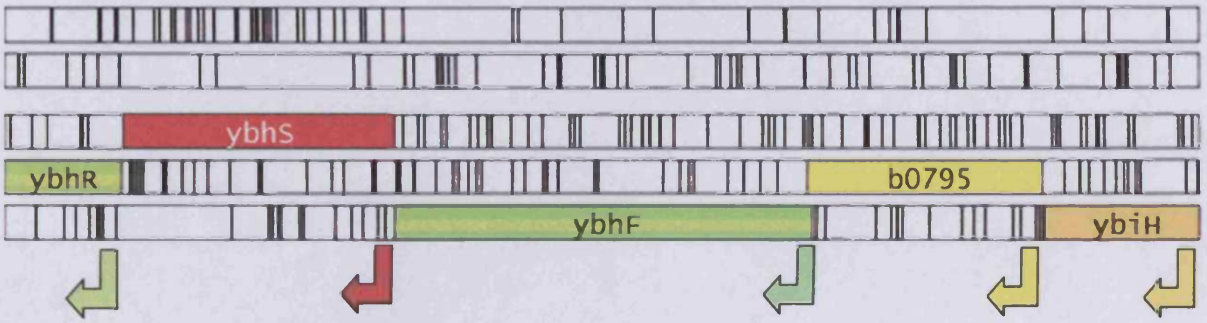


Fig. 5.1 Schematic of the ORFs in the *ybh* cluster (http://colibase.bham.ac.uk/). This cluster is located on the reverse strand of the *E. coli* genome. The arrows refer to the direction of transcription.

Fig. 5.2 Primary amino acid sequences of the proteins encoded by the *ybh* cluster are shown in the order of their genetic location within the cluster i.e. *ybiH* is proceeded by b0795, *ybhF*, *ybhS* and *ybhR*. The blue highlighted sequence in YbiH represents the predicted helix-turn-helix motif (Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hth.html)).

Putative transmembrane regions predicted using TMpred (www.ch.embnet.org/software/TMPRED_form.html) in b0795, YbhS and YbhR are highlighted red. Additionally, the signal peptide in b0795 is underlined and the hyphen shows the suggested cleavage site (Nielsen *et al.*, 1997).

The classical consensus motifs found in an ABC domain of an ABC system are shown in white font in YbhF. These are Walker site A, followed by the linker sequence, and Walker site B) (Higgins, 1992). Possible metal-ion binding cysteines and histidines are highlighted black and the fonts coloured cyan and yellow, respectively.

by Boos and Shuman, 1998, Linton and Higgins, 1998, respectively). A unique feature of the bacterial ABC importers that distinguish them from the exporters is that they all have a periplasmic binding protein (hence the term 'binding protein-dependent systems') which binds the incoming ligand and presents it to the rest of the import complex in the inner membrane. The periplasmic binding protein is the main determinant of substrate specificity (Ehrmann *et al.*, 1998). In gram-negative bacteria the periplasmic binding protein is located in the periplasm but in gram-positive bacteria these receptors are anchored to the cytoplasmic membrane and exposed on the cell surface (Gilson *et al.*, 1988).

In prokaryotes, ABC transporters consist of many subunits. They are also found in eukaryotes but the protein subunits are usually fused. Aside from metal-ions, the ABC importers are capable of importing a wide range of products such as oligopeptides, amino acids, sugars and phosphate (Fath and Kolter, 1993). The energy released from ATP hydrolysis brings about a conformational change enabling ABC transporters to pump substrates across the membrane against a concentration gradient (Higgins, 1992).

One of the characteristic features of ABC transporters is the presence of a conserved ATP-binding domain, better known as an ATP-binding cassette (ABC). This ABC domain is responsible for ATP hydrolysis and is located within a hydrophilic, cytoplasmic protein known as the ABC subunit (fig. 5.3). The ABC domains are approximately 200 amino acids long and display 30-50% sequence identity to subunits of similar transporters (Higgins, 1992).

The ABC subunits are associated tightly with two integral membrane-spanning proteins. These integral proteins are believed to form the pore or pathway through which substrates cross the membrane, and perhaps play a role in determining substrate specificity of each ABC transport system.

The *adc* operon of gram-positive *Streptococcus pneumoniae* encodes an ABC import system containing three ORFs designated *adcC, adcB* and *adcA* (Dintilhac *et al.*, 1997). These ORFs encode the ABC subunit, integral-membrane proteins and the substrate-

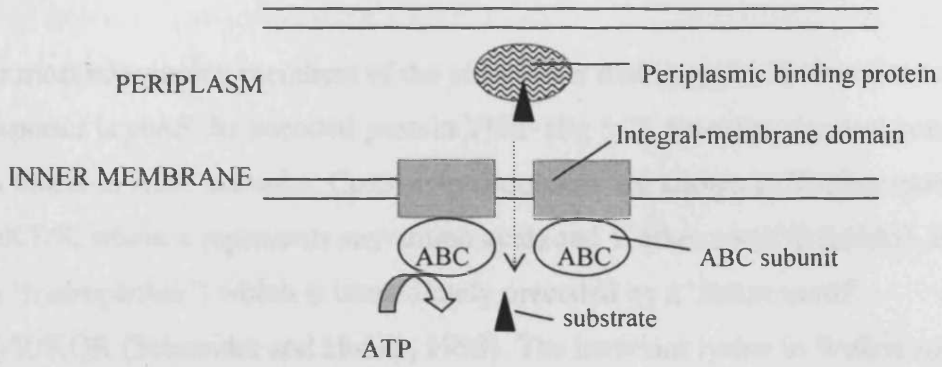


Fig. 5.3 Simplistic representation of an ABC importer from a gram-negative bacterium, such as *E. coli.* It is composed of a dimer of two six-pass integral membrane-bound proteins, associated with two ABC subunits. The periplasmic binding protein acts as the initial receptor for the incoming substrate. Adapted from Schneider and Hunke, 1998.

binding receptor respectively (Dintilhac and Claverys, 1997). Transcription of ABC transporters can be positively or negatively regulated (Ehrmann *et al.*, 1998).

Six putative membrane spanning regions were identified in the protein sequences of *ybhR* and *ybhS* (fig. 5.2). Transmembrane regions are usually present in the integral membrane components of a bacterial ABC transport system (Higgins, 1992). In each of these, the N terminal of the first of these transmembrane regions is predicted to be positioned in the inner membrane in an N-in orientation. Homo- or heterodimers of YbhR and YbhS are thought to form the transport channel. YbhR and YbhS are similar to one another and to NatB, the integral membrane protein of an ABC-type Na(I) transporter in *Bacillus subtilis* (Cheng *et al.*, 1997) (appendix II, fig. 2). YbhR bears 46% similarity and 16% identity to

NatB. Both YbhR and YbhS contain histidines and cysteines in crucial areas for binding metal-ions (fig. 5.2).

One of the most convincing members of the *ybh* cluster that supports its function as an ABC transporter is *ybhF.* Its encoded protein YbhF (fig 5.2), contains classical consensus sequences found in ABC subunits. Consensus sequences are known as Walker motif A (GxxGxGKT/S, where x represents any amino acid) and Walker motif B (hhhhD, h represents 'hydrophobic') which is immediately preceded by a 'linker motif' LSGGQQ/R/KQR (Schneider and Hunke, 1998). The invariant lysine in Walker motif A is required for binding the β- and γ- phosphates of ATP as mutational analysis has shown that ATP hydrolysis does not occur without this lysine (Delepelaire, 1994) even though nucleotide binding can still occur (Schneider *et al.*, 1994). The importance of the conserved aspartate in Walker motif B was similarly demonstrated as alterations to it abolished ATPase activity and nucleotide binding since the aspartate is crucial for binding the Mg(II) ion that accompanies ATP (Panagiotidis *et al.*, 1993).

YbhF bears strong similarity to CcmA (*E. coli*), the ABC subunit of a heme transporting ABC complex (Schulz *et al.*, 1999) and ZnuC, the ABC component of the ZnuABC Zn(II) import system in *E. coli* (detailed in section 1.3.1.1.1). Sequence identity and similarity is high particularly in the ABC domain consensus regions (appendix II, fig. 3).

YbiH contains a predicted helix-turn-helix motif in the N-terminal region (fig. 5.2) and constitutes a putative regulator of the *ybh* cluster. YbiH contains 4 cysteines and 7 histidines that may be implicated in metal-ion coordination. Alignments (appendix II, fig. 4) with the drug responsive transcriptional repressors TetR (*Salmonella typhimurium*) and AcrR (*E. coli*) demonstrate homology. Both TetR and AcrR contain helix-turn-helix motifs and their transcription is autoregulated in response to stress signals (Ma *et al.*, 1996).

The TetR homodimer was crystallised in complex with its palindromic DNA operator and comparison of the structure to TetR complexed with its transcriptional inducer tetracycline allowed an induction mechanism to be deduced (Orth *et al.*, 2000). Binding of tetracycline

to TetR initiates a conformational change causing dissociation from the operator site (Orth *et al.*, 2000). It is of interest to note that the promoter of the *ybh* cluster, designated P*ybh*, contains two inverted repeat sequences (fig 5.4) which may assist binding of a YbiH-type homodimer via its helix-turn-helix domains thereby regulating *ybh* in a manner similar to the regulation of the tetracycline efflux genes by TetR.

The structure of this putative promoter P*ybh* (fig. 5.4) has many other interesting components. As well as containing the conserved σ^{70} promoter elements –10 (TAAATT (4/6 bp homology with TATAAT consensus)) and –35 (GTAGCA (3/6 bp homology with TTGACA consensus)) (Hawley and McClure, 1983) several other features are apparent. A σ^{54} or RpoN recognition element, CGTTGACNNNNNTTGCT (N is a non consensus nucleotide) is present that is very close to the consensus sequence CTGGCACNNNN NTTGCA (Hunt and Magasanik, 1985). The σ^{54}-factor binds to these consensus sequences to form a transcriptionally inactive closed complex. The resulting σ^{54}-holoenzyme remains in this state and is unable to spontaneously isomerise to form a transcriptionally active open complex unlike the σ^{70}-holoenzyme.

Transcriptional initiation by a σ^{54}-holoenzyme requires interaction with a transcriptional activator known as a bacterial EBP (enhancer binding protein) which binds upstream of the σ^{54} recognition element. EBPs share in common a σ^{54} interaction domain as well as an additionally conserved domain and a DNA binding helix-turn helix domain. The interaction of the EBP with a σ^{54}-holoenzyme may require DNA bending (reviewed by Studholme and Dixon, 2003).

Whereas one of the inverted repeats in P*ybh* at postion –24 to –1 (fig. 5.4) may represent a binding site for a regulator such as YbiH, the direct repeat at –194 to –177 or the inverted repeat at –179 to –169 (fig. 5.4) may constitute a binding site for an EBP.

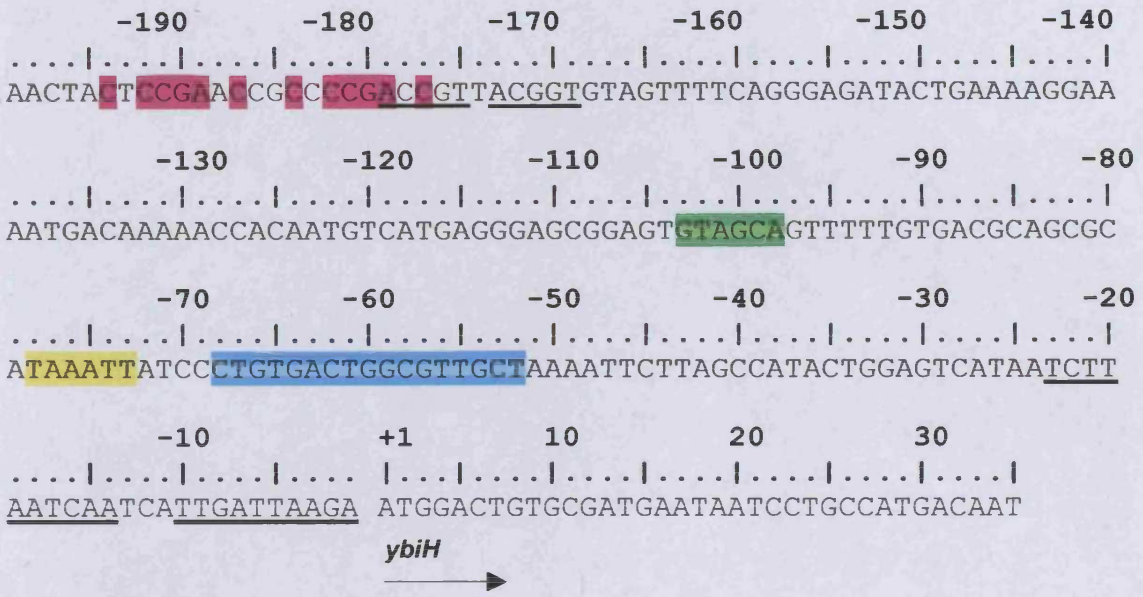


Fig. 5.4 Nucleotide sequence of the promoter of the *ybh* cluster, P*ybh*. The arrow labelled *ybiH* represents the translational start site of *ybiH*, the first ORF of the *ybh* cluster at position +1. Regions highlighted in yellow and green show potential –10 and –35 σ^{70} promoter elements, respectively.

The sequence highlighted in cyan is a σ^{54} (RpoN) recognition element. A direct repeat can be seen and is highlighted magenta. Two sets of inverted repeats are present in this promoter and these are underlined.

Transcription by σ^{54} has mostly been associated with nitrogen starvation conditions for example, several different amino acid synthesis or catabolism operons are activated by σ^{54} (reviewed by (Reitzer and Schneider, 2001)) however this is not always the case, an exception being the two component *zraSR* system. The *zraSR* genes contain a σ^{54} promoter in addition to a σ^{70} promoter and activate ZRAP, a periplasmic zinc resistance protein (detailed in section 1.3.1.2) (Leonhartsberger *et al.*, 2001). It seems that σ^{54} dependent transcription may not strictly be related to just nitrogen starvation but to other stressful stimuli too, however, these may be constrained within a narrow range and may be inter-related.

5.3.2 The introduction of P*ybh* into pUCD615

The promoter region of the *ybh* cluster from -173 bp to +35 bp (fig. 5.4) was amplified by PCR (section 2.2.6) using genomic DNA purified from *E. coli* TG2 (section 2.2.2.3) and primers P*ybh* For (*Bam*HI) and P*ybh* Rev (*Eco*RI) (section 2.1.3.7.1) at a T_m of 67.9°C. The size of the PCR product, visualised by agarose (2%) gel electrophoresis (section 2.2.5.1) was about 210 bp and this confirmed successful amplification of P*ybh*.

The PCR product was purified as described in section 2.2.3.4 and ligated (section 2.2.3.7) into linearised pCR®-Blunt (section 2.1.2). After completion of ligation, the reaction mixture was transformed (section 2.2.1.1.2) into competent TOP10 cells (section 2.1.1) with Km selection and incubated at 37°C, overnight.

Recombinant colonies were detected by PCR screening (section 2.2.6) using M13 forward and M13 reverse primers (section 2.1.3.7.1) followed by visualisation on an agarose gel (section 2.2.5.1). Colonies of TOP10 cells containing recombinant pCR®-Blunt-P*ybh* plasmids were picked, the plasmid DNA isolated (section 2.2.2.1) and the nucleotide sequences determined (section 2.2.8) to confirm the accuracy of P*ybh*.

Liberation of P*ybh* from pCR®-Blunt-P*ybh* was achieved by cleavage with *Bam*HI and *Eco*RI (section 2.2.3.1), sites for which were present in the original primers used for initial amplification of P*ybh* from TG2 ('P*ybh* For (*Bam*HI)' and 'P*ybh* Rev (*Eco*RI)'). The P*ybh* fragment was separated from other fragments by agarose gel electrophoresis (section 2.2.5.1) and purified (section 2.2.3.5) followed by ligation (section 2.2.3.7) into similarly cleaved pUCD615 (section 2.1.2).

After completion of ligation, the reaction mixture was transformed (section 2.2.1.1.2) into competent TG2 cells (section 2.1.1) with Cb and Km selection and incubated at 37°C, overnight. Plasmids from the resultant colonies were isolated (section 2.2.2.1) and the plasmids were digested with *Bam*HI and *Eco*RI (section 2.2.3.1). The resultant fragments were visualised on a 2% agarose gel (section 2.2.5.1) to identify pUCD615 derivatives carrying a band of approximately 220 bp indicating successful creation of pUCDP*ybh* plasmids.

5.3.3 Transcriptional activity at P*ybh* in response to metal-ions

Competent TG2 cells were transformed (section 2.2.1.1.2) with pUCDP*ybh* to Cb and Km resistance. pUCDP*zntA* was used as a control. Luciferase assays were performed on the transformants as detailed in section 2.2.10.1.

The luciferase readings of pUCDP*ybh* were measured in response to Zn(II), Cd(II), Co(II), Ni(II) and Pb(II) at the following concentrations in 0.1 mM increments except for Cd(II) which was added in increments of 50 μM:

$ZnSO_4$	0 – 1.2 mM
$CdSO_4$	0 – 500 μM
$CoSO_4$	0 – 1.0 mM
$NiSO_4$	0 – 1.0 mM
$Pb(C_2H_3O_2)_2$	0 – 1.0 mM

The results for the luciferase assays of TG2 [pUCDP*ybh*] in response to these metal-ions and TG2 [pUCDP*zntA*] in response to Zn(II) only, are shown in fig. 5.5. The figures presented in this section show luminescence measurements that have been adjusted for final D_{600} values to take into account any effects of toxicity from the metal-ions and so that an inaccurate representation of luminescence values is not given due to the cumulative effects of basal transcription that may be caused by differences in cell numbers rather than transcriptional activity. The results are presented as fold-inductions with respect to luminescence of each strain without the addition of any metal-ions. Each assay was repeated at least five times and a representative induction profile is shown for each metal.

P*zntA* was assayed for its response to Zn(II) in order to check that the system was working. The response of P*zntA* to Zn(II) was the usual, expected profile, which peaked at 1.1 mM as seen previously (section 3.3.3.1). P*ybh* appeared to be induced by Zn(II) up to 0.7 mM with transcription increasing up to about 1.8 fold, being followed by a sharp fall in transcription from 0.8 - 1.2 mM Zn(II). A similar transcriptional profile was observed with Cd(II); transcription from P*ybh* increased to almost 2-fold in response to Cd(II) at 0 - 250 μM and decreased sharply from 300 – 500 μM.

The transcriptional response of P*ybh* to Co(II) was the most dramatic with transcription steadily decreasing by the addition of 0.1 mM Co(II) with almost no detectable transcription from 0.5 – 1.0 mM Co(II). Ni(II) did not seem to affect transcription of P*ybh* up to 0.7 mM but the addition of 0.8 – 1.0 mM Ni(II) decreased transcription to below 0.5-fold. The effect of Pb(II) on P*ybh* was negligible.

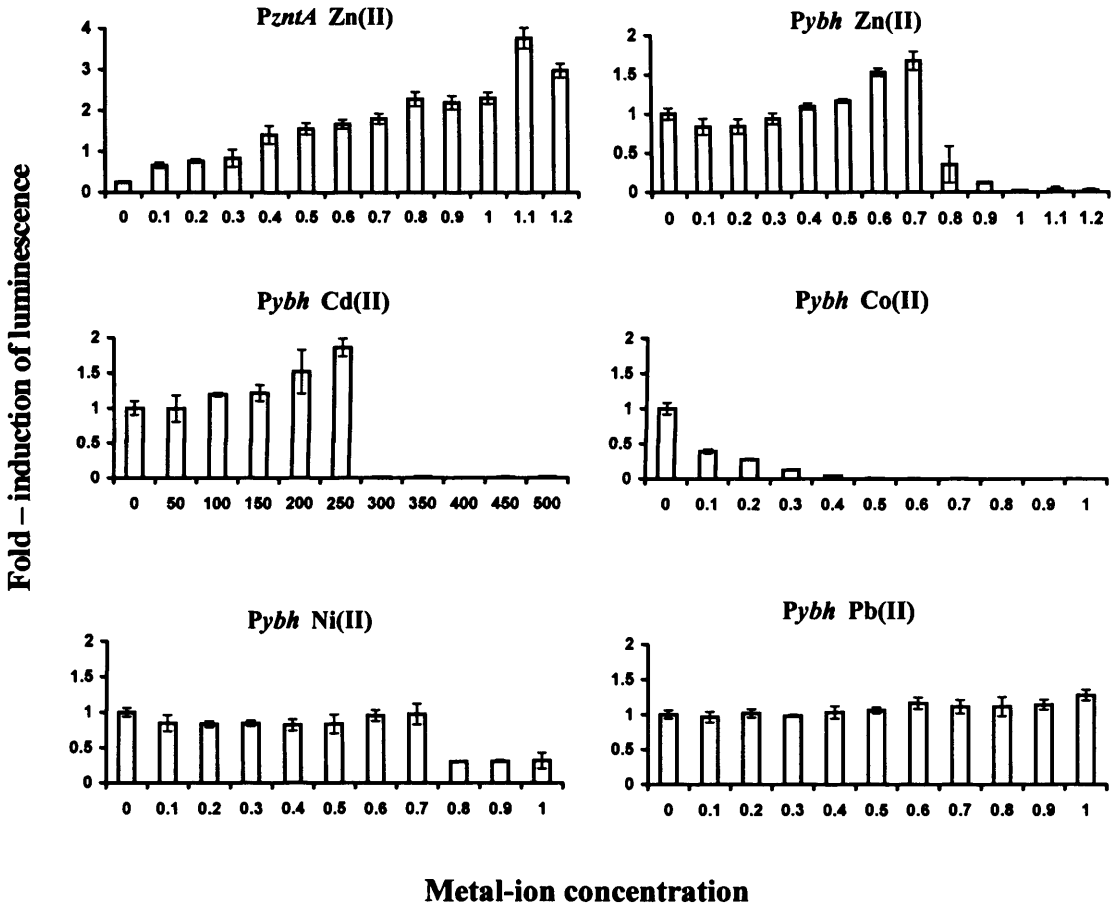


Fig. 5.5 Transcriptional response of P*zntA* in the presence of Zn(II) and P*ybh* in response to Zn(II), Cd(II), Co(II), Ni(II) and Pb(II).

The data are presented as fold-induction values (open columns) as derived from the corrected luminescence of *E. coli* TG2 [P*zntA*] and *E. coli* TG2 [P*ybh*] in media that had not been supplemented with any metal-ions.

The standard error (vertical bars) in each case is derived from a triplicate data set. The y-axis represents the fold-induction of transcription from P*zntA* or P*ybH* plotted against the metal-ion concentration in mM for each metal-ion except Cd(II) for which the units are μM. Note that the scale of the y-axis of the P*zntA* (Zn(II)) graph is different to the others.

5.3.4 Construction of *E. coli* W3110 (*ybhR*::*Km*) strain

The W3110 knockout strain, W3110 (*ybhR*::*Km*) was prepared as described in section 2.2.10.2. Primers '*ybhR* K.O. For' and '*ybhR* K.O. Rev' (section 2.1.3.7.1) were used for original amplification of the kanamycin cassette from plasmid pKD4 (section 2.1.2) and were designed to include nucleotide sequences complementary to regions flanking the YbhR gene in W3110, as required in the method (Datsenko and Wanner, 2000).

Primers '*ybhR* K.O. For - CHECK' and '*ybhR* K.O. Rev - CHECK' were used in combination with primers 'k1', 'k2' or 'kt' (section 2.1.3.7.1) to confirm creation of a successful knockout strain by PCR screening (section 2.2.6). The resulting PCR products were resolved by agarose gel electrophoresis (section 2.2.5.1). An example gel is shown (fig. 5.6) where the insertion of the kanamycin cassette into the genome was checked by PCR screening of W3110 wild-type and W3110 (*ybhR*::*km*) using primers '*ybhR* K.O. For-CHECK' in combination with k1.The primer '*ybhR* K.O. For-CHECK' was designed to anneal to a sequence upstream of the original *ybhR* in the genome. k1 was designed to anneal to a sequence within the kanamycin cassette (kT was designed to anneal to a different sequence within the kanamycin cassette). As expected, no amplification was observed in W3110 wild-type which was used as a control. Bands were visible for W3110 (*ybhR*::*km*) when PCR screened using primer combinations '*ybhR* K.O. For-CHECK' + k1 and '*ybhR* K.O. For-CHECK' + kT. This confirmed successful inactivation of *ybhR* by the kanamycin cassette.

5.3.5 MIC assays of *E. coli* W3110 (w.t.) and W3110 (*ybhR*::*Km*)

MIC assays on gradient plates were performed as described in section 2.2.10.4 on agar containing 3.5 or 7 mM $ZnSO_4$; 350 or 700 μM $CdSO_4$; 3.5 or 7 mM $CoSO_4$; 3.5 or 7 mM $NiSO_4$; 0.5 or 1 mM $Pb(C_2H_3O_2)_2$. Plates were incubated at 37°C and monitored every 24 hours up to 72 hours, however there appeared to be no significantly different patterns of growth between W3110 (w.t.) and W3110 (*ybhR*::*Km*).

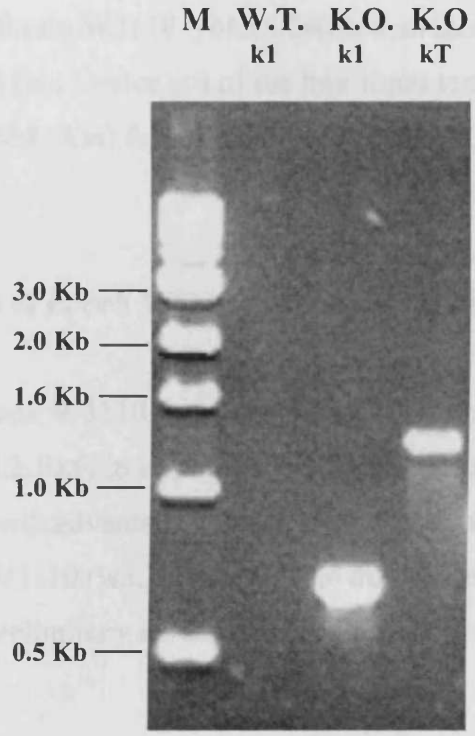


Fig. 5.6 PCR screen of W3110 wild-type (W.T.) and W3110 (*ybhR*::*km*) (knockout (K.O.)) strains. The lanes marked M, W.T. and K.O. indicate the 1 Kb ladder DNA marker, PCR products of W3110 wild-type and W3110 (*ybhR*::*km*) (knockout), respectively. The PCRs shown here were performed using the primer '*ybhR* K.O. For-CHECK' in combination with k1 for W3110 wild-type and k1 or kT for W3110 (*ybhR*::*km*).

Liquid MIC assays were carried out as described in section 2.2.10.5 with $ZnSO_4$, $CoSO_4$, and $NiSO_4$ at concentrations up to 6 mM, in 0.1 mM increments. These were carried out a maximum of four times for each metal due to time constraints but were non-reproducable for Zn(II) and Ni(II). Strain W3110 (*ybhR*::*Km*) had an increased MIC with Co(II) in comparison to W3110 (w.t.) twice out of the four times tested with the MIC being raised to 1.7 mM in W3110 (*ybhR*::*Km*) from 1.2 mM in W3110 (w.t.).

5.3.6 Growth studies of *E. coli* W3110 (w.t.) and W3110 (*ybhR*::*Km*)

Growth studies of *E. coli* W3110 (w.t.) and W3110 (*ybhR*::*Km*) were performed as described in section 2.2.10.6, in response to Co(II), Zn(II) and Ni(II). W3110 (*ybhR*::*Km*) showed a definite growth advantage in response to Co(II) at 0.5 – 1.5 mM and to Ni(II) at 1 mM as compared to W3110 (w.t.). There was no difference in growth in response to Zn(II) or Cd(II). These are preliminary results and need further validation (Khan, Wilkinson, Morby, 2003).

5.4 Discussion

The bioinformatic analysis of the *ybh* cluster revealed several interesting features which indicate that it encodes an ABC-type transporter. These data, together with the experimental results obtained with the pUCDP*ybh* construct and the *ybhR* knockout strain in response to certain metal-ions confirms to a great degree the original hypothesis that b0795 is an ORF that is part of a metal-ion importer (Brocklehurst and Morby, 2000). More precisely, the regulatory data suggest that the *ybh* cluster appears to encode proteins that import Co(II) primarily and to some degree Ni(II).

The bioinformatic data predicted that b0795 encodes a membrane bound periplasmic protein due to a predicted transmembrane domain in the N-terminal region with the rest of the protein being hydrophilic and the presence of a signal peptide. It also bears strong similarity to other membrane anchored periplasmic proteins. b0795 probably acts as the periplasmic binding protein, a component that is specific to ABC importers in gram-negative bacteria and recognise, co-ordinate and direct the incoming ligand to the integral membrane protein domains of the ABC importer (Gilson *et al.*, 1988).

YbhF is an excellent ATP-binding candidate of an ABC transport complex. Its primary amino acid sequence contains the typical consensus motifs found in bacterial ABC subunits such as the Walker site A, linker peptide and Walker site B. Additionally, it was found to have similarity to other ABC subunits. YbhF is abundant in cysteine and histidine residues. YbhS and YbhR represent the integral inner-membrane import components that may function as a heterodimer and may be loosely associated with b0795 on the periplasmic side and YbhF on the cytoplasmic side of the membrane. Predicted transmembrane regions were found in both proteins and the suggested orientation of the first (out of six) transmembrane regions was N-in. Similarity with another integral membrane protein was found.

YbiH probably regulates the operon. A predicted-helix-turn helix was found that may bind to one of the two inverted repeat regions within the promoter of the *ybh* cluster, P*ybh*. It was found to have strong similarity to two other transcriptional regulators that repress

transport of certain ligands as YbiH is predicted to do. YbiH also contains many histidine and cysteine residues. The transcriptional control of this region is likely to be more complicated given the presence of a σ^{54} binding site in addition to the σ^{70}.

The transcriptional response of P*ybh* to Zn(II), Cd(II), Co(II), Ni(II) and Pb(II) revealed important information about the regulation of the *ybh* cluster. It appeared to be most sensitive to Co(II) as transcription was strongly repressed by small quantities of Co(II) such as 0.1 mM. Zn(II), Cd(II) and Ni(II) also repressed transcription of P*ybh* but only at higher concentrations (0.8 mM for Zn(II) and Ni(II), 300 μM for Cd(II)). These results suggest that the *ybh* cluster is primarily involved in Co(II) import however its transcription is affected by Zn(II), Cd(II) and Ni(II) also. One explanation as to why Zn(II), Cd(II) and Ni(II) repress transcription of P*ybh* only at higher concentrations may be that these metal-ions slowly compete and displace Co(II) from the Co(II) binding site in the repressor at increased concentrations.

Further support for the *ybh* cluster being characterised as an ABC-importer of Co(II), came from the preliminary liquid MICs and growth studies which showed that the strain in which *ybhR* had been deactivated survived better in Co(II) elevated media. This was observed for Ni(II) also but only at high Ni(II) concentrations. Zn(II) or Cd(II) did not affect growth rates of the knockout strain versus the wild-type. The reason for this may be that the resistance systems in place for Zn(II) are more abundant and complex than for Co(II) or Ni(II) in *E. coli* and therefore resistance to Zn(II) does not solely rely on this importer.

Concluding remarks

The bioinformatic and experimental data on the *ybh* cluster obtained thus far suggests that it is an ABC-type transporter involved in the import of metal-ions, primarily Co(II) and Ni(II), although its transcription is also affected somewhat by Zn(II) and Cd(II). Future work is required to confirm these findings and exploration of the regulation of the *ybh* cluster would be useful in further defining the mechanisms and the conditions under which this importer functions.

CHAPTER SIX

GENERAL DISCUSSION

6.1 Site-directed mutagenesis of *zntR*

The transcriptional regulation of the primary Zn(II) exporter, *zntA*, in *E. coli* is under the control of ZntR, which was modified by site-directed mutagenesis, and the regulatory effects studied. The results obtained (section 3.3) showed that cysteines C79, C114, C115 and C124 were absolutely critical for optimum function of ZntR in response to Zn(II). Histidines H29, H53 and H119 were equally important with regards to Zn(II). The final amino acid in ZntR, C141 was found to be important for the sensitivity of this regulator in response to Zn(II), Cd(II) and Pb(II).

Interestingly, ZntR responded better to Cd(II) and Pb(II) when C115 was mutated to alanine as was the case with H29 and H76. Modification of H77 produced a slightly diminished response to Cd(II) and Pb(II). Overall, it was concluded that all 5 histidines and the 5 cysteines in ZntR were important for optimum functioning and a model was constructed (fig 3.9A).

Recently, after the publication of these results (Khan *et al.*, 2002), the crystal structure of the metal-binding region in ZntR was solved (Changela *et al.*, 2003). The structure (fig. 6.1) harmoniously backed up the results obtained in chapter 3. The structure showed that ZntR co-ordinated 2 Zn(II) ions between each metal-binding region on one monomer and the N-terminal region of the dimerisation domain of the other monomer (4 Zn(II) ions per dimer), in tetrahedral co-ordination environments. The first Zn(II) ion was found to be co-ordinated via the thiol groups of C114 and C124 from the ligand-binding region of one monomer, an oxygen from a phosphate group and C79 from the N-terminal region of the coiled-coil dimerisation domain of the other monomer. The second Zn(II) ion was co-ordinated by C115 and H119, another oxygen from the phosphate group and again C79 from the other monomer. The phosphate group and C79 in effect, act as bridging agents between the 2 Zn(II) ions.

The reported structure of ZntR is consistent with the effects of the mutagenesis of C79, C114, C115, C124 and H119, causing a detrimental effect on transcriptional regulation of

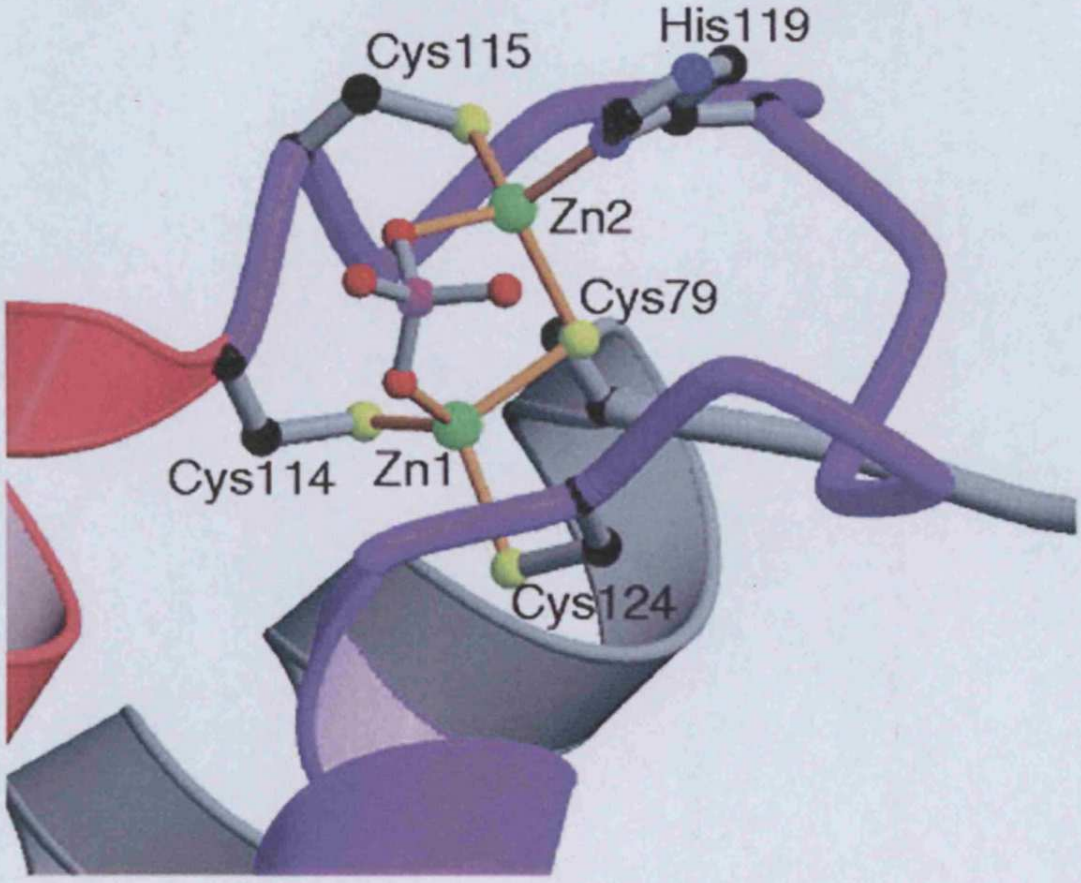


Fig 6.1 The metal-binding region of ZntR is shown, chelating two Zn(II) ions depicted as green spheres. Zn1 is coordinated to Cys^{114} and Cys^{124} of the metal-binding loop, and to Cys^{79} from the other monomer. Zn2 is coordinated to Cys^{115} and His^{119} of the metal-binding loop and Cys^{79} of the other monomer. The co-ordinating cysteines and histidine are highlighted in ball-and-stick representation. Each Zn(II) atom is also coordinated by an oxygen atom of a bridging phosphate ion, shown in ball-and-stick representation with the phosphate atom coloured magenta. The coordinate-covalent bonds to the metal-ions are shown in orange. The metal-binding domain is shown in blue ribbon, the dimerisation domain in red ribbon and the dimerisation domain from the other monomer is shown in grey ribbon. Reproduced from Changela *et al.*, 2003.

zntA by ZntR (section 3.3).

One interesting aspect of the ZntR structure solved by Changela *et al* (2003), was the involvement of a phosphate group in coordinating the Zn(II) ions. Closer inspection of the coordinates of the solved protein revealed that a truncated version had been crystallised, from aspartate-44 to glutamine-132. Therefore, some of the residues that were found to be significant for Zn(II) induced transcriptional regulation of P*zntA* by ZntR in my results were not included in this structure. These are, H29 and C141. Rather than 2 oxygen atoms from a phosphate group coordinating the Zn(II) ions, it may be more credible to suggest that H29 and C141 are involved.

Another interesting aspect of the work presented by Changela *et al* (2003), was the selectivity of MerR-family metalloregulators for +1 or +2 metal-ions. The structure of CueR (*E. coli*), the Cu(I)/Ag(I)/Au(I)-responsive analogue of ZntR, was solved at the same time as ZntR Changela *et al.*, 2003. CueR was found to coordinate one Cu(I) ion via 2 cysteines C112 and C120, both from the metal-binding region of the same monomer, however another residue, a serine, at the N-terminal region of the dimerisation domain of the other monomer was found to be involved. This serine, S77 was found to be equivalent to C79 of ZntR, and although S77 was not directly involved in binding Cu(I), it was found to extend across the dimer interface to contact the metal-binding loop. Its position is such that it is within hydrogen-bonding distance to several main chain atoms of the metal binding-loop and these interactions may be important in maintaining the conformation of the metal-binding loop. In CueR, a net negative charge arises from 2 thiolate anions and one positively charged Cu(I) ion which may be neutralised by the specialised electrostatic– and hydrogen bonding interactions occurring when a +1 ion, but not a +2 ion, binds to the metal-binding region (Changela *et al.*, 2003).

Alignments generated (Changela *et al.*, 2003) of MerR metalloregulatory homologues allowed characterisation of certain features, which may help to predict whether they would bind +1 or +2 metal-ions. For example, a serine or cysteine at the N-terminal end of the dimerisation helix provides selectivity for +1 or +2 metal-ions respectively.

In ZntR, histidines H76 and H77 are also located near C79 at the N-terminal end of the dimerisation helix and may have some effect in maintaining the conformation of the metal-binding loop in ZntR.

This structure of ZntR may also help to explain the effect of site-directed mutagenesis on the responses to Cd(II) and Pb(II), which were improved after mutagenesis of C115 to alanine. Considering that Cd(II) and Pb(II) can bind more avidly to the sulphydryl groups of cysteine residues than Zn(II) (Lippard and Berg, 1994; Harrison and Hoare, 1980), the dependency of Cd(II) and Pb(II) on all four cysteines may not be as strong as for Zn(II). It is not known how many Cd(II) and Pb(II) ions ZntR can bind. Another consideration in the case of Cd(II) is that it is larger than Zn(II) (cationic radii are 152 and 137 pm, respectively) (Jones and Atkins, 2000), and therefore the replacement of C115 by the smaller amino acid alanine allows the creation of a metal-binding cavity more accommodating to Cd(II) than for Zn(II). Pb(II) is also larger than Zn(II) (Jones and Atkins, 2000).

Much has now been revealed about the metal-ion specificity and selectivity of ZntR. However, reciprocal studies of CadR, another member of the MerR family of transcriptional regulators, that responds primarily to Cd(II), revealed that the metal-ion specificity and selectivity may not be solely dependent on the characteristics of the regulator alone.

Brocklehurst *et al,* (2003) identified a chromosomally located divergon in *Pseudomonas aeruginosa,* comprising homologues of *E. coli zntA* and *zntR*, called *cadA* and *cadR*. CadA is a P-type ATPase which primarily effluxes Cd(II) and Zn(II) (Lee *et al.*, 2001). CadR has 43% sequence identity with ZntR, contains an N-terminal helix-turn-helix motif and other features placing it in the MerR family of transcriptional regulators. Since Cd(II) serves no biological function in *E. coli,* it is toxic even at very low concentrations. CadR mediates Cd(II) responsive induction of *cadA* at the promoter P*cadA* (Lee *et al.*, 2001).

As with other MerR-type promoters, a structurally supraoptimal spacer region is apparent in P*cadA*, which is 19 bp in length, as well as an inverted repeat region in the form of a 9-2-9 bp dyad. It is likely that induction will involve the MerR DNA distortion mechanism.

Reciprocal experiments were performed by Brocklehurst *et al* (2003), in which the activity of CadR was assessed at promoter P*zntA*. The spacer length of P*zntA* is 1 bp longer than the spacer of P*cadA*. The results showed that CadR was able to induce transcription at P*zntA* although the primary inducer here was Hg(II), and induction was not as strong as that seen with CadR at P*cadA*. However when ZntR activity was assayed at P*cadA*, no metal induction was detected. It seems that CadR has a broader promoter binding specificity range than ZntR.

Brocklehurst *et al.*, 2003, created a P*zntA* deletion derivative, P*zntA*19, in which a single nucleotide was deleted from the centre of the inverted repeat. P*zntA*19 mimics the architecture of P*cadA*, both of which have a spacer length of 19 bp. At this promoter, CadR was most responsive to Cd(II), just as it is at P*cadA* (Brocklehurst *et al.*, 2003).

These results demonstrate that metal-ion specificity lies not only in the regulatory protein, but is also influenced by the promoter-regulator complex, with promoter spacing and the inverted repeat playing a fundamental role. Also, the type of metal-ion may play a role in twisting promoters to different degrees.

6.2 Whole-genome transcriptional analysis of *E. coli* (*zntR*::*Km*)

The global effect of elevated Zn(II) on *E. coli* lacking a normally functioning ZntR-ZntA resistance system was the focus of Chapter 4. Many interesting observations were made. An excess of Zn(II) appeared to interfere with systems utilising other metal-ions, namely Cu(II) and Fe. An attempt at increasing the rate of cell division was apparent and many stress-responsive genes were induced. The results also showed that alterations that are expected or predicted do not always occur, as was demonstrated in *E. coli* (*zntR*::*Km*) at

elevated Zn(II), because of observed inductions of *zupT, znuB* and *znuC* which are all related to Zn(II) import.

In general, an up-regulation of transcripts encoding potential Zn(II)-binding proteins was apparent in *E. coli* (*zntR*::*Km*) such as *ybbM*, a putative metal-ion transporter containing many cysteines and histidines. This type of up-regulation was also seen in the metal-ion adapted strains where the transcript abundance of *insA*-7 was found to have increased (Brocklehurst and Morby, 2000). Furthermore, *insA*-7 was over-expressed and conferred increased Zn(II) tolerance probably by virtue of two C-X-X-C motifs. Therefore, up-regulation of transcripts encoding proteins that could assist in metal-ion tolerance by means of export or chelation, would appear to be the general response of a bacterium subjected to heavy-metal stress.

6.3 Phenotypic characterisation of a novel ABC-type metal-ion importer

The bioinformatic data presented in section 5.3 and previous macroarray data (Brocklehurst and Morby, 2000) indicted that the *ybh* cluster encodes a metal-ion importer belonging to the ABC-family of transporters. Further evidence for this suggestion was obtained through studies of P*ybh* transcriptional fusions (section 5.3.3) and a knockout strain of *E. coli* W3110 (*ybhR*::*Km*) (sections 5.3.4, 5.3.5 and 5.3.6).

Primarily, this novel transporter responded to Co(II) and may represent the first known Co(II) importer in *E. coli.* Co(II) is a transition metal that has two naturally occurring oxidation states, Co(II) and Co(III), and it is an important cofactor in vitamin B_{12} dependent enzymes since vitamin B_{12} contains Co(III) (Battersby *et al.*, 1977). The enzyme methionine aminopeptidase that cleaves the N-terminal methionine from newly translated polypeptides utilises Co(II) also (Roderick and Matthews, 1993).

Co(II) has a cationic radius of 74 pm which is very close to that of Zn(II) (75 pm). The two cations are similar both in size and in preferred ligand type and co-ordination geometry

(Harrison and Hoare, 1980). As a result, the two can often be substituted. This could explain the results seen in section 5.3.3, where the activity of P*ybh* gradually increases in response to Zn(II) up to a certain point (0.8 mM), after which a sudden fall is observed.

6.4 Concluding remarks

The work presented in this thesis throws light on the nature of metalloregulatory proteins from the MerR family of transcriptional regulators. The dependence of metal-ion specificity on cysteine –115 and cysteine –141 in ZntR was particularly interesting.

Further work would be required to explore the regulatory activity of ZntR in greater detail and may take the form of further mutagenesis in other regions of this protein. This may help us to understand which amino acids are crucial for dimerisation and promoter recognition. The *ybh* cluster will definitely need to be investigated further to establish its precise role and its importance within *E. coli*.

The extensive role that metal-ions play in the biochemistry of all organisms can be seen by the examples presented in Chapter 1. Additionally, a significant amount of scientific literature is available on this subject. The usage of metal-ions in metabolism is becoming increasingly apparent as more research is directed towards this area of science. They are required for structural stability, as cofactors and as redox catalysts in some of the most crucial metabolic pathways such as the electron transport chain. The importance of metal-ions cannot be overstated.

BIBLIOGRAPHY

Ahmed, M., Borsch, C.M., Taylor, S.S., Vazquez-Laslop, N., and Neyfakh, A.A. (1994) A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *Journal of Biological Chemistry* 269: 28506-28513.

Ahmed, M., Lyass, L., Markham, P.N., Taylor, S.S., Vazquez-Laslop, N., and Neyfakh, A.A. (1995) Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. *Journal of Bacteriology* 177: 3904-3910.

Aiking, H., Kok, K., van Heerikhuizen, H., and van Riet, J. (1982) Adaptation to Cadmium by *Klebsiella aerogenes* growing in continuous culture proceeds mainly via formation of cadmium sulphide. *Applied and Environmental Microbiology* 44: 938-944.

Amabile-Cuevas, C.F., and Demple, B. (1991) Molecular characterisation of the *soxRS* genes of *Escherichia coli* : 2 genes control a superoxide stress regulon. *Nucleic Acids Research* 19: 4479-4484.

Anraku, Y., Kin, E., and Tanaka, Y. (1975) Transport of sugars and amino acids in bacteria. XV. Comparative studies on the effects of various energy poisons on the oxidative and phosphorylating activities and energy coupling reactions for the active transport systems for amino acids in *E. coli*. *Journal of Biochemistry (Tokyo)* 78: 165-179.

Ansari, A.Z., Bradner, J.E., and O'Halloran, T.V. (1995) DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* 374: 371-375.

Ansari, A.Z., Chael, M.L., and O'Halloran, T.V. (1992) Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* 355: 87-89.

Axelsen, K.B., and Palmgren, M.G. (1998) Evolution of substrate specificities in the P-type ATPase superfamily. *Journal of Molecular Evolution* 46: 84-101.

Banci, L., Bertini, I., Ciofi-Baffoni, S., Finney, L.A., Outten, C.E., and O'Halloran, T.V. (2002) A new zinc-protein coordination site in intracellular metal trafficking: solution structure of the Apo and Zn(II) forms of ZntA(46- 118). *Journal of Molecular Biology* 323: 883-897.

Bartolome, B., Jubete, Y., Martinez, E., and Delacruz, F. (1991) Construction and Properties Of a Family Of pACYC184-Derived Cloning Vectors Compatible With pBR322 and Its Derivatives. *Gene* 102: 75-78.

Bartsevich, V.V., and Pakrasi, H.B. (1995) Molecular identification of an ABC transporter complex for manganese: analysis of a cyanobacterial mutant strain impaired in the photosynthetic oxygen evolution process. *EMBO Journal* 14: 1845-1853.

Battersby, A.R., McDonald, E., Hollenstein, R., Ihara, M., Satoh, F., and Williams, D.C. (1977) Biosynthesis of porphyrins and related macrocycles. Part 10. Vitamin B12: biochemical derivation of cobyrinic acid from uroporphyrinogen III. Studies with corresponding ring c methyl heptacarboxylic prophyrinogen, and proof of seven intact methyl transfers. *Journal of the Chemical Society [Perkin 1]* 2: 166-178.

Beard, S.J., Hashim, R., Membriool-Hernandez, J., Hughes, M.N., and Poole, R.K. (1997) Zinc(II) tolerence in *Escherichia coli* K-12: evidence that the *zntA* gene (o732) encodes a cation transport ATPase. *Molecular Microbiology* 25: 883-891.

Beard, S.J., Hashim, R., Wu, G., Binet, M.R., Hughes, M.N., and Poole, R.K. (2000) Evidence for the transport of zinc(II) ions via the *pit* inorganic phosphate transport system in *Escherichia coli*. *FEMS Microbiology Letters* 184: 231-235.

Beard, S.J., Hughes, M.N., and Poole, R.K. (1995) Inhibition of the cytochrome bd-terminated NADH oxidase system in *Escherichia coli* K-12 by divalent metal cations. *FEMS Microbiology Letters* 131: 205-210.

Beynon, J., Cannon, M., Buchanan-Wollaston, V., and Cannon, F. (1983) The nif promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* 34: 665-671.

Bhriain, N.N., and Foster, T.J. (1986) Polypeptides specified by the mercuric resistance (*mer*) operon of plasmid R100. *Gene* 42: 323-330.

Blattner, F.R., Plunkett, G., 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* 277: 1453-1474.

Blencowe, D.K. (2002) Zn(II) Metabolism in *Escherichia coli*. In *Cardiff School of Biosciences (2)* Cardiff: Cardiff University.

Blencowe, D.K., Marshall, S.J., and Morby, A.P. (1997) Preliminary characterisation of *zntA*, a gene which encodes a Zn(II)/Cd(II)-export protein in *Escherichia coli*. *Biotechnology et alia* 2: 1-6.

Blindauer, C.A., Harrison, M.D., Robinson, A.K., Parkinson, J.A., Bowness, P.W., Sadler, P.J., and Robinson, N.J. (2002) Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Molecular Microbiology* 45: 1421-1432.

Boos, W., and Shuman, H. (1998) Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiology and Molecular Biology Reviews* 62: 204-229.

Borremans, B., Hobman, J.L., Provoost, A., Brown, N.L., and van Der Lelie, D. (2001) Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. *Journal of Bacteriology* 183: 5651-5658.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Braun, M., Killmann, H., Maier, E., Benz, R., and Braun, V. (2002) Diffusion through channel derivatives of the *Escherichia coli* FhuA transport protein. *European Journal of Biochemistry* 269: 4948-4959.

Brendel, V., Hamm, G.H., and Trifonov, E.N. (1986) Terminators of transcription with RNA polymerase from *Escherichia coli*: what they look like and how to find them. *Journal of Biomolecular Structure and Dynamics* 3: 705-723.

Brissette, J.L., Weiner, L., Ripmaster, T.L., and Model, P. (1991) Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. *Journal of Molecular Biology* 220: 35-48.

Brocklehurst, K.R., and Morby, A.P. (2000) Metal-ion tolerance in *Escherichia coli*: analysis of transcriptional profiles by gene-array technology. *Microbiology* 146 (Pt 9): 2277-2282.

Brocklehurst, K.R., Hobman, J.L., Lawley, B., Blank, L., Marshall, S.J., Brown, N.L., and Morby, A.P. (1999) ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. *Molecular Microbiology* 31: 893-902.

Brocklehurst, K.R., Megit, S.J., and Morby, A.P. (2003) Characterisation of CadR from *Pseudomonas aeruginosa*: a Cd(II)-responsive MerR homologue. *Biochemical and Biophysical Research Communications* 308: 234-239.

Brown, K.L., and Hughes, K.T. (1995) The role of anti-sigma factors in gene regulation. *Molecular Microbiology* 16: 397-404.

Bruins, M.R., Kapil, S., and Oehme, F.W. (2000) Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety* 45: 198-207.

Bucheder, F., and Broda, E. (1974) Energy-dependent zinc transport by *Escherichia coli*. *European Journal of Biochemistry* 45: 555-559.

Bull, P.C., and Cox, D.W. (1994) Wilson disease and Menkes disease : New handles on heavy-metal transport. *Trends in Genetics* 10: 246-252.

Burgess, R.R., Travers, A.A., Dunn, J.J., and Bautz, E.K. (1969) Factor stimulating transcription by RNA polymerase. *Nature* 221: 43-46.

Caguiat, J.J., Watson, A.L., and Summers, A.O. (1999) Cd(II)-responsive and constitutive mutants implicate a novel domain in MerR. *Journal Of Bacteriology* 181: 3462-3471.

Carballes, F., Bertrand, C., Bouche, J.P., and Cam, K. (1999) Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. *Molecular Microbiology* 34: 442-450.

Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W., and Gros, P. (1995) Nramp defines a family of membrane proteins. *Proceedings of the National Academy of Science, U.S.A.* 92: 10089-10093.

Chamberlin, M.J. (1974) The selectivity of transcription. *Annual Review of Biochemistry* 43: 721-775.

Changela, A., Chen, K., Xue, Y., Holschen, J., Outten, C.E., O'Halloran, T.V., and Mondragon, A. (2003) Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* 301: 1383-1387.

Cheng, J.,Guffanti, A.A. and Krulwich, T.A. (1997) A two-gene ABC-type transport system that extrudes Na+ in *Bacillus subtilis* is induced by ethanol or protonophore. *Molecular Microbiology* 23:6 1107-1120

Chou, A.Y., Archdeacon, J., and Kado, C.I. (1998) *Agrobacterium* transcriptional regulator Ros is a prokaryotic zinc finger protein that regulates the plant oncogene *ipt*. *Proceedings Of the National Academy Of Science, USA* 95: 5293-5298.

Comess, K.M., Shewchuk, L.M., Ivanetich, K., and Walsh, C.T. (1994) Construction of a synthetic gene for the metalloregulatory protein MerR and analysis of regionally mutated proteins for transcriptional regulation. *Biochemistry* 33: 4175-4186.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings Of the National Academy Of Science, USA* 97: 6640-6645.

de la Cruz, F., and Grinsted, J. (1982) Genetic and molecular characterization of Tn*21*, a multiple resistance transposon from R100.1. *Journal of Bacteriology* 151: 222-228.

deHaseth, P.L., Zupancic, M.L., and Record, M.T., Jr. (1998) RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. *Journal of Bacteriology* 180: 3019-3025.

Delepelaire, P. (1994) PrtD, the integral membrane ATP-binding cassette component of the *Erwinia chrysanthemi* metalloprotease secretion system, exhibits a secretion signal-regulated ATPase activity. *Journal of Biological Chemistry* 269: 27952-27957.

Diels, L., Dong, Q., van der Lelie, D., Baeyens, W., and Mergeay, M. (1995) The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. *Journal of Industrial Microbiology* 14: 142-153.

Dintilhac, A., Alloing, G., Granadel, C., and Claverys, J.P. (1997) Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Molecular Microbiology* 25: 727-739.

Dintilhac, A., and Claverys, J.P. (1997) The adc locus, which affects competence for genetic transformation in *Streptococcus pneumoniae*, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. *Research in Microbiology* 148: 119-131.

Ehrmann, M., Ehrle, R., Hofmann, E., Boos, W., and Schlosser, A. (1998) The ABC maltose transporter. *Molecular Microbiology* 29: 685-694.

Endo, G., and Silver, S. (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphlococcus aureus* plasmid pI258. *Journal of Bacteriology* 177: 4437-4441.

Epstein, W., and Beckwith, J.R. (1968) Regulation of gene expression. *Annual Review of Biochemistry* 37: 411-436.

Fath, M.J., and Kolter, R. (1993) ABC transporters: bacterial exporters. *Microbiological Reviews* 57: 995-1017.

Fernandez, S.V., Xing, J., Kapur, V., Libby, S.J., Barletta, R.G., and Moxley, R.A. (1998) Regulation of the *Escherichia coli sheA* gene and characterization of its encoded hemolytic activity. *FEMS Microbiology Letters* 168: 85-90.

Frantz, B., and O'Halloran, T.V. (1990) DNA distortion accompanies transcriptional activation by the metal-responsive gene-regulatory protein MerR. *Biochemistry* 29: 4747-4751.

Genevaux, P., Wawrzynow, A., Zylicz, M., Georgopoulos, C., and Kelley, W.L. (2001) DjlA is a third DnaK co-chaperone of *Escherichia coli*, and DjlA-mediated induction of colanic acid capsule requires DjlA-DnaK interaction. *Journal of Biological Chemistry* 276: 7906-7912.

Gentschev, I., Dietrich, G., and Goebel, W. (2002) The *E. coli* hemolysin secretion system and its use in vaccine development. *Trends in Microbiology* 10: 39-45.

Gibson, T.J. (1984) Studies on the Epstein-Barr virus genome. Cambridge, UK: Cambridge University.

Gilbert, H.F. (1990) Molecular and cellular aspects of thiol-disulfide exchange. *Advances in Enzymology and Related Areas of Molecular Biology* 63: 69-172.

Gilson, E., Alloing, G., Schmidt, T., Claverys, J.P., Dudler, R., and Hofnung, M. (1988) Evidence for high affinity binding-protein dependent transport systems in gram-positive bacteria and in Mycoplasma. *EMBO Journal* 7: 3971-3974.

Gitschier, J., Moffat, B., Reilly, D., Wood, W.I., and Fairbrother, W.J. (1998) Solution structure of the fourth metal-binding domain from the Menkes copper-transporting ATPase. *Nature Structure Biology* 5: 47-54.

Grass, G., and Rensing, C. (2001) CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochemical and Biophysical Research Communications* 286: 902-908.

Grass, G., Fan, B., Rosen, B.P., Franke, S., Nies, D.H., and Rensing, C. (2001) ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli*. *Journal of Bacteriology* 183: 4664-4667.

Grass, G., Wong, M.D., Rosen, B.P., Smith, R.L., and Rensing, C. (2002) ZupT is a Zn(II) uptake system in *Escherichia coli*. *Journal of Bacteriology* 184: 864-866.

Grosse, C., Grass, G., Anton, A., Franke, S., Santos, A.N., Lawley, B., Brown, N.L., and Nies, D.H. (1999) Transcriptional organization of the *czc* heavy-metal homeostasis determinant from *Alcaligenes eutrophus*. *Journal of Bacteriology* 181: 2385-2393.

Guerinot, M.L. (2000) The ZIP family of metal transporters. *Biochimica et Biophysica Acta* 1465: 190-198.

Guex, N., Diemand, A., and Peitsch, M.C. (1999) Protein modelling for all. *Trends in Biochemical Sciences* 24: 364-367.

Guffanti, A.A., Wei, Y., Rood, S.V., and Krulwich, T.A. (2002) An antiport mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K+ and H+. *Molecular Microbiology* 45: 145-153.

Guzzo, J., and Dubow, M.S. (2000) A novel selenite- and tellurite-inducible gene in *Escherichia coli*. *Applied and Environmental Microbiology* 66: 4972-4978.

Hamer, D.H. (1986) Metallothionein. *Annual Review of Biochemistry* 55: 913-951.

Hanahan, D. (1983) Studies on the transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* 166: 557-580.

Hantke, K. (2001) Bacterial zinc transporters and regulators. *Biometals* 14: 239-249.

Harley, C.B., and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Research* 15: 2343-2361.

Harrison, P.M., and Hoare, R.J. (1980) *Metals in biochemistry*: Chapman and Hall.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Research* 11: 2237-2255.

Heldwein, E.E., and Brennan, R.G. (2001) Crystal structure of the transcription activator BmrR bound to DNA and a drug. *Nature* 409: 378-382.

Helmann, J.D., and Chamberlin, M.J. (1988) Structure and function of bacterial sigma factors. *Annual Review of Biochemistry* 57: 839-872.

Heltzel, A., Lee, I.W., Totis, P.A., and Summers, A.O. (1990) Activator-dependent preinduction binding of sigma-70 RNA polymerase at the metal-regulated *mer* promoter. *Biochemistry* 29: 9572-9584.

Heroven, A.K., and Dersch, P. (2002) Two different open reading frames named *slyA* in the *E. coli* sequence databases. *Trends in Microbiology* 10: 267-268.

Higgins, C.F. (1992) ABC transporters: from microorganisms to man. *Annual Reviews in Cell Biology* 8: 67-113.

Higham, D.P., Sadler, P.J., and Scawen, M.D. (1986) Cadmium-binding proteins in *Pseudomonas putida*: pseudothioneins. *Environmental Health Perspectives* 65: 5-11.

Hobman, J.L., and Brown, N.L. (1997) Bacterial mercury-resistance genes. *Metal Ions in Biological Systems* 34: 527-568.

Holmes, D.J., Caso, J.L., and Thompson, C.J. (1993) Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. *EMBO Journal* 12: 3183-3191.

Hosie, A.H., Allaway, D., Jones, M.A., Walshaw, D.L., Johnston, A.W., and Poole, P.S. (2001) Solute-binding protein-dependent ABC transporters are responsible for solute efflux in addition to solute uptake. *Molecular Microbiology* 40: 1449-1459.

Hoyle, B., and Beveridge, T.J. (1983) Binding of metallic ions to the outer membrane of *Escherichia coli*. *Applied and Environmental Microbiology* 46: 749-752.

Hunt, T.P., and Magasanik, B. (1985) Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. *Proceedings Of the National Academy Of Science, USA* 82: 8453-8457.

Jackson, W.J., and Summers, A.O. (1982) Polypeptides encoded by the *mer* operon. *Journal of Bacteriology* 149: 479-487.

Jacquamet, L., Aberdam, D., Adrait, A., Hazemann, J.L., Latour, J.M., and Michaud-Soret, I. (1998) X-ray absorption spectroscopy of a new zinc site in the *fur* protein from *Escherichia coli*. *Biochemistry* 37: 2564-2571.

Jap, B.K., and Walian, P.J. (1996) Structure and functional mechanism of porins. *Physiological Reviews* 76: 1073-1088.

Jasper, P., and Silver, S. (1997) Magnesium transport in microorganisms. In Microorganisms and Minerals. Vol. 3. Weinberg, E.D. (ed). New York and Basel: Marcel Dekker, Inc., pp. 7-45.

Jellen-Ritter, A.S., and Kern, W.V. (2001) Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants Selected with a fluoroquinolone. *Antimicrobial Agents and Chemotherapy* 45: 1467-1472.

Ji, G., and Silver, S. (1995) Bacterial resistance mechanisms for heavy metals of environmental concern. *Journal of Industrial Microbiology* 14: 61-75.

Jones, L. and Atkins, P. (2000) Chemistry: Molecules, Matter, and Change. 4th Edition. W H Freeman & Co.

Kehres, D.G., Zaharik, M.L., Finlay, B.B., and Maguire, M.E. (2000) The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. *Molecular Microbiology* 36: 1085-1100.

Khan, S., Brocklehurst, K.R., Jones, G.W., and Morby, A.P. (2002) The functional analysis of directed amino-acid alterations in ZntR from *Escherichia coli*. *Biochemical and Biophysical Research Communications* 299: 438-445.

Khazaeli, M.B., and Mitra, R.S. (1981) Cadmium-binding component in *Escherichia coli* during accommodation to low levels of this ion. *Applied Environmental Microbiology* 41: 46-50.

Kidd, S.P., and Brown, N.L. (2003) ZccR--a MerR-like regulator from *Bordetella pertussis* which responds to zinc, cadmium, and cobalt. *Biochemical and Biophysical Research Communications* 302: 697-702.

Kita, K., Konishi, K., and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. I. Purification and properties of cytochrome b562-o complex from cells in the early exponential phase of aerobic growth. *Journal of Biological Chemistry* 259: 3368-3374.

Koh, Y.S., and Roe, J.H. (1995) Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the *soxRS* locus in *Escherichia coli*. *Journal of Bacteriology* 177: 2673-2678.

Korbashi, P., Katzhendler, J., Saltman, P., and Chevion, M. (1989) Zinc protects *Escherichia coli* against copper-mediated paraquat-induced damage. *Journal of Biological Chemistry* 264: 8479-8482.

Kung, F.C., Raymond, J., and Glaser, D.A. (1976) Metal ion content of *Escherichia coli* versus cell age. *Journal of Bacteriology* 126: 1089-1095.

Kyte, J., and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157: 105-132.

Lane, T.W., and Morel, F.M. (2000) A biological function for cadmium in marine diatoms. *Proceedings Of the National Academy Of Science, USA* 97: 4627-4631.

Lee, S.M., Grass, G., Haney, C.J., Fan, B., Rosen, B.P., Anton, A., Nies, D.H., and Rensing, C. (2002) Functional analysis of the *Escherichia coli* zinc transporter ZitB. *FEMS Microbiology Letters* 215: 273-278.

Lee, S.W., Glickmann, E., and Cooksey, D.A. (2001) Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR family response regulator. *Applied and Environmental Microbiology* 67: 1437-1444.

Leonhartsberger, S., Huber, A., Lottspeich, F., and Bock, A. (2001) The *hydH/G* genes from *Escherichia coli* code for a zinc and lead responsive two-component regulatory system. *Journal of Molecular Biology* 307: 93-105.

Linton, K.J., and Higgins, C.F. (1998) The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Molecular Microbiology* 28: 5-13.

Lippard, S.J., and Berg, J.M. (1994) *Principles of bioinorganic chemistry*: University Science.

Livrelli, V., Lee, I.W., and Summers, A.O. (1993) In vivo DNA-protein interactions at the divergent mercury resistance (*mer*) promoters. I. Metalloregulatory protein MerR mutants. *Journal of Biological Chemistry* 268: 2623-2631.

Lomovskaya, O., and Lewis, K. (1992) Emr, an *Escherichia coli* locus for multidrug resistance. *Proceedings Of the National Academy Of Science, USA* 89: 8938-8942.

Lund, P.A., and Brown, N.L. (1989a) Regulation of transcription in *Escherichia coli* from the *mer* and *merR* promoters in the transposon Tn*501*. *Journal of Molecular Biology* 205: 343-353.

Lund, P.A., Ford, S.J., and Brown, N.L. (1986) Transcriptional regulation of the mercury-resistance genes of transposon Tn*501*. *Journal of General Microbiology* 132: 465-480.

Lutkenhaus, J.F. (1977) Role of a major outer membrane protein in *Escherichia coli*. *Journal of Bacteriology* 131: 631-637.

Ma, D., Alberti, M., Lynch, C., Nikaido, H., and Hearst, J.E. (1996) The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Molecular Microbiology* 19: 101-112.

Makino, K., Shinagawa, H., Amemura, M., Kawamoto, T., Yamada, M., and Nakata, A. (1989) Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. *Journal of Molecular Biology* 210: 551-559.

Makui, H., Roig, E., Cole, S.T., Helmann, J.D., Gros, P., and Cellier, M.F. (2000) Identification of the *Escherichia coli* K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter. *Molecular Microbiology* 35: 1065-1078.

McHugh, J.P., Rodriguez-Quinones, F., Abdul-Tehrani, H., Svistunenko, D.A., Poole, R.K., Cooper, C.E., and Andrews, S.C. (2003) Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. *Journal of Biological Chemistry* 278: 29478-29486.

Megit, S. (2002) Identification and characterisation of prokaryotic environmentally responsive promoters. Cardiff, UK: Cardiff University.

Morby, A.P., Turner, J.S., Huckle, J.W., and Robinson, N.J. (1993) SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA* : identification of a Zn inhibited DNA-protein complex. *Nucleic Acids Research* 21: 921-925.

Mukhopadhyay, D., Yu, H.R., Nucifora, G., and Misra, T.K. (1991) Purification and functional characterization of MerD. A coregulator of the mercury resistance operon in gram-negative bacteria. *Journal of Biological Chemistry* 266: 18538-18542.

Murata, K., Fukuda, Y., Shimosaka, M., Watanabe, K., Saikusa, T., and Kimura, A. (1985) Phenotype character of the methylglyoxal resistance gene in *Saccharomyces cerevisiae*: expression in *Escherichia coli* and application to breeding wild-type yeast strains. *Applied and Environmental Microbiology* 50: 1200-1207.

Newlands, J.T..Josaitis, C.A., Ross, W and Gourse, R.L. (1992) Both fis-dependent and factor-independent upstream activation of the *rrnB* P1 promoter are face of the helix dependent. *Nucleic Acids Research* 20:4 719-726.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* 10: 1-6.

Nies, D.H. (1999) Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* 51: 730-750.

Noll, M., Petrukhin, K., and Lutsenko, S. (1998) Identification of a novel transcription regulator from *Proteus mirabilis*, PMTR, revealed a possible role of YJAI protein in balancing zinc in *Escherichia coli*. *Journal of Biological Chemistry* 273: 21393-21401.

Nucifora, G., Chu, L., Misra, T.K., and Silver, S. (1989c) Cadmium resistance from *Staphlococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. *Proceedings of the National Academy of Science, U.S.A.* 86: 3544-3548.

Nucifora, G., Silver, S., and Misra, T.K. (1989b) Down regulation of the mercury resistance operon by the most promoter-distal gene *merD*. *Molecular Gen Genet* 220: 69-72.

O'Halloran, T.V., and Culotta, V.C. (2000) Metallochaperones, an intracellular shuttle service for metal ions. *Journal of Biological Chemistry* 275: 25057-25060.

O'Halloran, T.V., Frantz, B., Shin, M.K., Ralston, D.M., and Wright, J.G. (1989) The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* 56: 119-129.

Okkeri, J., and Haltia, T. (1999) Expression and mutagenesis of ZntA, a zinc-transporting P-type ATPase from *Escherichia coli. Biochemistry* 38: 14109-14116.

Okkeri, J., Bencomo, E., Pietila, M., and Haltia, T. (2002) Introducing Wilson disease mutations into the zinc-transporting P-type ATPase of *Escherichia coli*. The mutation P634L in the 'hinge' motif (GDGXNDXP) perturbs the formation of the E2P state. *European Journal of Biochemistry* 269: 1579-1586.

Olafson, R.W., McCubbin, W.D., and Kay, C.M. (1988) Primary- and seconary-structural analysis of a unique prokaryotic metallothionein from a *Synechoccus* sp. cyanobacterium. *Biochemical Journal* 251: 691-699.

Omori, K., Akatsuka, H., and Komatsubara, S. (1994) Versatile *Escherichia coli* expression vectors for production of truncated proteins. *Plasmid* 31: 297-299.

Orth, P., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nature Structure Biology* 7: 215-219.

Outten, C.E., and O'Halloran, T.V. (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* 292: 2488-2492.

Outten, C.E., Outten, F.W., and O'Halloran, T.V. (1999) DNA distortion mechanism for transcriptional activation by ZntR, a Zn(II)-responsive MerR homologue in *Escherichia coli. Journal of Biological Chemistry* 274: 37517-37524.

Outten, C.E., Tobin, D.A., Penner-Hahn, J.E., and O'Halloran, T.V. (2001) Characterization of the metal receptor sites in *Escherichia coli* Zur, an ultrasensitive zinc(II) metalloregulatory protein. *Biochemistry* 40: 10417-10423.

Outten, F.W., Outten, C.E., Hale, J., and O'Halloran, T.V. (2000) Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, *cueR. Journal of Biological Chemistry* 275: 31024-31029.

Palmiter, R.D. (1987) Molecular biology of metallothionein gene expression. *Experientia Suppl* 52: 63-80.

Panagiotidis, C.H., Reyes, M., Sievertsen, A., Boos, W., and Shuman, H.A. (1993) Characterization of the structural requirements for assembly and nucleotide binding of an ATP-binding cassette transporter. The maltose transport system of *Escherichia coli*. *Journal of Biological Chemistry* 268: 23685-23696.

Panne, D., Raleigh, E.A., and Bickle, T.A. (1998) McrBs, a modulator peptide for McrBC activity. *EMBO Journal* 17: 5477-5483.

Park, M.H., Wong, B.B., and Lusk, J.E. (1976) Mutants in three genes affecting transport of magnesium in *Escherichia coli*: genetics and physiology. *Journal of Bacteriology* 126: 1096-1103.

Parkhill, J., and Brown, N.L. (1990) Site-specific insertion and deletion mutants in the *mer* promoter-operator region of Tn*501*; the nineteen base-pair spacer is essential for normal induction of the promoter by MerR. *Nucleic Acids Research* 18: 5157-5162.

Parkhill, J., Ansari, A.Z., Wright, J.G., Brown, N.L., and O'Halloran, T.V. (1993) Construction and characterization of a mercury-independent MerR activator (MerRAC): transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion. *EMBO Journal* 12: 413-421.

Parkhill, J., Lawley, B., Hobman, J.L., and Brown, N.L. (1998) Selection and characterization of mercury-independent activation mutants of the Tn*501* transcriptional regulator, MerR. *Microbiology* 144 (Pt 10): 2855-2864.

Patzer, S.I., and Hantke, K. (2000) The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. *Journal of Biological Chemistry* 275: 24321-24332.

Patzer, S.I., and Hantke, K. (2001) Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. *Journal of Bacteriology* 183: 4806-4813.

Paulsen, I.T., and Saier, M.H., Jr. (1997) A novel family of ubiquitous heavy metal ion transport proteins. *Journal of Membrane Biology* 156: 99-103.

Rapisarda, V.A., Montelongo, L.R., Farias, R.N., and Massa, E.M. (1999) Characterization of an NADH-linked cupric reductase activity from the *Escherichia coli* respiratory chain. *Archives of Biochemistry and Biophysics* 370: 143-150.

Reitzer, L., and Schneider, B.L. (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. *Microbiology and Molecular Biology Reviews* 65: 422-444.

Rensing, C., Fan, B., Sharma, R., Mitra, B., and Rosen, B.P. (2000) CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proceedings of the National Academy of Science, U.S.A.* 97: 652-656.

Rensing, C., Ghosh, M., and Rosen, B.P. (1999) Families of soft-metal-ion-transporting ATPases. *Journal of Bacteriology* 181: 5891-5897.

Rensing, C., Mitra, B., and Rosen, B.P. (1997a) The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-typre ATPase. *Proceedings of the National Academy of Science, U.S.A.* 94: 14326-14331.

Rensing, C., Pribyl, T., and Nies, D.H. (1997b) New functions for the three subunits of the Czc CBA cation-proton antiporter. *Journal of Bacteriology* 179: 6871-6879.

Rensing, C., Mitra, B., and Rosen, B.P. (1998) A Zn(II)-translocating P-type ATPase from *Proteus mirabilis. Biochemistry and Cellular Biology* 76: 787-790.

Roberts, J.W. (1969) Termination factor for RNA synthesis. *Nature* 224: 1168-1174.

Roderick, S.L., and Matthews, B.W. (1993) Structure of the cobalt-dependent methionine aminopeptidase from *Escherichia coli*: a new type of proteolytic enzyme. *Biochemistry* 32: 3907-3912.

Rogowsky, P.M., Close, T.J., Chimera, J.A., Shaw, J.J., and Kado, C.I. (1987) Regulation of *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *Journal of Bacteriology* 169: 5101-5112.

Ross, W., Park, S.J., and Summers, A.O. (1989) Genetic analysis of transcriptional activation and repression in the Tn*21 mer* operon. *Journal of Bacteriology* 171: 4009-4018.

Rouch, D.A., Lee, B.T.O., and Morby, A.P. (1995) Understanding cellular rersponses to toxic agents : a model for mechanism-choice in bacterial metal resistance. *Journal of Industrial Microbiology* 14: 132-141.

Rutherford, J.C., Cavet, J.S., and Robinson, N.J. (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. *Journal of Biological Chemistry* 274: 25827-25832.

Sadowsky, M.J., Cregan, P.B., Gottfert, M., Sharma, A., Gerhold, D., Rodriguez-Quinones, F., Keyser, H.H., Hennecke, H., and Stacey, G. (1991) The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans. *Proceedings Of the National Academy Of Science, USA* 88: 637-641.

Salehizadeh, H., and Shojaosadati, S.A. (2003) Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. *Water Research* 37: 4231-4235.

Samartzidou, H., and Delcour, A.H. (1998) *E. coli* PhoE porin has an opposite voltage-dependence to the homologous OmpF. *EMBO Journal* 17: 93-100.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning : A laboratory manual*: Cold Spring Harbour Laboratory Press.

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

Schneider, E., and Hunke, S. (1998) ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiology Reviews* 22: 1-20.

Schneider, E., Wilken, S., and Schmid, R. (1994) Nucleotide-induced conformational changes of MalK, a bacterial ATP binding cassette transporter protein. *Journal of Biological Chemistry* 269: 20456-20461.

Schulz, H., Fabianek, R.A., Pellicioli, E.C., Hennecke, H., and Thony-Meyer, L. (1999) Heme transfer to the heme chaperone CcmE during cytochrome c maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB. *Proceedings Of the National Academy Of Science, USA* 96: 6462-6467.

Scott, J.A., and Palmer, S.J. (1990) Sites of cadmium uptake in bacteria used for biosorption. *Applied Microbiology and Biotechnology* 33: 221-225.

Serres, M.H., Goswami, S., and Riley, M. (2004) GenProtEC: an updated and improved analysis of functions of *Escherichia coli* K-12 proteins. *Nucleic Acids Research* 32 Database issue: D300-302.

Shewchuk, L.M., Helmann, J.D., Ross, W., Park, S.J., Summers, A.O., and Walsh, C.T. (1989a) Transcriptional switching by the MerR protein: activation and repression mutants implicate distinct DNA and mercury(II) binding domains. *Biochemistry* 28: 2340-2344.

Shewchuk, L.M., Verdine, G.L., and Walsh, C.T. (1989c) Transcriptional switching by the metalloregulatory MerR protein: initial characterization of DNA and mercury (II) binding activities. *Biochemistry* 28: 2331-2339.

Shewchuk, L.M., Verdine, G.L., Nash, H., and Walsh, C.T. (1989b) Mutagenesis of the cysteines in the metalloregulatory protein MerR indicates that a metal-bridged dimer activates transcription. *Biochemistry* 28: 6140-6145.

Shi, W., Wu, J., and Rosen, B.P. (1994) Identification of a putative metal binding site in a new family of metalloregulatory proteins. *Journal of Biological Chemistry* 269: 19826-19829.

Silver, S. (1969) Active transport of magnesium in *Escherichia coli*. *Proceedings of the National Academy of Science, U.S.A.* 62: 764-771.

Silver, S., and Phung, L.T. (1996) Bacterial heavy metal resistance: New surprises. *Annual Review of Microbiology* 50: 753-789.

Smith, R.L., and Maguire, M.E. (1995) Distribution of the CorA Mg^{2+} transport system in gram-negative bacteria. *Journal of Bacteriology* 177: 1638-1640.

Sofia, H.J., Burland, V., Daniels, D.L., Plunkett III, G., and Blattner, F.R. (1994) Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 - 81.5 min. *Nucleic Acids Research* 22: 2576-2586.

Solioz, M., and Vulpe, C. (1996) CPx-type ATPases: A class of P-type ATPases that pump heavy metals. *Trends in Biochemical Sciences* 21: 237-241.

Stanisich, V.A., Bennett, P.M., and Richmond, M.H. (1977) Characterization of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 129: 1227-1233.

Staudenmaier, H., Van Hove, B., Yaraghi, Z., and Braun, V. (1989) Nucleotide sequences of the *fecBCDE* genes and locations of the proteins suggest a periplasmic-binding-protein-dependent transport mechanism for iron(III) dicitrate in *Escherichia coli*. *Journal of Bacteriology* 171: 2626-2633.

Stoyanov, J.V., and Brown, N.L. (2003) The *Escherichia coli* copper-responsive *copA* promoter is activated by gold. *Journal of Biological Chemistry* 278: 1407-1410.

Stoyanov, J.V., Hobman, J.L., and Brown, N.L. (2001) CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Molecular Microbiology* 39: 502-511.

Studholme, D.J., and Dixon, R. (2003) Domain architectures of sigma54-dependent transcriptional activators. *Journal of Bacteriology* 185: 1757-1767.

Summers, A.O., and Silver, S. (1978) Microbial transformations of metals. *Annual Reviews in Microbiology* 32: 637-672.

Szoke, P.A., Allen, T.L., and deHaseth, P.L. (1987) Promoter recognition by *Escherichia coli* RNA polymerase: effects of base substitutions in the -10 and -35 regions. *Biochemistry* 26: 6188-6194.

Tao, T., Snavely, M.D., Farr, S.G., and Maguire, M.E. (1995) Magnesium transport in *Salmonella typhimurium*: *mgtA* encodes a P-type ATPase and is regulated by Mg^{2+} in a manner similar to that of the *mgtB* P-type ATPase. *Journal of Bacteriology* 177: 2654-2662.

Taylor, D.E. (1999) Bacterial tellurite resistance. *Trends in Microbiology* 7: 111-115.

Travers, A.A., and Burgessrr (1969) Cyclic re-use of the RNA polymerase sigma factor. *Nature* 222: 537-540.

Tsaneva, I.R., and Weiss, B. (1990) *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *Journal of Bacteriology* 172: 4197-4205.

Turner, R.J., Weiner, J.H., and Taylor, D.E. (1995) The tellurite-resistance determinants *tehA tehB* and *klaA klaB telB* have different biochemical requirements. *Microbiology* 141 (Pt 12): 3133-3140.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings Of the National Academy Of Science, USA* 98: 5116-5121.

Vallee, B.L., and Auld, D.S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29: 5647-5659.

van Veen, H.W., Abee, T., Kortstee, G.J., Konings, W.N., and Zehnder, A.J. (1994) Translocation of metal phosphate via the phosphate inorganic transport system of *Escherichia coli*. *Biochemistry* 33: 1766-1770.

Weber, A., and Jung, K. (2002) Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. *Journal of Bacteriology* 184: 5502-5507.

Wu, F.Y., Huang, W.J., Sinclair, R.B., and Powers, L. (1992) The structure of the zinc sites of *Escherichia coli* DNA-dependent RNA polymerase. *Journal of Biological Chemistry* 267: 25560-25567.

Youderian, P., Bouvier, S., and Susskind, M.M. (1982) Sequence determinants of promoter activity. *Cell* 30: 843-853

Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T.M., Chiancone, E., and Chasteen, N.D. (2002) Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *Journal of Biological Chemistry* 277: 27689-27696.

Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *Journal of Bacteriology* 183: 4562-4570.

APPENDIX I

Appendix I

Table 1 Complete list of genes for which transcript levels were altered in strain TG1 (w.t.) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (w.t.) to transcript levels for untreated TG1 (w.t.). Data has been presented according to increasing Blattner numbers (Gene ID). Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array_Info.xls

Gene	Gene ID	Function	Fold Change
yaaF	b0030	Unknown	2.73
yabH	b0055	Chaperone	7.11
yabI	b0065	Unknown	3.89
leuC	b0072	Amino acid biosynthesis: Leucine	-2.05
leuB	b0073	Amino acid biosynthesis: Leucine	-1.79
b0100 (f44)	b0100	Unknown	3.67
nadC	b0109	Biosynthesis of cofactors, carriers: Pyridine nucleotide	3.56
yacH	b0117	Unknown	5.83
yacL	b0119	Unknown	5.00
yadE	b0130	Unknown	5.93
hemL	b0154	Biosynthesis of cofactors, carriers: Heme, porphyrin	3.17
yafB	b0207	Degradation of carbohydrates	2.75
afuC	b0262	Transporter (putative): Ferric transport	2.71
yagF	b0296	Ribosomal protein (putative)	2.78
yahB	b0316	Unknown	7.60
mhpC	b0349	Degradation of small molecules: Carbon compounds	3.90
ybaO	b0447	Unknown	2.99
amtB	b0451	Central intermediary metabolism: Pool, multipurpose conversions	2.99
ybbK	b0489	Unknown	-2.70
ybbP	b0496	Unknown	5.64
ybcO	b0549	Unknown	2.67
ybcT	b0556	Phage-related functions and prophages	8.21
ybdJ	b0580	Unknown	3.73
ybdK	b0581	Unknown	3.02
entF	b0586	Biosynthesis of cofactors, carriers: Enterochelin	7.24
entA	b0596	Biosynthesis of cofactors, carriers: Enterochelin	3.96
ybdB	b0597	Unknown	3.05
cstA	b0598	Global regulatory functions	3.06
b0609 (o153)	b0609	Unknown	2.83
citG	b0613	Unknown	2.73
citF	b0615	Central intermediary metabolism: Pool, multipurpose conversions	10.57
ybeG	b0622	Unknown	2.79
ybeH	b0625	Unknown	3.00
lipA	b0628	Biosynthesis of cofactors, carriers: Lipoate	4.82
lipB	b0630	Biosynthesis of cofactors, carriers: Lipoate	2.78
ybeB	b0637	Unknown	3.02
ybeU	b0648	Unknown	-2.24
gltK	b0653	Transport of small molecules: Amino acids, amines	5.74
b0671 (o50)	b0671	Unknown	8.65
clpA	b0882	Degradation of proteins, peptides, glycopeptides	3.56
mukF	b0922	Cell killing	3.63
ompA	b0957	Outer membrane constituents	-2.26
wrbA	b1004	Amino acid biosynthesis: Tryptophan	2.69
rimJ	b1066	Ribosomes - maturation and modification	2.99
rne	b1084	Degradation of RNA	-2.26
fabD	b1092	Fatty acid and phosphatidic acid biosynthesis	-2.58
ycfF	b1103	Unknown	3.06
ycfR	b1112	Unknown	3.07
ymfA	b1122	Unknown	3.20
pepT	b1127	Degradation of proteins, peptides, glycopeptides	2.88
ymfP	b1152	Unknown	3.18
ymgA	b1165	Unknown	3.94
b1170 (o338)	b1170	Unknown	3.96

ymgD	b1171	Unknown	5.11
ycgN	b1181	Unknown	4.51
hlyE	b1182	Cell killing	3.94
yciQ	b1268	Unknown	2.80
ycjN	b1310	Transporter (putative), ABC-type	2.31
pgmB	b1317	Unknown	3.50
ycjV	b1318	Transporter (putative): sugars	4.45
ompG	b1319	Outer membrane constituents: pore protein	3.20
ycjF	b1322	Unknown	3.31
ydaA	b1333	Unknown	3.44
abgB	b1337	Unknown	3.07
abgA	b1338	Degradation of carbohydrates	3.00
b1341 (f430)	b1341	Unknown	3.06
ydaQ	b1346	Unknown	3.02
ydbL	b1383	Unknown	4.07
paaZ	b1387	Degradation of phenyl acetic acid	4.09
paaA	b1388	Unknown	2.28
paaI	b1396	Unknown	4.33
paaK	b1398	Unknown	2.86
paaX	b1399	Unknown	3.01
paaY	b1400	Degradation of phenyl acetic acid	3.26
b1408 (o203)	b1408	Unknown	2.97
b1410 (o585)	b1410	Unknown	4.68
ynbD	b1411	Enzyme (putative): phosphatase	5.00
ydcF	b1414	Unknown	3.32
ydcA	b1419	Unknown	4.44
b1420 (f55)	b1420	Unknown	3.97
b1431 (o222)	b1431	Unknown	4.23
ydcW	b1444	Enzyme (putative): aldehyde dehydrogenase	2.78
b1522 (f315)	b1522	Unknown	6.98
yneH	b1524	Degradation of small molecules (putative): Amino acids	3.13
cspF	b1558	Stress adaptation	4.56
ydfX	b1568	Unknown	3.50
ynfB	b1583	Unknown	2.84
ynfK	b1593	Unknown	3.39
ydgF	b1600	Transport protein: multidrug	6.19
b1601 (o344)	b1601	Transporter (putative)	4.89
ydgH	b1604	Unknown	3.77
slyA	b1642	Adaptations, atypical conditions	4.08
sodC	b1646	Detoxification	-2.66
phsC	b1670	Unknown	3.18
yhdV	b1673	Unknown	3.31
sufA	b1684	Unknown	4.52
ydiK	b1688	Unknown	2.91
ydiA	b1703	Unknown	2.70
ydiE	b1705	Unknown	3.50
pheS	b1714	Aminoacyl tRNA synthetases, tRNA modification	4.58
rpmI	b1717	Ribosomal proteins - synthesis, modification	4.75
ymiA	b1725	Unknown	3.43
cho	b1741	DNA repair	3.64
astE	b1744	Unknown	3.18
ydjZ	b1752	Unknown	3.01
ynjB	b1754	Unknown	3.21
ynjC	b1755	Transporter (putative), ABC-type	2.99
ansA	b1767	Degradation of small molecules: Amino acids	3.16
ydjE	b1769	Transporter (putative)	3.81
ydjG	b1771	Unknown	2.63
yeaH	b1784	Unknown	3.50
yeaI	b1785	Unknown	3.51
yeaJ	b1786	Unknown	4.54
yeaN	b1791	Transporter (putative)	3.70
yeaQ	b1795	Unknown	5.01
fadD	b1805	Degradation of small molecules: Fatty acids	3.49
aspS	b1866	Aminoacyl tRNA synthetases, tRNA modification	4.69
pgsA	b1912	Macromolecule synthesis, modification: Phospholipids	2.96
yedS	b1964	Outer membrane constituents: pore protein	3.56
yeeO	b1985	Unknown	3.58
yi22_3	b1996	Transposon-related functions	6.68
yeeZ	b2016	Degradation of carbohydrates	2.70
hisL	b2018	Amino acid biosynthesis: Histidine	4.09
hisG	b2019	Amino acid biosynthesis: Histidine	3.71
hisC	b2021	Amino acid biosynthesis: Histidine	2.73

hisH	b2023	Amino acid biosynthesis: Histidine	3.79
ugd	b2028	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.62
wzxC	b2046	Protein, peptide secretion	6.30
wcfC	b2051	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	3.26
wcfB	b2052	Biosynthesis of extracellular polysaccharide colanic acid	2.43
wcaF	b2054	Biosynthesis of extracellular polysaccharide colanic acid	2.83
wcaE	b2055	Biosynthesis of extracellular polysaccharide colanic acid	2.90
wcaB	b2058	Biosynthesis of extracellular polysaccharide colanic acid	3.22
wcgA	b2064	Outer membrane constituents: lipopolysaccharide biosynthesis	7.32
b2071 (o153)	b2071	Unknown	2.79
b2081 (o453)	b2081	Unknown	5.87
wcgX	b2102	Unknown	3.32
thiD	b2103	Biosynthesis of cofactors, carriers: Thiamin	3.76
yohM	b2106	Unknown	3.31
moiR_3	b2117	Biosynthesis of cofactors, carriers: Molybdopterin	5.48
mirA	b2127	Biosynthesis of extracellular matrix and curli: regulator (MerR)	3.98
yohF	b2137	Enzyme (putative): oxidoreductase	2.74
yeiK	b2162	Unknown	4.16
yeiP	b2171	Unknown	-3.04
rtn	b2176	Unknown	3.02
yejB	b2178	Transporter (putative), ABC-type: Oligopeptides	3.87
yejG	b2181	Unknown	3.19
yojF	b2207	Unknown	3.92
yojH	b2210	Unknown	2.73
yojL	b2214	Unknown	3.05
atoA	b2222	Degradation of small molecules: Fatty acids	3.74
atoB	b2223	Degradation of small molecules: Fatty acids	3.10
b2224 (o394)	b2224	Degradation of small molecules: Fatty acids	3.19
nrdA	b2234	2'-Deoxyribonucleotide metabolism	6.66
yfaW	b2247	Enzyme (putative): dehydratase	4.50
b2249 (f400)	b2249	Unknown	2.80
yfaO	b2251	Unknown	4.00
b2256 (o296)	b2256	Unknown	4.91
arnT	b2257	Unknown	6.58
nuoI	b2281	Energy metabolism, carbon: Aerobic respiration	-1.86
pdxB	b2320	Biosynthesis of cofactors, carriers: Pyridoxine	-2.63
dsdC	b2364	Degradation of small molecules: Amino acids	6.58
b2375 (f211)	b2375	Unknown	2.90
cysM	b2421	Amino acid biosynthesis: Cysteine	2.94
yfeT	b2427	Unknown	2.94
b2444 (o81)	b2444	Unknown	5.37
ypfG	b2466	Unknown	3.02
yffG	b2468	Enzyme (putative): oxidoreductase	5.73
glnB	b2553	Amino acid biosynthesis: Glutamine	5.06
era	b2566	Global regulatory functions	2.38
yfiC	b2575	Enzyme (putative): methyltransferase	2.97
b2596 (o68)	b2596	Unknown	4.01
yfiN	b2604	Unknown	3.72
yfiB	b2615	Unknown	3.00
yfiR	b2634	Unknown	3.19
yfjT	b2637	Unknown	2.68
yfjU	b2638	Unknown	3.57
b2650 (f160)	b2650	Unknown	3.26
b2658 (o90)	b2658	Unknown	4.85
ygaQ	b2689	Unknown	4.61
surE	b2744	Cell protection	2.68
ygbO	b2745	Enzyme (putative): dehydrogenase	2.68
ispD	b2747	Unknown	3.44
cysD	b2752	Central intermediary metabolism: Sulfur metabolism	9.89
b2755 (f305)	b2755	Unknown	3.04
ygcJ	b2758	Unknown	6.03
b2760 (f502)	b2760	Unknown	4.07
cysI	b2763	Central intermediary metabolism: Sulfur metabolism	5.42
chpA	b2782	Cell growth regulation	3.47
b2790 (f149)	b2790	Unknown	5.10
ppdA	b2826	Biosynthesis of macromolecules: fimbria, pili	4.15
yi21_4	b2861	Transposon-related functions	3.27
xerD	b2894	DNA - replication, repair, restriction/modification	-2.38
yggD	b2941	Unknown	7.67
yghU	b2989	Unknown	2.83
b3007 (f51)	b3007	Unknown	4.74
plsC	b3018	Macromolecule synthesis, modification: Phospholipids	2.77

ygiW	b3024	Unknown	5.22
ygiC	b3042	Unknown	4.04
ygjR	b3087	Unknown	3.63
ygjD	b3098	Unknown	2.84
yhaL	b3107	Unknown	4.08
yhaP	b3111	Degradation of amino acids: L-serine	4.16
yhaC	b3121	Unknown	3.01
yhaV	b3130	Unknown	3.61
yraL	b3146	Unknown	3.29
yraM	b3147	Unknown	-1.83
yraR	b3152	Unknown	3.51
yhbE	b3184	Unknown	3.03
yrbA	b3190	Unknown	3.44
yhcF	b3219	Unknown	4.73
yhcG	b3220	Unknown	3.24
yhcO	b3239	Unknown	4.30
b3254 (f33)	b3254	Unknown	6.55
yhdT	b3257	Unknown	5.68
yrdB	b3280	Unknown	3.52
rplV	b3315	Ribosomal proteins - synthesis, modification	-2.40
yheD	b3323	Transporter (putative): protein export	4.75
kefB	b3350	Transport of small molecules: K(I) / H(I) antiporter	3.07
yrfC	b3394	Unknown	3.08
yhgJ	b3419	Unknown	4.03
yhgN	b3434	Unknown	3.80
zntA (o732)	b3469	Transport of small molecules: Zn(II), Cd(II), Pb(II)	4.23
yhjW	b3546	Unknown	3.97
yhjY	b3548	Enzyme (putative): lipase	3.55
yicI	b3656	Unknown	4.55
yicM	b3662	Transporter (putative)	4.21
ivbL	b3672	Amino acid biosynthesis: Isoleucine, Valine	4.32
yidG	b3675	Unknown	3.60
glvG	b3681	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.53
yidR	b3689	Unknown	2.37
yieF	b3713	Unknown	2.59
yieJ	b3717	Unknown	3.94
yieL	b3719	Degradation of carbohydrates (putative)	3.31
rbsR	b3753	Degradation of small molecules: Carbon compounds	-2.80
pssR	b3763	Macromolecule synthesis, modification: Phospholipids	2.87
yigA	b3810	Unknown	2.90
b3814 (f99)	b3814	Unknown	3.18
yigE	b3815	Unknown	2.55
yigL	b3826	Unknown	3.42
yigM	b3827	Unknown	3.39
tatA	b3836	Unknown	4.33
tatC	b3839	Unknown	3.37
tatD	b3840	Unknown	4.03
yigW 2 (o113)	b3841	Unknown	4.04
yigC	b3843	Biosynthesis of cofactors, carriers: Menaquinone	2.58
fadA	b3845	Degradation of small molecules: Fatty acids	2.50
mobB	b3856	Central intermediary metabolism: Nucleotide interconversions	3.57
yihF	b3861	Unknown	3.15
yihA	b3865	Unknown	3.23
yihL	b3872	Unknown	2.91
yiiE	b3889	Unknown	4.92
yiiF	b3890	Unknown	6.98
yiiQ	b3920	Unknown	4.77
ppc	b3956	Energy metabolism, carbon: Fermentation	-1.72
yjbJ	b4045	Unknown	2.77
zur	b4046	Transport of Zn(II): high-affinity Zn(II) import, repressor	3.08
yjbO	b4050	Unknown	4.20
yjcD	b4064	Unknown	2.96
phnQ	b4091	Unknown	3.13
phnN	b4094	Central intermediary metabolism: Phosphorus compounds	3.30
phnM	b4095	Central intermediary metabolism: Phosphorus compounds	4.17
phnK	b4097	Central intermediary metabolism: Phosphorus compounds	4.03
phnF	b4102	Unknown	3.65
yjeH	b4141	Transporter (putative)	4.99
yjfR	b4192	Unknown	3.64
ytfB	b4206	Unknown	4.31
ytfI	b4215	Unknown	12.91
chpS	b4224	Cell growth regulation	4.87

yjgX	b4275	Unknown	3.86
yjgZ	b4277	Unknown	2.51
yjhD	b4281	Unknown	4.01
b4286 (o137)	b4286	Unknown	3.63
yjhH	b4298	Phage-related functions and prophages	3.24
yjhO	b4305	Phage-related functions and prophages	3.29
yjiC	b4325	Unknown	3.00
yjiD	b4326	Unknown	2.46
yjiG	b4329	Unknown	2.80
yjiH	b4330	Unknown	3.01
yjiN	b4336	Unknown	2.78
yjiP	b4338	Unknown	6.16
yjiS	b4341	Unknown	2.99
yjiW	b4347	Unknown	4.61
yjiX	b4353	Unknown	4.17
yjiS	b4367	Unknown	4.06
yjiX	b4394	Unknown	2.59
arcA	b4401	Global regulatory functions	-2.32

Appendix I

Table 2 Complete list of genes for which transcript levels were altered in strain TG1 (*zntR* ::*Km*) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (*zntR* ::*Km*) strain to transcript levels for untreated TG1 (*zntR* ::*Km*) strain. Down-regulated genes are indicated by a minus sign (-) in the 'Fold change' column. Data has been presented according to increasing Blattner numbers (Gene ID). Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array_Info.xls

Gene	Gene ID	Function	Fold Change
thrB	b0003	Amino acid biosynthesis: Threonine	2.92
yaaJ	b0007	Transport of small molecules: Amino acids, amines	3.50
yaaD	b0028	Proteins - translation and modification	3.79
caiF	b0034	Central intermediary metabolism: Pool, multipurpose conversions	6.83
leuC	b0072	Amino acid biosynthesis: Leucine	2.27
yadG	b0127	Transporter (putative): ABC-type, ATP-binding component	3.48
fhuA	b0150	Outer membrane constituents: ferric uptake	2.90
yadS	b0157	Unknown	3.33
glnD	b0167	Amino acid biosynthesis: Glutamine	4.52
cdsA	b0175	Fatty acid and phosphatidic acid biosynthesis	2.68
phoE	b0241	Outer membrane constituents: pore protein; phosphate uptake	3.56
ykfA	b0253	Phage-related functions and prophages	3.21
afuB	b0263	Transporter (putative): membrane component of ferric transport system	2.53
argF	b0273	Amino acid biosynthesis: Arginine	2.65
b0299 (o288)	b0299	Transposon-related functions	3.88
yahO	b0329	Unknown	2.85
mhpR	b0346	Degradation of fatty acids	3.75
tauC	b0367	Transport of small molecules: Amino acids, amines	4.27
psiF	b0384	Central intermediary metabolism: Phosphorus compounds	2.47
tgt	b0406	Aminoacyl tRNA synthetases, tRNA modification	4.64
secF	b0409	Protein, peptide secretion	-2.27
tsx	b0411	Transport of small molecules: Nucleosides, purines, pyrimidines	-1.89
bolA	b0435	Murein sacculus, peptidoglycan	3.15
ybaX	b0444	Unknown	9.90
maa	b0459	Degradation of carbohydrates	2.70
ybaN	b0468	Phage-related functions and prophages	5.09
cueR	b0487	Transport of Cu(II) : Transcriptional regulator of Cu(II)-exporting ATPase	2.65
ybbM	b0491	Transporter (putative): metal resistance	4.33
ybbD	b0500	Unknown	3.82
allP	b0511	Transporter (putative): allantoin transport	2.90
ybcG	b0534	Pilin-like protein (putative)	4.59
b0540 (o102)	b0540	Unknown	3.51
tra5_2	b0541	Transposon-related functions	3.67
ybcY	b0562	Unknown	2.66
ybeK	b0651	Not classified	-2.55
gltJ	b0654	Transport of small molecules: Amino acids, amines	4.77
ybgP	b0717	Pilin chaperone, periplasmic (putative)	2.88
hrsA	b0731	Proteins - translation and modification	-1.95
ybhA	b0766	Enzyme (putative): phosphatase	3.90
moaB	b0782	Biosynthesis of cofactors, carriers: Molybdopterin	3.13
dps	b0812	Global regulatory functions, iron storage	3.34
ybiT	b0820	Transporter (putative): ABC-type, ATP-binding component	3.36
potH	b0856	Transport of small molecules: Amino acids, amines	3.90
ybjR	b0867	Enzyme (putative): amidase	2.57
dmsC	b0896	Energy metabolism, carbon: Anaerobic respiration- dimethyl sulphoxide	3.13
ycaC	b0897	Unknown	3.32
pqiA	b0950	Detoxification: paraquat inducible protein	4.66
pqiB	b0951	Detoxification: paraquat inducible protein	3.48
ycbG	b0956	Unknown	2.80
hyaD	b0975	Energy metabolism, carbon: Aerobic respiration	2.55

cspH	b0989	Stress adaptation - cold shock	4.12
cbpA	b1000	Chaperones	3.09
tra5_3	b1026	Transposon-related functions	3.67
ycdU	b1029	Unknown	7.32
yceK	b1050	Unknown	3.21
msyB	b1051	Protein, peptide secretion	6.49
yceI	b1056	Unknown	4.02
yceB	b1063	Unknown	2.91
grxB	b1064	Biosynthesis of cofactors, carriers: Thioredoxin, glutaredoxin, glutathione	3.22
flgM	b1071	Surface structures: biosynthesis of flagella	2.41
flgI	b1080	Surface structures: biosynthesis of flagella	4.21
ycfL	b1104	Unknown	3.03
ycfT	b1115	Unknown	2.47
lit	b1139	Phage-related functions and prophages	5.26
b1145 (f224)	b1145	Phage-related functions and prophages	3.27
treA	b1197	Stress adaptation - osmotic shock	2.94
prfA	b1211	Proteins - translation and modification	2.68
b1213 (o130)	b1213	Unknown	2.28
ychA	b1214	Unknown	4.00
narJ	b1226	Energy metabolism, carbon: Anaerobic respiration- nitrate reductase	2.52
yciU	b1248	Unknown	3.30
yciI	b1251	Unknown	3.73
yciF	b1258	Unknown	3.71
yciG	b1259	Unknown	3.08
trpC	b1262	Amino acid biosynthesis: Tryptophan	-2.25
yciK	b1271	Unknown	5.10
sohB	b1272	Degradation of proteins, peptides, glycopeptides	4.40
pgpB	b1278	Macromolecule synthesis, modification: Phospholipids	2.43
yciS	b1279	Unknown	4.17
yciH	b1282	Unknown	6.64
yciW	b1287	Unknown	3.40
sapB	b1293	Transport of proteins, peptides	2.94
aldH	b1300	Energy metabolism, carbon: Fermentation- aldehyde dehydrogenase	2.37
pspA	b1304	Stress adaptation - osmotic shock: *psp* operon	4.49
pspB	b1305	Stress adaptation - osmotic shock: *psp* operon	2.69
pspE	b1308	Stress adaptation - osmotic shock: *psp* operon	2.82
ycjT	b1316	Unknown	3.46
rarC	b1356	Unknown	5.22
b1369 (o51)	b1369	Unknown	2.70
ynaC	b1373	Unknown	10.08
ompN	b1377	Outer membrane constituents: pore protein, non-specific	3.00
gapC_1	b1417	Energy metabolism, carbon: Glycolysis	2.62
ydcI	b1422	Unknown	2.19
b1425 (f67)	b1425	Unknown	2.93
tehB	b1430	Transporter: tellurite resistance	3.66
ydcQ	b1438	Unknown	2.52
ydcX	b1445	Unknown	3.12
yncC	b1450	Unknown	3.18
yncH	b1455	Unknown	4.66
yddH	b1462	Unknown	4.19
ddpC	b1485	Transporter (putative): dipeptide transport, ABC-type	4.40
ddpX	b1488	Unknown	3.25
yddC	b1494	Biosynthesis of cofactors, carriers: Ubiquinone	2.75
ydeS	b1504	Pilin-like protein (putative)	8.43
b1506 (f59)	b1506	Unknown	3.83
ydeU	b1509	Unknown	11.64
b1527 (o371)	b1527	Unknown	4.33
ydeD	b1533	Unknown	4.28
ydeI	b1536	Unknown	3.67
ydfK	b1544	Unknown	4.27
gnsB	b1550	Unknown	4.64
ynfN	b1551	Unknown	11.96
cspI	b1552	Stress adaptation - cold shock	3.75
ydfT	b1559	Unknown	7.84
relE	b1563	Unknown	4.97
ftxA	b1566	Unknown	4.10
ydjN	b1629	Enzyme (putative): oxidoreductase	4.57
ydgO	b1630	Unknown	2.91
ydgP	b1631	Unknown	2.61
ydhL	b1648	Unknown	2.57
cfa	b1661	Fatty acid and phosphatidic acid biosynthesis	2.40

sufE	b1679	Unknown	3.96
sufB	b1683	Unknown	3.13
b1685 (f89)	b1685	Unknown	3.08
ydiV	b1707	Unknown	2.96
infC	b1718	Proteins - translation and modification	4.68
yniC	b1727	Unknown	11.93
ydjX	b1750	Unknown	4.38
yoaG	b1796	Unknown	2.85
yeaR	b1797	Unknown	5.35
yeaZ	b1807	Unknown	4.09
b1810 (o119)	b1810	Unknown	3.70
yobG	b1826	Unknown	5.37
yebR	b1832	Unknown	4.11
b1836 (o83)	b1836	Unknown	3.72
b1843 (o218)	b1843	Unknown	4.26
yebF	b1847	Unknown	2.76
znuC	b1858	Transport of Zn(II): high-affinity Zn(II) import	3.59
znuB	b1859	Transport of Zn(II): high-affinity Zn(II) import	2.59
yecM	b1875	Unknown	2.92
yecT	b1877	Unknown	2.80
yecC	b1918	Transport of small molecules (putative): Amino acids, amines	3.08
yedN	b1934	Unknown	3.77
yedM	b1935	Unknown	2.58
fliE	b1937	Surface structures: biosynthesis of flagella	2.85
yodD	b1953	Unknown	3.51
yedA	b1959	Unknown	3.52
flu	b2000	Outer membrane constituents: fluffing protein	3.14
yeeV	b2005	Unknown	3.46
wzzB	b2027	Outer membrane constituents: modification of polyliposaccharide chains	3.29
wcaK	b2045	Biosynthesis of extracellular polysaccharide colanic acid	2.83
yegD	b2069	Stress adaptation (putative)	2.81
mdtC	b2076	Unknown	3.62
baeS	b2078	RNA synthesis, modification, DNA transcription	3.14
yegP	b2080	Unknown	3.09
b2084 (f88)	b2084	Unknown	5.92
tra5_4	b2089	Transposon-related functions	2.54
fbaB	b2097	Unknown	3.62
yegU	b2099	Unknown	2.71
yohL	b2105	Unknown	2.61
yohC	b2135	Unknown	3.65
dsbE	b2195	Energy metabolism, carbon: Electron transport	5.09
rcsC	b2218	Cell division: capsule biosynthesis	5.72
b2227 (f161)	b2227	Unknown	8.39
yfbE	b2253	Enzyme (putative): aminotransferase	3.34
yfbJ	b2258	Unknown	2.54
yfbM	b2272	Unknown	3.49
yfbN	b2273	Unknown	3.65
yfcE	b2300	Unknown	5.70
b2334 (f162)	b2334	Unknown	3.86
b2345 (o352)	b2345	Unknown	7.02
yfdC	b2347	Transporter (putative)	2.78
b2350 (o120)	b2350	Unknown	8.09
crr	b2417	Transport of small molecules: Carbohydrates, organic acids, alcohols	5.60
b2432 (f191)	b2432	Unknown	2.60
eutL	b2439	Unknown	2.75
mae B	b2463	Enzyme (putative): bifunctional oxidoreductase/phosphotransacetylase	-2.36
talA	b2464	Central intermediary metabolism: Non-oxidative branch, pentose pathway	3.70
hyfB	b2482	Energy metabolism, carbon: Anaerobic respiration	2.49
hyfF	b2486	Energy metabolism, carbon: Anaerobic respiration	2.36
iscU	b2529	Unknown	3.13
suhB	b2533	Global regulatory functions	3.89
yfhA	b2554	Unknown	3.03
purL	b2557	Purine ribonucleotide biosynthesis	2.91
yfhH	b2561	Unknown	3.56
ypjE	b2612	Unknown	3.63
b2649 (f263)	b2649	Unknown	2.90
b2680 (o88)	b2680	Unknown	3.05
emrR	b2684	Plasmid-related functions: repressor of multidrug resistance pump	2.70
hycI	b2717	Degradation of proteins, peptides, glycopeptides	4.46
b2790 (f149)	b2790	Unknown	-2.04
truC	b2791	Unknown	3.11

csdA	b2810	Unknown	3.78
ygdL	b2812	Unknown	6.15
ygdB	b2824	Unknown	3.57
aas	b2836	Fatty acid and phosphatidic acid biosynthesis	2.29
b2859 (f141)	b2859	Unknown	3.53
ygeP	b2862	Unknown	3.61
ygeV	b2869	Degradation of nucleotide triphosphates (putative)	3.01
ygqB	b2924	Stress adaptation (putative) - osmotic shock	3.24
cmtA	b2933	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.70
ygqG	b2936	Unknown	4.39
ygqJ	b2946	Unknown	3.85
yqiI	b3048	Unknown	2.36
yqiM	b3055	Unknown	2.81
yqiG	b3058	Biosynthesis of cofactors, carriers: Folic acid	4.06
yqjH	b3070	Unknown	5.88
yqjV	b3090	Unknown	3.40
exuT	b3093	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.82
yhaQ	b3112	Degradation of amino acids: L-serine	3.58
tdcB	b3117	Degradation of small molecules: Amino acids	-2.11
b3122 (o54)	b3122	Unknown	2.68
agaR	b3131	Central intermediary metabolism:Amino sugar conversions	2.64
yraP	b3150	Transporter (putative)	4.90
yhbO	b3153	Unknown	3.40
truB	b3166	Aminoacyl tRNA synthetases, tRNA modification	2.69
yrbG	b3196	Transporter (putative): Na(I)/Ca(I) antiporter	-3.35
gltD	b3213	Central intermediary metabolism: Pool, multipurpose conversions	2.98
yhdP	b3245	Degradaton of proteins, peptides, glycopeptides	-3.24
rplB	b3317	Ribosomal proteins - synthesis, modification	-2.90
gspE	b3326	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	2.56
gspL	b3333	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	3.38
gspM	b3334	Transporter (putative): proteins for pili biosynthesis - *gsp* operon	4.00
yheL	b3343	Unknown	3.43
yheU	b3354	Unknown	4.83
fic	b3361	Cell division: filamentation, induced in stationary phase	2.57
yhfC	b3364	Transporter (putative)	3.81
yhfU	b3378	Unknown	3.76
yhfY	b3382	Unknown	3.07
yhfZ	b3383	Unknown	4.79
mrcA	b3396	Murein sacculus, peptidoglycan	2.94
yhgF	b3407	Unknown	3.06
glpE	b3425	Energy metabolism, carbon: Anaerobic respiration	2.39
glgB	b3432	Macromolecule synthesis, modification: Polysaccharides - (cytoplasmic)	2.48
yhhY	b3441	Unknown	4.10
yhhZ	b3442	Unknown	2.68
yrhA	b3443	Unknown	3.99
ugpE	b3451	Transport of small molecules: Carbohydrates, organic acids, alcohols	2.71
ugpA	b3452	Transport of small molecules: Carbohydrates, organic acids, alcohols	3.46
livF	b3454	Transport of small molecules: Amino acids, amines	2.88
livM	b3456	Transport of small molecules: Amino acids, amines	2.59
yhhF	b3465	Unknown	2.95
gadA	b3517	Central intermediary metabolism: Pool, multipurpose conversions	5.80
xylR	b3569	Degradation of small molecules: Carbon compounds	3.41
rfaK	b3623	Macromolecule metabolism: Lipopolysaccharide	2.44
kdtB	b3634	Biosynthesis of cofactors, carriers: Coenzyme A	3.77
spoU	b3651	Aminoacyl tRNA methyltrasferase, tRNA modification	2.62
yicK	b3659	Transporter (putative): sugars	2.71
yidF	b3674	Unknown	2.72
yidI	b3677	Unknown	3.84
yieE	b3712	Unknown	2.52
pstA	b3726	Transport of small molecules: phosphate (phosphatase)	3.12
pstS	b3728	Transport of small molecules: phosphate (phosphate binding protein)	4.63
yifE	b3764	Unknown	2.71
ilvL	b3766	Amino acid biosynthesis: Isoleucine, Valine	3.59
rhlB	b3780	Not classified	-2.89
rfe	b3784	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.78
rffD	b3787	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	3.45
rffH	b3789	Central intermediary metabolism: Sugar-nucleotide biosynthesis, conversions	2.31
xerC	b3811	Cell division: chromosomal segregation	2.92
yigG	b3818	Unknown	4.41
glnG	b3868	Amino acid biosynthesis: Glutamine	2.54
yihW	b3884	Unknown	2.30

glpK	b3926	Central intermediary metabolism: Pool, multipurpose conversions	-2.35
yijE	b3943	Unknown	3.09
yijC	b3963	Unknown	3.01
yijD	b3964	Unknown	3.66
trmA	b3965	Aminoacyl tRNA synthetases, tRNA modification	5.61
nusG	b3982	RNA synthesis, modification, DNA transcription	5.02
rplL	b3986	Ribosomal proteins - synthesis, modification	3.70
yjaD	b3996	Unknown	3.06
hydH	b4003	Enzyme: kinase, Zn(II) sensor	7.99
yjaB	b4012	Unknown	3.60
yjbA	b4030	Unknown	2.41
yjbM	b4048	Unknown	2.83
yjbR	b4057	Unknown	3.53
yjcQ	b4081	Transporter (putative): Pit family	3.88
phnP	b4092	Central intermediary metabolism: Phosphorus compounds	2.53
phnB	b4107	Unknown	3.43
basR	b4113	RNA synthesis, modification, DNA transcription	3.39
yjdL	b4130	Transporter (putative): di-/ tripeptides	3.09
fxsA	b4140	Unknown	3.09
mopB	b4142	Chaperones	-1.54
ampC	b4150	Drug/analog sensitivity	5.11
frdC	b4152	Energy metabolism, carbon: Anaerobic respiration- fumarate reductase	2.48
yjeR	b4162	Unknown	3.44
yjfJ	b4182	Unknown	9.31
yjfK	b4183	Unknown	3.00
yjgA	b4234	Transporter (putative): ABC-type	2.89
yjgM	b4256	Unknown	4.68
fecC	b4289	Transport of small molecules: Fe(III)	4.71
fecB	b4290	Transport of small molecules: Fe(III)	2.81
yjhL	b4302	Phage-related functions and prophages	3.33
fimC	b4316	Surface structures: biosynthesis of pili	2.97
yjiO	b4337	Transporter: multidrug	2.46
mcrC	b4345	Degradation of DNA: methylation dependent endonuclease	2.90
mcrB	b4346	Degradation of DNA: methylation dependent endonuclease	8.55
dnaT	b4362	DNA replication: assembly of primosome	2.99
yjjT	b4371	Aminoacyl tRNA methylase, tRNA modification	3.52

Appendix I

Table 3 Complete list of genes for which transcript levels were altered in strains TG1 (w.t.) and TG1 (*zntR* ::*Km*) at 0.3 mM Zn(II). The fold-change levels represent the ratio of transcript levels for Zn(II)-treated TG1 (w.t.) and TG1 (*zntR* ::*Km*) strain to transcript levels for untreated TG1 (w.t.) and TG1 (*zntR* ::*Km*) strains, respectively. Data has been presented according to increasing Blattner numbers (Gene ID). Function descriptions were taken from http://www.sigma-genosys.com/media/Ecoli_Array_Info.xls

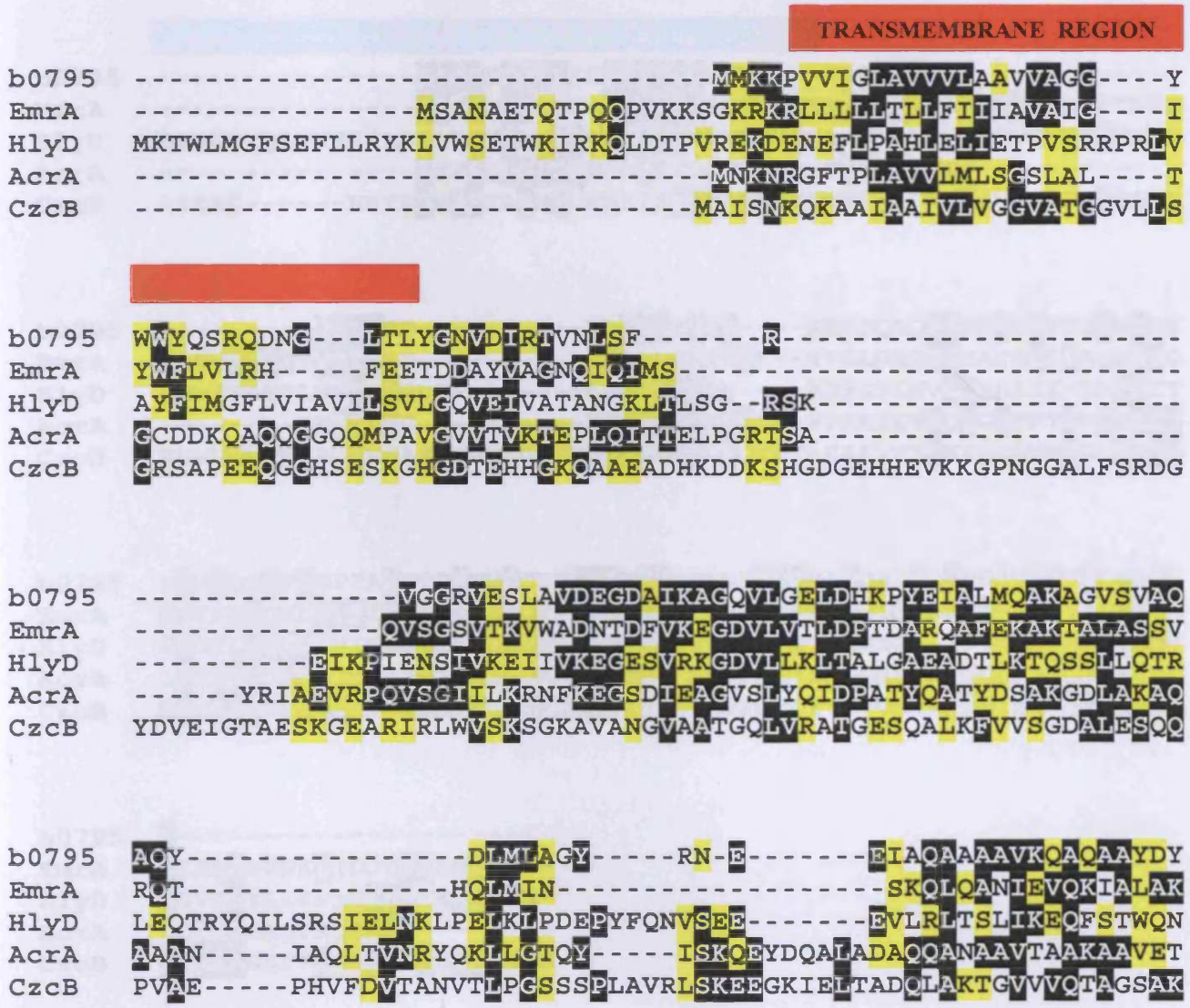
Gene	Gene ID	Function	Fold Change TG1	Fold change TG1 (*zntR* ::*Km*)
yadD	b0132	Unknown	5.44	3.05
yaiA	b0389	Unknown	6.15	3.55
aroM	b0390	Amino acid biosynthesis: Chorismate	2.50	3.12
ybcW	b0559	Unknown	4.32	2.87
cusF	b0573	Unknown	4.47	3.90
ycdF	b1005	Unknown	5.60	5.20
intE	b1140	Phage-related functions and prophages: integrase	4.26	19.21
ymfR	b1150	Unknown	3.52	4.06
ymfO	b1151	Unknown	2.92	2.58
ychG	b1239	Unknown	7.00	7.04
ycjL	b1269	Unknown	3.58	3.0
ynaJ	b1332	Unknown	3.56	4.79
ydaD	b1352	Unknown	3.52	3.61
ydaG	b1355	Unknown	4.95	4.16
racR	b1357	Unknown	4.69	3.40
ydaW	b1361	Unknown	4.38	3.57
ynaK	b1365	Unknown	5.61	6.04
ydaY	b1366	Unknown	6.46	4.13
b1367 (o77)	b1367	Unknown	7.15	4.82
nfnB	b1378	Enzyme (putative): oxidoreductase	3.32	3.56
ynbE	b1382	Unknown	7.11	7.07
ydcP	b1435	Enzyme (putative): collagenase	3.45	3.53
yncA	b1448	Drug/analog sensitivity	3.90	4.0
ydcD	b1457	Unknown	5.89	6.88
b1458 (o248)	b1458	Unknown	4.49	5.25
b1459 (o66)	b1459	Unknown	5.17	5.08
rpsV	b1480	Ribosomal proteins - synthesis, modification	3.34	4.57
yneI	b1525	Enzyme (putative): aldehyde dehydrogenase	2.98	2.34
ydeJ	b1537	Unknown	3.69	3.98
b1672 (f215)	b1672	Unknown	3.71	3.58
sufD	b1681	Unknown	3.39	3.25
ydiJ	b1687	Enzyme (putative): oxidase	4.24	3.22
ydiL	b1689	Unknown	3.72	2.96
ydiZ	b1724	Unknown	4.87	9.88
yniB	b1726	Unknown	4.00	2.83
ydjM	b1728	Unknown	4.49	2.97
ves	b1742	Unknown	5.43	5.07
spy	b1743	Spheroblast formation	5.73	3.64
astA	b1747	Unknown	4.27	2.85
ynjA	b1753	Unknown	4.70	3.38
yeaL	b1789	Unknown	4.15	3.5
yecH	b1906	Unknown	4.61	5.4
yeeE	b2013	Transport of small molecules: Amino acids, amines	6.65	4.45
wzxC	b2046	Protein, peptide secretion	6.30	7.25
mdtB	b2075	Unknown	5.04	5.26
yeqQ	b2081	Unknown	5.87	4.31
yeqW	b2101	Unknown	3.01	2.35
yehE	b2112	Unknown	3.71	2.88
metG	b2114	Aminoacyl tRNA synthetases, tRNA modification	3.36	3.5
yehU	b2126	Unknown	2.93	2.56
yehY	b2130	Transporter (putative): glycine, betaine, choline	7.08	3.66

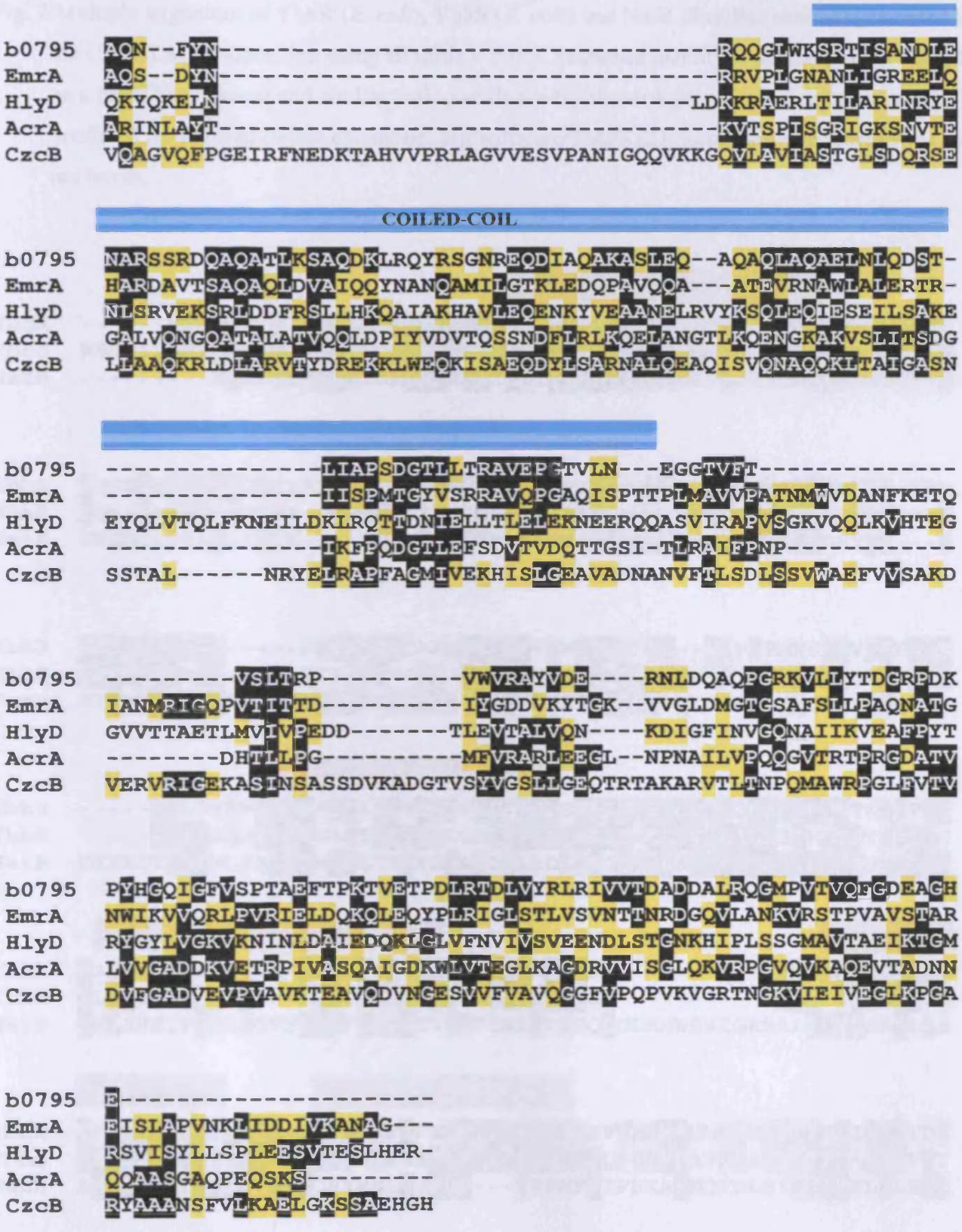
yeiA	b2147	Enzyme (putative): dehydrogenase	6.15	3.78
b2229 (f216)	b2229	Unknown	6.74	4.02
yfaA	b2230	Unknown	8.82	4.97
elaB	b2266	Unknown	4.11	4.42
cysK	b2414	Amino acid biosynthesis: Cysteine	8.34	4.79
yffO	b2446	Unknown	3.14	3.21
b2450 (o279)	b2450	Unknown	5.08	6.28
yfiK	b2578	Unknown	6.24	6.27
yfiP	b2583	Unknown	5.38	2.9
yfiL	b2602	Unknown	3.58	2.78
yfiR	b2603	Unknown	6.84	7.49
yfjG	b2619	Unknown	3.95	4.13
yfjM	b2629	Unknown	4.41	3.81
ypjK	b2635	Unknown	3.30	4.29
yfjZ	b2645	Unknown	3.12	3.17
ypjF	b2646	Unknown	4.07	3.74
b2656 (o62)	b2656	Unknown	5.03	4.74
b2659 (o360)	b2659	Unknown	6.22	4.06
ygaC	b2671	Unknown	5.34	3.4
ygaM	b2672	Unknown	5.32	3.39
yggC	b2928	Enzyme (putative): kinase	2.83	3.15
yghE	b2969	Unknown	4.37	3.73
zupT	b3040	Transport of Zn(II): low-affinity Zn(II) import	4.56	4.47
ebgA	b3076	Degradation of small molecules: Carbon compounds	3.85	3.55
yqjM	b3082	Unknown	3.71	3.86
yqjT	b3088	Unknown	4.90	5.04
yqjA	b3095	Unknown	3.87	3.34
yhaK	b3106	Unknown	4.74	4.65
yhbQ	b3155	Unknown	4.46	3.28
yhcD	b3216	Unknown	3.21	2.31
yhcE	b3217	Unknown	4.76	2.8
yhfL	b3369	Unknown	3.53	3.26
yhhL	b3466	Unknown	5.72	4.37
yhhP	b3470	Unknown	6.23	4.75
xylF	b3566	Transport of small molecules: Carbohydrates, organic acids	5.46	4.55
yibG	b3596	Unknown	7.65	4.92
yidC	b3705	Transport of proteins across inner membrane	4.59	3.38
yifA	b3762	Unknown	4.46	4.95
b3776 (f91)	b3776	Unknown	10.64	4.38
aslB	b3800	Unknown	10.36	11.46
yigF	b3817	Unknown	7.52	4.45
yigP	b3834	Unknown	3.96	2.93
yibD	b4023	Unknown	3.06	2.63
yjcB	b4060	Unknown	4.34	4.04
cysQ	b4214	Central intermediary metabolism: Sulfur metabolism	4.08	3.12
chpB	b4225	Cell killing	4.06	4.08
yigW	b4274	Unknown	4.05	3.12
yjhS	b4309	Unknown	5.23	4.41
yjiI	b4331	Unknown	3.79	3.48
lasT	b4403	Unknown	5.01	5.49

APPENDIX II

Appendix II

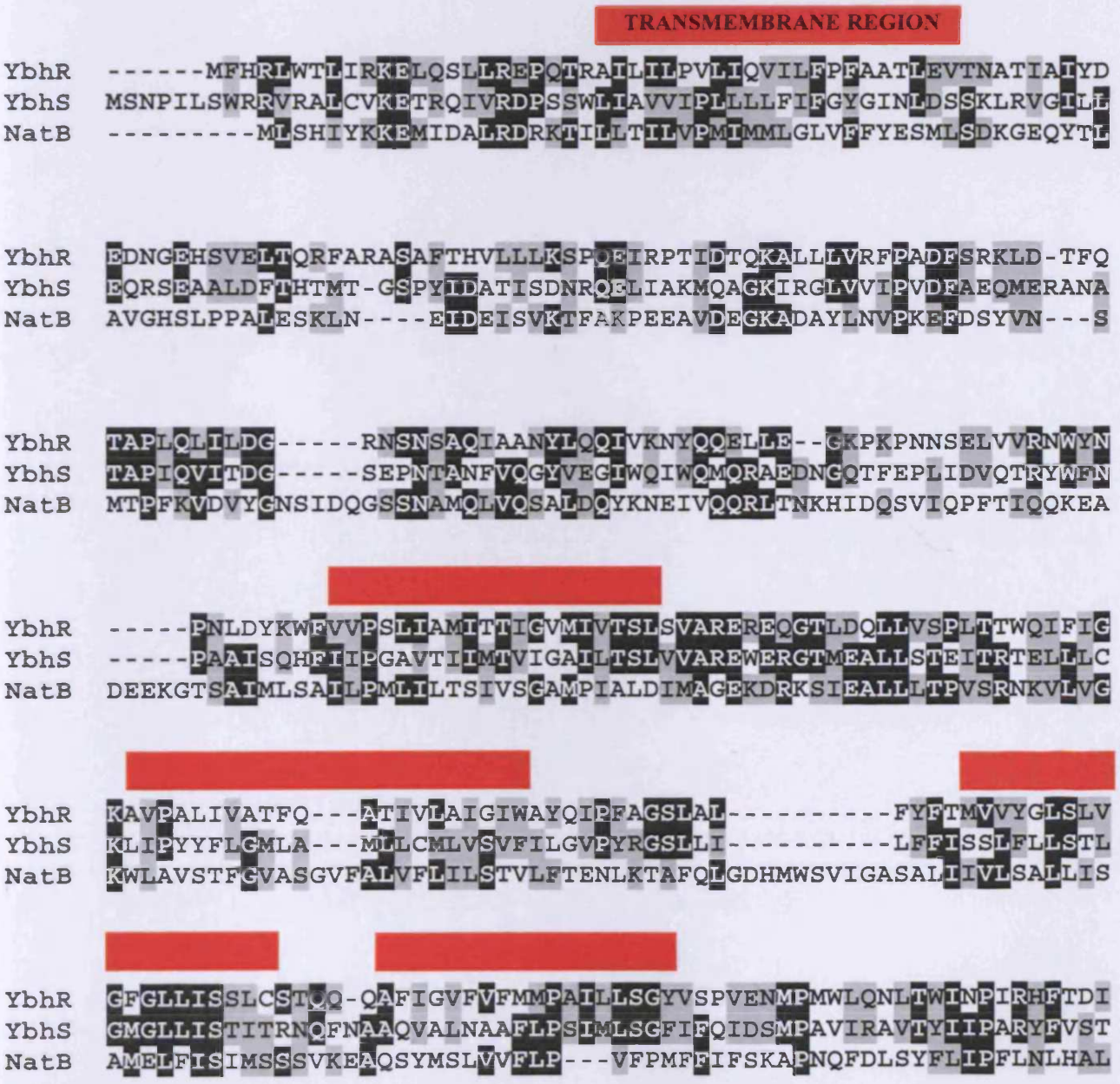
Fig. 1 Multiple alignment of b0795 (*E. coli*), EmrA (*E. coli*), HlyD (*E. coli*), AcrA (*E. coli*) and CzcB (*Ralstonia metallidurans*) generated by the CLUSTALW algorithm using BioEdit V 5.0.6. Sequence identity is displayed by a white font on a black background and similar amino acids are highlighted yellow. The transmembrane regions as predicted by TMpred (www.ch.embnet.org/ software /TMPRED_form.html) are overlaid with red boxes. Coiled-coils in each protein lie within the region indicated by the cyan boxes. They vary in length depending on the regulator and were predicted using PAIRCOIL (http://www.paircoil.lcd.mit.edu/cgi-bin/paircoil).

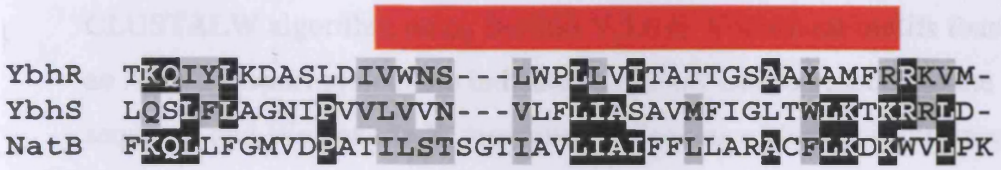



COILED-COIL
b0795
EmrA
HlyD
AcrA
CzcB

Appendix II

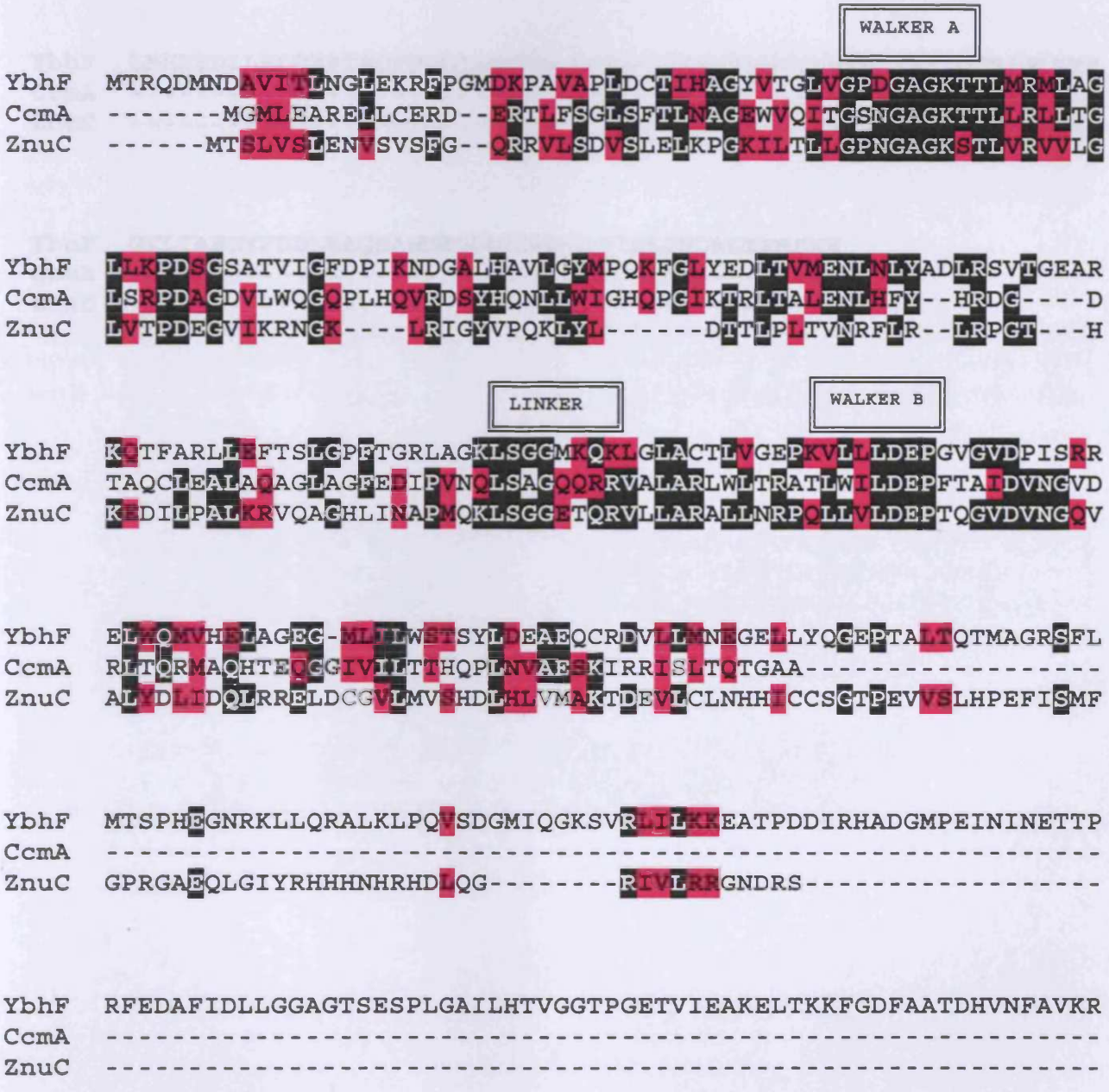
Fig. 2 Multiple alignment of YbhR (*E. coli*), YbhS (*E. coli*) and NatB (*Bacillus subtilis*) generated by the CLUSTALW algorithm using BioEdit V 5.0.6. Sequence identity is displayed by a white font on a black background and similar amino acids are highlighted grey. Transmembrane regions as predicted by TMpred (www.ch.embnet.org/software/TMPRED_form.html) are represented by red boxes.




YbhR TKQIYLKDASLDIVWNS---LWPLLVITATTGSAAYAMFRRKVM-
YbhS LQSLFLAGNIPVVLVVN---VLFLIASAVMFIGLTWLKTKRRLD-
NatB FKQLLFGMVDPATILSTSGTIAVLIAIFFLLARACFLKDKWVLPK

Appendix II

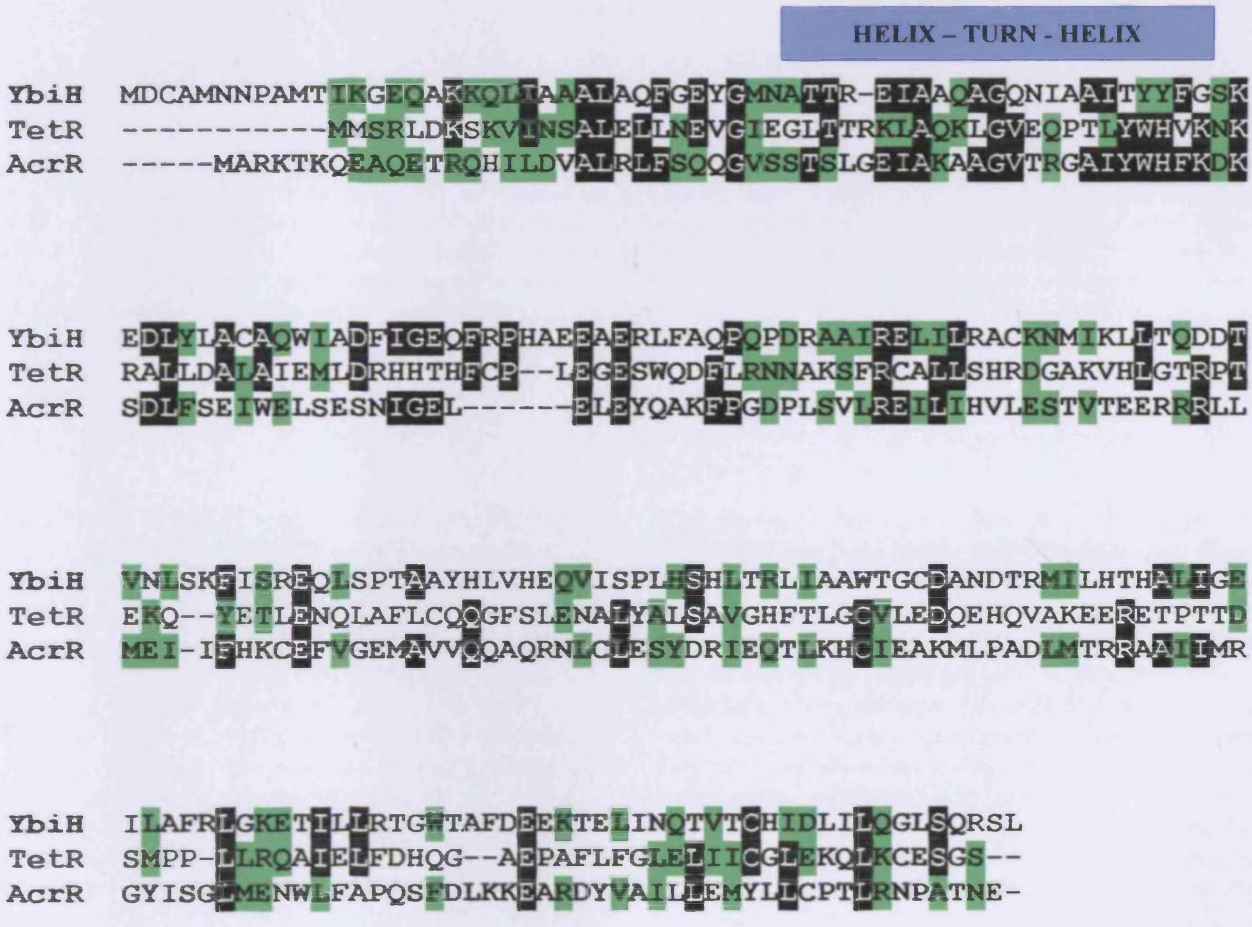
Fig. 3 Multiple alignment of YbhF (*E. coli*), CcmA (*E. coli*) and ZnuC (*E.* coli) generated by the CLUSTALW algorithm using BioEdit V 5.0.6. Consensus motifs found in the ABC subunit of an ABC transport system are indicated by boxes and correspond to the Walker site A, the linker sequence and Walker site B, respectively. Sequence identity is displayed by a white font on a black background and similar amino acids are highlighted magenta.



```
YbhF  GEIFGLLGPNGAGKSTTFKMMCGLLVPTSGQALVLGMDLKESSGKARQHLGYMAQKFSLY
CcmA  ------------------------------------------------------------
ZnuC  ------------------------------------------------------------

YbhF  GNLTVEQNLRFFSGVYGLRGRAQNEKISRMSEAFGLKSIASHATDELPLGFKQRLALACS
CcmA  ------------------------------------------------------------
ZnuC  ------------------------------------------------------------

YbhF  LMHEPDILFLDEPTSGVDPLTRREFWLHINSMVEKGVTVMVTTHFMDEAEYCDRIGLVYR
CcmA  ------------------------------------------------------------
ZnuC  ------------------------------------------------------------

YbhF  GKLIASGTPDDLKAQSANDEQPDPTMEQAFIQLIHDWDKEHSNE
CcmA  ----------------------------------------------
ZnuC  ----------------------------------------------
```

Appendix II

Fig. 4 Multiple alignment of YbiH (*E. coli*), TetR (*Salmonella typhimurium*) and AcrR (*E. coli*) generated by the CLUSTALW algorithm using BioEdit V 5.0.6. Sequence identity is displayed by a white font on a black background and similar amino acids are highlighted green. Helix-turn-helix regions as predicted by Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/ NPSA/npsa_hth.html) are represented by a blue box.



PUBLICATIONS

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 299 (2002) 438–445

BBRC

www.academicpress.com

The functional analysis of directed amino-acid alterations in ZntR from *Escherichia coli*

Saira Khan,[a] Kathryn R. Brocklehurst,[b] Gareth W. Jones,[a] and Andrew P. Morby[a,*]

[a] *School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, UK*
[b] *Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK*

Received 17 October 2002

Abstract

The ZntR protein from *Escherichia coli* is a member of the MerR-family of transcriptional regulatory proteins and acts as a hyper-sensitive transcriptional switch primarily in response to Zn(II) and Cd(II). The binding of metal-ions to ZntR initiates a mechanism that remodels the cognate promoter, increasing its affinity for RNA polymerase. We have introduced site-directed mutations into *zntR* and shown that cysteine and histidine residues are important for transcriptional control and have an effect on metal-ion preference, sensitivity and magnitude of induction. We propose a three-dimensional model of the N-terminal region of ZntR based upon the coordinates of the MerR-family regulator BmrR.

Keywords: ZntR; MerR; Zn(II); Metal transport; Regulation; Site-directed mutagenesis

Zn(II) is an abundant regulator and cofactor in all living systems [1–3], but until recently the control of cellular Zn(II) status was poorly understood in prokaryotes.

A Zn(II), Cd(II), and Pb(II) specific P-type-ATPase, ZntA, has been identified in *Escherichia coli* which exports ions from the cytosol [4–7].

The region upstream of *zntA* contains a σ^{70} promoter (P*zntA*) similar in structure to the mercury resistance (*mer*) promoters of Tn*501* and Tn*21* [8,9].

The cognate regulatory gene has been designated *zntR* that encodes a polypeptide of 141 amino acids and has been shown to regulate P*zntA* [10]. Each monomer contains an N-terminal DNA-binding domain, characterised by the presence of a helix-turn-helix (H-T-H) structure, and a C-terminal "signal recognition" domain although the functional protein acts as a dimer. The predicted primary sequence of ZntR shows 34% identity with MerR, containing five cysteine and five histidine residues, and is a member of the MerR-family of regulators which includes SoxR from *E. coli* (the superoxide stress response [11]); $TipA_L$ from *Streptomyces lividans* (thiostrepton-regulated gene expression [12]; NolA from *Bradyrhizobium japonicum* (nodulation gene expression; [13] and BmrR and BltR from *Bacillus subtilis* (expression of multidrug resistance [14,15]. In contrast to the majority of the metal-responsive MerR-family members, which are transcribed divergently from the regulated gene, *zntR* is distant on the chromosome (74.1 min) from its associated structural gene (*zntA* at 77.7 min) [10].

ZntR mediates transcriptional induction primarily in response to Zn(II) (binding two ions per monomer [16]) and Cd(II), however, Hg(II) and Pb(II) induce to a lesser extent [10].

ZntR has been shown to induce transcription via a DNA-distortion (torsional) mechanism [16] analogous to that of MerR [17,18] and is very sensitive to Zn(II), responding to femtomolar levels [19].

Here, we report the analysis of transcriptional regulation of P*zntA* by derivatives of ZntR which contain single directed alterations in the primary amino-acid sequence. We show that single amino-acid alterations can dramatically change the metal-ion specificity and dynamic range of the P*zntA*/ZntR regulatory complex.

* Corresponding author. Fax: +44-29-2087-4116.
E-mail address: morby@cf.ac.uk (A.P. Morby).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02660-8

Materials and methods

Bacterial strains and plasmids. All experiments were carried out using as host TG1 (*zntR*::*kan*) [10] which is a derivative of TG1 (K12, *lac-pro supE thi hsdD5* [F′ *traD36 proA* + *B* + *lacIq lacZ*M15]) [20] that carries an insertion in the *zntR* gene. *E. coli* TG2 (*sup*E, *hsd*Δ5, *thi*, Δ(lac-proAB) F′[*tra*D36, *pro*AB$^+$, *lacI*^{q}, *lac*Δ15] Δ(*srl-recA*)306::Tn*10*(*tet*^{r}) [21] was used to propagate plasmid DNA for nucleotide sequence determination and storage.

The plasmids used were: pUCD615 [22], pSU18 [23], pUCDP*zntA* pUCDP*zntA*-1, and pSU*zntR* [10]. All strains were cultured in Luria broth unless otherwise stated and antibiotics were added to media at standard concentrations [21].

DNA manipulations. Competent *E. coli* cells were prepared for transformation using the rubidium chloride method (adapted from [24]. Plasmid purification was performed using a HiSpeed Plasmid Purification kit (Qiagen) as described by the manufacturer.

All other DNA manipulations were carried out according to [21].

Luciferase assays. Promoter activity was assayed with a luciferase based reporter gene system (pUCD615 derivatives) and TG1 (*zntR*::*kan*) [10] using logarithmically growing cells (D_{600} = 0.2 to 0.3) at a range of metal-ion concentrations and in triplicate to generate an induction profile for each ZntR derivative (*zntR* and its derivatives were expressed *in trans* from pSU18). Otherwise, luciferase assays were performed as described in [10] except that a Lumino luminometer (Stratec) was used to measure light emission.

Site-directed mutagenesis. Site-directed mutants of *zntR* (with the exception of *zntR* (H119A) were created using pSU-*zntR* as template DNA with Stratagene's QuickChange Site-Directed Mutagenesis Kit. The *zntR* (H119A) mutant was constructed by PCR using M13-reverse primer in combination with H119A primer. The resulting PCR product was gel purified with a Concert gel extraction kit (Gibco-BRL) and used as a "primer" in another PCR with M13-forward primer. The PCR product was purified, cleaved with *EcoR*I/*Bam*H1 (New England Biolabs) and ligated into similarly digested pSU18.

Oligonucleotides used for site-directed mutagenesis were synthesised by Invitrogen Life Technologies (Table 1).

The nucleotide sequences of all *zntR* mutants were confirmed using an ABI Prism BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit (supplied by Perkin–Elmer Life Science Products). Electrophoresis and visualisation of the extension products were accomplished using an ABI Prism 373 DNA sequencer.

Results and discussion

The luminescence measurements have been adjusted for final optical attenuation and variation in the assay has been normalised by presenting the results as a percentage value based on the maximal level of induction derived from P*zntA*/ZntR + Zn(II) for any given assay set. The assays have been repeated many times for each ZntR derivative and a representative induction profile is displayed for each one.

Transcriptional activity of ZntR derivatives at PzntA-1

All of the ZntR derivatives created in this work were assayed for their ability to elicit transcription from a P*zntA* derivative promoter (P*zntA*-1 [10]) in which the spacer region between the −10 and −35 regions has been shortened from the 20 bp seen in the w.t sequence to 19 bp. The critical feature of this promoter is that ZntR w.t. induces transcription in the absence of Zn(II), i.e., interaction of ZntR w.t. with P*zntA*-1 alone can be measured [10]. The structural integrity of each of the ZntR derivatives was measured by their capacity to induce transcription at P*zntA*-1. All of the ZntR derivatives were active at P*zntA*-1 in the absence or presence of

Table 1
Primers used for mutagenesis written in a 5′–3′ orientation

Primer name	Sequence
H29A	CAGCAGATGATGGAGGCTGAGGTGCGTACTGAAGG
H53A	CCAGCGATTGAAATTTATCCGGGCCGCCAGACAACTAGG
H76A	GCATCGATCCTGAGGCCCATACCTGTCAGGAGTCAAAAGGC
H77A	GCATCGATCCTGAACACGCTACATGTCAGGAGTCAAAAGGC
C79A	CGATCCTGAACACCATACCGCTCAGGAGTCAAAAGGC
C79S	CGATCCTGAACACCATACCTCACAGGAGTCAAAAGGC
C114A	CGCCTTAACGATGCCGCATGTGGGACTGCTCATAGC
C114S	CGCCTTAACGATGCCTCATGTGGGACTGCTCATAGC
C115A	CGCCTTAACGATGCCTGTGCAGGGACTGCTCATAGC
C115S	CGCCTTAACGATGCCTGTTCAGGGACTGCTCATAGC
C124A	GGGACTGCTCATTAGCAGTGTTTATGCATCGATTCTTGAAGC
C124S	GGGACTGCTCATTAGCAGTGTTTATTCATCGATTCTTGAAGC
C141A	GGCGTTAAGAGTGGCGCTTGATTTTTTGCACTGGCAGG
C141S	GGCGTTAAGAGTGGTTCATGATTTTTTGCACTGGCAGG
C141H	GGCGTTAAGAGTGGTCATTGATTTTTTGCACTGGCAGG
C141L	GGCGTTAAGAGTGGTCTCTAATTTTTTGCACTGGCAGG
C141Y	GGCGTTAAGAGTGGTTACTAGTTTTTTGCACTGGCAGG
C141G	GGCGTTAAGAGTGGTGGTTGATTTTTTGCACTGGCAGG
C141D	GGCGTTAAGAGTGGTGATTGATTTTTTGCACTGGCAGG
H119A	GCCTGTTGTGGGACTGCTGCTAGCCAGTGTTTATTGTTCG
M13-Forward	CGCCAGGGTTTTCCCAGTCACGAC
M13-Reverse	AGCGGATAACAATTTCACACAGGA

Only one of the primer pairs is shown as the second primer was an exact complement. Underlined sequence shows the altered bases.

Zn(II) except those in which cysteine at position 124 was replaced with either alanine or serine (C124A and C124S) (data not shown).

Transcriptional activity of ZntR derivatives H29A, H53A, H76A, H77A, and H119A at PzntA

The transcriptional profiles of ZntR(H29A), ZntR(H53A), and ZntR(H119A) (Fig. 1) show that no significant induction is mediated by these ZntR derivatives in response to Zn(II). The alterations ZntR(H76A) and ZntR(H77A) appeared to diminish ZntR-mediated Zn(II) induction to some extent but each shows a similar profile to that for ZntR w.t., this is surprising given the conservation seen between ZntR [10], MerR [8] and CueR [25,26] in this region (Fig. 5). These results suggest that H29A, H53A, and H119A play a role in the mechanism of ZntR either as ligands for Zn(II) or as mediators of the torsional restructuring of P*zntA*.

Surprisingly, whilst ZntR(H29A) shows little response to Zn(II) it now responds to both Cd(II) and Pb(II) to a greater degree than w.t., exhibiting a 6-fold increase in transcriptional response to Cd(II) (Figs. 4B–D). The model of ZntR (Fig. 6A) predicts that H29 lies close to the DNA-binding H-T-H region of ZntR and is distant from a proposed binding site at the dimer interface although our data show that this residue has a significant effect on the selectivity and dynamic range of ZntR. Both ZntR(H53A) and ZntR(H119A) respond at almost w.t. levels to Cd(II) and Pb(II) (Figs. 4A–C) but not to Zn(II) suggesting that both of these residues play a role in defining the substrate range of this regulator. This result is of particular interest given that H53 is conserved in ZntR, CadR [27], and PbrR [28] yet only Zn(II) induction is affected by its replacement. Whilst exhibiting little alteration in its response to Zn(II), ZntR(H76A) induces P*zntA* to a greater extent in the presence of Cd(II) and Pb(II) (Figs. 4B–D). A coiled-coil structure (Fig. 6B, shown in magenta) is present in BmrR [29] and a similar structure is thought to link the two domains of ZntR (and other MerR-like regulators) [30]. The predicted position of H76 at the proximal end of the coiled-coil structure places this residue in the centre of the proposed dimer-interface region known to

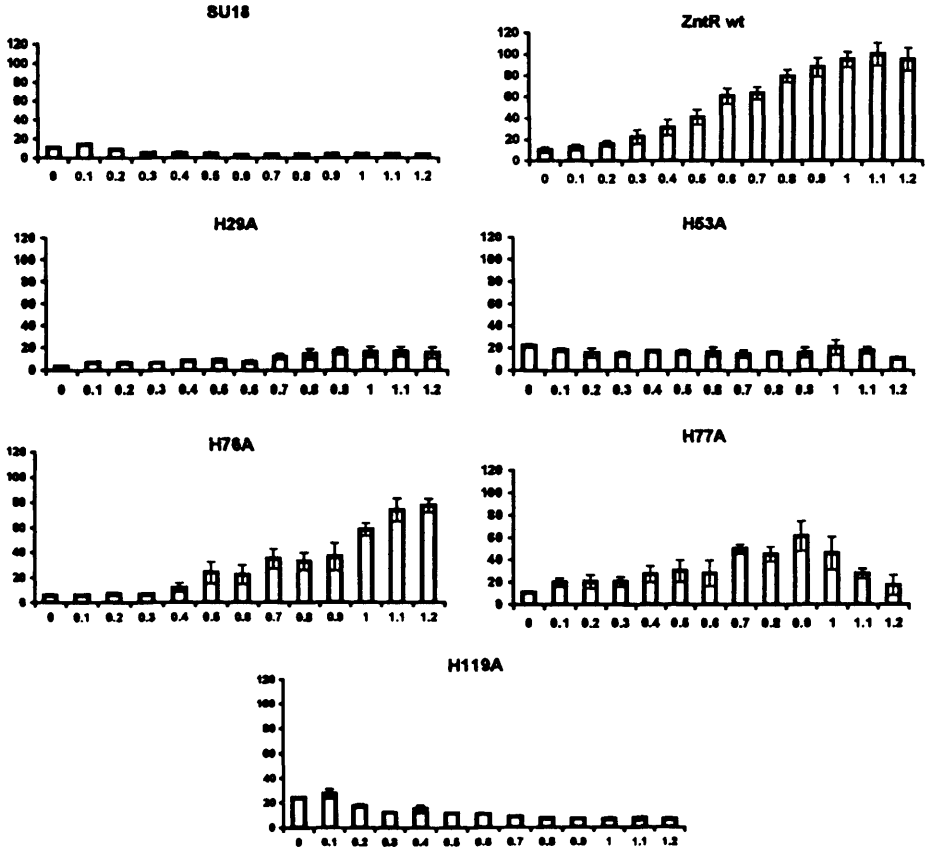


Fig. 1. Transcriptional response of P*zntA* with the ZntR-histidine derivatives. Luminescence was measured in *E. coli* TG1(*zntR*::*kan*)[pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Zn(II) ranging from 0 to 1.2 mM (open columns). The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. The standard error (vertical bars) in each case is derived from a triplicate data set.

be important for substrate binding in BmrR [29]. In contrast, ZntR(H77A) shows no increased response to Cd(II) and Pb(II). It is surprising that there is a substantial difference between the properties of ZntR(H76A) and ZntR(H77A) since they are adjacent residues.

It is tempting to suggest that since alterations in these residues appear to affect the substrate range of ZntR that they act as ligands for one of the two Zn(II)-ions predicted to bind each ZntR monomer, perhaps acting in a structural role rather than a primary driver of DNA distortion. Irrespective of the detailed function of these residues, these results show that all five histidine residues are important for ZntR function.

Transcriptional activity of ZntR derivatives C79A/S, C114A/S, C115A/S, and C124A/S at PzntA

In contrast to the histidine modified ZntR derivatives, replacement of the first four cysteine residues resulted in a complete loss of activity with Zn(II) as the inducer whether replaced with alanine or serine (Fig. 2) and ZntR modified at positions C79, C114, and C124 also shows no activity with Cd(II) or Pb(II) (Figs. 4A–C). Surprisingly, both C115S and C115A derivatives of ZntR mediated induction at P*zntA* in response to Cd(II) and Pb(II) (Figs. 4A–D) despite showing no activity with Zn(II), indeed ZntR(C115A) induced the promoter 2.5- and 3-fold greater than w.t. with Cd(II) and Pb(II), respectively. Of all the ZntR derivatives, only the C124A/S derivatives did not give a response at P*zntA* -1 (see above), suggesting that this polypeptide is either unstable or lacks the required structure to interact productively with the promoter.

In MerR, from Tn*21* and Tn*501*, four cysteines are present. Three of these cysteines, C82, C117, and C126 have been shown, by mutational analysis of MerR in Tn*21* [31,32] and Tn*501* [33–35], to serve as ligands for Hg(II) binding. These three cysteine residues are conserved with respect to other MerR family members (Fig. 5), and correspond to residues C79, C114, and C124, respectively, in ZntR. It is unsurprising that alterations of these residues abrogate the transcriptional response of ZntR. This work demonstrates that these residues are important for ZntR function and structure, as they are in MerR. The complete loss of function in many of these

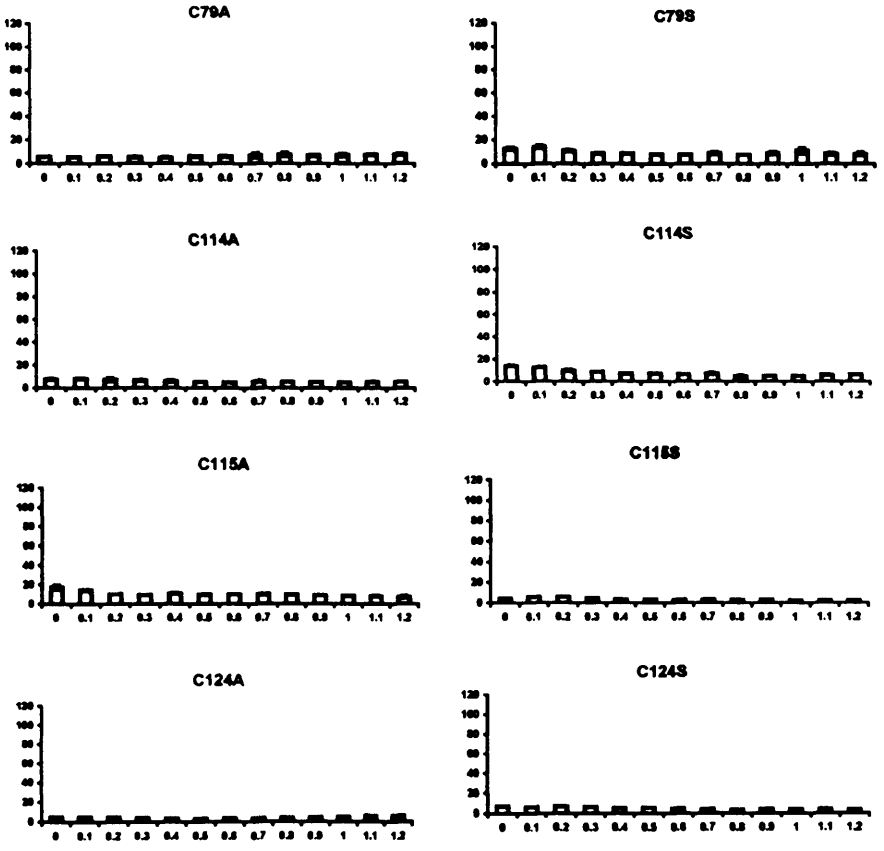


Fig. 2. Transcriptional response of PzntA with the ZntR-cysteine derivatives. Luminescence was measured in *E. coli* TG1(*zntR*::*kan*)[pUCDP*zntA*] with the control, ZntR or its derivatives expressed in trans in the presence of IPTG (1 mM) and Zn(II) ranging from 0 to 1.2 mM (open columns). The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. The standard error (vertical bars) in each case is derived from a triplicate data set.

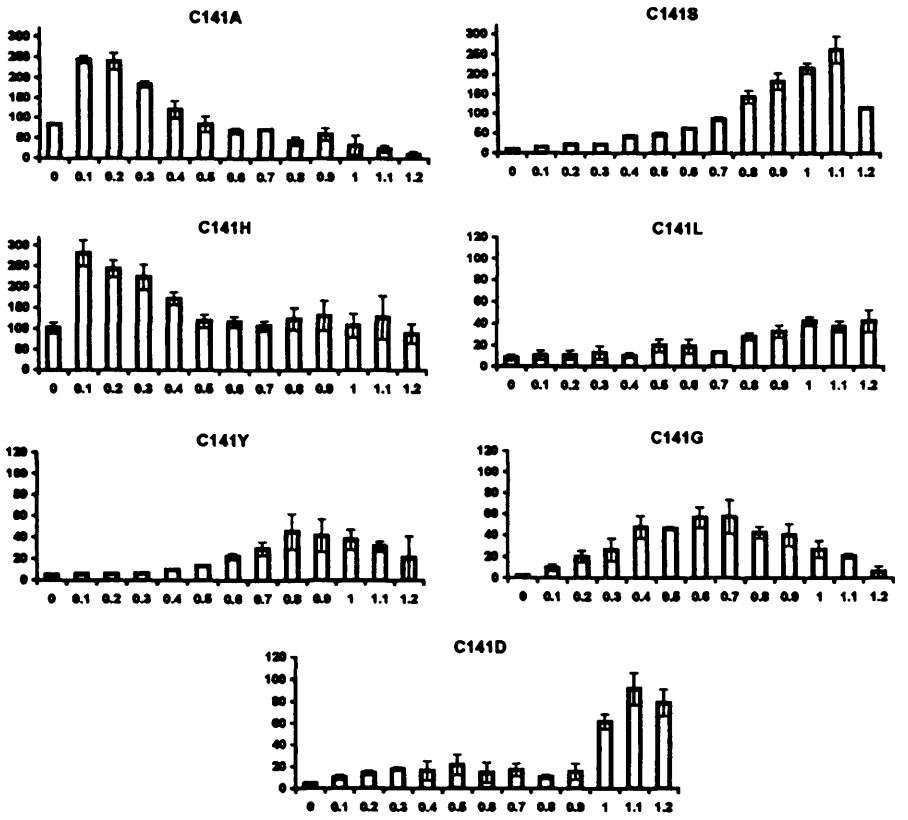


Fig. 3. Transcriptional response of *PzntA* with the ZntR-cysteine 141 derivatives. Luminescence was measured in *E. coli* TG1(*zntR*::*kan*) [pUCDP*zntA*] with the control, ZntR or its derivatives expressed in trans in the presence of IPTG (1 mM) and Zn(II) ranging from 0 to 1.2 mM (open columns). The data are presented as a percentage of the maximal induced value for P*zntA*/ZntR w.t. The standard error (vertical bars) in each case is derived from a triplicate data set. Note that the value axes are of different ranges.

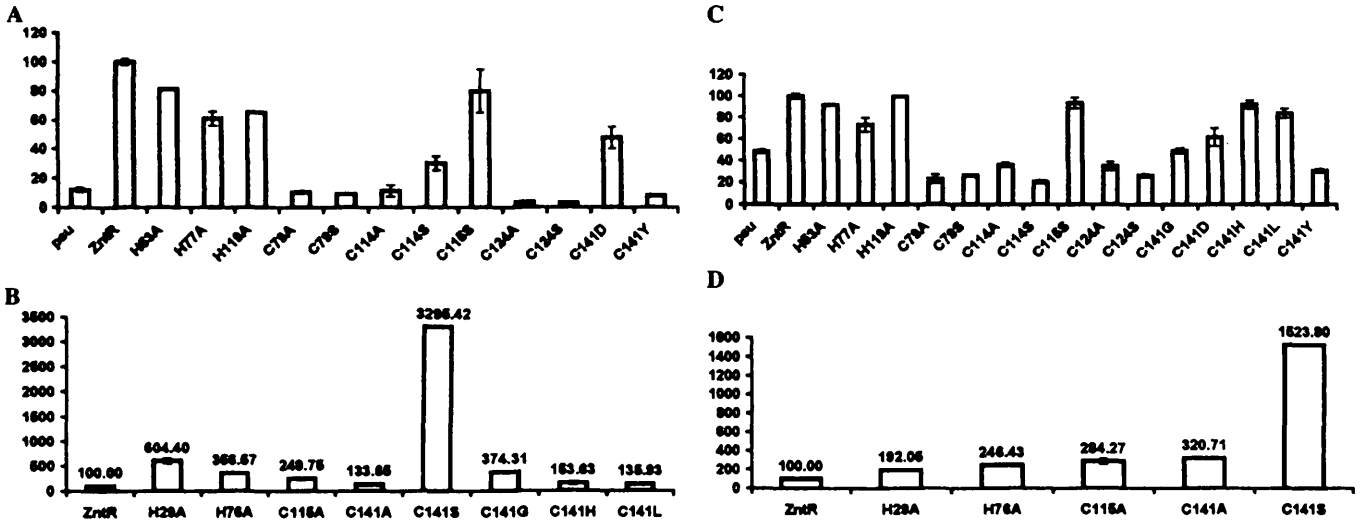


Fig. 4. Transcriptional response of P*zntA* to ZntR and its derivatives. Luminescence was measured in *E. coli* TG1(*zntR*::*kan*)[pUCDP*zntA*] with the control, ZntR or its derivatives expressed *in trans* in the presence of IPTG (1 mM) and Cd(II) at 50 μM (A (100% ZntR w.t. activity or less) and B (greater than 100% ZntR w.t. activity) or Pb(II) at 0.5 mM (C (100% ZntR w.t. activity or less) and D (greater than 100% ZntR w.t. activity)) (open columns). The data are presented as a percentage of the maximal induced value for *PzntA*/*Znt*R w.t. The standard error (vertical bars) in each case is derived from a triplicate data set. Note that the value axes are of different ranges.

ZntR derivatives suggests that the conserved cysteine residues play a central role in the mechanism of induction and are likely to act as ligands for metal-ions.

Transcriptional activity of C141 ZntR derivatives at PzntA

The C-terminal residue of ZntR (position 141) is a cysteine and shows no sequence conservation with other MerR-family members except CueR (Fig. 5). It is of interest that the most diverse region in the MerR family (Fig. 5) is at the C-terminal region where many MerR-like proteins have putative metal-binding ligands such as thiols and imidazoles. It is perhaps in this C-terminal region where some measure of specificity is directed.

Initial experimentation demonstrated that replacement of C141 in ZntR with either an alanine or serine residue had very different effects on function. ZntR(C141A) showed dramatic differences in its Zn(II)-induction profile with respect to w.t. ZntR. Most interestingly, replacement of C141 with alanine increased the maximal induction of ZntR in response to Zn(II) (2.5-fold) but surprisingly changed the sensitivity of the regulator/promoter complex, with maximal induction now occurring at 0.1 mM Zn(II) whereas ZntR w.t. (and most of the active derivatives) showed maximal induction in the 0.9–1.2 mM range. ZntR(C141A) was 3-fold more responsive to Pb(II) but little altered with respect to Cd(II) (Figs. 4A and B). In contrast, ZntR(C141S) retained the usual transcriptional profile (maximal induction 1.1 mM) but was massively more responsive to Cd(II) and Pb(II) (33- and 16-fold, respectively) and more responsive to Zn(II) (3-fold) (Figs. 3 and 4A and B).

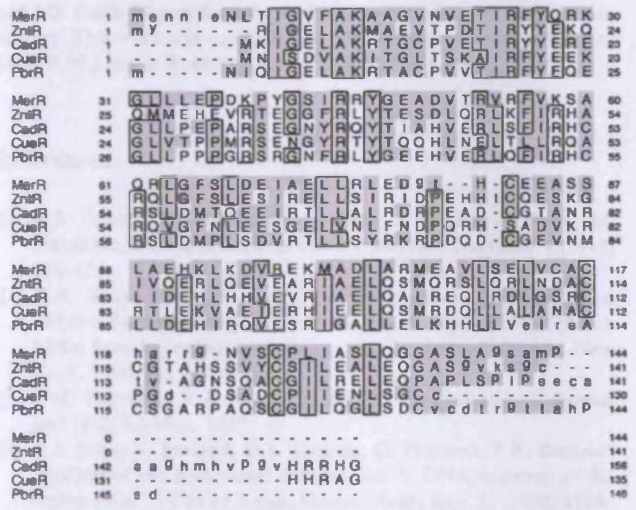


Fig. 5. Multiple alignment of MerR [8], ZntR [10], CadR [27], CueR [25,26], and PbrR [28]. The alignment was generated using DIALIGN [39] (http://bibiserv.techfak.uni-bielefeld.de/dialign/) and displayed using SeqVu (The Garvan Institute of Medical Research). Sequence identity is represented by boxes and similarity by shading.

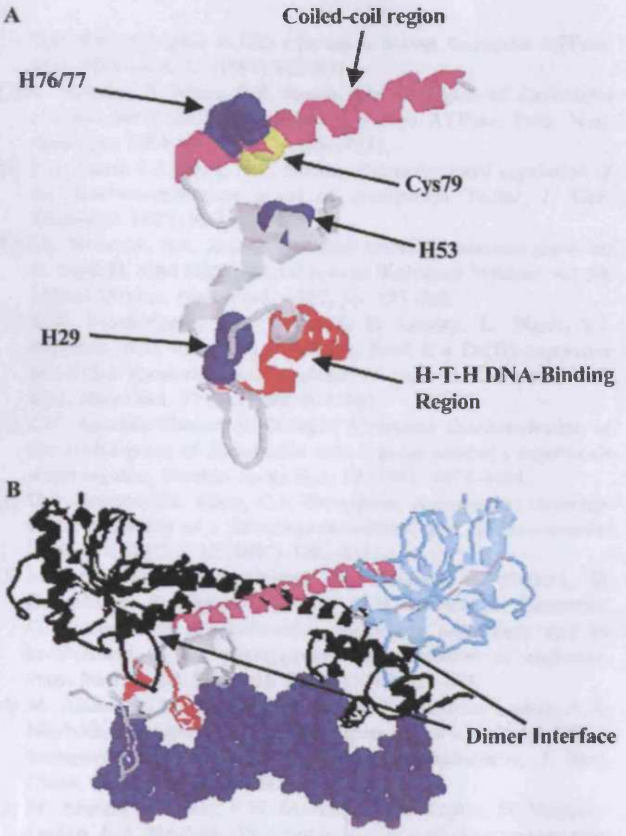


Fig. 6. A structural model for the first 95 amino acids of ZntR was created (A) using SWISS-MODEL [40] (http://swissmodel.expasy.org/SWISS-MODEL.html) with BmrR coordinates as the template. Histidine residues are shown in blue and spacefill, cysteine 79 is shown in yellow and spacefill. A dimer of BmrR [29] complexed with DNA (blue/spacefill) is shown, for simplicity, one monomer is in black, the second shows the N-terminal DNA-binding domain (grey) containing the H-T-H region (red), the coiled-coil structure (magenta), and the C-terminal region (cyan). Equivalent structure/model features between BmrR (B) and ZntR (A) are shown in red (H-T-H) and magenta (coiled-coil region).

Residue C141 has also been replaced with histidine, tyrosine, leucine, glycine, and aspartate, the latter four derivatives show a slightly diminished response to Zn(II) with ZntR(C141G) being more responsive to Cd(II) (4-fold) and exhibiting altered profiles and sensitivities (Figs. 3 and 4A–C). ZntR(C141H) showed an increased maximal induction (3-fold) with Zn(II) and an alteration in sensitivity with maximal induction occurring at 0.1 mM similar to that seen in C141A. Induction was slightly increased with Cd(II) but little changed with Pb(II) for ZntR(C141H).

Taken together, these data show that both cysteine and histidine residues are important in the function of ZntR and that alteration of the C-terminal amino acid can have a profound effect on the properties of this regulator. It is unclear why the C141A or C141H mutations alter the function of ZntR to such a degree

although we would hypothesise that the presence of a thiol group at this position may alter the access of metal-ions to other binding sites which drive the torsional mechanism of induction. It is unsurprising, given the chemical similarity of Zn(II), Cd(II), and Pb(II), that simplistic interpretations of the structure–function relationships within ZntR are not easily defined, although the three-dimensional structure of BmrR [29] has allowed us to create a model of the first 95 amino acids of ZntR (Fig. 6A) which positions three of the histidine (H53, H76, and H77) and one of the cysteine residues (C79) in regions expected to interact with metal-ions, e.g., at the proposed dimer interface (see Fig. 6B).

An interesting aspect of this research is the mutability of the specificity, sensitivity, and dynamic range of ZntR, with 7 of the 10 derivatives showing substantial differences from w.t. This mutability of ZntR is in sharp contrast to that seen during mutational analysis of MerR [31,36–38] which appears to be rather more refractory to functional alteration [30]. Perhaps the differences in the strength of interaction with biological molecules between Zn(II) and Hg(II) have allowed MerR to become more selective and less open to change than we see here for ZntR.

Further study of this family of transcriptional regulators will yield important information regarding the evolution of specificity and mechanism, particularly with respect to metal-ion dependent transcription in prokaryotes.

Acknowledgments

We thank Prof. N.L. Brown and Dr. J.L. Hobman for discussion and Ms. Faith Miyagi for help with this work whilst visiting the laboratory. This work was supported by BBSRC Grants G05844/G09282 (to A.P.M.) and a Wellcome award 058466 (to A.P.M.).

References

[1] A.S. Prasad, J.T. Fitzgerald, J. Hess, J. Kaplan, F. Pelen, M. Dardenne, Zinc-deficiency in elderly patients, Nutrition 9 (1993) 218–224.

[2] D.A. Suhy, T.V. O'Halloran, Metal-responsive gene-regulation and the zinc metalloregulatory model, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 32, Marcel Dekker, New York, 1996, pp. 557–578.

[3] P.M. Harrison, R.J. Hoare, Metals in Biochemistry, Chapman and Hall, London, 1980.

[4] H.J. Sofia, V. Burland, D.L. Daniels, G. Plunkett, F.R. Blattner, Analysis of the *Escherichia coli* genome. 5. DNA-sequence of the region from 76.0 to 81.5 min, Nucleic Acids Res. 22 (1994) 2576–2586.

[5] D.K. Blencowe, S.J. Marshall, A.P. Morby, Preliminary characterisation of *zntA*, a gene which encodes a Zn(II)/Cd(II)-export protein in *Escherichia coli*, Biotech. et alia 2 (1997) 1–6.

[6] S.J. Beard, R. Hashim, J. Membrillo-Hernandez, M.N. Hughes, R.K. Poole, Zinc(II) tolerance in *Escherichia coli* K-12: evidence that the *zntA* gene (o732) encodes a cation transport ATPase, Mol. Microbiol. 25 (1997) 883–891.

[7] C. Rensing, B. Mitra, B.P. Rosen, The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase, Proc. Natl. Acad. Sci. USA 94 (1997) 14326–14331.

[8] P.A. Lund, S.J. Ford, N.L. Brown, Transcriptional regulation of the mercury-resistance genes of transposon Tn*501*, J. Gen. Microbiol. 132 (1986) 465–480.

[9] J.L. Hobman, N.L. Brown, Bacterial mercury-resistance genes, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 34, Marcel Dekker, New York, 1997, pp. 527–568.

[10] K.R. Brocklehurst, J.L. Hobman, B. Lawley, L. Blank, S.J. Marshall, N.L. Brown, A.P. Morby, ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*, Mol. Microbiol. 31 (3) (1999) 893–902.

[11] C.F. Amabile-Cuevas, B. Demple, Molecular characterisation of the *soxRS* genes of *Escherichia coli*—2 genes control a superoxide stress regulon, Nucleic Acids Res. 19 (1991) 4479–4484.

[12] D.J. Holmes, J.L. Caso, C.J. Thompson, Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*, EMBO J. 12 (1993) 3183–3191.

[13] M.J. Sadowsky, P.B. Cregan, M. Gottfert, A. Sharma, D. Gerhold, F. Rodriguez-Quinones, H.H. Keyser, H. Hennecke, G. Stacey, The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans, Proc. Natl. Acad. Sci. USA 88 (2) (1991) 637–641.

[14] M. Ahmed, C.M. Borsch, S.S. Taylor, N. Vazquez-Laslop, A.A. Neyfakh, A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates, J. Biol. Chem. 269 (1994) 28506–28513.

[15] M. Ahmed, L. Lyass, P.N. Markham, S.S. Taylor, N. Vazquez-Laslop, A.A. Neyfakh, Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated, J. Bacteriol. 177 (1995) 3904–3910.

[16] C.E. Outten, F.W. Outten, T.V. O'Halloran, DNA distortion mechanism for transcriptional activation by ZntR, a Zn(II)-responsive MerR homologue in *Escherichia coli*, J. Biol. Chem. 274 (53) (1999) 37517–37524.

[17] A.Z. Ansari, M.L. Chael, T.V. O'Halloran, Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR, Nature 355 (1992) 87–89.

[18] A.Z. Ansari, J.E. Bradner, T.V. O'Halloran, DNA bend modulation in a repressor-to-activator switching mechanism, Nature 374 (1995) 371–375.

[19] C.E. Outten, T.V. O'Halloran, Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis, Science 292 (5526) (2001) 2488–2492.

[20] T.J. Gibson, Ph.D. Thesis, Cambridge University, UK, 1984.

[21] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, New York, 1989.

[22] T.M. Rogowsky, T.J. Close, J.A. Chimera, J.J. Shaw, C.I. Kado, Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTIC58, J. Bacteriol. 169 (1987) 5101–5112.

[23] B. Bartolome, Y. Jubete, E. Martinez, F. de la Cruz, Construction and properties of a family of pACYC184 derived cloning vectors compatible with pBR322 and its derivatives, Gene 102 (1991) 75–78.

[24] D. Hanahan, Studies on transformation of *Escherichia coli* with plasmids, J. Mol. Biol. 166 (1983) 557–580.

[25] J.V. Stoyanov, J.L. Hobman, N.L. Brown, CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA, Mol. Microbiol. 39 (2001) 502–512.

[26] F.W. Outten, C.E. Outten, J.A. Hale, T.V. O'Halloran, Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal *merR* homologue, *cueR*, J. Biol. Chem. 275 (2000) 31024–31029.

[27] S.W. Lee, E. Glickman, D.A. Cooksey, Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR family response regulator, Appl. Environ. Microbiol. 67 (2001) 1437–1444.

[28] B. Borremans, J.L. Hobman, A. Provoost, N.L. Brown, D. van de Lelie, Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34, J. Bacteriol. 183 (2001) 5651–5658.

[29] E.E. Zheleznova Heldwein, R.G. Brennan, Crystal structure of the transcription activator BmrR bound to DNA and a drug, Nature 409 (2001) 378–381.

[30] J.J. Caguiat, A.L. Watson, A.O. Summers, Cd(II)-responsive and constitutive mutants implicate a novel domain in MerR, J. Bacteriol. 181 (1999) 3462–3471.

[31] W. Ross, S.J. Park, A.O. Summers, Genetic analysis of transcriptional activation and repression in the Tn*21 mer* operon, J. Bacteriol. 171 (1989) 4009–4018.

[32] V. Livrelli, I.W. Lee, A.O. Summers, In vivo DNA–protein interactions at the divergent mercury resistance (*mer*) promoters: metalloregulatory protein MerR mutants, J. Biol. Chem. 268 (1993) 2623–2631.

[33] L.M. Shewchuck, J.D. Helmann, W. Ross, S.J. Park, A.O. Summers, C.T. Walsh, Transcriptional switching by the MerR protein-activation and repression mutants implicate distinct DNA and mercury(II) binding domains, Biochemistry 28 (1989) 2340–2344.

[34] L.M. Shewchuck, G.L. Verdine, H. Nash, C.T. Walsh, Mutagenesis of the cysteines in the metalloregulatory protein MerR indicates that a metal-bridged dimer activates transcription, Biochemistry 28 (1989) 6140–6145.

[35] L.M. Shewchuck, G.L. Verdine, C.T. Walsh, Transcriptional switching by the metalloregulatory MerR protein initial characterisation of DNA and mercury(II) binding activities, Biochemistry 28 (1989) 2331–2339.

[36] J. Parkhill, A.Z. Ansari, J.G. Wright, N.L. Brown, T.V. O'Halloran, Construction and characterization of a mercury-independent MerR activator ($MerR^{AC}$): transcriptional activation in the absence of Hg(II) is accomplished by DNA-distortion, EMBO J. 12 (1993) 413–421.

[37] K.M. Comess, L.M. Shewchuk, K. Ivanetich, C.T. Walsh, Construction of a synthetic gene for the metalloregulatory protein MerR, Biochemistry 33 (1994) 4175–4186.

[38] J. Parkhill, B. Lawley, J.L. Hobman, N.L. Brown, Selection and characterization of mercury-independent activation of the Tn*501* transcriptional regulator MerR, Microbiology 144 (1998) 2855–2864.

[39] B. Morgenstern, DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment, Bioinformatics 15 (1999) 211–218.

[40] N. Guex, A. Diemand, M.C. Peitsch, Protein modelling for all, TIBS 24 (1999) 364–367.