Metal-ion tolerance in *Escherichia coli*: analysis of transcriptional profiles by gene-array technology

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***Escherichia coli* was adapted to grow in medium containing substantially elevated concentrations of either Zn(II), Cd(II), Co(II) or Ni(II). Whole-genome transcriptional profiles were generated from adapted strains and analysed for significant alteration in transcript abundance with reference to a wild-type strain. Similar alterations in specific message levels were observed for strains adapted to the four metal ions. One unexpected trend was the increase in transcript level of genes involved in transposition of IS elements, particularly *insA*. Subsequent expression of *insA-7* from a heterologous promoter in *E. coli* conferred tolerance to Zn(II).**

Keywords: *Escherichia coli*, metal ion, adaptation, gene array, *insA*

INTRODUCTION

Many mechanisms solely responsible for specific metal-ion resistance in prokaryotes have been described at the molecular level (recently reviewed by Nies, 1999; Xu *et al.*, 1998). The effect of metal-ion stress on microbial cells/communities has been investigated and suggests that individual strains adapt to elevated metal-ion concentrations; however, no analysis of the resultant strains was initiated at the molecular level (reviewed by Giller *et al.*, 1998; Kelly *et al.*, 1999). Limited genetic analysis has been performed for a Cd(II)-tolerant strain of *Synechococcus* PCC 7942 that demonstrated an increase in the chromosomal gene copy number of *smtA*, which encodes a prokaryotic metallothionein capable of binding Cd(II) and other divalent metal ions (Gupta *et al.*, 1992). In addition, the regulatory gene *smtB* was shown to carry a deletion rendering the *smt* repressor protein non-functional (Gupta *et al.*, 1993). The molecular bases for the increase in Cd(II) tolerance in *Synechococcus* are almost certainly more extensive than the amplification/deletion within the *smt* locus, although at the time of the original experiments further characterization of other chromosomal alterations was not possible. As far as we are aware, genomic analysis of metal tolerance in bacterial cells has not been undertaken.

The recent advent of array technology has allowed the global study of all 4255 genes in *Escherichia coli*. This work details the investigation of metal-ion tolerance in *E. coli* adapted to Zn(II), Cd(II), Co(II) and Ni(II), and

the results demonstrate that the transcript abundance of a range of genes was altered in tolerant strains. In particular, the transcript level of *insA* was increased in Zn(II)-, Cd(II)- and Co(II)-adapted strains. The subsequent expression of *insA-7* from a heterologous promoter conferred Zn(II) tolerance on *E. coli*.

METHODS

Bacterial strains and plasmids. All experiments were carried out using as host *Escherichia coli* TG1 (described in Sambrook *et al.*, 1989) [K12, *lac-pro supE thi hsdD5* (F' *traD36 proA⁺B⁺ lacI^a lacZ M15*)] or *E. coli* TG2 (described in Sambrook *et al.*, 1989) (which is *E. coli* TG1 carrying the *recA::Tn10* mutation). pBAD24 (Guzman *et al.*, 1995) was used for *insA-7* expression. All strains were cultured in Luria–Bertani medium and antibiotics were added to the medium at standard concentrations unless otherwise specified (Sambrook *et al.*, 1989).

Adaptation of *E. coli* TG1 to metal-ion tolerance. A 10 ml volume of LB medium was inoculated with 150 µl of an overnight culture of *E. coli* TG1 and grown with shaking for 1.5 h, before 200 µl aliquots were transferred into a 96-well plate containing an increasing range of metal-ion concentrations of Zn(II) (ZnSO₄), Cd(II) (CdSO₄), Co(II) (CoSO₄) and Ni(II) (NiSO₄). The plates were incubated at 37 °C for 24–48 h with shaking (Camlab Microtherm; 500 r.p.m.) and the OD₆₀₀ of cell cultures was measured (Molecular Devices Thermo max microplate reader). A 150 µl volume was taken from the well containing the highest concentration of each individual metal ion where the OD₆₀₀ was ≥0.100, and this was used to inoculate 10 ml LB medium for further adaptive incubation. This stepwise adaptation generated strains with

increased tolerance to Zn(II) (strain EZn), Cd(II) (strain ECd), Co(II) (strain ECo) and Ni(II) (strain ENi). Adaptation was concluded when cultures reached a stable tolerance maximum over two serial culture steps. The resultant cultures were either diluted 50 % (v/v) with glycerol for storage or used for the isolation of RNA.

Measurement of metal-ion tolerance (MICs). A 30 ml volume of LB medium was inoculated with the individual strains of *E. coli* TG1, EZn, ECd, ECo and ENi, and grown with shaking for 1 h at 37 °C. A 200 µl sample of each culture was transferred into separate wells of a microtitre plate which contained increasing ranges of metal-ion concentrations, and incubated at 37 °C for 24 h with shaking (Camlab Microtherm; 500 r.p.m.). The OD₆₀₀ of cell cultures was then measured (Molecular Devices Thermo max microplate reader). MICs for each strain were measured for the four metal ions Zn(II), Cd(II), Co(II) and Ni(II). In some cases it was impossible to accurately determine an MIC as the strains grew in medium in which the given metal ion was at the limit of solubility.

Whole-genome transcriptional analysis. *E. coli* Panorama arrays were used throughout this work (Sigma-Genosys). Metal-ion-tolerant *E. coli* strains were grown to stationary phase in LB containing the maximum permissive concentration of the appropriate metal salt. RNA was extracted using a Qiagen RNeasy kit according to the manufacturer's instructions. cDNA probes were generated as described by Sigma-Genosys, using primers and reverse transcriptase provided. Hybridized probes were visualized by autoradiography using a phosphorimager (Bio-Rad, Personal FX). Hybridization intensity was quantified and compared using Quantity One (Bio-Rad) and Excel (Microsoft). Transcriptional profiles for each strain of cells were compared to that of the wild-type strain grown to stationary phase in the absence of metal ions. LB medium was used to allow future array experiments involving alternative challenges to be comparable with this data set.

Amplification of the *insA-7* coding region and plasmid construction. All DNA manipulations were carried out according to Sambrook *et al.* (1989). The *insA-7* coding region was amplified from the Co(II)-tolerant strain ECo using primers A7(V)N-term (5'-CGGAATTCTGCGTGGCTTCC-ATTTCCATCAGATGTCC-3') and A7C-term (5'-GCTC-TAGACGCTGACGTGATTTAGACCCGACG-3') (synthesized by Gibco-BRL). The nucleotide sequence of the amplified *insA* was determined and was identical to that reported, except for one base change (+150 from start codon: C to A). This change does not affect the primary amino acid sequence. The derivative of pBAD24 (Guzman *et al.*, 1995) carrying the *insA-7* coding region was created by digesting the PCR product with *Eco*RI and *Xba*I and ligating together with identically cut pBAD24 to generate pBAD*insA-7*.

***insA-7* expression.** Overnight cultures of TG2(pBAD24) or TG2(pBAD*insA-7*) were used to inoculate 20 ml fresh LB medium containing arabinose at a final concentration of 0.5 % (w/v). A 200 µl sample of each culture was transferred into separate wells of a microtitre plate containing increasing concentration ranges of Zn(II), Cd(II), Co(II) or Ni(II). The cultures were incubated at 37 °C with shaking, and the OD₆₀₀ was measured (Molecular Devices Thermo max microplate reader) every 1 or 2 h over a 30 h period. Carbenicillin was added to medium to allow selection of pBAD24 and pBAD*insA-7* which carry the *bla* marker gene.

RESULTS AND DISCUSSION

Adaptation of *E. coli* to metal tolerance

E. coli TG1 was grown in LB medium supplemented with a range of either Zn(II), Cd(II), Co(II) or Ni(II) and the MICs were determined as 2.2, 1.2, 1.7 and 4.0 mM, respectively (Fig. 1). The MIC values for the four metal ions were also determined for derivative strains individually adapted for growth in elevated concentrations of Zn(II) (strain EZn), Cd(II) (strain ECd), Co(II) (strain ECo) and Ni(II) (strain ENi). Despite the diminished solubility of metal ions in a rich medium, a clear increase in tolerance was observed for all the adapted strains. EZn was tolerant to Zn(II) at 9.0 mM (Fig. 1) and also exhibited slight cross-tolerance to Co(II) and Ni(II) but sensitivity to Cd(II) (data not shown). ECd exhibited an MIC to Cd(II) of 4.0 mM (Fig. 1) and this strain showed increased tolerance to Co(II) and Ni(II) (data not shown). ECo is dramatically increased in tolerance, exhibiting growth at over 23.0 mM supplementary Co(II) (Fig. 1) and showing an increase in tolerance to Ni(II) (data not shown). The MIC of ENi was 19.0 mM (Fig. 1) and significant cross-tolerance to Co(II) was observed with an increase in sensitivity to Cd(II) (data not shown). That metal-ion cross-tolerance/dependence was observed is unsurprising given the chemical similarities that exist between these divalent cations.

Transcriptional analysis of adapted strains

Transcriptional profiles were generated using macroarrays (Sigma-Genosys) representing all 4255 ORFs present in the *E. coli* genome (Blattner *et al.*, 1997). The genes of interest which were significantly modulated (more than two standard deviations from the mean) are detailed (Tables 1 and 2). For brevity, not all significant results are shown here (complete listings can be accessed via URL <http://www.cf.ac.uk/biosi/staff/people/morby.html>).

Analysis of genes for which transcriptional levels are decreased (Table 1) reveals a significant commonality. Of those with a known function, *yfiA* (translational stimulator; Bylund *et al.*, 1997), *tufA/tufB* (EF-Tu; reviewed by Weijland *et al.*, 1992) and *yjbC* (putative pseudouridylate synthase, shows similarity to *rsuA*; Wrzesinski *et al.*, 1995) encode proteins involved in translation; this adaptation may comprise a general response to stress or may merely be a function of lower growth rates in the presence of a toxic agent. In addition, *tnaA* (tryptophanase; Deeley & Yanofsky, 1981) and *aspA* (aspartase; Guest *et al.*, 1984) are reduced, suggesting the cell is less catabolic in nature.

Consistent with tolerance to cations, *ompC* and *ompA*, which encode outer-membrane porins (Ried *et al.*, 1990), are reduced in most of the adapted strains. b0795, which shows similarity to CzcB (a component of a cation efflux pump; Nies *et al.*, 1989), is reduced in all four strains. Analysis of the b0795-encoded sequence shows the presence of a signal peptide, suggesting it is periplasmic in location. In addition, the operon which contains

Fig. 1. Liquid MICs for Zn(II), Cd(II), Co(II) and Ni(II), shown for *E. coli* TG1 adapted for tolerance to Zn(II) (strain EZn), Cd(II) (strain ECd), Co(II) (strain ECo) and Ni(II) (strain ENi), respectively. The OD₆₀₀ was determined for each strain (□); wild-type *E. coli* TG1 is shown on each graph as a comparison (●). The graphs show OD₆₀₀ against metal-ion concentration. Assays were performed in triplicate and the sds are shown.

Table 1. Transcriptional profiles of metal-ion-tolerant strains: genes of interest for which transcriptional levels were decreased are listed

The fold decrease in transcript abundance is shown for each gene in each strain where appropriate.

Gene	Gene product description	Fold decrease in strain:			
		EZn	ECd	ECo	ENi
<i>yfiA</i>	Putative translational stimulator	9.7	16.2	11.4	15.0
<i>tnaA</i>	Tryptophanase	9.2	15.4	9.8	15.3
<i>tufB</i>	Elongation factor EF-Tu (duplicate gene)	7.3	11.4	12.0	11.5
<i>yjbC</i>	Putative pseudouridylate synthase	5.6	5.0	10.2	10.4
<i>aspA</i>	Aspartase	4.0	8.8	7.9	7.7
<i>tufA</i>	Protein chain elongation factor EF-Tu (duplicate of <i>tufB</i>)	3.4	5.5	8.9	12.1
b0795 (f332)	Putative periplasmic protein, sequence similarity to <i>czcB</i>	6.7	4.5	6.9	7.0
<i>ompC</i>	Outer-membrane protein C precursor	5.4	6.4	4.8	3.9
<i>ompA</i>	Outer-membrane protein A	5.0	3.5		5.8

b0795 also contains genes whose products are similar to ABC-transporter proteins. It is possible that this operon is involved in cation import and therefore it is consistent for one or more components to be diminished in metal-adapted cells.

Genes which show increased transcriptional levels (Table 2) also have some commonality between strains. Some induced genes have a functional association with metal cations, including *nikD* [Ni(II) export; Navarro *et al.*, 1993], *yfeC* [putative chelated-Fe(II) export; Bearden

et al., 1998], *yeaJ* (shows sequence similarity to *hmsT*, a putative regulator of haem storage; Jones *et al.*, 1999) and *bisC* (biotin sulfoxide reductase, binds molybdenum; Pierson & Campbell, 1990). These gene products could be involved in the chelation of excess cytosolic metal ions which may generate tolerance or perhaps represent compensatory alterations which preserve cation [e.g. Mn(II) or Fe(II)] metabolism in the presence of excess Zn(II), Cd(II), Co(II) or Ni(II).

The majority of the characterized genes identified in this

Table 2. Transcriptional profiles of metal-ion-tolerant strains: genes of interest for which transcriptional levels were increased are listed

The fold increase in transcript abundance is shown for each gene in each strain where appropriate.

Gene	Gene product description	Fold increase in strain:			
		<i>EZn</i>	<i>ECd</i>	<i>ECo</i>	<i>ENi</i>
<i>insA-7</i>	Insertion element IS1 protein InsA	3.5	3.8	2.2	
<i>insA-6</i>	Insertion element IS1 protein InsA	3.5	3.4	3.8	
<i>insA-1</i>	Insertion element IS1 protein InsA		3.3	3.6	
<i>insA-2</i>	Insertion element IS1 protein InsA	3.3		3.0	
<i>insA-5</i>	Insertion element IS1 protein InsA	4.0	6.6		
<i>insB-1</i>	Insertion element IS1 protein InsB	2.6		4.2	
<i>insB-6</i>	Insertion element IS1 protein InsB	2.8		3.1	
<i>nikD</i>	Nickel transport ATP-binding protein NikD	8.2	21.5		
<i>yfeC</i>	Putative chelated Fe(II)-export protein	8.0	20.1		
<i>bisC</i>	Biotin sulfoxide reductase			3.1	2.9
<i>yeaJ</i>	Putative haem storage regulator	3.1		4.3

Fig. 2. Graphs showing growth curves for TG2(pBAD24) (●) and TG2(pBAD*insA-7*) (△). (a) Growth medium containing no additional metal ions. (b) Growth medium containing 0.6 mM Cd(II). (c) Growth medium containing 1.0 mM Zn(II). The graphs show OD₆₀₀ against time. Assays were performed in triplicate and the SDs are shown.

experiment encode proteins involved in the transposition of IS1, 2, 3, 5 and 30 (see data at URL <http://www.cf.ac.uk/biosi/staff/people/morby.html>).

It is well documented that IS genes may be induced by cellular stress and it is hypothesized that the movement of IS elements is capable of increasing genetic diversity (Naas *et al.*, 1994).

The most consistently induced genes are those for *insA* (IS1 transposition), for which transcripts are elevated in three out of the four strains. It is impossible to determine from array analyses which of the *insA/B* sequences were induced, given the close sequence similarity within the gene families, which may have resulted in cross-hybridization during the experiment. The repeated increase in transcript abundance of *insA* genes led to the investigation of metal tolerance in a wild-type strain expressing *insA-7* from a heterologous promoter.

Increase in metal tolerance by overexpression of *insA-7*

E. coli TG2 carrying pBAD*insA-7* was generated in which gene expression was controlled by the level of arabinose in the medium. *insA-7* was used since all *insA* sequences are almost identical and this gene has no apparent associated *insB*. When grown in medium containing arabinose, but no additional metal ions, TG2(pBAD*insA-7*) showed a slight growth advantage over TG2(pBAD24) (Fig. 2a). In contrast, when grown in the presence of arabinose plus 0.6 mM Cd(II) and 1.0 mM Zn(II), TG2(pBAD*insA-7*) shows a slight increase in tolerance to Cd(II) but a marked increase in tolerance to Zn(II) (Fig. 2b, c). No increase in tolerance to Co(II) or Ni(II) was observed (data not shown). The *insA*-dependent increase in tolerance to Zn(II) is consistent with the observation that *EZn* showed increases in transcript abundance for genes encoded by IS1.

InsA alone binds to *pinsL* to negatively auto-regulate and inhibit transposition of IS1 (Zerbib *et al.*, 1987; Machida & Machida, 1989; Matsutani, 1997). The

heterologous expression of *insA-7* should therefore reduce transposition of *IS1* in TG2(pBAD*insA-7*), which suggests that the metal-tolerance phenotype is directly conferred by an increase of *InsA-7* within the cell. This result contradicts the dogma that IS elements cannot directly enhance the fitness of their host, but merely carry genes for transposition functions (Blot *et al.*, 1993). Analysis of the *InsA-7* primary sequence shows the presence of two cysteine-X-X-cysteine motifs which are known to bind metal ions in both prokaryotic and eukaryotic proteins containing HMA motifs (GXXC-XXC) (Bull & Cox, 1994); such motifs also occur in group I, II and III metallothioneins (reviewed by Kille *et al.*, 1994). The CXXC motifs in *InsA-7* may enable this protein to bind metal ions in the cell cytosol. Given the spacing between the two CXXC motifs it is possible that these proteins bind to DNA using a Zn(II)-finger motif rather than the helix–turn–helix motif previously identified by weak similarity (24% identity) to known helix–turn–helix motifs (Zerbib *et al.* 1987).

Concluding remarks

In response to toxic concentrations of Zn(II), Cd(II), Co(II) or Ni(II), *E. coli* exhibited varying degrees of tolerance (3–14-fold greater than wild-type) both to the adaptive metal and its congeners. All of the adapted strains showed similar patterns of diminished gene expression, with particular bias towards genes whose products are involved in translation. No single gene whose expression increased was common to the four adapted strains, but surprisingly *insA* was increased in the Zn(II)-, Cd(II)- and Co(II)-adapted strains. Subsequent expression of *insA-7* in *E. coli* demonstrated that this gene can confer tolerance to Zn(II), suggesting that it may be capable of binding divalent metal ions by virtue of two paired cysteine motifs. This study shows the utility of an array-based approach to functional genomics in *E. coli* and the capacity for the generation of novel avenues of research using these techniques.

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