

ORIGINAL PAPER

Stefania Spada · J. Tony Pembroke · J. Gerard Wall

## Isolation of a novel *Thermus thermophilus* metal efflux protein that improves *Escherichia coli* growth under stress conditions

Received: August 8, 2001 / Accepted: October 18, 2001 / Published online: March 19, 2002

**Abstract** The mechanisms of metal ion transport in thermophilic organisms are poorly understood. Phage display-based screening of a *Thermus thermophilus* genomic library in *Escherichia coli* led to the identification of a novel metal cation efflux protein. The *Thermus* protein showed extensive sequence and putative structural conservation to Czc and Czc proteins in mesophilic bacterial and mammalian species. Expression of the gene in *E. coli* led to increased resistance to zinc and cadmium ions, but not to cobalt, in an effect that was apparently caused by increased efflux of metals from the cell. This increased resistance was inducible by zinc and cadmium and, to a lesser extent, by cobalt. Furthermore, *E. coli* cells containing the *Thermus* gene exhibited improved cell physiology and delayed cell lysis during recombinant protein production, leading to accumulation of higher levels of recombinant protein. The molecular basis and potential application of the findings are discussed.

**Key words** CzcB · Heavy metal efflux · *Thermus thermophilus* · Stress reduction

### Introduction

Many heavy metals, although toxic to microorganisms at high concentrations, are essential at lower levels for cellular growth and survival. Metal ions such as Fe, Cu, Ni, Mg, and Co are required for redox reactions and various enzymatic activities in the cell, whereas others, such as Cd and Hg, have no physiological function (Nies 1999). Zinc in particular plays an essential role in almost all aspects of

metabolism, including catalysis, structural stabilization, and regulatory functions, but is nevertheless toxic at higher concentrations (Silver et al. 1989; Hughes and Poole 1991; Nies 1999). Microorganisms, therefore, must have a finely tuned mechanism for sensing the level of heavy metals present in their growth environment and controlling the intracellular concentrations of these metal ions according to their biochemical needs.

Heavy metal uptake mechanisms consist of two distinct types: a fast, constitutively expressed, and generally substrate-nonspecific group of transporters that are usually proton motive force-driven and are of low energy cost to the cell, and a slower, substrate-specific, and ATP-hydrolytic family of transporter that is induced in times of metal starvation (Nies and Silver 1995; Nies 1999). Easy entry of metal ions into the cell via the former has necessitated development of mechanisms to counteract their accumulation to toxic levels in the cell. Because degradation is not a viable option and detoxification (in the form of reduction or complexation) tends to postpone rather than solve the problem, efflux systems play a critical role in maintaining cellular metal ion concentrations (Silver and Phung 1996; Nies 1999).

Four major families of proteins that catalyze the transport of heavy metal ions in microorganisms have been identified: P-type ATPases that transport  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$ , as well as  $\text{H}^+$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$ , both into and out of the cell; ABC-type transporters, which transport  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mo}^{2+}$ ; RND transporters that efflux  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Zn}^{2+}$  from the cell; and a novel cation diffusion facilitator (CDF) family that typically transport  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Co}^{2+}$  heavy metal ions (Paulsen and Saier 1997). During the past two decades, our understanding of the specificity, control, polarity, and efficiency of resistance mechanisms such as these has advanced considerably. Metal transport processes have primarily been characterized in mesophilic organisms to date, however, and our understanding of metal resistance mechanisms in species living at physiological extremes is poor. Furthermore, many of the best studied systems are plasmid-encoded and, although genome sequencing efforts increasingly reveal evidence for

Communicated by G. Antranikian

S. Spada · J.T. Pembroke · J.G. Wall (✉)  
Department of Chemical and Environmental Sciences, University of  
Limerick, Plassey Technological Park, Limerick, Ireland  
Tel. +353-61-202296; Fax +353-61-202568  
e-mail: gerard.wall@ul.ie

chromosomal homologues (Kuroda et al. 1999; Stover et al. 2000), our knowledge of chromosome-mediated metal resistance remains minimal.

*Thermus thermophilus* is a eubacterial thermophile that is commonly found in thermal springs and geothermal power plants. It requires metals such as zinc, cobalt, and copper for its growth, but its mechanisms of metal uptake and regulation are poorly understood. In this study, we report identification and functional characterization for the first time of a metal ion efflux protein from a thermophilic bacterium, *T. thermophilus*, and its expression and functional analysis in *Escherichia coli*. The protein protected *E. coli* cells from the toxic effects of heavy metal ions and exhibited a beneficial effect during bacteriophage and recombinant protein production.

## Materials and methods

### Bacterial strains and plasmids

*Escherichia coli* XL1-Blue (Bullock et al. 1987) was used for DNA cloning, sequencing, bacteriophage production, and growth experiments. *E. coli* JM83 (Yanisch-Perron et al. 1985) was the host for determination of minimal inhibitory concentrations (MICs) and zinc accumulation analysis. Phagemid pHB102 (Bothmann and Plückthun 1998) was used for *T. thermophilus* library construction and screening (Spada et al. 2001); pUC19 was used for DNA sequencing and MIC determinations.

### Manipulation of DNA

All DNA manipulation, if not otherwise stated, was carried out using standard methods (Sambrook et al. 1989).

### Construction of *Thermus thermophilus* genomic library

A 5-ml culture of *T. thermophilus* KT8 was harvested at an OD<sub>600</sub> of 1.8 and the cell pellet was resuspended in 0.5 ml STE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0). RNase A was added to a final concentration of 100 µg/ml, SDS to 8.5 mg/ml, and proteinase K to 100 µg/ml. Incubation for 2 h at 37°C was followed by two phenol extractions, three phenol:chloroform:isoamyl alcohol extractions, ethanol precipitation, and resuspension in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The DNA was partially digested using *Sau3AI*, and fragments in the 1- to 5-kb range were purified using a QIAEXII agarose gel DNA extraction kit (Qiagen) and cloned into a *Bgl*II-digested pHB102 phagemid vector containing an anti-fluorescein isothiocyanate (anti-FITC) scFv antibody fragment (Bothmann and Plückthun 1998). The library was transformed into *E. coli* XL1-Blue cells, and library size and diversity were estimated by *NotI* digestion.

### DNA sequencing and analysis

Double-stranded DNA was prepared using a Wizard Plus SV Minipreps DNA kit (Promega). DNA sequencing reactions were performed using the Sequitherm EXCEL II Long Read DNA Sequencing kit (Epicentre) and oligonucleotides PHBFOR (5'-AGTGTGACCGTGTGCTTCTC-3') and PHBREV (5'-GGAGAGCCTGAGCAAAGCGG-3'). Reactions were run on a Long ReadIR 4200 automated DNA sequencer (Licor), and sequences of both strands were determined before deposition in the EMBL database. Sequences were analyzed by BLASTx (Altschul et al. 1997) query of the EMBL database using the default parameters provided, and multiple sequence alignments were generated using CLUSTALW (Thompson et al. 1994). PSORT II (Nakai and Kanehisa 1992) was employed for subcellular localization prediction analysis, and a modified hidden Markov model was utilized for prediction of transmembrane helices (TMHMM; Krogh et al. 2001).

### Recloning the *czrB* gene

The *czrB* gene, with 100 bp upstream and 78 bp downstream, was amplified from *T. thermophilus* genomic DNA using oligonucleotides CZRBFOR1 (5'-CTGGATCCGCC TCGCCATGCTCCTCACC-3') and CZRBREV1 (5'-CTC AAGCTTAGAGCCAGTCGTGCTTCCTGCGC-3'). The amplification product was cloned into pUC19 and resequenced.

### Determination of MICs

*Escherichia coli* clones were grown in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and 25 µg/ml streptomycin for 90 min at 37°C. This growth was carried out with and without addition of 165 µM ZnCl<sub>2</sub>, 220 µM CoCl<sub>2</sub>, or 80 µM CdCl<sub>2</sub> (chosen as approximately 10% of MICs). Following dilution in LB, 10<sup>3</sup>–10<sup>4</sup> cells were spread on LB agar (plus ampicillin and streptomycin) containing ZnCl<sub>2</sub> (at concentrations ranging from 1.4 to 2.9 mM at 0.1-mM intervals), CoCl<sub>2</sub> (from 1.7 to 2.2 mM with 0.1-mM steps), or CdCl<sub>2</sub> (from 0.6 to 1.2 mM with 0.1-mM steps). Growth of *E. coli* was measured after 24 and 40 h, with MICs of the three metals defined as the lowest concentrations not allowing detectable *E. coli* growth after 40 h at 37°C. MIC determinations were carried out three times with each clone and metal ion; results shown are representative of three independent experiments.

### Analysis of metal accumulation

Intracellular zinc concentrations were measured using a protocol modified from Beard et al. (1997). *E. coli* clones were grown at 37°C in LB medium (100 µg/ml ampicillin, 25 µg/ml streptomycin) in the presence or absence of 165 µM ZnCl<sub>2</sub> until an OD<sub>600</sub> of 1.0 was reached. After addition of 1, 2, or 5 mM ZnCl<sub>2</sub>, growth was continued for

30 min, 1 h, or 2 h, with control cultures grown in the absence of  $\text{ZnCl}_2$ . Samples (20 ml) of each culture were centrifuged at 8,000 g for 20 min at 4°C, and cell pellets were washed in 4 ml LB and in 4 ml 0.1 N  $\text{HNO}_3$ . Following 15 min at 121°C, pellets were dissolved in 500  $\mu\text{l}$   $\text{H}_2\text{SO}_4$ , and approximately 150  $\mu\text{l}$   $\text{HNO}_3$  was added dropwise until the solution became clear; then, 6 ml of water was added, followed by centrifugation at 8,000 g for 25 min. The zinc concentration was measured in the supernatant using an atomic absorption spectrophotometer (Varian SpectraAA-400 Plus), with standard solutions prepared immediately before use from commercial standards (Fisher Scientific). A calibration curve relating  $\text{OD}_{600}$  to cell dry weight was used to calculate intracellular zinc concentrations at time of harvesting.

### Phage production and library screening

*Escherichia coli* cells harboring the *Thermus* library were inoculated to an  $\text{OD}_{600} \leq 0.05$  in 10 ml 2×YT (yeast-tryptone) medium containing tetracycline (15  $\mu\text{g}/\text{ml}$ ), additional salts (8.6 mM NaCl, 2.5 mM KCl, 10 mM  $\text{MgCl}_2$ ), and 0.4% glucose. After 1 h at 37°C, 30  $\mu\text{g}/\text{ml}$  chloramphenicol was added, followed by  $10^{10}$  plaque-forming units (pfu) of helper phage (VCSM13 helper phage; Stratagene) at an  $\text{OD}_{600}$  of 0.5. Incubation at 50°C for 5 min was followed by the addition of 50 ml 2×YT containing tetracycline, chloramphenicol, additional salts, and 0.5 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG). The culture was shaken at 40°C for 2 h and, after the addition of 30  $\mu\text{g}/\text{ml}$  kanamycin, grown for a further 12–14 h at 40°C.

Phage particles were precipitated from culture supernatants by two polyethylene glycol (PEG) precipitation steps and resuspended in 1 ml phosphate-buffered saline (PBS) (8 g NaCl, 0.24 g  $\text{KH}_2\text{PO}_4$ , 1.44 g  $\text{Na}_2\text{HPO}_4$ , 0.2 g KCl in 1 l, pH 7.4). Immunotubes (Nunc) were coated overnight at 4°C with 1  $\mu\text{g}/\text{ml}$  fluorescein isothiocyanate coupled to bovine serum albumin (FITC-BSA) (Bothmann and Plückthun 1998) in PBS. Blocking was with 5% skimmed milk in PBST (PBS containing 0.05% Tween-20) for 2 h at 37°C, followed by dilution of 800  $\mu\text{l}$  of the phage solution in 3.3 ml 2% skimmed milk in PBST and incubation in the tubes for 2 h at 25°C. Twenty washes with PBST and two with PBS were followed by elution of bound particles for 10 min at room temperature using 1 ml 0.1 M glycine/HCl (pH 2.2). The eluate was neutralized immediately with 60  $\mu\text{l}$  2 M Tris and used for reinfection of *E. coli*; this procedure was repeated for five rounds of binding and reinfection ("panning"). After each panning round, DNA from the phage pool was digested with *NotI* to check for enrichment of *Thermus* DNA inserts. After the fifth panning round, insert sizes of 30 isolated clones were determined by *NotI* digestion.

### Growth experiments and phage titering

*Escherichia coli* cells infected with phage were grown as described for phage production. The  $\text{OD}_{600}$  was read at hourly intervals for the first 8–10 h after induction, and cul-

ture samples were collected to determine phage titers (Sambrook et al. 1989) and the amount of antibody on the phage surface. Individual clones were analyzed by restriction digestion after 22 h of induction to confirm they contained the *Thermus* insert and the recombinant antibody gene. Clones were analyzed in at least three independent experiments and, although absolute OD values, phage titers, and enzyme-linked immunosorbent assay (ELISA) readings varied between experiments, the respective patterns of growth and production exhibited by individual clones remained highly consistent throughout.

### Enzyme-Linked Immunosorbent Assay

Immunoplate wells (Nunc) were coated with 100  $\mu\text{l}$  FITC-BSA and blocked with 5% skimmed milk in PBST. After washing, 100- $\mu\text{l}$  phage solutions containing 0.5% skimmed milk were added and incubated for 2 h at 25°C. Phage particles were detected using a peroxidase-conjugated anti-M13 antibody (1:3,000 in PBST; Amersham Pharmacia Biotech), and development was carried out using a BM Blue POD soluble substrate (Roche Diagnostics). After stopping the reaction using 25  $\mu\text{l}$  1 N  $\text{H}_2\text{SO}_4$ , the absorbance was read at 405 nm.

### Nucleotide sequence accession number

The *cztB* sequence data were submitted to the EMBL database under accession number AJ307316.

## Results and discussion

Phage display technology is the technique of expressing proteins or peptides in functional form on the surface of bacteriophage particles. These bacteriophages each display the recombinant molecule encoded in their genome and phage particles with a high affinity for a ligand of interest are selected on the immobilized ligand and propagated in *E. coli* cells (McCafferty et al. 1990). The approach has generally been used to isolate high-affinity binders, typically antibodies, from large, combinatorial libraries. In this work, all phage particles expressed the same antibody molecule so that the strength of the antibody–ligand interaction was identical for all bacteriophage particles. Thus, selection of a bacteriophage from the phage population was dictated, not by antibody affinity, but by the efficiency with which it, and its displayed protein in particular, was produced by the host *E. coli* cells (Bothmann and Plückthun 1998). Many recombinant molecules are, however, poorly folded or expressed in *E. coli* (Wall and Plückthun 1999). To identify novel factors that might improve antibody folding in *E. coli*, we introduced a *T. thermophilus* genomic library into the phage-producing cells, resulting in a selection for proteins from the thermophile that aid expression of the recombinant antibody in *E. coli*. *T. thermophilus* was chosen for the

work in an attempt to identify novel chaperone-like molecules that enable this thermostable organism to efficiently fold its proteins at elevated temperatures.

#### Construction and screening of *Thermus thermophilus* library

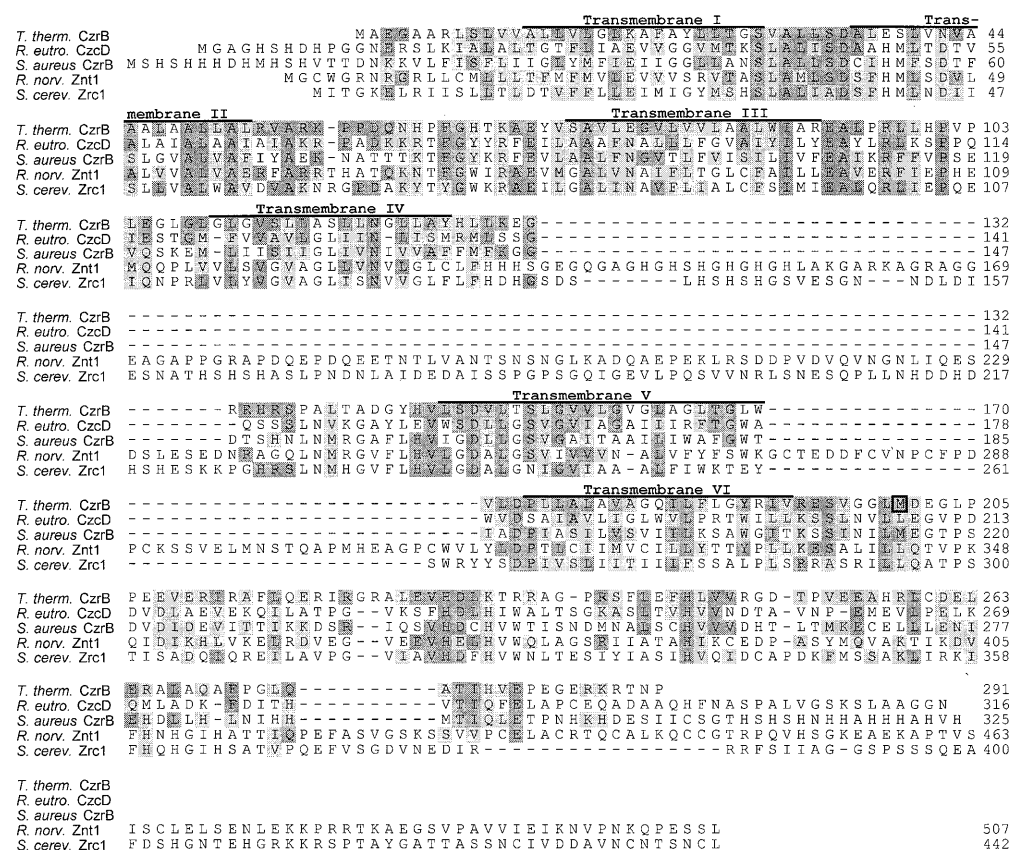
Cloning of *T. thermophilus* genomic DNA yielded a library of  $1.1 \times 10^4$  clones. Restriction analysis of the library pool and of isolated clones indicated that 90% of clones contained an insert and that insert diversity was high. The library was calculated to be tenfold the size necessary to include all *T. thermophilus* genes.

Phage production was induced overnight in *E. coli* cells harboring the *Thermus* library. As these cells also expressed an antibody fragment on the phage surface, five rounds of phage selection on immobilized FITC, the antigen recognized by the displayed antibody fragment, were carried out. After the third round, a DNA fragment of approximately 1.2 kb began to appear in the digested library pool, as well as a less intense band of 1.8 kb that became considerably enriched by the fifth panning round (data not shown). After the fifth round, 30 isolated clones were analyzed by *NotI* digestion. One clone had an insert of approximately 1.8 kb, 2 clones had inserts of 1.2 kb, 25 clones between 50 and 280 bp, and the remaining 2 clones no insert. Clones containing the 3 larger fragments were sequenced to identify the isolated *Thermus* genes.

#### Identification and analysis of *Thermus thermophilus* *czrB*

Sequencing of the isolated 1.8-kb clone revealed an insert of 1,743 bp, containing a single complete open reading frame (ORF) of 876 bp. BLASTx analysis (Altschul et al. 1997) of the complete ORF using the EMBL database revealed homology to cation efflux system proteins, mostly termed Czc (for cadmium-zinc resistance) or CzcD (for cadmium-zinc-cobalt resistance), from a variety of organisms (Pfam 01545; Protein Families database, <http://www.sanger.ac.uk/Software/Pfam/>; Paulsen and Saier 1997). Based on experimental analysis, we propose naming the *T. thermophilus* gene *czrB*, after the *Staphylococcus aureus* gene (Kuroda et al. 1999). Of the homologous proteins, only those from rat (Palmiter and Findley 1995), *Saccharomyces cerevisiae* (Kamizono et al. 1989; Conklin et al. 1994), *Ralstonia eutropha*-like CH34 (previously *Alcaligenes eutrophus* CH34; Nies et al. 1989; van der Lelie et al. 1997), and *S. aureus* (Xiong and Jayaswal 1998; Kuroda et al. 1999) have been phenotypically characterized, with the main structural difference between proteins being the extended loops between putative transmembrane segments in eukaryotic species (Fig. 1). Prediction analysis of the *Thermus* protein envisaged it to be a cytoplasmic membrane protein also, of molecular mass 31,233 Da. It contained six putative membrane-spanning  $\alpha$ -helices, of which the 4 N-terminal spanners were highly hydrophobic (Krogh et al. 2001), features conserved in other CzcD-like proteins (Fig. 1) (Paulsen and Saier 1997; Anton et al. 1999). The *Thermus*

**Fig. 1.** Alignment of the *Thermus thermophilus* CzcB amino acid sequence with homologues from *Ralstonia eutropha* (CzcD, accession number P13512), *Staphylococcus aureus* (CzcB, Q9ZNF5), rat (Znt1, Q62720), and *Saccharomyces cerevisiae* (Zrc1, P20107). Dark shading, residues identical to the *T. thermophilus* sequence; lighter shading, residues homologous to the *Thermus* sequence. Predicted transmembrane domains from the *T. thermophilus* protein are indicated above the aligned sequences, and the putative translation start site for the partial *czrB* gene isolated from the library (Met<sub>200</sub>) is boxed



protein, however, lacked both the C-X-X-C putative metal-binding motif and the histidine-rich N- and C-termini found in a number of other CzcD-like proteins and, although none of these features is strictly conserved (Singh et al. 1999), the mechanism of its interaction with metal cations remains unclear. Helices II, V, and VI generally each contain a conserved aspartyl residue, the  $\beta$ -carboxyls of which may form a cation-binding site in the membrane, but the *T. thermophilus* protein also exhibits a D42N change from this consensus in helix II (Fig. 1).

The *Thermus* structural gene had a GC content of 72% overall and 94% in the third nucleotide position, typical of genes from thermophilic species (Wilquet and van de Castele 1999). Amino acid compositional analysis revealed a number of characteristics of thermostability in the *Thermus* protein (Kumar et al. 2000), such as significantly ( $\pm 2$  SD) higher arginine and lower serine contents than in the other prokaryotic members of the Pfam 01545 cation efflux family. Protein modeling would now be necessary to determine whether the overall compactness of the molecule is increased compared to its mesophilic homologues.

Sequencing of the two 1.2-kb clones isolated during library screening revealed identical partial copies of *czrB*, encoding the 108 C-terminal amino acids of this 291-residue protein. With the next consensus ATG in the *czrB* gene (Met<sub>200</sub>) closely preceded by a putative ribosome-binding site, it is likely that translation begins in these clones at residue 200 and yields a 92-amino-acid peptide corresponding to the C-terminal, cytoplasmic tail of the mature CzcB molecule (see Fig. 1). Subcellular localization analysis of this putative polypeptide (Nakai and Kanehisa 1992) predicted it to form a soluble, cytoplasmic molecule in the cell.

### Subcloning of *czrB*

Partial ORFs that showed homology to lysyl-tRNA synthetase and methylmalonyl-CoA mutase chain A were identified flanking *czrB* in the 1.8-kb *Thermus* DNA insert. Thus, the *czrB* gene was recloned from the *Thermus* genome to eliminate potential interference of the flanking genes in phenotypic analysis of CzcB in *E. coli*. Following cloning into pUC19, the original *czrB* sequence was confirmed.

### Determination of minimal inhibitory concentrations of heavy metals

CzcD homologues were originally thought to act as membrane-bound sensors that detect divalent metal cations in the cellular environment, leading to efflux of metal ions via a CzcCB<sub>2</sub>A zinc efflux-proton antiporter channel (Nies 1992; Paulsen and Saier 1997). More recently, it has become apparent with a number of organisms that the protein also carries out metal efflux directly (Palmiter and Findley 1995; Anton et al. 1999; Grass et al. 2001). *E. coli* cells containing the *T. thermophilus czrB* gene exhibited a significantly higher minimal inhibitory concentration (MIC) for Zn<sup>2+</sup> than cells containing pUC alone, whereas the cadmium MIC increased only slightly and cobalt resistance was unaf-

ected by the presence of *czrB* (Table 1). The substrate specificity is more similar to that of CzcB of *S. aureus* (Kuroda et al. 1999) and ZRC1 of *S. cerevisiae* (Kamizono et al. 1989), in which separate cobalt export systems also exist, than to the well-characterized *R. eutropha* CzcD, which uses cobalt as a substrate (Nies and Silver 1995), suggesting that an independent cobalt efflux system also exists in *T. thermophilus*. Homologues of *czrB* are frequently (van der Lelie et al. 1997; Kuroda et al. 1999), although not exclusively (Sturr et al. 1997), coregulated with additional, contiguous genes in other species; these gene products, such as components of the CzcCB<sub>2</sub>A efflux pump, are also involved in metal pumping and are responsive to *czrB*. There appear to be no such functionally related genes flanking *czrB* in *T. thermophilus*, and it is currently unclear whether these genes exist in a noncontiguous location in the *T. thermophilus* genome. Alternatively, because thermophilic species generally have relatively small genomes, the *T. thermophilus czr* system may by necessity be less complex than its mesophilic homologues, with CzcB carrying out sensing and efflux in the absence of the dedicated efflux pump found in other species.

### Induction of metal resistance

Induction of metal resistance in *E. coli* cells carrying *czrB* was studied by preincubation with sub-MIC (~10%) levels of metal ions for 90 min, followed by analysis of growth on increasing metal ion concentrations after 24 and 40 h. This relatively short induction period, designed to drive production of the transporter from its multicopy plasmid, was found to have a significant effect on metal tolerance of cells over the subsequent growth period, but with no difference observed between relative MICs measured after 24 or 40 h. Resistance to zinc mediated by the *Thermus czrB* could be induced by preincubation of *E. coli* cells with zinc, cadmium, or cobalt (see Table 1). Thus, curiously, the *Thermus* protein appears to recognize cobalt and yet not to transport the cation. The ability to induce zinc tolerance was considerable, with the zinc MIC increasing from 1.6 mM (no *czrB*)

**Table 1.** Minimal inhibitory concentrations (MICs) of ZnCl<sub>2</sub>, CoCl<sub>2</sub>, and CdCl<sub>2</sub> determined for *Escherichia coli* JM83, JM83 containing pUC, and JM83 containing pUC with cloned *czrB*

Clone	Preinduction	Zinc (mM)	Cobalt (mM)	Cadmium (mM)
JM83	None	2.0	2.1	0.9
+pUC	None	1.6	2.1	0.9
+pUC	Zinc	1.6	2.1	0.9
+pUC	Cobalt	1.6	2.1	0.9
+pUC	Cadmium	1.6	2.1	0.7
+pUC- <i>czrB</i>	None	1.9	2.1	1.0
+pUC- <i>czrB</i>	Zinc	2.5	2.1	1.0
+pUC- <i>czrB</i>	Cobalt	2.1	2.1	1.1
+pUC- <i>czrB</i>	Cadmium	2.2	2.1	0.9

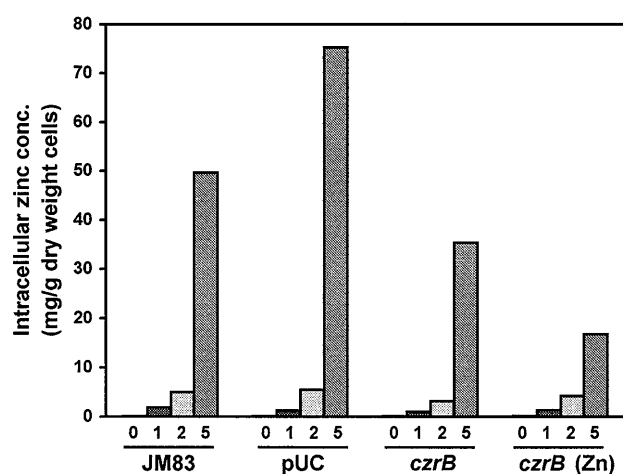
MICs were determined for each metal with and without preexposure of clones to ZnCl<sub>2</sub> (165  $\mu$ M), CoCl<sub>2</sub> (220  $\mu$ M), or CdCl<sub>2</sub> (80  $\mu$ M). Results shown are representative of three independent experiments

to 1.9 mM (with *cztB*) to 2.5 mM (with *cztB* preinduced by zinc) in the *E. coli* cells. The ability to induce cadmium resistance was poor, however, with only slight, statistically non-significant, increases in MIC inducible with cadmium or cobalt, and no detectable effect with zinc, whereas zinc and cadmium commonly induce resistance to each other via the *czc/czt* efflux systems in other organisms (Nies and Silver 1995; Hassan et al. 1999) in a process mediated by increased transcription of efflux protein-encoding genes (Conklin et al. 1994; Xiong and Jayaswal 1998).

No increase in zinc MICs was seen on preincubation of wild-type *E. coli* cells with zinc (see Table 1), in spite of reports in the literature of a number of zinc-inducible transporters in the organism. Functional analysis of such transporters has generally involved identification of metal toxicity in gene-deleted or gene-interrupted strains (Beard et al. 1997; Grass et al. 2001), with increased metal tolerance arising from expression of the genes on multicopy plasmids (Grass et al. 2001). It appears likely that the increased tolerance to metals observed with *cztB* in this work is dependent on the high copy number of the gene, as observed by other groups with homologous zinc transporters (Xiong and Jayaswal 1998; Grass et al. 2001) and that the short preinduction step incorporated into our experimental setup is not sufficient to produce a detectable effect on MICs via induction of chromosomally encoded genes.

#### Measurement of intracellular zinc concentrations

Investigation of intracellular zinc concentrations was carried out to determine whether *cztB*-mediated resistance was the result of metal sequestering, which would increase the level of metal ions in the cell, or variations in transport, which should reduce intracellular levels. The presence of *cztB* was found to significantly reduce the levels of zinc in *E. coli* cells, whereas pUC alone led to elevated intracellular



**Fig. 2.** Quantification of intracellular zinc levels in *Escherichia coli* clones. Intracellular zinc concentrations were determined by atomic absorption spectroscopy for *E. coli* JM83 (JM83), JM83 containing pUC (pUC), and JM83 containing pUC-*cztB* without (*cztB*) and with [*cztB* (Zn)] a zinc preinduction step. Results are shown for 1-h incubations in 0, 1, 2, and 5 mM extracellular zinc concentrations

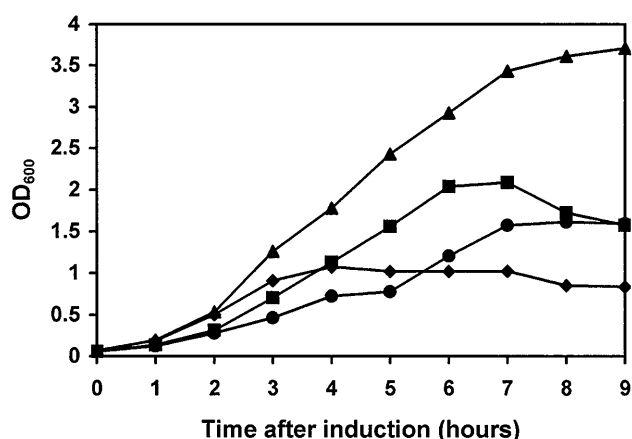
zinc levels (Fig. 2), indicating that increased metal resistance was mediated by modification of either influx or efflux activity. This effect was particularly evident at high extracellular zinc concentrations that led to elevated initial intracellular concentrations in *E. coli* cells (see Fig. 2). Preexposure of cells to zinc further reduced cellular levels in *cztB* clones in high-zinc environments, indicating an inducible resistance mechanism, as observed in MIC experiments.

A time course experiment was carried out to distinguish between reduced influx and increased efflux as the cause of this reduced cellular accumulation of zinc. Intracellular zinc concentrations decreased significantly over the analysis period, with the rate of ion removal increased on preexposure of cells to zinc (data not shown), indicating that the protective mechanism of *cztB* involves an inducible metal efflux process. CztB-like proteins in other organisms have been found to act as metal sensors as well as efflux proteins per se. Because little or no benefit might be expected to accrue to host cells from increased numbers of sensor molecules at the very high metal concentrations used in our work, we propose that the *T. thermophilus* CztB protein effluxes metals directly, as has been reported in rat (Palmiter and Findley 1995), *R. eutropha* (Anton et al. 1999), and, more recently, *E. coli* (Grass et al. 2001).

#### Effects on growth of *Escherichia coli* under stress conditions

The strong selection of clones that had eliminated all or part of their *Thermus* insert during phage panning (27 of 30 clones analyzed had inserts <300 bp) indicated that *cztB* exerted a strong positive effect on its host cells merely to be retained throughout library screening. In addition, the *cztB* gene was contained in all three large-insert clones selected from the library, in one case as a full-length molecule and in the other two as identical partial sequences. Because an increased metal efflux capability was of no apparent advantage in the screening process, *E. coli* clones were analyzed under recombinant antibody and phage production conditions to investigate the basis of the effect of CztB. This study revealed that *cztB*-containing clones grew considerably better than cells containing only the phagemid vector (Fig. 3), with delayed cell lysis and attained cell densities greater than twofold higher. These cell density differences were maintained after 22 h of induction (data not shown), and this improved growth in the presence of *cztB* is believed to account for selection of the gene from the library.

Isolated clones containing the partial *cztB* insert (thought to synthesize a 92-amino-acid cytoplasmic tail of CztB) exhibited growth characteristics intermediate between the full-length *cztB* clone and cells containing the phagemid vector (see Fig. 3), whereas a randomly selected poorer growth than clones with the vector, providing an insight into how the original library became biased in favor of clones containing small-sized or no *Thermus* inserts in the absence of a phenotypic benefit associated with the cloned DNA.



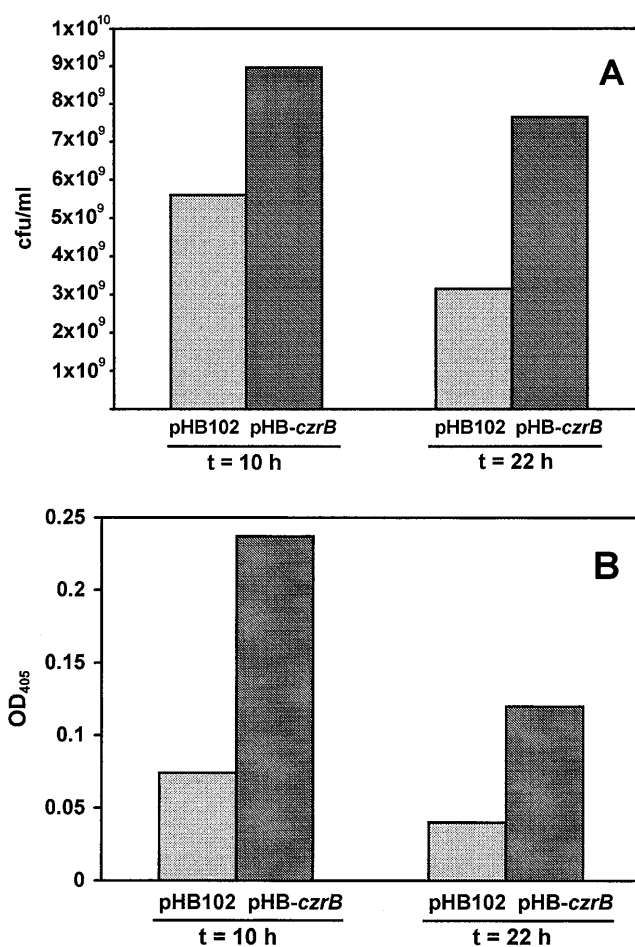
**Fig. 3.** Growth of *E. coli* clones containing *T. thermophilus czrB*. Growth characteristics are shown for *E. coli* clones containing pHB102 phagemid vector alone (circles) or with *czrB* (triangles), a partial *czrB* gene (squares), or random *T. thermophilus* DNA (diamonds) as an insert. The experiment was carried out during bacteriophage and recombinant antibody production in the *E. coli* cells

#### Analysis of effects on bacteriophage and recombinant protein production

*Escherichia coli* cultures containing *czrB* were found to exhibit twofold-higher phage titers and to produce more than twice the amount of functional antibody as cultures containing the vector alone (Fig. 4). These increased yields could be attributed to the increased cell densities observed with *czrB*, such that the *czrB* gene product appeared to have no effect on cellular antibody expression or bacteriophage production. To investigate this further, *czrB* was expressed from a *lac* promoter in a standard pUC-based expression vector. The same pattern of *E. coli* cell growth, relative to cells containing the vector alone, was observed on  $P_{lac}$  induction (data not shown), confirming that the effect of CzcB appears to be to improve *E. coli* physiology, at least in the presence of pUC-based vectors, rather than to interact directly with the antibody or bacteriophage molecules in the cell. Cells containing the partial *czrB* gene also showed intermediate signals in both phage titer and ELISA studies (data not shown), leading to the conclusion that, although the transmembrane domains of the *T. thermophilus* protein are required for its full effect to be achieved, the putative cytoplasmic tail of CzcB alone makes a significant contribution to the beneficial effects of the protein in *E. coli*. Although our truncated CzcB construct was not tested in efflux experiments, other workers have found that the C-terminal 62 and 72 amino acids from rat ZnT-1 (Palmiter and Findley 1995) and *R. eutropha* CzcD (Nies et al. 1989), respectively, are not essential for the role of the protein in cation efflux, suggesting that the roles of *T. thermophilus* CzcB in metal efflux and in facilitating *E. coli* growth occur via distinct mechanisms.

#### Biotechnological potential of CzcB

Polypeptide misfolding and stress-induced lysis of host cells are phenomena frequently encountered when expressing



**Fig. 4A,B.** Phage titers and ELISA analysis of clones expressing *czrB*. **A** Phage titers of clones containing pHB102 phagemid vector or pHB102-*czrB* were determined 10 h (left) and 22 h (right) after induction; cfu, colony-forming units. **B** Anti-FITC ELISAs were carried out on the same clones to investigate functionality of bacteriophage-displayed FITC-binding antibody fragments. Results are shown for samples taken 10 h (left) and 22 h (right) after induction

heterologous proteins in *E. coli*. Rational engineering has generally provided only protein-specific solutions to this problem to date (Knappik and Plückthun 1995; Wall and Plückthun 1999), whereas coexpression of folding-assisting molecules, although apparently highly promising, has also failed thus far to provide a general means of improving protein yields. This study has identified a *T. thermophilus* protein that improves, by a mechanism which is apparently independent of the heterologous protein being expressed, *E. coli* growth characteristics under recombinant protein production conditions. We speculate that overproduction of exported proteins in *E. coli* leads to pressure on the cytoplasmic membrane translocation machinery, resulting in agglomeration of polypeptides at the membrane and interference with metal extrusion systems. Coexpression of this *T. thermophilus* gene thus may have general application in increasing production of heterologous proteins in *E. coli* and, in particular, molecules that impose a severe physiological strain on the producing cell.

**Acknowledgements** The authors wish to thank Ray Hanamy for help with atomic adsorption analysis. Stefania Spada was funded by Enterprise Ireland Science and Technology Development Agency award PD/1997/026.

## References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Anton A, Grosse C, Reissmann J, Pribyl T, Nies DH (1999) CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. *J Bacteriol* 181:6876–6881
- Beard SJ, Hashim R, Membrillo-Hernández J, Hughes MN, Poole RK (1997) Zinc(II) tolerance in *Escherichia coli* K-12: evidence that the *zntA* gene (*o732*) encodes a cation transport ATPase. *Mol Microbiol* 25:883–891
- Bothmann H, Plückthun A (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat Biotechnol* 16:376–380
- Bullock WO, Fernandez JM, Short JM (1987) XL1-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with  $\beta$ -galactosidase selection. *BioTechniques* 5:376–379
- Conklin DS, Culbertson MR, Kung C (1994) Interactions between gene products involved in divalent cation transport in *Saccharomyces cerevisiae*. *Mol Gen Genet* 244:303–311
- Grass G, Fan B, Rosen BP, Franke S, Nies DH, Rensing C (2001) ZitB (YbgR), a member of the cation diffusion facilitator family, is an essential zinc transporter in *Escherichia coli*. *J Bacteriol* 183:4664–4667
- Hassan M-e-T, van der Lelie D, Springael D, Römmling U, Ahmed N, Mergeay M (1999) Identification of a gene cluster, *czt*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. *Gene (Amst)* 238:417–425
- Hughes MN, Poole RK (1991) Metal speciation and microbial growth – the hard (and soft) facts. *J Gen Microbiol* 137:725–734
- Kamizono A, Nishizawa M, Teranishi Y, Murata K, Kimura A (1989) Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. *Mol Gen Genet* 219:161–167
- Knappik A, Plückthun A (1995) Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Eng* 8:81–89
- Krogh A, Larsson B, von Heijne G, Sonnhammer ELL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305:567–580
- Kumar S, Tsai C-J, Nussinov R (2000) Factors enhancing protein thermostability. *Protein Eng* 13:179–191
- Kuroda M, Hayashi H, Ohta T (1999) Chromosome-determined zinc-responsive operon *czt* in *Staphylococcus aureus* strain 912. *Microbiol Immunol* 43:115–125
- McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies – filamentous phage displaying antibody variable domains. *Nature (Lond)* 348:552–554
- Nakai K, Kanehisa M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 14:897–911
- Nies DH (1992) CzcR and CzcD, gene products affecting regulation of resistance to cobalt, cadmium and zinc (*czc* system) in *Alcaligenes eutrophus*. *J Bacteriol* 174:8102–8110
- Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* 51:730–750
- Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. *J Ind Microbiol* 14:186–199
- Nies DH, Nies A, Chu L, Silver S (1989) Expression and nucleotide sequences of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proc Natl Acad Sci USA* 86:7351–7355
- Palmiter RD, Findley SD (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J* 14:639–649
- Paulsen IT, Saier MH Jr (1997) A novel family of ubiquitous heavy metal ion transport proteins. *J Membr Biol* 156:99–103
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Press, New York
- Silver S, Phung LT (1996) Bacterial heavy metal resistances: new surprises. *Annu Rev Microbiol* 50:753–789
- Silver S, Misra TK, Laddaga RA (1989) Bacterial resistance to toxic heavy metals. In: Beveridge TJ, Doyle RJ (eds) *Metal ions and bacteria*. Wiley, New York, pp 121–139
- Singh VK, Xiong A, Usgaard TR, Chakrabarti S, Deora R, Misra TK, Jayaswal RK (1999) ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon *znt* of *Staphylococcus aureus*. *Mol Microbiol* 33:200–207
- Spada S, Gilbert Y, Pembroke JT, Wall JG (2001) Isolation and characterisation of the *ylmE* homologue of *Thermus thermophilus*. *DNA Sequence* 11:507–514
- Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GKS, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature (Lond)* 406:959–964
- Sturr MG, Ablooglu AJ, Krulwich TA (1997) A *Bacillus subtilis* locus encoding several gene products affecting transport of cations. *Gene (Amst)* 188:91–94
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 22:4673–4680
- van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, Baeyens W, Mergeay M, Nies DH (1997) Two-component regulatory system involved in transcriptional control of heavy-metal homeostasis in *Alcaligenes eutrophus*. *Mol Microbiol* 23:493–503
- Wall JG, Plückthun A (1999) The hierarchy of mutations influencing the folding of antibody domains in *Escherichia coli*. *Protein Eng* 12:605–611
- Wilquet V, van de Casteele M (1999) The role of the codon first letter in the relationship between genomic GC content and protein amino acid composition. *Res Microbiol* 150:21–32
- Xiong A, Jayaswal RK (1998) Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. *J Bacteriol* 180:4024–4029
- Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene (Amst)* 33:103–119