

Preliminary Characterization of *zntA*, a Gene Which Encodes a Zn(II)/Cd(II)-Export Protein in *Escherichia coli*

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Abstract

An increasing number of proteins from both prokaryotic and eukaryotic organisms which transport and/or bind transition metal ions contain sequences corresponding to the conserved heavy metal associated motif (GMTCXXC). Those proteins in this group which translocate metal ions across membranes also show primary sequence similarity to the P-type ATPase family of membrane transport proteins. Database analysis identified a gene encoding a candidate metal ion transport ATPase in the *E. coli* genome (*yhho*). The gene has been isolated and expressed in *E. coli* from a heterologous promoter. Data supports the function of this polypeptide as a Zn(II)/Cd(II) export protein and we designate the gene, *zntA*.

Introduction

Zn(II) is an important trace metal ion, being almost as abundant as iron in the human body (Harrison and Hoare, 1980), and is required by all organisms for both catalytic and structural purposes. The control of cellular Zn(II) status is essential for efficient metabolism. In addition, the control of cellular Zn(II) availability within cells has recently been implicated in "switching" whole classes of genes on and off due to alterations in the metal ion occupancy of Zn(II) requiring transcription factors. This proposed role places this simple cation at the center of a range of developmentally programmed changes and extends its importance in the context of cellular control (Suhy and O'Halloran, 1996).

Little is known about the control of Zn(II) status in prokaryotes, only the plasmid-borne Cd(II)/Zn(II) resistance determinants (Nies and Silver, 1995) and the cyanobacterial metallothionein SmtA, (Turner and Robinson, 1995) are known to be involved in the modulation of content and/or availability of Zn(II).

The *E. coli* genome sequencing program has identified a gene (*yhho*) (Sofia *et al.*, 1994) whose predicted gene product contains a cysteine-rich N-terminus and which has primary sequence similarity to a range of proteins known to be involved in the metabolism of, or resistance to, the metal ions (Hg(II), Cd(II), Cu(II)) in prokaryotes and eukaryotes. Of particular interest is the conservation among these proteins of an N-terminal "heavy metal associated" motif (GMTCXXC) (Sofia *et al.*, 1994), which is thought to be involved in metal ion binding (Bull and Cox, 1994).

Here we report the heterologous expression of this gene. Our data show that this gene product mediates alterations in cellular tolerance to Zn(II) and Cd(II). The data are consistent with the designation of this gene product as a Zn(II)/Cd(II)-export protein.

Materials and Methods

Bacterial Strains and Plasmids. The following *E. coli* strains were used: JM109 (Yanish-Perron *et al.*, 1985) and TOP10 F' (Invitrogen). Plasmids used were pCRBlunt (Invitrogen) and pBAD18 (Guzman *et al.*, 1995). All strains were cultured in LB-medium at 37°C. Antibiotics were added to media at standard concentrations (Sambrook *et al.*, 1989).

DNA Manipulations. All DNA manipulations were carried out according to Sambrook *et al.*, 1989.

Plasmid Construction. pCRzntA was created by the ligation of the zntA PCR product (using primers zit3 and zit4, see below) with pCR-Blunt according to manufacturers instructions (Invitrogen). pBADzntA was created by the ligation of an XbaI/HindIII fragment from pCRzntA into XbaI/HindIII cut pBAD18.

PCR Amplification of zntA. Vent DNA polymerase (NEB) was used for DNA amplification according to manufacturers instructions using a Techne Progene Thermal Cycler. Primers used to amplify the structural gene were zit3 (5'-GAGGATGCCATGTCGACTC-3') and zit4 (5'-GATGGTGCGCAAACCGTG-3'). The nucleotide sequence of the zntA PCR product was monitored for alterations using DNA Sequencing Kit (PE Applied Biosystems) and analyzed on an ABI Prism automated sequence analyzer (PE Applied Biosystems), and no alterations were found.

Minimum Inhibitory Concentration Assays. Overnight liquid cultures were initiated from single colonies in media containing antibiotic selection. The resulting cultures were diluted 1:100 in fresh media and grown for 6 hours with and without 0.5% arabinose. Cultures were then diluted 1:50 into sterile isosaline and 10µl samples placed onto selective agar containing increasing amounts of Zn(II) or Cd(II). After overnight incubation the minimal inhibitory concentration for each strain was defined as the lowest metal ion concentration which completely inhibited growth. These assays were performed in triplicate; standard deviations are shown.

Results

Identification of a Metal Ion ATPase Primary Sequence. TBLASTN was used with the MerP (Ni'Bhriain and Foster 1986) sequence as a probe to search the non-redundant DNA database at NIH. zntA was within a range of sequences encoding heavy metal associated motifs and having a strong statistical similarity. Figure 1 shows an alignment of a reduced set of those sequences with similarity to MerP. A consensus sequence is shown.

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E.coli ZntA      VSGMDCCAACARKVENAVRQLA-GVNQVQVLFA
Tn501 MerP      VPGMTCSACPITVKKAISEVE-GVSKVDVTFE
Tn501 MerA      ITGMTCDCSCAAHVKEALEKVP-GVQSALVSY-
S.aureus CadA  VQGFTCANCAGKFEKNVKKIP-GVQDAKVNFG
Human Menkes   VEGMTCNSCVWTIEQQIGKVN-GEHHIKVSLE
Yeast Atx1     -VVMTCSGCSGAVNKVLTkLEPDVSKIDIS--
Rat Wilsons    --GMTCASCVANIERNLRREE-GIYSV-----
Hum Wilsons    YEGMTCQSCVSSIEGKYRKLQ-GVVRVKVSL-
Rice Cu ATPase --GMSCQGCAGAVRRVLTkME-GVETFDIDME
H.pylori Cu ATPase VPSITCSHCVDKIEKFVGEIE-GVSFIDANVE

Consensus      --gMTC--C-----GV---d---e
    
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Fig. 1. Sequence similarity of ZntA with metal binding/transport proteins. GCG Pileup (Devereux *et al.*, 1984) was used to optimally align a sub-set of proteins that contain the heavy metal associated (HMA) motif. The HMA consensus is shown in bold.

Expression of zntA from a Heterologous Promoter. The complete *zntA* coding region, ribosome binding site and predicted terminator sequence was introduced into the high copy number expression vector pBAD18 under control of the arabinose promoter to create pBAD*zntA*. *E. coli* JM109 cells containing pBAD18 and pBAD*zntA* were assayed for tolerance to Zn(II) and Cd(II) using minimal inhibitory concentration assays (MIC). The MIC values for JM109(pBAD18) were taken to represent an arbitrary 0-100% scale and all other readings were normalized to that control scale.

In cells containing pBAD18 or pBAD*zntA* which were cultured under inducing conditions (+ arabinose) prior to use in MIC assays, the presence of *zntA* conferred an increased tolerance to Zn(II) (130 % +/- 5 % of w.t.) and an increased tolerance to Cd(II) (169 % +/- 13% of w.t.) (Table 1). Cultures grown without additional arabinose (uninduced) showed no alteration in tolerance to Zn(II) and only a slight increase in tolerance to Cd(II) (110% +/- 0 of w.t.). In comparison, previous work detailing the introduction of a Cd(II)-resistance mechanism (*cadAC*) from *Staphylococcus aureus* into *E. coli* (Yoon and Silver, 1991) conferred a maximal resistance approximately 400% that of cells expressing *zntA* (*E. coli* (pBAD*zntA*) and *E. coli* (*cadAC*) gave maximal Cd(II) resistance of 700 µM and 2,560 µM ,respectively).

Culture conditions	Zn(II)-MIC (% of control cell)	Cd(II)-MIC (% of control cell)
Non-induced	100 (0)	110 (0)
Induced	130 (5)	169 (13)

Table 1. The data presented are mean values derived from MIC assays of *E. coli* JM109 (pBAD*zntA*) performed in triplicate and normalized as a percentage against the mean MIC values obtained for the control strain JM109 (pBAD18). Standard deviations are shown in brackets. The non-linear nature of the assay resulted in no error values being generated in some data sets.

Sequence Analysis of zntA. The encoded polypeptide of *zntA* is 732 amino acid residues in length and has good primary sequence similarity to CadA (36% identity/58% similarity with *cada_bacfi.sw* (Ivey *et al.*, 1992)), a Cd(II)-export protein which confers resistance in a range of prokaryotic organisms. In addition, ZntA contains an N-terminal region with six cysteine residues, two arranged in the heavy metal associated motif GMDCAAC (residues 56-62) with the other four lying closer to the N-terminus and arranged as CCCXXXC (residues 29-35). Analysis of hydropathy and acid/base distribution allows us to propose a topology for ZntA (Figure 2) similar to that proposed for CadA (Nucifora *et al.*, 1989)

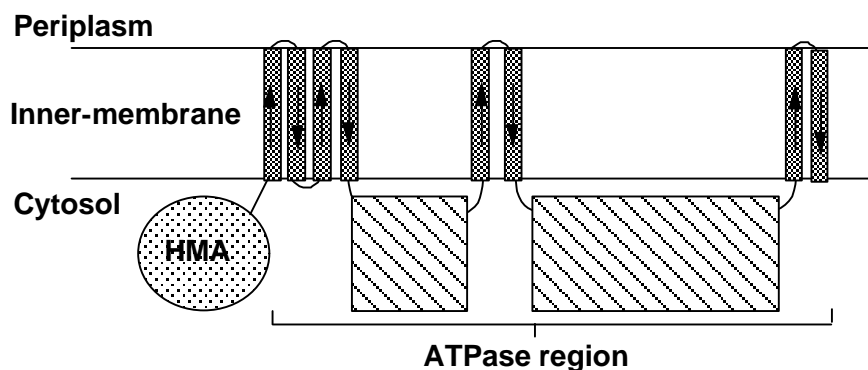


Fig. 2 Schematic representation of the predicted topology for ZntA. The HMA region is indicated by the oval.

Discussion

In this report we describe preliminary functional assignment of *zntA* (*yhho*), which was first identified in the *E. coli* genome sequencing program by Sofia *et al.*, 1994 and classified as a putative metal-binding protein. Database analysis, using the MerP primary sequence as a probe, first brought this gene to our attention and confirms the value of the increasing number of genomics programs in progress. The predicted ZntA primary sequence differs from other metal ion transporting P-type ATPases in having an extended N-terminus which contains four cysteine residues arranged as a CCCXXXC motif. The usage of sulfhydryl-groups to coordinate metal ions, including Zn(II) is well documented and we propose that the N-terminus of ZntA is a site for metal ion interaction. The N-terminal region may be directly involved in the movement of bound metal ions across the membrane or act as a switch to trigger the ATPase region.

The aim of this work was to define the function of *zntA* by expressing the gene in a non-Zn(II)-dependent manner. Expression of ZntA from the inducible arabinose promoter confers an increased tolerance to both Zn(II) and Cd(II) on *E. coli* JM109 when pre-induced cells are used in MIC assays. Non-induced cells show little difference in tolerance between the control and cells containing pBAD*zntA*. Whilst the greatest ZntA-mediated increase in tolerance is seen to Cd(II), analysis of *zntA* transcriptional induction demonstrates that the primary inducer is Zn(II) (Brocklehurst *et al.*, in preparation). We propose that the action of this gene product is to modulate Zn(II)-concentrations within the cell by exporting excess metal ions from the cytoplasm in an ATP-dependent fashion, and the data presented here are consistent with this interpretation.

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References

- Bull, P.C. and Cox, D.W. (1994) *Trends in Genetics* **10**:246-252.
- Devereux, J., Haeberli, P. and Smithies, O. (1984) *Nucl. Acids Res.* **12**:387-395.
- Guzman, L.-M., Belin, D., Carson, M., Beckwith, J. (1995) *J. Bacteriol.* **177**:4121-4130.
- Harrison, P.M. and Hoare, R.J. (1980) *Metals in Biochemistry*. London: Chapman and Hall
- Morby, A.P., Hobman, J.L. and Brown, N.L. (1995) *Molec. Microbiol.* **17**:25-35.
- Ivey, D.M., Guffanti, A.A., Shen, Z., Kudyan, N. and Krulwich T.A. (1992) *J. Bacteriol.* **174**:4878-4884.
- Ni'Bhriain, N. and Foster, T.J. (1986) *Gene* **42**:232-330.
- Nies, DH and Silver, S. (1995) *J. Ind. Microbiol.* **14**:186-199.
- Nucifora, G., Chu, L., Misra, T.K. and Silver, S. (1989) *Proc. Nat. Acad. Sci. U.S.A.* **86**:3544-3548.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press.
- Sofia, H.J., Burland, V., Daniels, D.L., Plunkett, G. and Blattner, F.R. (1994) *Nucl. Acids Res.* **22**:2576-2586.
- Suhy, D.A. and O'Halloran, T.V. (1996) *Metal Ions in Biological Systems* **32**:557-578.
- Turner, J.S. and Robinson, N.J. (1995) *J. Ind. Microbiol.* **14**:119-125.
- Yanish-Perron, C., Viera, J. and Messing, J. (1985) *Gene* **33**:103-199.
- Yoon, K.P. and Silver, S. (1991) *J. Bacteriol.* **173**: 7636-7642.