Identification of a Novel Transcription Regulator from *Proteus mirabilis*, PMTR, Revealed a Possible Role of YJAI Protein in Balancing Zinc in *Escherichia coli*

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Zinc is an essential trace element required for structural integrity and functional activity of numerous proteins, yet mechanisms by which cells regulate zinc concentration are poorly understood. Here, we identified a gene from *Proteus mirabilis* that encodes a 135-amino acid residue protein, PMTR (*P. mirabilis* transcription regulator), a new member of the MerR family of transcription activators. Transformation of *Escherichia coli* with PMTR-carrying vectors specifically increases cell tolerance to zinc, suggesting the role of PMTR in zinc homeostasis. In response to zinc, PMTR-containing cells robustly accumulate a 12-kDa protein, the amount of which correlates with the cells’ ability to grow at high zinc concentrations. The 12-kDa protein is not induced in the presence of Ni2+, Co2+, Cd2+, Mn2+, or Fe2+, indicating that the PMTR-dependent expression of the 12-kDa protein is specifically regulated by zinc. The 12-kDa protein was identified as the C-terminal fragment of *E. coli* protein YJAI, and was shown to contain two zinc-binding motifs. Metal-affinity chromatography and 65Zn blotting assay confirmed the ability of the 12-kDa protein to bind zinc specifically (zinc > cobalt >> cadmium). We propose that YJAI is an important component of the zinc-balancing mechanism in *E. coli*, the up-regulation of which with PMTR results in an increased tolerance to zinc.

Heavy metals play an important role in the metabolism of eukaryotic and prokaryotic cells. Zinc, copper, cobalt, and nickel are essential for functional activity and/or structural stability of a large variety of proteins (1–3), while other metals, such as mercury and lead, are toxic, and their accumulation in the cell has inhibitory effects on various cell functions (4, 5). High concentrations of essential heavy metals could also be deleterious; consequently, cells must precisely regulate their availability (6). Two basic mechanisms of heavy metal resistance have been identified: intracellular sequestration through formation of complexes with metal-specific proteins (such as phytochelatins in plants and yeast, or metallothioneins in animals, plants, yeast, and cyanobacteria), and reduced accumulation based on an active efflux of the cation (found in both eukaryotes and prokaryotes) (7). Some of the heavy metal binding and transporting proteins, including metallothioneins and copper-transporting P-type ATPases of mammals, bacteria, and yeast were remarkably preserved during evolution, suggesting that certain ways of regulation of essential microelements are very similar for eukaryotic and prokaryotic cells (8).

Zinc plays a particularly important role in cell homeostasis. More than 300 known enzymes require zinc for their catalytic functions. The essential role of zinc in protein structure stabilization and folding has been illustrated by the discovery and characterization of the eukaryotic zinc finger transcription factors and the large family of hormone receptor proteins (8). Appreciation of the importance of zinc for cell metabolism has stimulated genetic studies aimed at the identification of zinc-binding and zinc-transporting proteins in various cells and organisms. During the last 5 years, a number of novel genes encoding zinc-transporting and zinc-binding proteins have been identified (9–11), but still very little is known as to how the corresponding proteins work and how their expression is regulated.

Recently, transposon mutagenesis was used to show that ZntA, a novel member of the heavy metal transporting subfamily of P-type ATPases, is essential for zinc and cadmium resistance in *Escherichia coli* (12). In everted vesicles, ZntA transports both cadmium and zinc with efficiency that is 4 times higher for zinc than for cadmium (6). Whether this dual specificity is encoded in the ZntA structure or whether other protein(s) modulate ZntA specificity toward transported metals is still unknown. Thus, it remains unclear how ZntA removes excess of cadmium from the cytosol without depleting the cell of zinc. It seems very likely that fine-tune regulation of cellular zinc concentration requires participation of zinc-specific proteins, which would bind to rather nonselective transporters and modulate their cation specificity.

Gene disruption followed by zinc sensitivity assay was proven to be a very useful approach for identification of the genes essential for zinc and cadmium efflux (6, 12). However, genes encoding regulatory or signaling molecules important for fine-tune balancing of zinc that do not belong to the same operon as a transporter could easily be missed in such a screen, since it is unlikely that disruption of these genes would completely eliminate zinc resistance. Consequently, for the identification of proteins that are not primary transporters but otherwise are intimately involved in zinc metabolism, certain biochemical criteria may be used. These criteria include specific regulation of protein expression by zinc, ability of proteins to bind zinc selectively, correlation between the expression of protein of interest and cells’ ability to maintain optimal zinc concentration, etc.

In this paper, we provide biochemical evidence that YJAI, a protein with previously unknown function, could be an important component of zinc-balancing machinery in *E. coli*. The identification of this protein was a result of cloning, heterologous expression and characterization of PMTR (*Proteus mirabilis* transcription regulator), a novel MerR-like transcription...
regulator from *P. mirabilis* that specifically increases endogenous zinc resistance in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids Used for the Identification and Sequence Analysis of PMTR—*P. mirabilis* strain CDC PR 14 was obtained from ATCC (catalog no. 29906) and cultivated as described previously (13). *P. mirabilis* genomic DNA was purified using established protocols (14), digested with EcoRI, and ligated into the appropriately digested pUC18 vector. This procedure yielded the PROT1 plasmid, which corresponds to the original JWPM110 plasmid described previously (15). PROT1 contains 13.5-kb EcoRI fragment of *P. mirabilis*, of which 3.8 kb was unknown.

For all further experiments, the Epicurian Coli Sure II *E. coli* strain from Stratagene was used. PROT1 was digested with PstI restriction endonuclease, and the fragment including the 3.8-kb segment of unknown region was cloned into PstI-digested pUC18 vector. This procedure yielded the PROT2 plasmid. PROT2 was used as a template for DNA sequence analysis of the unknown portion of the insert. DNA sequence of both strands was carried out by the “primer walking” DNA approach (16) using the Amersham Sequenase version 2.0 DNA sequencing kit. Sequence comparison and identification of proteins was performed using the NCBI BLAST Search. The 2.5-kb region was shown to contain a new *pmtr* gene; the complementary chain included a fragment of an open reading frame, with 90–95% identity to the *secA* gene of *E. coli*. Only a fragment of the SecA coding sequence was present in PROT2; consequently, *secA* gene has not been analyzed further.

In order to obtain a pUC18-PMTR plasmid, the 540-bp region of the *pmtr* gene corresponding to the 405-bp PMTR protein coding sequence and its 135-bp 5′ nontranslated region were amplified by PCR using the following primers: 5′-TCTAGAATTCTGGCTGCCAGCAATG-3′ as the forward primer and 5′-CCCTGATTCCTCAGATGCAGCTAGA-3′ as the reverse primer. The PCR product was purified from the agarose gel, digested with EcoRI restriction endonuclease, and cloned into EcoRI site of the pUC18 vector using standard procedures. *E. coli* were transformed with either vector (pUC18) or vector with insert (PROT2 or pUC18-PMTR), and the resistance of cells to various heavy metals was analyzed.

**Measurements of Heavy Metal Resistance—**Cells bearing either pUC18 or PROT2 were grown overnight in liquid broth (LB) containing 75 μg/ml ampicillin. 15 μl of the overnight cultures were transferred into 5 ml of fresh LB-ampicillin medium containing heavy metal ion (for resistance measurements) or without added ions (control). Chloride salts of zinc, nickel, cobalt, manganese, or copper were added to a final concentration of 1 mM, and CdCl₂ was added to a final concentration of 200 μM or 1 mM. The cells were then placed in a shaker at 37 °C, and their growth was monitored by measuring the optical density of cultures at 600 nm (OD₆₀₀) after 2, 4, 6, 8, and 24 h. The heavy metal resistance of pUC18-PMTR cells was measured following a 6- or 24-h incubation in the presence or absence of zinc as described above.

**Analysis of the PMTR/Heavy Metal-dependent Protein Expression—** *E. coli* cells transformed with either pUC18 or PROT2 were grown in the presence or absence of heavy metals as described above, and aliquots of 1 ml were removed after 6 or 24 h of growth. Cells were pelleted by centrifugation at 5,000 × g for 15 min, and the pellets were resuspended in different volumes of Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 10% SDS, 8M urea (1:1:1, v/v), 1% β-mercaptoethanol) to obtain similar protein concentration for control and heavy metal-treated cell samples. Protein concentration was measured according to Lowry et al. (17). The cell lysates were then separated by 15% polyacrylamide Laemmli gel (18); the gels were stained in 0.1% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid.

In order to characterize zinc concentration dependence of the 12-kDa protein expression, 10 μl of overnight culture of PROT2-containing cells were transferred to 3 ml of LB-ampicillin medium, containing increasing concentrations of zinc (0–1.0 mM zinc) (legend for Fig. 4). Cells were grown for 6 or 24 h at 37 °C in a shaker. The optical density of cultures was measured, and aliquots containing equal amounts of cells were pelleted by centrifugation at 3000 × g for 10 min at 4 °C. Cell pellets were dissolved in the Laemmli sample buffer, containing 1% β-mercaptoethanol and analyzed by 15% Laemmli gel. The gel was either stained by 0.1% Coomassie Brilliant Blue R-250 or proteins were transferred to polyvinylidene difluoride membrane (PVDF, Millipore) and processed for the N-terminal amino acid sequencing.

**The N-terminal Amino Acid Sequence Analysis of PMTR/Zinc-inducible Proteins—**Cell lysates or periplasmic fraction (see below) were separated by 15% Laemmli polyacrylamide gel, and proteins were transferred to PVDF membrane as described by Matsudaira (19). The transfer was carried out at 180 mA for 1 h in 10 mM CAPS buffer, pH 11.0, and the membrane was stained by a solution of 0.1% Coomassie R-250 in 50% methanol and destained by 40% methanol in 10% acetic acid. Protein bands were excised from the membrane and sequenced at the Microchemical Facility in Winship Cancer Center (Emory University, Atlanta, GA). N-terminal amino acid sequence analysis for the PMTR/zinc-induced 12-kDa protein was repeated three times with identical results, and the protein corresponding to the 20-kDa band was sequenced twice.

For the 12-kDa protein, the homogeneous and unambiguous sequence HGGHGMWQQNAAPLTT was consistently obtained. The 20-kDa band contained two protein sequences, the major ADTTTAAPADAKPM sequence and the minor sequence SGTIEERVK. In order to confirm that major sequence corresponds to the zinc-induced 20-kDa protein, the adjacent band from the parallel sample of cells grown without zinc was analyzed by N-terminal amino acid sequencing. In the case of ADTTTAAPADAKPM sequence, the only sequence SGTIEERVK was found, confirming that the ADTTTAAPADAKPM sequence corresponds to the 20-kDa protein expressed in the presence of zinc.

**Localization of the 12-kDa Protein in the Cellular Fractions—** LB media containing 1 mM ZnCl₂ or no zinc (control) were inoculated with the overnight cultures of cells bearing either pUC18 or PROT2, and the cells were grown for 6 h at 37 °C to reach an OD₆₀₀ of 0.9–1.0. The cells were then harvested by centrifugation at 5,000 × g for 20 min, and cell fractions were prepared. Periplasmic fraction was obtained essentially as described by Harris et al. (20). Cells were resuspended in 200 mM Tris-HCl, pH 8.0 to a final concentration of 40 mg/ml. The suspension was diluted with an equal volume of 200 mM Tris-HCl, pH 8.0, containing 1 mM sucrose, to which 0.5% of 100 mM EDTA, pH 7.6 was added. Lysozyme was added to a final concentration of 60 μg/ml. The suspension was diluted 2-fold in water and incubated at room temperature until the spheroplasts were formed. In order to monitor the formation of the spheroplasts, aliquot of the suspension was diluted 1:100 with H₂O and change in the absorbance at 450 nm (A₄₅₀) was measured. Spheroplast formation was considered to be complete when the A₄₅₀ fell 80–85%. Cells were added to a final concentration of 20 μM to stabilize the spheroplasts, and the spheroplasts were sedimented by centrifugation at 5000 × g for 20 min. The supernatant containing the periplasmic proteins was concentrated by centrifugation using Centricon 10 unit (Beckman GS-15R Centrifuge) and kept at −20 °C until further use. The pellet containing spheroplasts was used to isolate the cytoplasmic proteins and the membrane fraction. The pellet of the spheroplasts obtained above was resuspended in 200 mM Tris-HCl, pH 8.0 (25 ml/g), 50 mM 4-2-aminoethylbenzenesulfonyl fluoride was added to a final concentration of 0.16 mM, and the suspension was incubated on ice for 20 min with occasional stirring. Deoxyribonuclease was added to a final concentration of 8 μg/ml, and the suspension was incubated at room temperature until it was no longer viscous. The cell lysate was centrifuged at 100,000 × g for 10 min to separate the cytoplasmic proteins from the membrane fraction. The supernatant containing cytosolic proteins was saved, and the pellet was resuspended in the volume of H₂O equivalent to the volume of the supernatant.

**Protein composition of the periplasmic, cytosolic, and membrane fractions from both zinc induced and control cells was analyzed by 15% Laemmli polyacrylamide gel.**

Metal-chelate affinity chromatography was carried out using imino-diacetic acid-agarose (Sigma) equilibrated with different heavy metals. The washes and elutions of the resin were done by centrifugation at 10,000 × g for 10 min in a Fisher microcentrifuge. Affinity resin (50 μl/sample) was washed with 1 ml of 150 mM sodium phosphate buffer, pH 7.2 (Na-P buffer). The resin was then equilibrated for 5 min at room temperature with 1 ml of Na-P buffer containing 1 mM chloride salt of heavy metal (zinc, nickel, cobalt, cadmium) or no heavy metal (control). The equilibrated resin was washed with 1 ml of Na-P buffer to remove an excess of heavy metals and resuspended in 50 μl of iminodiacetic acid-resin. Samples were incubated for 15 min at room temperature, vortexing.
ing every 3–5 min. The unbound protein was removed by centrifugation at 10,000 \( \times g \) for 10 min, and the resin was washed three times with 1 ml of Na-P buffer. The bound protein was eluted with 30 \( \mu l \) of Laemmli buffer containing 1% \( \beta \)-mercaptoethanol and electrophoresed on 15% Laemmli polyacrylamide gel.

\[ \text{Zinc blotting and autoradiography was performed by the method described by DiDonato et al. (21). PROT2 cultures were grown overnight in the presence and absence of zinc (1 \( \mu M \)), and their periplasmic fractions were prepared as described above (20). Periplasmic fractions containing 20 \( \mu g \) of total protein were electrophoresed on 15% polyacrylamide Laemmli gels. Following electrophoresis, the proteins were electroblotted onto PVDF membranes using CAPS transfer buffer (10 mM DTT) on a shaker for 2 h. Then the equilibration buffer was replaced with 20 \( ml \) of Buffer B (100 mM Tris-HCl, pH 7.0, 50 mM NaCl, 30 mM ZnCl\(_2\)) to which 100 \( \mu Ci \) of \( ^{65}\text{Zn} \) was added. Membranes were incubated in this buffer for 1 h, then washed twice for 15 min each in the same buffer. The membranes were then exposed either to Kodak XAR film for 8 h at \( -70 \) °C with intensifying screen or overnight at room temperature in the phosphoimager (Bio-Rad GS505) using Bio-Rad imaging screen-CS.}

Competition with other metals was performed as described above except that the membranes were probed with \( ^{65}\text{Zn} \) in the presence of a nonradioactive competitor ion, i.e. 100 \( \mu M \) nickel, cobalt, cadmium, or manganese.

**RESULTS**

**PMTR, a Novel Member of the MerR Family of Transcription Activators, Is Associated with an Increased Tolerance to Zinc in *E. coli*—**We have recently demonstrated that the 13.5-kb genomic fragment of *P. mirabilis*, containing a 3.8-kb unknown region, was associated with an increased resistance to zinc in *E. coli* (22). Here, the 13.5-kb fragment was further digested with *Pst*I restriction endonuclease, and the fragment containing the 2.5-kb portion of the unknown sequence was cloned into pUC18 vector to yield the plasmid PROT2. *E. coli* cells were then transformed with either control pUC18 vector or with PROT2, and their ability to grow at increasing concentrations of various heavy metals was tested.

Incubations with various concentrations of cobalt, cadmium, nickel, copper, or iron revealed little or no difference in the rates of growth for control and PROT2 cells (data not shown). In contrast, in the presence of elevated zinc (>0.6 \( \mu M \)), PROT2 cells grew markedly better than control cells at all time intervals and all concentrations of zinc tested (Fig. 1A). Moreover, zinc resistance induced by the PROT2 plasmid was about 10–20% higher than the resistance conferred by the original plasmid with 2.5-kb unknown region of the insert is sufficient to provide high tolerance to zinc.

DNA sequence analysis of the 2.5-kb unknown segment demonstrated that it contained a single open reading frame of 405 bp (Fig. 2A). The putative promoter site (score 0.73) and ribosome-binding site were identified immediately upstream the protein coding sequence (Fig. 2A).

In order to verify the role of the identified gene in zinc resistance, the 540-bp segment including the protein-coding and promoter region for a new gene, was amplified by PCR and cloned into pUC18 vector to yield a pUC18-PMTR construct. Transformation of *E. coli* with this construct led to zinc resistance that was 3 times higher than the resistance of control pUC18-transformed cells (Fig. 1B), confirming that the new gene in enhancement of zinc resistance in *E. coli*.

Transformation of the protein coding region revealed that the identified gene encodes a 135-amino acid residue protein with significant homology (30–60%) to the transcription activators, members of the MerR family (Fig. 2B). The best characterized member of this family, MerR, is a dimeric DNA-binding protein that mediates the Hg(II)-dependent induction of mercury resistance operons (23). Interestingly, two hypothetical MerR-like *E. coli* proteins, YBBI and YHDM, were found to be very close homologs of the identified *P. mirabilis* protein (53–57% similarities, 39% identities) (see Fig. 2B), suggesting that PMTR may mimic functions of one of these proteins.

Based on the significant sequence homology of PMTR with MerR-like transcription activators and the fact that expression of PMTR in *E. coli* specifically increases cell resistance to zinc, we hypothesized that PMTR could be a zinc-dependent transcription factor that up-regulates the expression of endogenous proteins involved in balancing zinc concentration in *E. coli*. In order to test this hypothesis, protein composition of control and PMTR-containing cells grown in the presence or absence of zinc was compared.

**Increase in Zinc Resistance Is Associated with Accumulation**

![Graph A](image1.png)

**Graph A** shows the growth curves for the PROT2-containing cells and control pUC18-transformed cells in the presence and absence of 1 mM zinc. Growth of pUC18 or PROT2-containing cells in the absence of zinc was essentially similar; thus, both are represented by the same curve. Aliquots of 1 ml were taken out following 4, 6, and 24 h of incubation at 37 °C, and optical density of cell cultures at 600 nm (OD\(_{600}\)) was measured. Similar difference in growth between the pUC18 and PROT2 cells was observed at other zinc concentrations (0.6–2 mM). B, zinc resistance of control (pUC18) cells and PMTR-containing cells (PROT2 and pUC18-PMTR). Cells were grown in the presence or absence of 1 mM ZnCl\(_2\) for 24 h at 37 °C, and the OD\(_{600}\) of the corresponding cell cultures was compared.
of the 12-kDa Protein—Electrophoretic analysis of lysates of the PMTR and pUC18 cells revealed a marked increase of the 12-kDa protein band in PMTR cells grown in the presence of zinc (Fig. 3A). The 12-kDa protein was also present in control cells, although at much lower level. This suggested that (i) PMTR stimulates the expression of this protein directly or indirectly in the presence of zinc, and (ii) that the increased zinc tolerance of PMTR-containing cells could be associated at least partially, with the overexpression of the 12-kDa protein.

No significant differences in the protein patterns of control and PROT2 cell were observed in the absence of zinc, in agreement with the expected lack of transcription activation by PMTR in the absence of ligand (Fig. 3A).

In the presence of zinc, changes in the intensity of some other protein bands were also observed. The 20-kDa protein was markedly induced in the presence of zinc (Fig. 3A). However, the amount of this protein was essentially identical for control and PROT2-containing cells, indicating that the 20-kDa protein is unlikely to be associated with the increase in zinc resistance. A diminished intensity was seen for the 24-kDa band in PROT2 cell lysate (Fig. 3A). Further analysis demonstrated that the expression of this protein was largely dependent on aeration conditions rather than on the presence of PMTR, and the presence or absence of this protein had no correlation with zinc resistance (data not shown). Changes in intensity of other bands were inconsistent, and therefore the corresponding proteins were not analyzed.

The Amount of the 12-kDa Protein Produced in the Cell Correlates with the Cell's Ability to Grow at Elevated Zinc Concentrations—Some control cells had higher resistance to zinc than others, although their tolerance to zinc was always at least 2 times lower than the resistance of PMTR-containing cells. Thus, several different clones of control pUC18-transformed cells and PMTR-cells were used to determine whether the difference in their ability to grow at elevated zinc correlates with the expression of the 12-kDa protein. Cells were grown in the presence of 1 mM ZnCl$_2$, and their growth was characterized by the optical density of cultures at 600 nm. Cell lysates containing the same amount of total protein were analyzed by gel electrophoresis, and quantities of the 12-kDa protein were compared.

Fig. 2. A, the nucleotide sequence and deduced amino acid sequence of PMTR. The predicted promoter region is marked by lowercase letters, and the putative –35 and –10 regions are underlined. A potential ribosome binding site (GGAGA) is located at the 98–102 region. B, amino acid sequence alignment of PMTR, YHDM (Swiss-Prot accession no. P36676), YBBI (Swiss-Prot accession no. P77565), and MerR (Swiss-Prot accession no. P22853). Conserved amino acid residues are boxed.

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these samples were compared. The densitometry of Coomassie-stained gels revealed an excellent correlation between the intensity of the 12-kDa protein band and cells’ resistance to zinc (Fig. 4) suggesting a direct association of this protein with the ability of cells to grow at high zinc concentrations.

PMTR-dependent Expression of the 12-kDa Protein Is Specifically Stimulated by Zinc—If 12-kDa protein is indeed important for the tolerance of the cell to zinc, then one would expect that the expression of the 12-kDa protein is regulated by zinc specifically. In order to test this assumption, cells were grown in the presence of various heavy metals for 6–8 h, and protein composition of these cells was analyzed by gel-electrophoresis. Neither nickel, copper, manganese, or cobalt at 1 mM concentration nor cadmium at 200 μmol (the concentration of cadmium at which the cells can still grow) induced the appearance of the 12-kDa protein (see Fig. 5B for nickel and cobalt as examples), confirming that the PMTR-dependent expression of the 12-kDa protein is in fact specifically regulated by zinc.

Importantly, zinc induces the expression of the 12-kDa protein in a concentration-dependent manner at concentrations much lower than 1 mM ZnCl₂ (Kₘ ~ 5 μmol) (Fig. 5A), in contrast to the 20-kDa protein which was produced only at very high zinc concentrations (data not shown). Thus, appearance of the 12-kDa protein seems to reflect a specific cellular reaction to zinc, which was enhanced by PMTR.

In order to further verify that PMTR/zinc-dependent induction of the 12-kDa protein represents an amplified physiological response of E. coli cells to zinc, zinc-dependent induction of this protein was compared for control cells, and cells transformed with PMTR containing plasmid. Fig. 5A illustrates that, in both pUC18 and PROT2 containing cells, the increase in zinc concentration was accompanied by the intensification of the 12-kDa band. Moreover, the concentration-dependent response was similar for both samples (Fig. 5B). A 2.5-fold difference in intensity of the 12-kDa band between the pUC18 and PMTR samples observed in this experiment was in good agreement with the OD₆₆₀ of corresponding cultures (0.56 for PROT2 and 0.23) for pUC18 at 1 mM zinc.

The 12-kDa Protein Is a C-terminal Fragment of the YJAI Protein of E. coli—The PMTR/zinc-dependent expression of the 12-kDa protein (Fig. 3), and particularly direct correlation between the amount of this protein and the acquired zinc resistance (Fig. 4), pointed to the possible role of the 12-kDa protein in balancing zinc in E. coli. In order to identify the molecular nature of this protein, the 12-kDa product was transferred to PVDF membrane and sequenced. The N-terminal amino acid sequence of the 12-kDa protein, HGHHGMWQQNAAPLT, was found to be identical to the His⁷⁴-Thr⁸⁸ segment of the YJAI protein of E. coli (GenBank accession no. P32682). The open reading frame encoding this protein was described during E. coli genome sequencing, but no functional information was available for this gene product. The calculated molecular mass of the C-terminal fragment of YJAI protein, which begins at His⁷⁴, is 12.5 kDa, which is in good agreement with the apparent molecular mass of the induced 12-kDa product. Thus, the 12-kDa protein is most likely to represent the entire C-terminal fragment of the YJAI protein.

Based on the DNA sequence analysis, the molecular mass of the full-length YJAI protein has been reported to be 20.4 kDa. Since we observed two bands overexpressed in the presence of zinc (20- and 12-kDa protein, Fig. 3), we investigated whether the 20-kDa band was the full-length YJAI protein and if the 12-kDa protein was its proteolytic product. The N-terminal sequence analysis demonstrated that the 20-kDa band contained the major sequence ADTTTAAPADAKPMM. This sequence corresponds to the periplasmic spy protein of E. coli (25) and is structurally unrelated to YJAI.
The accumulation of the 12-kDa protein in the presence of zinc coincided with the decrease in intensity of 24-kDa protein (see Fig. 3A). This effect was seen clearly after 24 h of growth, but was significantly less noticeable when cells were analyzed at the logarithmic stage (data not shown). In order to verify that the 24-kDa protein does not represent the full-length YJAI, this protein purified by gel-electrophoresis was sequenced. The presence of two hydrophobic stretches (20 and 19 amino acid residues long) at the N-terminal portion of the molecule, indicating that the full-length YJAI is a transmembrane protein. The 12-kDa segment begins immediately after the major hydrophobic stretch and is entirely hydrophilic (Fig. 6).

Thus, we hypothesized that the YJAI protein was overexpressed due to the combined action of PMTR and zinc, and was then translocated to the membrane, where it became cleaved. In the membrane, this protein can either form an oligomer and participate in zinc export, or more likely it may interact with transmembrane zinc-transporters and regulate their function. The selective cleavage and release of the C-terminal hydrophilic 12-kDa fragment of YJAI (presumably into the periplasm) seems to be an important step in the mechanism of action of this protein. In order to obtain a further insight into the function of YJAI, experiments were carried out on the subcellular localization of the 12-kDa fragment and its ability to bind heavy metals.

**Subcellular Localization of the 12-kDa Protein and Analysis of Its Metal Binding Specificity**—The periplasmic, cytosolic, and membrane fractions of the PROT2 and pUC18-containing cells grown in zinc were prepared and analyzed by gel electrophoresis (Fig. 7). The 12-kDa protein was recovered in the supernatant obtained after osmotic shock, indicative that the 12-kDa protein was indeed located in the periplasm (Fig. 7, lane P). The amount of the 12-kDa protein released to the periplasm was higher for the PROT2 cells than for the pUC18 cells, as predicted (Fig. 7). These data ascertained that most of the expressed YJAI protein relocates to the membrane where it presumably functions. The induced 20-kDa protein (periplasmic spy protein) was also found in the same fraction, providing us with an excellent internal control for further experiments on metal-binding specificity of the zinc-induced proteins (see below).

The 12-kDa fragment contains two zinc-binding motifs, suggesting that it may bind zinc. In order to determine the metal-binding properties of the 12-kDa protein, the periplasmic fraction containing this protein was incubated with inimodiacetic acid agarose equilibrated with one of the following metal ions (Zn$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Cd$^{2+}$, or Mn$^{2+}$). Fig. 8 demonstrates that the 12-kDa protein strongly binds to the zinc- and nickel-equilibrated columns (zinc, nickel > cobalt) and does not bind to cadmium (Fig. 8) or manganese (data not shown) equilibrated resins. In contrast, the 20-kDa protein, which was similarly overexpressed and which contains a similar amount of His residues, was not bound to any of these columns (compare Figs. 7 and 8). No binding was observed to the resin lacking heavy metals or when zinc-equilibrated resin was incubated with the periplasmic proteins from PROT2 cells grown in the absence of zinc.

The ability of 12-kDa protein to bind zinc specifically was further confirmed by direct zinc-binding studies using radioactive $^{65}$Zn. The periplasmic fraction of *E. coli* cells grown in the presence of zinc was separated by gel electrophoresis, transferred onto PVDF membrane, and equilibrated with $^{65}$Zn in the presence (competition binding) or absence of other metals. Fig. 9A illustrates that the 12-kDa proteins strongly binds zinc. Presence of a 3-fold excess of manganese or cadmium in the binding buffer does not have any effect on zinc binding, while nickel and cobalt compete with zinc to some extent (Fig. 9B), in agreement with earlier data on metal-chelate chromatography (see above, Fig. 8). As expected, no radioactive 12-kDa band was seen in the periplasmic fraction obtained from the cells grown without zinc (Fig. 9A, last lane).

**DISCUSSION**

*E. coli* has an endogenous mechanism of regulation of zinc availability, the first important component of which, ZntA, a zinc/cadmium-transporting P$_1$-type ATPase, had been recently...
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identified (6). Although ZntA was shown to be a key enzyme providing ATP-dependent zinc efflux, it was also demonstrated to lack the unique selectivity to zinc. Other proteins that could be involved in specific zinc-signaling events, selective uptake of zinc, zinc-dependent regulation of transcription, or transport activity remain uncharacterized. In this study we report identification and characterization of two proteins, expression of which in \textit{E. coli} is associated with specific increase in cell resistance to zinc.

The endogenous regulation of zinc availability in \textit{E. coli} seems rather effective since cell growth is essentially unaffected when concentration of zinc in the medium is increased up to 0.5 mM. Further increase of zinc concentration (>0.6 mM) leads to inhibition of growth, indicating that the regulatory capacity of the system has been saturated. However, \textit{E. coli} cells can grow normally even at 1 mM zinc if transformed with the PROT2 plasmid. We demonstrate that this effect is due to the presence of a small gene in PROT2, which encodes a 135-
Analysis of the deduced amino acid sequence revealed that PMTR is a new member of a large family of MerR-like transcription activators (Fig. 2). MerR represses transcription by binding tightly to the "mer" operator region in the absence of mercury. When mercury is present, the dimeric MerR complex binds a single ion and becomes a potent transcriptional activator. In Streptomyces lividans, transcriptional MerR-like activator TipA regulates transcription upon specific binding of antibiotic thiostrepton at concentrations of thiostrepton as low as $10^{-10}$ to $10^{-9}$ M (27). In Bacillus subtilis, the other member of MerR family, BmrR, activates transcription from the multi-drug transporter gene, bmr, after binding either rhodamine or tetraphenylphosphonium (28). All these various members of the MerR family are highly homologous in their N-terminal portion, which includes the characteristic "helix-turn-helix" DNA-binding motif. The variable C-terminal portions of these proteins are involved in specific binding of the stimulating ligand and are structurally dissimilar.

The N-terminal half of the PMTR protein sequence has high homology to the DNA-binding region of MerR-like proteins (Fig. 2), which strongly points to the role of PMTR in DNA-binding and transcription regulation. The C-terminal ligand-binding region does not have strong homology with the ligand-binding sites of MerR or other biochemically characterized members of the family, suggesting that PMTR is regulated by its own specific ligand.

Our data suggest that PMTR could be a zinc-dependent transcription activator involved in zinc homeostasis in bacterial cells. The transformation with PMTR plasmid specifically confers resistance to zinc and not to any other heavy metals tested. In addition, PMTR stimulates expression of the 12-kDa protein only in the presence of zinc, the amount of which correlates with the cell's ability to tolerate high concentrations of this metal (Fig. 4). Future studies on characterization of the DNA-binding and metal-binding properties of recombinant PMTR will precisely determine the DNA and ligand specificity of this transcription regulator.

It is quite possible that PMTR or its functional *E. coli* analogs regulate a number of genes, the coexpression and cooperation of which lead to the observed increase in zinc tolerance. The experiments are currently under way to identify all genes that are expressed due to the simultaneous presence of transcription regulator and zinc. Our current results suggest that one of such genes is *yjai*, and offer some clues to the possible function of the corresponding protein.

It is evident that the expression of *YJAI* protein in both control and PMTR-containing cells is specifically induced by increasing concentration of zinc in the medium (Fig. 5A), and that the amount of C-terminal fragment of the *YJAI* released to the periplasm correlates with the ability of cells to grow at elevated concentrations of zinc (Fig. 4). Zinc-dependent expression of the full-length *YJAI* has not been demonstrated directly; however, zinc-dependent accumulation of the 12-kDa fragment and the absence of the corresponding amounts of the full-length precursor in the zinc-free media strongly suggests that the expression of the *YJAI*, and not just cleavage, are stimulated by zinc. To the best of our knowledge, such a highly selective effect of zinc on protein expression, as we see for *YJAI*, has not been demonstrated for any other *E. coli* protein including ZntA, pointing to the unique role of *YJAI* for zinc homeostasis.

We demonstrated that the 12-kDa C-terminal fragment of *YJAI* is released to the periplasm and can bind zinc with significant selectivity (zinc $>$ cobalt $>$ cadmium) (Fig. 8). The 20-kDa periplasmic spy protein, which was produced at 1 mM ZnCl$_2$ and which contains comparable amounts of His residues, does not show any binding to metal-equilibrated resin, confirming that the observed metal binding of the 12-kDa protein is specific. The dramatic difference in binding of the 12-kDa protein to zinc- and cadmium-equilibrated columns is particularly important. Chemical properties of zinc and cadmium are similar, and cadmium is known to be able to substitute for zinc in many enzymes (29). However, cadmium is bigger than zinc, whereas zinc, nickel, and cobalt are similar in size. Consequently, the preferential binding of the 12-kDa protein to one column and not to the other indicates that specific protein structure rather than mere presence of histidine residues governs the observed protein-metal interactions. The $^{65}$Zn-binding and competition studies with various metals further confirmed the ability of the C terminus of YJAI to bind zinc specifically (Fig. 9, A and B).

Some properties of YJAI, such as its specific induction in the presence of zinc, the localization of the C-terminal domain of this protein in the periplasm, and the ability of the 12-kDa fragment to bind zinc much more strongly than cadmium, make the YJAI protein an excellent candidate for the role of the modulator of cation selectivity of metal transporters, such as ZntA. Alternatively, zinc complex of the 12-kDa protein can serve as an inhibitor of low affinity metal uptake proteins, such as the manganese transporter, which has broad specificity for various divalent cations including zinc (30). Future studies of the protein-protein interactions and characterization of the mutant strain lacking YJAI would verify which of these hypothesis is correct.

Currently, the data demonstrate that cell response to the increasing zinc concentration may include zinc-dependent expression of YJAI, followed by relocation of this protein to the membrane, binding of zinc by the C-terminal fragment of YJAI, accompanied by selective proteolytic cleavage and release of the protein-zinc complex into the periplasm. It is interesting that cleavage of YJAI occurs at the HGGHGM site. It is possible that binding of zinc at this region induces a conformational change and exposes this segment for the proteolytic (autolytic) attack. Alternatively, cleavage of YJAI may not require zinc, and the 12-kDa C-terminal fragment is released to periplasm in a metal-free form and binds zinc later.

The name "YJAI" is an abbreviation that was initially used to describe this putative *E. coli* protein, and does not reflect functional properties of the protein. It is also very difficult to pronounce; thus, in future we would like to refer to YJAI as "zinc resistance-associated protein" or "ZRAP."

In conclusion, we have identified a novel transcription regulator, PMTR, responsible for the increased zinc resistance in *E. coli* and found that PMTR has significant sequence homology to two endogenous *E. coli* proteins, YHDM and YBBI. We demonstrate that in the presence of zinc, PMTR induces accumulation of the previously uncharacterized protein YJAI (ZRAP). Characterization of YJAI revealed that it can selectively bind zinc in the periplasm, and that the expression of this protein is precisely stimulated by zinc in a concentration-dependent manner. All data together indicate that YJAI could be an important component of zinc-balancing mechanism in *E. coli*.

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