

## ZntB Is a Novel Zn<sup>2+</sup> Transporter in *Salmonella enterica* Serovar Typhimurium

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**A Zn<sup>2+</sup> transport system encoded by the *zntB* locus of *Salmonella enterica* serovar Typhimurium has been identified. The protein encoded by this locus is homologous to the CorA family of Mg<sup>2+</sup> transport proteins and is widely distributed among the eubacteria. Mutations at *zntB* confer an increased sensitivity to the cytotoxic effects of Zn<sup>2+</sup> and Cd<sup>2+</sup>, a phenotype that suggests that the encoded protein mediates the efflux of both cations. A direct analysis of transport activity identified a capacity for Zn<sup>2+</sup> efflux. These data identify ZntB as a zinc efflux pathway in the enteric bacteria and assign a new function to the CorA family of cation transporters.**

Zinc is the second most abundant transition metal in biological systems (6, 11, 24). It is an essential element that is employed in a wide range of biochemical and biophysical roles. It is required to maintain the structural stability of macromolecules and to serve as a cofactor for more than 300 metabolic enzymes. It also plays a prominent role in gene expression as a structural component in a large number of Zn<sup>2+</sup>-dependent transcription factors (4, 19). While the cellular requirement for zinc is absolute, excess concentrations of the cation are highly toxic. Consequently, the ability to maintain the intracellular Zn<sup>2+</sup> concentration within very narrow limits is a fundamental property of all living cells. Enteric bacteria are currently thought to maintain Zn<sup>2+</sup> homeostasis through the activities of at least four Zn<sup>2+</sup>-specific transport systems. Zinc uptake is facilitated by the combined activities of the ZnuABC and ZupT transport systems (10, 13). The ZnuABC system is the primary Zn<sup>2+</sup> influx pathway and is induced in response to zinc deprivation (16). Bacteria respond to high levels of exogenous Zn<sup>2+</sup> by increased expression of the ZntA and ZitB efflux systems (2, 5, 9, 17). The previously uncharacterized open reading frame *b1342* of *Escherichia coli* encodes a protein that is homologous to the CorA family of cation transporters (3, 20). In *Salmonella enterica* serovar Typhimurium and *E. coli*, CorA functions as the primary influx pathway for Mg<sup>2+</sup> and is distinguished by its unique ability to mediate both the influx and efflux of Mg<sup>2+</sup> (8, 22, 23). In an effort to ascertain the function of the peptide encoded by *b1342*, our laboratory identified the corresponding allele in *S. enterica* serovar Typhimurium and disrupted it by the insertion of an antibiotic resistance cassette. In this report, we show that the serovar Typhimurium homolog of *b1342* encodes an additional zinc transport system. Mutations at this locus resulted in an increased sensitivity to cytotoxic levels of Zn<sup>2+</sup> and Cd<sup>2+</sup> and a reduced capacity for zinc efflux. Consequently, the mutated gene and its protein product were designated ZntB.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were maintained in *E. coli* Top10F' unless indicated otherwise.

**Culture media and reagents.** Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) medium or on LB agar plates (14), except when otherwise stated. Minimal medium was based on the N medium of Nelson and Kennedy (15), supplemented with 0.25% glucose, 0.02% Casamino Acids, and 1 mM MgSO<sub>4</sub>. Antibiotics were added to complex and minimal culture media, respectively, at the following concentrations: sodium ampicillin salt (100 and 30 μg/ml), kanamycin sulfate (50 and 100 μg/ml), chloramphenicol (25 and 10 μg/ml). Restriction endonucleases, T4 DNA ligase, and shrimp alkaline phosphatase were obtained from Promega Corporation. Sequencing enzymes and associated biochemicals were obtained from Epicentre Technologies. <sup>65</sup>ZnCl<sub>2</sub> was purchased from Perkin Elmer Life Sciences, Inc. Additional chemicals were obtained from standard suppliers.

**DNA manipulations and *zntB* mutant construction.** Construction of the serovar Typhimurium *zntB* mutant strain was performed as described previously by Datsenko and Wanner (7) with the following modifications: pKD46 was first moved into serovar Typhimurium 14028s by electroporation. The *zntB1::ΩCm* mutagenic cassette was gel purified and transformed into 14028s/pKD46. Chloramphenicol-resistant transformants were subsequently cured of pKD46 by growth at 40°C. Chromosomal integration of the mutagenic cassette was confirmed by PCR and sequenced using oligonucleotides external to the integrated cassette. Plasmid DNA was prepared from 5-ml cultures using the Qiaprep Spin Miniprep kit obtained from Qiagen. Genomic DNA was isolated using a PureGene purification kit obtained from Gentra Systems. Restriction endonuclease digestion, DNA ligation, and transformation of linear and plasmid DNA were performed as described previously (1).

**Quantitation of cation sensitivity.** Susceptibility profiles were generated by disk diffusion and growth curve analyses. Bacterial lawns were spread on N-minimal agar plates, and 8-mm sterile filter disks were placed in the center. Lethal levels of ZnSO<sub>4</sub>, CoCl<sub>2</sub>, NiCl<sub>2</sub>, MnCl<sub>2</sub>, and CdCl<sub>2</sub> (100 mM, 100 mM, 1 M, 1 M, and 100 mM, respectively) were added to the disks. Zones of inhibition were measured after 24 h of growth at 37°C. In addition, growth was monitored over time in N-minimal media containing various levels of ZnSO<sub>4</sub>. Overnight cultures were diluted 1:100 into appropriate growth assay media and readings of the optical density at 600 nm (OD<sub>600</sub>) were made every hour until stationary phase was reached.

**<sup>65</sup>Zn<sup>2+</sup> uptake.** Overnight cultures were diluted 100-fold into supplemented N-minimal medium and grown at 37°C with aeration to an OD<sub>600</sub> of 0.4. Cells were then washed twice with ice-cold N-minimal medium without MgSO<sub>4</sub>, Casamino Acids, and glucose supplementation. The pellet was resuspended in wash buffer to a final OD<sub>600</sub> of between 1.0 and 2.0. Uptake was measured at 37°C in N-minimal medium containing 10 mM MgSO<sub>4</sub>, 0.25% glucose, and 6 μM <sup>65</sup>ZnCl<sub>2</sub>. The reaction was initiated by addition of prewarmed cells. At timed intervals, 100-μl samples were removed and filtered through nitrocellulose filters (0.45-μm pore size; Whatman). Filters were washed with 8 ml of cold wash buffer containing 0.5 mM EDTA. Radioactivity was analyzed by gamma counting. A

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
Serovar Typhimurium		
RS404	Nominal wild-type strain 14028s	ATCC
RS1100	<i>zntB1::ΩCm</i>	This study
MM281	<i>corA45::Tn10Δ16Δ17(Tc) mgtCB::Mu dJ mgtA::Mu dJ</i>	21
<i>E. coli</i>		
GR480	<i>E. coli ΔzntA ΔzitB ΔzupD ΔznuABC ΔyiiP</i>	C. Rensing; 9, 10
RS1220	<i>E. coli ΔzntA ΔzitB ΔzupD ΔznuABC ΔyiiP zntB1::ΩCm</i>	This study
Top10F'	F' { <i>lacI<sup>q</sup> Tn10(Tc)</i> } <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR araΔ139 (ara-leu)7697 galK rpsL endA nupG</i>	Invitrogen
Plasmids		
pKD46	<i>oriR101 repA101(Ts) P-araB-gam-bet-exo</i>	7
pAJW54	Serovar Typhimurium <i>zntB</i> <sup>+</sup> native promoter in pLG338/IBI30 (low copy)	This study
pRS310	Serovar Typhimurium <i>corA</i> <sup>+</sup> cw in pCR2.1	21
pRS319	<i>M. jannaschii corA</i> <sup>+</sup> in pCR2.1	21

background value, obtained from 1 ml of assay mixture without the addition of cells, was subtracted from all readings.

**<sup>65</sup>Zn<sup>2+</sup> efflux.** Cells were grown and washed as stated above with the following changes. Washed cells were resuspended in N-minimal medium supplemented with glucose, MgSO<sub>4</sub>, and Casamino Acids and containing 100 μM MnCl<sub>2</sub>. Cells were incubated at 37°C for 1 h to allow Mn<sup>2+</sup> to bind intracellular sites and allow efflux. <sup>65</sup>ZnCl<sub>2</sub> was added to 600-μl cell suspension aliquots to a final concentration of 5 μM and incubated for 20 min. Cells were pelleted at 12,000 × g for 3 min, the supernatant was removed, and the cells were resuspended in 600 μl of assay buffer (N-minimal medium with nutrient supplements and 10 μM ZnSO<sub>4</sub>). At timed intervals, 100-μl aliquots of the cell suspension were removed and collected on nitrocellulose filters as described above. Radioactivity was analyzed by gamma counting.

**DNA sequencing and PCR.** DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (18) as modified by Tabor and Richardson (25), using T7 DNA polymerase obtained from Epicentre Technologies. Initial reactions were performed using M13 -20 promoter primers obtained from LI-COR Biosciences. Subsequent reactions employed synthetic oligodeoxynucleotide primers complementary or identical to segments within a previously sequenced segment. PCRs were performed using the Opti-Prime PCR optimization kit obtained from Stratagene and oligodeoxynucleotide primers obtained from Integrated DNA Technologies.

**Nucleotide sequence accession number.** The nucleotide sequence of the *S. enterica* serovar Typhimurium *zntB* locus was deposited in the nucleotide database of the National Center for Biotechnology Information with the accession number AF308568.

## RESULTS

**Disruption of *zntB* increases sensitivity to zinc.** Allelic replacement techniques were used to disrupt the *S. enterica* serovar Typhimurium homolog of *b1342* by the insertion of a chloramphenicol resistance cassette into the coding sequence, producing strain RS1100 (7). Bacteriophage P22-mediated cotransduction techniques were used to determine the position of the *zntB* locus on the serovar Typhimurium chromosome. The insertion mutation mapped to centisome 36 and was 100% linked to *oxrA(fnr)*, which is in agreement with the recently completed genome sequence. The effects of the insertion mutation on metal sensitivity were determined by disk diffusion assay. Disruption of this locus did not significantly alter the sensitivities to cobalt, nickel, or manganese compared to those of the wild-type strain RS404. However, the loss of this allele had a marked effect on resistance levels to zinc and cadmium (Fig. 1). The increased zones of sensitivity displayed by the mutant suggest that the product of *b1342* might participate in

the extrusion of Zn<sup>2+</sup> and possibly Cd<sup>2+</sup>. Consequently, *b1342* and its gene product were designated ZntB (for Zn<sup>2+</sup> transport). Expression of a wild-type *zntB* allele in the mutant background partially restored resistance to zinc but had no measurable effect on resistance to cadmium. Growth of the *zntB* mutant (RS1100) and wild-type (RS404) strains was measured in minimal growth media containing a range of inhibitory zinc concentrations (Fig. 2). The wild type displayed half-maximal growth in medium containing 60 μM Zn<sup>2+</sup>. By comparison, the

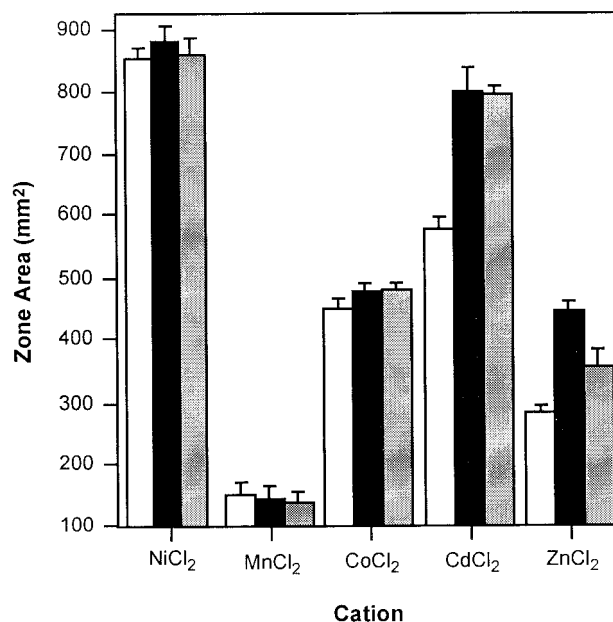


FIG. 1. Cation sensitivity profiles of serovar Typhimurium strains RS404 (white), RS1100 (black), and RS1100/pAJW54 (gray). Cells were grown overnight in supplemented N-minimal medium, and 100 μl of the culture was spread onto N-minimal agar plates. An 8-mm filter disk was placed in the center and saturated with 17 μl of a 100 mM cation solution of ZnSO<sub>4</sub>, CdCl<sub>2</sub>, and CoCl<sub>2</sub> and 1 M solutions of MnCl<sub>2</sub> and NiCl<sub>2</sub>. The areas of the resulting inhibition zones were determined after 18 h of incubation at 37°C. Values presented are averages of three independent trials, with standard deviations of the means indicated.

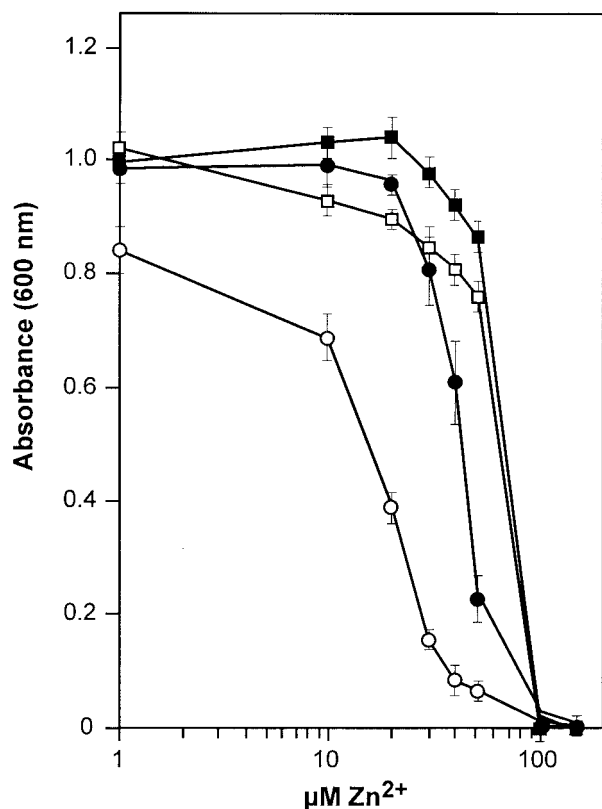


FIG. 2. Effects of zinc on growth of serovar Typhimurium strains RS404 (□), RS404/pAJW54 (■), RS1100 (○), and RS1100/pAJW54 (●). Overnight cultures were prepared in supplemented N-minimal medium and diluted 1:500 into fresh N-minimal medium with the indicated concentrations of  $ZnCl_2$ . Cell growth was measured as the  $OD_{600}$  as the cultures entered late log phase after 6 h of aerobic incubation at 37°C. The data are the averages of six independent trials, with standard deviations of the means indicated.

*zntB* mutant displayed half-maximal growth at 20  $\mu M Zn^{2+}$ . The zinc-sensitive phenotype displayed by RS1100 could be partially rescued through the introduction of a low-copy-number plasmid encoding the wild-type *zntB* allele (pAJW54). This strain (RS1100/pAJW54) attained half-maximal growth at 45  $\mu M Zn^{2+}$ .

**ZntB does not function as a  $Mg^{2+}$  uptake system.** ZntB is homologous to the CorA family of cation transporters (12, 20). In serovar Typhimurium and *E. coli*, CorA functions as the primary influx and efflux pathway for  $Mg^{2+}$  (22, 23). The ability of ZntB to function as a  $Mg^{2+}$  transport system was tested. Strain MM281 carries insertion mutations in each of the three known  $Mg^{2+}$  transport loci (*mgtA*, *mgtCB*, and *corA*) which render the cell unable to grow unless supplemented with 100 mM  $Mg^{2+}$ . The  $Mg^{2+}$ -dependent growth of MM281 can be rescued by introducing a plasmid encoding a functional  $Mg^{2+}$  transport system (21). Strain RS1225 (MM281/pAJW54) displayed a  $Mg^{2+}$  growth dependence that was indistinguishable from that exhibited by MM281 (Fig. 3). In stark contrast, strains expressing CorAs from serovar Typhimurium (MM281/pRS310) and the archaeon *Methanococcus jannaschii* (MM281/pRS319) displayed a pronounced leftward shift in

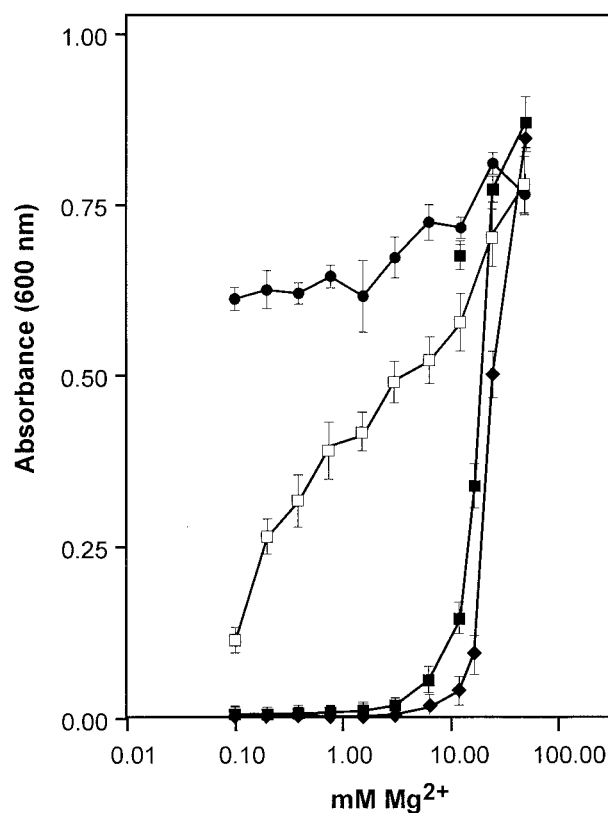


FIG. 3. Magnesium-dependent growth of serovar Typhimurium strains MM281 (◆), MM281/pRS310 (●), MM281/pRS319 (□), and MM281/pAJW54 (■). Overnight cultures were prepared in LB growth medium supplemented with 100 mM  $Mg^{2+}$ . Prior to the assay, the cell cultures were washed twice with medium containing no supplemental  $Mg^{2+}$  and diluted 1:500 into LB medium containing the indicated concentration of  $MgSO_4$ . Cell growth was measured as the  $OD_{600}$  after 8 h of aerobic incubation at 37°C. The data presented are the averages of five independent trials, with standard deviations of the means indicated.

$Mg^{2+}$  dependence for growth. Thus, at the level of growth, ZntB does not function as a  $Mg^{2+}$  uptake system.

**$^{65}Zn^{2+}$  transport assays.** To characterize the transport activity of ZntB, uptake of  $^{65}Zn^{2+}$  was measured in RS404 (wild-type serovar Typhimurium), RS1100 (*zntB* mutant), and the complementing strain RS1100/pAJW54. All three strains exhibited similar rates of uptake. The total amount of  $^{65}Zn^{2+}$  accumulated by the *zntB* mutant was 1.2-fold greater than the wild-type control accumulation. Expression of *zntB* from pAJW54 reduced the level of zinc accumulation to 1.1-fold of the levels demonstrated by the wild type (Fig. 4). These data indicate that ZntB does not facilitate zinc uptake, but rather suggest a possible role in the efflux of zinc. This was directly tested by introducing pAJW54 into a  $Zn^{2+}$  transport-deficient strain of *E. coli* and measuring the rate of  $^{65}Zn^{2+}$  efflux. GR480 contains mutations in each of the known  $Zn^{2+}$  transport loci (*zntA*, *zitB*, *zupD*, *znuABC*, and *yiiP*) but retains a functional *zntB* allele (9, 10). This gene was disrupted by the insertion of a chloramphenicol resistance cassette producing strain RS1220 (7). The presence of the single chromosomal *zntB* allele carried in strain GR480 resulted in an efflux rate

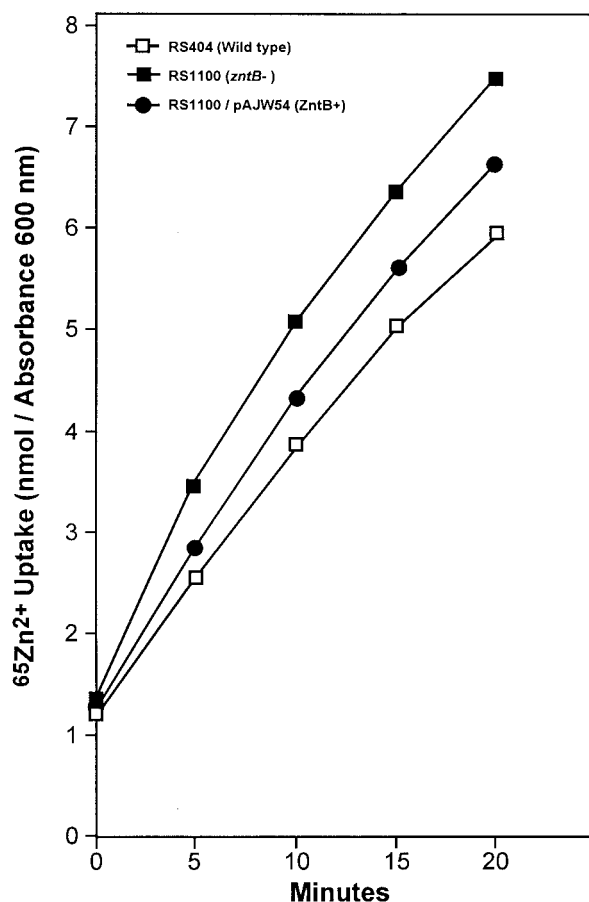


FIG. 4. Effects of *zntB* expression on  $\text{Zn}^{2+}$  uptake. Bulk uptake of  $^{65}\text{Zn}^{2+}$  was measured in serovar Typhimurium strains RS404 (wild type  $\square$ ), RS1100 (*zntB*<sup>-</sup>  $\blacksquare$ ), and RS1100/pAJW54 ( $\bullet$ ). Cation uptake was measured for 20 min at 37°C as described in Materials and Methods. The data shown are from a single representative experiment.

that was 5-fold greater than the rate for the transport-deficient strain (RS1220). Expression of ZntB from pAJW54 further increased the rate of  $^{65}\text{Zn}^{2+}$  efflux 8.8-fold (Fig. 5). Thus, ZntB is able to facilitate the efflux of zinc.

## DISCUSSION

In this report, we demonstrate that the *zntB* locus of *S. enterica* serovar Typhimurium encodes a protein that is involved in the transmembrane flux of zinc. Mutations at *zntB* render the cell hypersensitive to the cytotoxic effects of zinc, as indicated by disk diffusion analysis and growth characterization. This phenotype suggests that ZntB plays a role in the efflux of this cation. A direct examination of transport activity revealed that *zntB* mutations diminish the capacity to extrude  $\text{Zn}^{2+}$  without significantly affecting the uptake activity. Moreover, this transport deficiency could be complemented by a plasmid encoding a wild-type *zntB* allele. The ZntB transporter is homologous to the CorA  $\text{Mg}^{2+}$  transport protein, which is the defining member of a large class of unusual proteins that appear to be widely distributed throughout the bacteria and the archaea (20, 22). In *S. enterica* serovar Typhimurium and *E.*

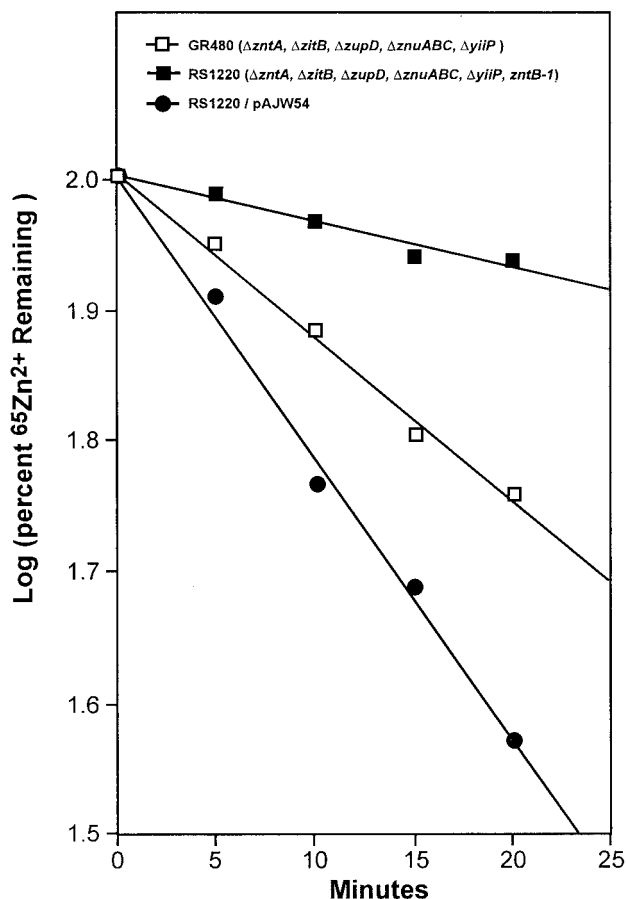


FIG. 5. Effects of *zntB* expression on  $\text{Zn}^{2+}$  efflux. Efflux was measured at 37°C in *E. coli* strains GR480 ( $\square$ ), RS1220 ( $\blacksquare$ ), and RS1220/pAJW54 ( $\bullet$ ) as described in Materials and Methods. GR480 carries chromosomal deletions in each of the known zinc transport loci ( $\Delta zntA, \Delta zitB, \Delta zupD, \Delta znuABC,$  and  $\Delta yjiP$ ) but retains a functional *zntB* allele. RS1220 was derived from GR480 by introducing an additional mutation in *zntB*. Plasmid pAJW54 encodes a wild-type *zntB* allele from serovar Typhimurium. The data shown are the averages of three independent experiments. Variability was less than  $\pm 5\%$  at each point. Correlation coefficients derived from linear regression analysis were  $>0.95$  for each individual experiment.

*coli*, CorA functions as the primary influx pathway for magnesium and is responsible for more than 95% of the total magnesium accumulated under normal growth conditions. The ZntB transporter, however, is not able to function as a  $\text{Mg}^{2+}$  uptake pathway. Our results identify ZntB as a novel transport system for  $\text{Zn}^{2+}$  in the enteric bacteria and, as such, assign a new function to the ubiquitous CorA family of cation transporters.

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