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ZitB (YbgR), a Member of the Cation Diffusion Facilitator Family, Is an Additional Zinc Transporter in *Escherichia coli*

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The *Escherichia coli zitB* gene encodes a Zn(II) transporter belonging to the cation diffusion facilitator family. ZitB is specifically induced by zinc. ZitB expression on a plasmid rendered *zntA*-disrupted *E. coli* cells more resistant to zinc, and the cells exhibited reduced accumulation of ^{65}Zn, suggesting ZitB-mediated efflux of zinc.

Zinc is an essential component of many proteins and is required for life in all organisms. However, excess zinc is toxic, and as a result, cells require homeostatic mechanisms to control intracellular zinc levels. In *Escherichia coli*, zinc deficiency induces expression of a specific zinc uptake system, ZnuABC, which is an ABC transporter for zinc uptake (22). Under conditions of zinc sufficiency, expression of the pump is repressed by the Fur homologue Zur, which presumably binds to the bidirectional promoter region of *znuA* and *znuBC*. However, under toxic conditions Zn(II) enters the cells by an unknown pathway. The phosphate uptake system has been implicated in uptake of Zn(II), possibly as a metal phosphate (3). Growth of *E. coli* in high concentrations of Zn(II), Cd(II), or Pb(II) resulted in induction of ZntA, a Zn(II)-Cd(II)-Pb(II)-translocating P-type ATPase. ZntR, a MerR homologue, is a transcriptional activator of *zntA* (4, 18). Disruption of *zntA* resulted in sensitivity to Zn(II), Cd(II), and Pb(II) (2, 24, 25). However, in addition to *zntA*, there are two uncharacterized genes, *ybgR* and *yiiP*, encoding gene products belonging to the cation diffusion facilitator (CDF) family of proteins (17, 23). The CDF family has common structural characteristics, with six transmembrane domains and containing histidine-rich motifs predicted to extend into the cytosol (1, 6). In addition, overproduction of eukaryotic members of this family confers resistance to zinc in *Saccharomyces cerevisiae* (6, 15).

In this report we show that *zitB* (formerly *ybgR*) encodes an additional zinc transporter belonging to the CDF family of proteins. Double disruption of *zitB* and *zntA* rendered *E. coli* cells more zinc sensitive than a single disruption in *zntA* alone. Furthermore, overexpression of ZitB resulted in a significant increase in zinc resistance and reduced uptake of zinc. Expression of both *zitB* and *yiiP* was inducible by zinc in a concentration-dependent manner. However, in contrast to *zitB*, the overexpression of *yiiP* did not confer additional zinc resistance, and disruption of *yiiP* in different strains did not alter zinc resistance, so the function of its gene product remains unknown.

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ZitB is an additional zinc transporter. *zitB* deletions were introduced into *E. coli* W3110 and *E. coli* RW3110 (*zntA*::Km), producing *E. coli* strains GG51 (Δ*zitB*::Cm) and GG48 (Δ*zitB*::Cm *zntA*::Km). Chromosomal deletions were performed as described by Datsenko and Wanner (5), and the gene of interest was replaced by a chloramphenicol cassette (Cm). The Δ*zitB*::Cm cassette was transduced into *E. coli* W3110 and RW3110 (*zntA*::Km) by P1 transduction. Mutants with a single Δ*zitB* deletion did not exhibit significant differences in metal sensitivity compared to *E. coli* W3110 (data not shown). However, *E. coli* strain GG48 (Δ*zitB*::Cm *zntA*::Km) was more zinc sensitive than *E. coli* RW3110 (*zntA*::Km), indicating that *zitB* (formerly *ybgR*) might encode a zinc transporter (Fig. 1). There was no effect on the MICs of cobalt and cadmium when *E. coli* strains GG48 and RW3110 were compared (data not shown). Since *zitB* appears to be selective for zinc, *ybgR* was renamed *zitB* (for "zinc transporter").

Zinc resistance and transport by ZitB. To determine whether ZitB transports zinc, the *zitB* gene was cloned into plasmid pASK-IBA3 (IBA Göttingen), leading to plasmid pZITB. Primer sequences are available on request. This plasmid was transferred into *E. coli* strain GG48 (Δ*zitB*::Cm *zntA*::Km). Induction of *zitB* on plasmid pZITB by addition of anhydrotetracycline (AHT) led to a significant increase in zinc resistance (Fig. 1). Induction by AHT was required to confer maximal zinc resistance. Expression of ZitB did not confer resistance to cobalt and cadmium (data not shown).

ZitB is homologous to members of the CDF family that have been implicated in transport of metal ions (6). Resistance mediated by a zinc transporter may be based on efflux, which decreases the intracellular concentration of metal ions. Uptake experiments were performed by filtration as described previously (16). When levels of cell-associated zinc ions in *E. coli* strain GG48 (Δ*zitB*::Cm *zntA*::Km) with and without expressed ZitB were compared, resistant cells accumulated significantly less zinc than the respective control cells (Fig. 2). Since it is a member of the CDF family, it is reasonable to propose that ZitB is located in the cytoplasmic membrane. Thus, reduced accumulation probably results from active transport of Zn(II) across the cytoplasmic membrane catalyzed by ZitB.

The *yiiP* gene product may also be involved in zinc homeostasis. The *yiiP* gene encodes a putative gene product also

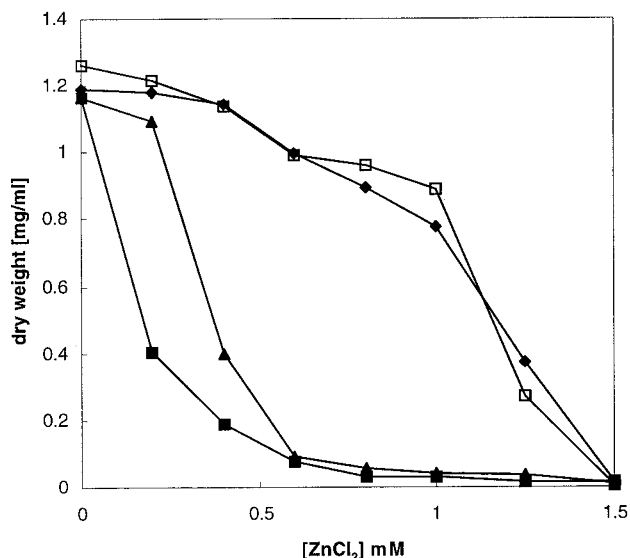

FIG. 1. Effect of zinc on growth *E. coli* W3110 (□), RW3110 (*zntA*::Km) (▲), GG48 (Δ*zitB*::Cm *zntA*::Km) (■), and GG48 (Δ*zitB*::Cm *zntA*::Km)/pZITB (◆). Growth curves with different $ZnCl_2$ concentrations are shown. Overnight cultures were diluted 1:500 into fresh Luria-Bertani medium with the indicated concentrations of $ZnCl_2$. Cell growth was monitored as the optical density at 600 nm after 15 h of incubation at 37°C with shaking and converted to dry weight. Experiments were performed in triplicate, values are averages.

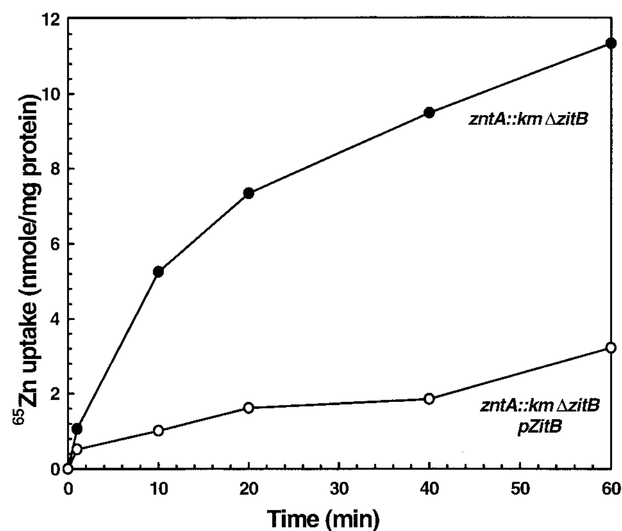

FIG. 2. ^{65}Zn(II) uptake by cells of *E. coli* GG48 (Δ*zitB*::Cm *zntA*::Km)/pZITB expressing *zitB*. Cells were grown overnight in Luria-Bertani medium and diluted 100-fold into fresh prewarmed Luria-Bertani medium. The cells were grown to an optical density at 600 nm of 0.8 and induced with 200 μg of AHT per liter. After growth for 2.5 h, the cells were washed with buffer A (10 mM Tris-HCl [pH 7.0], 2 g of glucose per liter, 10 mM Na_2HPO_4) and concentrated fourfold in the same buffer. $^{65}ZnSO_4$ was added to a final concentration of 5 μM. The cells were incubated at 37°C, and 0.1-ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and immediately washed with 10 ml of buffer B (10 mM Tris-HCl [pH 7.0], 10 mM $MgCl_2$). The membranes were dried, and radioactivity was measured using a liquid scintillation counter. The protein concentration was determined using the bicinchoninic acid kit (Sigma), and the amount of Zn(II) per milligram of protein was calculated.

belonging to the CDF family. Mutants with a Δ*yiiP* deletion were constructed from *E. coli* W3110, RW3110 (*zntA*::Km), and GG48 (Δ*zitB zntA*::Km), leading to strains GG180 (Δ*yiiP*::Cm), GG253 (Δ*yiiP*::Cm *zntA*::Km), and GG252 (Δ*yiiP*::Cm Δ*zitB zntA*::Km). Mutants with a Δ*yiiP* deletion did not show a decrease in zinc, cadmium, or cobalt resistance compared to the parental *E. coli* strains (data not shown). In contrast, strain GG253 (Δ*yiiP*::Cm *zntA*::Km) was slightly but significantly more zinc resistant than strain RW3110 (*zntA*::Km). However, overexpression of *yiiP* in plasmid pYIIP did not lead to an increase or decrease in zinc, cadmium, or cobalt tolerance (data not shown).

The *zitB* and *yiiP* genes are induced by zinc. To analyze metal-dependent expression of *zitB* and *yiiP*, transcriptional fusions using *lacZ* as a reporter gene were constructed. To construct the chromosomal Φ(*zitB-lacZ*) transcriptional fusion in strain *E. coli* GG161 (W3110 Δ*lacZYA*::Km), the 400 bp upstream and downstream of the *zitB* stop codon were separately amplified by PCR from chromosomal DNA of *E. coli* W3110. These fragments were digested with *Bam*HI, and both fragments were cloned into vector plasmid pGEM T-Easy (Promega, Madison, Wis.) in one step. As confirmed by control sequencing, this led to a plasmid harboring an 800-bp *zitB* fragment with a *Bam*HI and an *Xba*I site located directly downstream of the stop codon of *zitB*, mutating the sequence CATT**TAA**TGGGACAGC (the TAA stop codon of *zitB* is in boldface) to CATT**AA**<u>GGATCC</u>GGG<u>TCTAGA</u>GGCCATTC ACATCATCACCATTAA (underlining indicates restriction sites for *Bam*HI and *Xba*I). A promoterless *lacZ* gene was inserted into the *Bam*HI/*Xba*I site of this plasmid introduced by PCR, and the fragment containing *zitB-lacZ* was cloned as a *Not*I fragment into plasmid pKO3 (12). Finally, the pKO3 hybrid plasmid with Φ(*zitB-lacZ*) was used in a double-recombination event to insert the *lacZ* gene downstream of *zitB* on the chromosome of *E. coli* GG161 (W3110 Δ*lacZYA*::Km) as described previously (7). The correct insertion and orientation of *lacZ* in strain *E. coli* GG260 [W3110 Δ*lacZYA*::Km Φ(*zitB-lacZ*)] were verified by PCR. *E. coli* GG161 (W3110 Δ*lacZYA*::Km) was constructed by transfer of the *lacZYA*::Km replacement by generalized P1 transduction from strain *E. coli* BW25434 (5) into *E. coli* W3110. The β-galactosidase activity in permeabilized cells was determined as published previously (14). Likewise, a Φ(*yiiP-lacZ*) operon fusion was constructed, resulting in strain GG193 [W3110 Δ*lacZYA*::km Φ(*yiip-lacZ*)].

Expression of *zitB* was strongly induced by zinc and slightly induced by cadmium, while other metals did not significantly induce Φ(*zitB-lacZ*) (Table 1). The zinc concentration dependency of *zitB* expression was examined. Induction of *zitB* was observed with 50 μM $ZnCl_2$ and reached a maximum at 100 μM in mineral salts medium. Higher concentrations of Zn(II) led to a decrease of *zitB* expression (Fig. 3). Northern blot analysis (8, 9) also showed an increase in *zitB*-specific transcript after addition of zinc (data not shown). Expression of *yiiP* was also maximally induced by zinc and also to a lesser degree by cadmium (Table 1).

Conclusions. In this report we describe the identification of two genes, *ybgR* (*zitB*) and *yiiP*, on the *E. coli* chromosome that encode putative CDF proteins. Most CDF transporters analyzed thus far are responsible for zinc transport from the cy-

TABLE 1. Induction of *zitB* and *yiiP* by different metals

Addition	Avg β-galactosidase activity (Miller units)[a]	
	Φ(*zitB-lacZ*)	Φ(*yiiP-lacZ*)
None	21.7	36.9
$ZnCl_2$	223.5	212.5
$CdCl_2$	59.1	74.5
$CoCl_2$	24.5	30.7
$NiCl_2$	44.2	42.5
EDTA	25	35.9
$CuCl_2$	52.2	41.3

[a] Cells of either *E. coli* GG260 Φ(*zitB-lacZ*) or GG193 Φ(*yiiP-lacZ*) were diluted 1:100 into fresh mineral salts medium with 0.2% glycerol and 0.1% yeast extract containing no added metal or were induced after 3 h of growth with different metals or EDTA, each at 0.1 mM. Incubation was continued with shaking for 3 h at 30°C, and the β-galactosidase activity was determined (14). The averages of three independent experiments are shown.

tosol across different membranes. Four mammalian CDF transporters have been characterized: ZnT-1, ZnT-2, ZnT-3, and ZnT-4. ZnT-1 is responsible for zinc transport across the plasma membrane (19). ZnT-2 is responsible for zinc transport into lysosomes, and ZnT-3 is responsible for zinc transport into synaptic vesicles (20, 21). ZnT-4 is also thought to function in zinc efflux (10). Prokaryotic members of the CDF family include CzcD from *Ralstonia metallidurans* CH34 and CzrB (also named ZntA) from *Staphylococcus aureus* (1, 11, 26). In addition to zinc, these transporters were also shown to transport cobalt and cadmium (1). CzcD appears to have an additional regulatory function in repressing the CzC system by exporting inducing cations (1). However, in all CDF transporters characterized so far, neither the transport mechanism nor the actual substrate of the pump is known. It might therefore be premature to speculate about their physiological function.

In this study we examined the physiological role of the *yiiP* and *zitB* gene products in *E. coli*. No clear phenotype of a *yiiP*-disrupted strain was observed, so the physiological role of

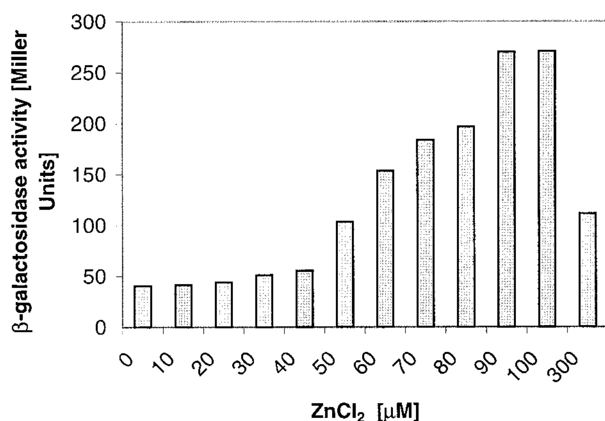

FIG. 3. Induction of *zitB*. Induction of β-galactosidase activity in a *zitB-lacZ* transcriptional fusion strain. Overnight cultures of *E. coli* GG260 containing a Φ(*zitB-lacZ*) operon fusion on the bacterial chromosome were diluted 1:100 into fresh minimal medium with 0.2% glycerol and 0.1% yeast extract containing no added metal or were induced after 3 h of growth by increasing concentrations of $ZnCl_2$. Incubation was continued with shaking for 3 h at 30°C, and the β-galactosidase activity was determined (14). Each experiment was performed in triplicate, and values are averages.

YiiP remains obscure. On the other hand, there was a clear relationship between expression of the *zitB* gene product and zinc tolerance in *E. coli*. Disruption of both *zitB* and *zntA*, which encodes a Zn(II)-translocating P-type ATPase (24), resulted in hypersensitivity to zinc. A strain disrupted only in *zitB* did not exhibit a decreased zinc tolerance, perhaps because ZntA could pump out zinc efficiently at high zinc concentrations. However, expression of *zitB* on a plasmid led to a significant increase in zinc resistance. It is possible that ZitB contributes to zinc homeostasis at low concentrations of zinc, while ZntA is required for growth at higher and more toxic concentrations. Additionally, zinc induction of a Φ(*zitB-lacZ*) transcriptional fusion showed a steady increase of transcription up to approximately 0.1 mM. Higher medium concentrations of zinc did not lead to a further increase in *zitB* transcription. This may reflect the fact that ZntA maintains the intracellular zinc concentration lower than the medium concentration. These studies indicate that zinc resistance is not due to a single transport system or any one factor but rather is due to many systems interacting in an as-yet-undefined way. The residual zinc resistance in a strain disrupted in both *zntA* and *zitB* suggests that there are additional factors or systems involved in zinc resistance.

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