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 65Zn, 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.

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 (∆zitB::Cm) and GG48 (∆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 ∆zitB::Cm cassette was transduced into E. coli W3110 and RW3110 (zntA::Km) by P1 transduction. Mutants with a single ∆zitB deletion did not exhibit significant differences in metal sensitivity compared to E. coli W3110 (data not shown). However, E. coli strain GG48 (∆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 (∆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 (∆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...
hybrid plasmid with $\Phi(zib-lacZ)$ was used in a double-recombination event to insert the $lacZ$ gene downstream of $zib$ on the chromosome of $E. coli$ GG161 ($W3110$ $\Delta lacZYA::Km$) as described previously (7). The correct insertion and orientation of $lacZ$ in strain $E. coli$ GG260 ($W3110$ $\Delta lacZYA::Km$ $\Phi(zib-lacZ)$) were verified by PCR. $E. coli$ GG161 ($W3110$ $\Delta lacZYA::Km$) was constructed by transfer of the $lacZYA::Km$ replacement by generalized P1 transduction from strain $E. coli$ BW25434 (5) into $E. coli$ W3110. The $\beta$-galactosidase activity in permeabilized cells was determined as published previously (14). Likewise, a $\Phi(yipP-lacZ)$ operon fusion was constructed, resulting in strain GG193 ($W3110$ $\Delta lacZYA::Km$ $\Phi(yipP-lacZ)$).

Expression of $zib$ was strongly induced by zinc and slightly induced by cadmium, while other metals did not significantly induce $\Phi(zib-lacZ)$ (Table 1). The zinc concentration dependency of $zib$ expression was examined. Induction of $zib$ was observed with 50 $\mu$M ZnCl$_2$ and reached a maximum at 100 $\mu$M in mineral salts medium. Higher concentrations of Zn(II) led to a decrease of $zib$ expression (Fig. 5). Northern blot analysis (8, 9) also showed an increase in $zib$-specific transcript after addition of zinc (data not shown). Expression of $yipP$ was also maximally induced by zinc and also to a lesser degree by cadmium (Table 1).
The physiological role of the zitB and yiiP gene products in *E. coli* 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.

<table>
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<th>Addition</th>
<th>Avg β-galactosidase activity (Miller units)</th>
<th>Φ(zitB-lacZ)</th>
<th>Φ(yiiP-lacZ)</th>
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<tr>
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*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 growth with shaking for 3 h at 30°C, and the β-galactosidase activity was determined (14). The averages of three independent experiments are shown.*

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