

## Insertional Inactivation of *dsbA* Produces Sensitivity to Cadmium and Zinc in *Escherichia coli*

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**In a search for genes that produce hypersensitivity to cadmium salts in *Escherichia coli*, random transposon mutagenesis with *TnphoA* was used. One of the mutant strains obtained was sensitive to  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$ . Sequence analysis showed that the *TnphoA* insertion was located in the *dsbA* gene coding for a periplasmic protein required for disulfide bond formation.**

Trace nutrients, such as zinc, copper, and nickel, are required for all living cells (14). However, these elements are also toxic in excess. *Escherichia coli* is intrinsically tolerant to high levels of  $\text{Cd}^{2+}$ . This high level of tolerance could be due to active efflux of cadmium. To identify the putative  $\text{Cd}^{2+}$  transporter, *E. coli* W3110 (1) was subjected to random *TnphoA* mutagenesis (10). This procedure has been used to identify the genes for transport proteins because such fusions can produce blue colonies on XP plates (15).

**Random *TnphoA*-mediated mutagenesis to obtain a  $\text{Cd}^{2+}$ -sensitive mutant.** *E. coli* W3110 was infected with  $\lambda$ b221 *rex::TnphoA* cI857 as previously described (10). Kanamycin-resistant colonies that also formed blue colonies on XP (20 mg of 5-bromo-4-chloro-3-indolylphosphate per ml) plates were then screened for  $\text{Cd}^{2+}$  sensitivity on Luria-Bertani (LB) agar plates containing 0.5 mM Cd acetate. One mutant was obtained that showed a large decrease in  $\text{Cd}^{2+}$  tolerance. Colonies of this mutant strain, CW3110, were light blue on XP plates, in contrast to the white colonies of W3110. Although the goal of the genetic selection was isolation of mutants defective in  $\text{Cd}^{2+}$  transport, no difference in the accumulation of  $^{109}\text{Cd}^{2+}$  in cells of CW3110 compared with W3110 was observed (data not shown). Thus, the nature of the mutation was investigated further.

**Cadmium sensitivity due to a single *TnphoA* insertion.** To determine whether the mutant strain carried the *TnphoA* insertion in a single locus, the kanamycin resistance phenotype was transduced back into strain W3110 by generalized transduction with P1 phage. All transductants were  $\text{Cd}^{2+}$  sensitive. Southern blot hybridization was performed with *Bam*HI-digested genomic DNA of CW3110, with DNA from W3110 as a control, by using a 485-bp *TnphoA*-specific probe. The result of the Southern blotting confirmed the existence of only a single *TnphoA* insertion (data not shown).

**Location of *TnphoA* insertion in the *dsbA* gene.** Since there is no *Bam*HI site between the site of fusion in *TnphoA* and the kanamycin phosphotransferase gene, and there is a *Bam*HI site immediately following the 3' end of the kanamycin phosphotransferase gene (7), chromosomal DNA of CW3110 was digested with *Bam*HI. The portion of DNA proximal to the fusion junction was cloned into the unique *Bam*HI site of pUC18 (16); the transformed colonies were screened for  $\text{Km}^r$ .

The resulting plasmid, pCGR4, contained a 6.2-kb insert composed of 1.3 kb of *E. coli* chromosomal DNA and 4.9 kb from *TnphoA*. Sequence analysis confirmed that the *TnphoA* insertion was located at 87.35 min on the *E. coli* chromosome, in the *dsbA* gene (2, 9).

**Metal sensitivity of *E. coli* CW3110 (*dsbA::TnphoA*).** The effects of various metals on the growth of *E. coli* CW3110 were tested (Table 1). CW3110 showed increased sensitivity to  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$  salts on agar plates compared to parent strain *E. coli* W3110. The mutant was 40-fold more sensitive to  $\text{Cd}^{2+}$  on solid medium. However, the lowest concentration of  $\text{Cd}^{2+}$  at which the mutant grew normally was 3  $\mu\text{M}$ ; at higher concentrations,  $\text{Cd}^{2+}$  produced a mucoid phenotype in the mutant. Cells of the mutant also became mucoid in the presence of  $\text{Zn}^{2+}$ . Although the mutant still grew on solid medium at 0.8 mM  $\text{ZnSO}_4$ , it displayed a mucoid phenotype starting at a concentration of 50  $\mu\text{M}$ . The mucoid phenotype was also observed at sublethal concentrations of sodium arsenite. A mucoid phenotype is often observed as a stress response in *E. coli*. The colonies were very small and were surrounded by a layer of excreted polysaccharide (5).

Since mucoidy made metal sensitivity on solid medium difficult to analyze, monitoring of growth in liquid culture with and without added metal salts was used to confirm sensitivity to  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  (Fig. 1); sodium arsenite and potassium antimonium tartrate also decreased the growth of CW3110 in liquid culture compared to that of W3110 but to a much smaller extent (data not shown). The  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  sensitivity of strain CW3110 was complemented by introducing a plasmid (p12-7) carrying the *dsbA* gene (2) (data not shown).

**Possibility that cadmium toxicity is due to accumulation of cadmium-induced formation of misfolded proteins in the periplasm.** The *dsbA* gene product is a periplasmic protein involved in disulfide bond formation in *E. coli* (2). It is presumably oxidized by DsbB (6, 12) and is required for proper folding of secreted proteins. Although the *dsbA* gene is not essential for growth on LB medium, protein folding is defective in a *dsbA* mutant strain (2). While DsbA is thought to be the oxidase that introduces the initial disulfide bonds, DsbC has been shown to be a disulfide bond isomerase that rearranges the bonds to allow proper protein folding, and mutations in this locus result in copper, but not cadmium, sensitivity (13). Under aerobic growth conditions, disulfide bond formation and proper protein folding in the periplasm may be slow in a *dsbA* mutant strain. In a wild-type strain, the thiols of periplasmic or membrane proteins are in the form of disulfide bonds

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TABLE 1. MICs of metal salts

| Metal salt        | Apparent MIC <sup>a</sup> (mM) |                                   |
|-------------------|--------------------------------|-----------------------------------|
|                   | W3110                          | CW3110<br>( <i>dsbA::TnphoA</i> ) |
| Cd acetate        | 1.2                            | 0.03 <sup>b</sup>                 |
| ZnSO <sub>4</sub> | 1.6                            | 1.0 <sup>c</sup>                  |
| HgCl <sub>2</sub> | 0.05                           | <0.01                             |
| CoCl <sub>2</sub> | 1.2                            | 1.6                               |
| CuSO <sub>4</sub> | 6                              | 6                                 |
| NiCl <sub>2</sub> | 5                              | 5                                 |

<sup>a</sup> Cells were grown overnight and streaked on LB plates containing various concentrations of metal salts. Growth was monitored after incubation for 24 h at 37°C. The apparent MIC is the concentration at which no colonies were detected.

<sup>b</sup> At concentrations below the apparent MIC, small, mucoid colonies were observed. The highest concentration at which normal colony morphology was detected was 3 μM.

<sup>c</sup> At concentrations below the apparent MIC, small, mucoid colonies were observed. The highest concentration at which normal colony morphology was detected was 50 μM.

and are not reactive with soft metals such as Cd<sup>2+</sup>. In contrast, the thiols of the proteins remain accessible to Cd<sup>2+</sup> or Zn<sup>2+</sup> in a *dsbA* mutant strain. Thus, the toxic effects of Cd<sup>2+</sup> and Zn<sup>2+</sup> in a strain lacking *dsbA* could be due to the binding of these

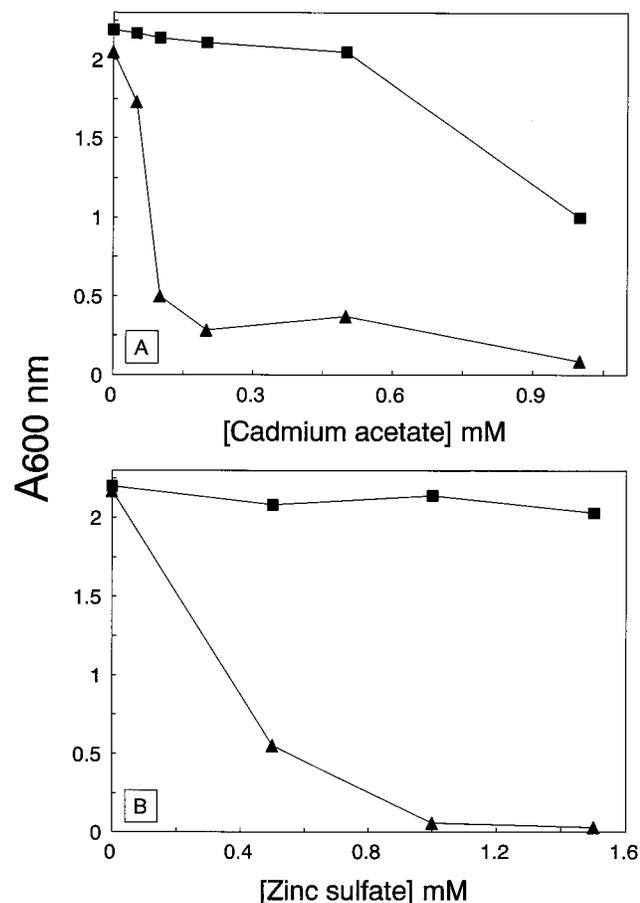


FIG. 1. Cadmium and zinc ion resistance. Metal ion resistance was assayed in cells of *E. coli* W3110 (wild type) (■) and CW3110 (*dsbA::TnphoA*) (▲). Cells were grown in LB medium with the indicated concentrations of cadmium acetate (A) or zinc sulfate (B) for 24 h at 37°C with shaking, and turbidity was measured at 600 nm.

metals to the free thiols of periplasmic proteins which are normally oxidized by the *dsbA* gene product.

Jungmann et al. (8) have shown that in yeast, mutants deficient in specific ubiquitin-conjugating enzymes are hypersensitive to Cd<sup>2+</sup>. Moreover, mutants in the proteasome were also hypersensitive to Cd<sup>2+</sup>. They propose that a major reason for Cd<sup>2+</sup> toxicity may be the accumulation of abnormally folded proteins induced by Cd<sup>2+</sup>. Cadmium, mercury, and, to a lesser extent, zinc, preferentially bind to thiol groups of proteins. Copper, nickel, and cobalt bind to thiol groups less tightly than does cadmium or zinc. This may explain why the *dsbA* disruption produces sensitivity to Cd<sup>2+</sup>, Zn<sup>2+</sup>, and Hg<sup>2+</sup> but not to copper or other metals.

Another possibility is that DsbA is required for proper folding of a Cd<sup>2+</sup>- or Zn<sup>2+</sup>-specific transporter. Without DsbA, the proposed transporter would fail to export zinc and cadmium, thus making the cells hypersensitive to cadmium and zinc. However, this seems unlikely, since mercury and arsenite have similar effects on the mutant strain. The effect of another putative protein disulfide isomerase, DsbD (also called CutA2 or DipZ), on the level of tolerance to copper and cadmium was shown by Fong et al. (4). Although they were unable to completely complement the Cu<sup>2+</sup>- and Cd<sup>2+</sup>-sensitive phenotype of their mutant, the effect of DsbD on copper and cadmium tolerance levels could be clearly demonstrated. DsbD is involved in the assembly of cytochromes in *E. coli* (3) and, if absent, might make the respiratory chain more susceptible to attack by metals. Although DsbA was also shown to be essential for cytochrome *c* synthesis (11), the phenotype of our mutant strain indicates that the *dsbA* and *dsbD* gene products introduce disulfide bonds in different classes of proteins since, in contrast to *dsbD* mutants, there was no detectable difference in the level of copper tolerance and a much larger decrease of cadmium and zinc tolerance in the *dsbA* mutant.

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## REFERENCES

- Bachmann, B. J. 1987. Deviations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
- Bardwell, J. C. A., K. McGovern, and J. Beckwith. 1991. Identification of a protein required for disulfide bond formation *in vivo*. *Cell* 67:581–589.
- Crooke, H., and J. Cole. 1995. The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulfide isomerase-like domain. *Mol. Microbiol.* 15:1139–1150.
- Fong, S., J. Camakaris, and B. T. O. Lee. 1995. Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12. *Mol. Microbiol.* 15:1127–1137.
- Gottesman, S., P. Trisler, A. S. Torres-Cabassa, and M. Maurizi. 1985. Regulation via proteolysis: the *Escherichia coli lon* system, p. 350–354. In L. Leive (ed.), *Microbiology—1985*. American Society for Microbiology, Washington, D.C.
- Guilhot, C., G. Jander, N. L. Martin, and J. Beckwith. 1995. Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. *Proc. Natl. Acad. Sci. USA* 92:9895–9899.
- Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* 177:65–72.
- Jungmann, J., H. Reins, C. Schobert, and S. Jentsch. 1993. Resistance to cadmium mediated by ubiquitin-dependent proteolysis. *Nature* 361:369–371.
- Kamitani, S., Y. Akiyama, and K. Ito. 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. *EMBO J.* 11:57–62.
- Manoil, C., and J. Beckwith. 1985. *TnphoA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* 82:8129–8133.
- Metheringham, R., L. Griffiths, H. Crooke, S. Forsythe, and J. Cole. 1995. An essential role for DsbA in cytochrome *c* synthesis and formate-dependent nitrite reduction by *Escherichia coli* K-12. *Arch. Microbiol.* 164:301–307.

12. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1993. Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds *in vivo*. Proc. Natl. Acad. Sci. USA **90**:7084–7088.
13. **Rietsch, A., D. Belin, N. Martin, and J. Beckwith.** 1996. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **93**:13048–13053.
14. **Rosen, B. P., and S. Silver (ed.).** 1987. Ion transport in prokaryotes. Academic Press, San Diego, Calif.
15. **Steffes, C., J. Ellis, J. H. Wu, and B. P. Rosen.** 1992. The *lysP* gene encodes the lysine-specific permease. J. Bacteriol. **174**:3242–3249.
16. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and their host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.