Evidence for the transport of zinc(II) ions via the Pit inorganic phosphate transport system in Escherichia coli

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Abstract

A locus involved in zinc(II) uptake in Escherichia coli K-12 was identified through the generation of a zinc(II)-resistant mutant by transposon (Tn10dCam) mutagenesis. The mutation was located within the pitA gene, which encodes the low-affinity inorganic phosphate transport system (Pit). The pitA mutant accumulated reduced amounts of zinc(II) when exposed to 0.5–2.0 mM ZnSO4 during growth in Luria–Bertani medium. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Zinc(II) is an essential trace metal ion for all organisms, but above optimal concentrations it is toxic. The intracellular concentration of zinc(II) must therefore be regulated within very narrow limits. Uptake and intracellular transport systems must ensure an adequate supply of zinc(II) ions to Zn(II)-dependent proteins, but mechanisms must also exist for the sequestration or efflux of zinc(II) when in excess. Recent research has led to a rapid increase in our understanding of both uptake and efflux systems.

Growth of Escherichia coli at high concentrations of zinc(II) results in Zn(II) export through ZntA, a cation transport (P-type) ATPase [1,2]. The expression of zntA is regulated by ZntR, a zinc(II)-responsive homologue of the transcriptional regulator MerR [3]. Homologues of ZntA have been identified in several bacteria such as Synchocystis sp. (ZiaA) [4], Proteus mirabilis (ZntA) [5], Staphylococcus aureus (ZntA) [6], Helicobacter pylori (CadA) [7], Alcaligenes eutrophus (the Czc plasmid-encoded system) [8] and Pseudomonas aeruginosa (Czr) [9].

Uptake systems for metal ions are frequently duplicated in bacteria: constitutive, low-affinity systems may co-exist with inducible high-affinity systems. High-affinity zinc(II) uptake systems have recently been identified in several bacteria. In E. coli, zinc(II) deficiency induces the expression of the ZnuABC Zn(II) uptake system, which belongs to the superfamily of ATP-binding cassette (ABC) transporters [10]. Under conditions of zinc(II) sufficiency, expression of the pump is repressed by the Fur homologue Zur. Genes encoding homologues of ZnuABC have been identified in a number of other bacteria such as Streptococcus pneumoniae [11], Streptococcus pyogenes [12], Haemophilus influenzae [13], Haemophilus ducreyi [14] and Listeria monocytogenes (in which a Zur-like protein was also identified [15]). In Bacillus subtilis, two gene loci have been implicated in zinc(II) uptake [16]: the ycdH-containing operon encodes a putative high-affinity zinc(II)-translocating ABC transporter, whereas yciC encodes an ATP-binding integral membrane protein that may form part of a low-affinity system.
Mutations in genes encoding components of metal uptake systems may confer a metal-resistant or metal-dependent phenotype (e.g. [11,13,14,16–19]). To identify other genetic loci involved in zinc(II) uptake in *E. coli*, we have sought mutations that confer a zinc(II)-resistant phenotype. We describe here the properties of a mutant that showed enhanced resistance to Zn(II). The mutation is located within *pitA*, which encodes the inorganic phosphate transport system (Pit) in *E. coli* [20].

2. Materials and methods

2.1. Bacterial strains and phages

Derivatives of *E. coli* K-12 were used throughout. Strain GE2515 was used for transposon mutagenesis [21], and strain DH5α [22] was used as a host for cloning. Strains GE2515 and MG1655 (wild-type) were kindly provided by H.D. Williams (Imperial College of Science and Technology, London, UK). Hfr and P1 mapping strains [23] were kindly provided by C.A. Gross (University of California, San Francisco, CA, USA). Bacteriophage P1*r*ir was used for generalised transductions.

2.2. Isolation of zinc(II)-resistant mutants

Random insertional mutagenesis of *E. coli* GE2515 was carried out using the Tn10dCam transposition system as described by Heath et al. [21]. The mutagenised cells were harvested by centrifugation, resuspended in 0.2 ml of Luria–Bertani (LB) medium, and serial dilutions were plated on LB agar containing chloramphenicol and ZnSO₄ (at a range of concentrations between 0 and 10 mM).

2.3. Analyses of metal sensitivity and intracellular zinc(II) accumulation

Metal sensitivity was assessed by monitoring the turbidity of cultures containing varying concentrations of ZnSO₄ or CdCl₂. Cultures were grown with shaking at 37°C for 30 h. Sensitivity to these metal ions was also assessed by streaking cells across metal gradient agar plates [1]. Amounts of intracellular Zn(II) were measured after cells were exposed to various concentrations of ZnSO₄ during exponential growth as described previously [1].

2.4. Molecular genetic techniques

Generalised transductions were carried out using bacteriophage P1*r*ir as described by Silhavy et al. [24]. Hfr matings were performed as described by Singer et al. [23]. Methods for the manipulation and analysis of DNA were performed as described in Sambrook et al. [25]. Nucleotide sequencing was performed using an ABI Prism 377 automated sequencer.

2.5. Cloning and sequencing of a DNA fragment containing Tn10dCam

Genomic DNA from SJB201 was digested with *Sal*I and ligated to *Sal*I-cut pBR322. This mixture was introduced into *E. coli* DH5α and chloramphenicol-resistant transfectants were selected. A plasmid (pRP1080) isolated from one Cm*R* transfectant contained a 4.6-kb insert, which was shown to contain Tn10dCam by restriction analysis. To construct a plasmid containing only one end of Tn10dCam for sequencing, plasmid pRP1080 was digested with *Eco*RI (which cuts once within Tn10dCam), religated and introduced into strain DH5α. A plasmid (pRP1081) was isolated from an Ap*R*, Cm*R* transformant, and it was found to contain a 1.6-kb insert. This was sequenced using an oligonucleotide primer RP57 (5'–GATATTTACCAAAAATCATAGGGG-3') which is complementary to a region at the end of Tn10dCam.

3. Results and discussion

3.1. Isolation of a zinc(II)-resistant mutant

Following Tn10dCam mutagenesis, zinc(II)-resistant mutants were identified as colonies growing on LB agar containing 2.5 mM added ZnSO₄; no colonies appeared after cells from a non-mutagenised culture were plated onto the same medium. The disrupted allele in one mutant was co-transduced with the Cm*R* marker into the wild-type strain MG1655. The resulting transductant (designated SJB201) appeared to exhibit increased resistance to both Zn(II) and Cd(II) ions when growth was assessed across metal gradient plates (Fig. 1). The zinc(II)-resistant phenotype of SJB201 was confirmed by comparing its growth with that of MG1655 in batch cultures containing added ZnSO₄ (Fig. 2). At the lowest Zn(II) concentration (1.5 mM), the wild-type strain began to grow after a considerably prolonged lag phase (>8 h), by which time the

![Fig. 1. Growth of SJB201 (pitA) and GE2515 (pitA−) across concentration gradients of Zn(II) and Cd(II). Freshly grown biomass was streaked across solidified glycerol-glycerophosphate minimal medium [1] containing chloramphenicol and concentration gradients of (A) ZnSO₄ (0.60 mM maximum) and (B) CdCl₂ (0.20 mM maximum). The plates were incubated at 37°C for 3 days.](https://academic.oup.com/femsle/article-abstract/184/2/231/607533/fig1/9295-2-3-00)
pitA mutant had reached stationary phase. We were unable to confirm the Cd(II)-resistant phenotype of SJB201 in batch culture.

3.2. Genetic mapping of the Tn10dCam insertion

Using a set of Hfr mapping strains that contain Tn10 [23], the Tn10dCam element was estimated to lie between 67 and 81.75 min on the chromosome of SJB201. Transduction with a P1vir lysate prepared from one (CAG18450) of a set of P1 mapping strains [23] that cover this region generated chloramphenicol-sensitive transductants (with concomitant loss of the zinc(II)-resistant phenotype) at a frequency of approximately 10%. The mutation in SJB201 was estimated to map between 74.5 and 75.5 min on the E. coli genetic map [26].

3.3. Identity of the mutated gene conferring resistance to Zn(II)

To identify the precise location of the Tn10dCam insertion in the genome of SJB201, a DNA fragment containing the transposon was cloned and sequenced. The first 279 nucleotides adjacent to one end of Tn10dCam showed 100% identity to the pitA gene from E. coli [27], which encodes Pit [20]. The Tn10dCam element was located at nucleotide 1169 in the 1497-bp pitA open reading frame (ORF). The downstream ORF yhiO is transcribed towards pitA, so it is unlikely that the Tn10dCam insertion affected its function. The predicted amino acid sequence of PitA indicates that it contains 10 potential transmembrane domains, and that it lacks any of the typical zinc(II)-binding motifs.

3.4. Intracellular accumulation of Zn(II) by strains SJB201 and MG1655

The amounts of intracellular Zn(II) accumulated by strains SJB201 (pitA) and MG1655 (pitA+) were measured after cells were exposed to various concentrations of ZnSO4 during exponential growth, as described previously [1] (Fig. 3). In these experiments, the minimal inhibitory concentrations (MICs) of ZnSO4 were 1.5 mM for MG1655 and 2.0 mM for SJB201. The amounts of intracellular Zn(II) accumulated by SJB201 were significantly lower than the amounts accumulated by MG1655 when ZnSO4 was added in the concentration range 0.5–2.0 mM (Fig. 3). At higher, growth inhibitory Zn(II) concentrations (2.5–9.5 mM), however, SJB201 was found to accumulate slightly higher levels of Zn(II) than the wild-type (data not shown). The amounts of Zn(II) accumulated by MG1655 were found repeatedly to be about one order of magnitude greater than the amounts accumulated by its RecA3 derivative, strain SJB124 (MG1655 recA::Tn10), as reported previously [1]. A possible explanation for this discrepancy may be that SJB124 exhibits enhanced Zn(II) efflux, since the Tn10-encoded TetA protein has been shown to transport divalent cation–tetracycline complexes [28].

3.5. Conclusions

We have demonstrated that insertional mutagenesis of the pitA gene in E. coli confers increased resistance to zinc(II), and that this is associated with reduced accumulation of Zn(II) when cells are exposed to concentrations of ZnSO4 below the MIC. This indicates that the Pit system represents a second, low-affinity uptake system for
zinc(II) in *E. coli*, in contrast to the ZnuABC high-affinity uptake system that has been described recently [10]. The Pit system is expressed constitutively in *E. coli* [29], whereas expression of ZnuABC is repressed at high concentrations of Zn(II) [10].

Previous studies [e.g. (17,30–32)] have shown that Pit of prokaryotes mediates the uptake of P, and divalent cations, although transport of zinc(II) ions via Pit has not been reported before. For example, Mg^2+\, Ca^{2+}, Mn^{2+}\text{ and Co}^{2+}\text{ were shown to be transported as neutral metal–phosphate (MeHPO}_4\text{) complexes via the Pit systems in *E. coli* [30] and *Acinetobacter johnsonii* [31]. In *B. subtilis*, uptake of these metal ions was stimulated by inorganic phosphate [17], and a Pit\text{ }^-\text{ mutant exhibited reduced transport of Ca}^{2+}\text{ and Co}^{2+}. Pit\text{ }^-\text{ mutants of *B. subtilis* and *E. coli* also exhibit increased resistance to arsenate [17,18], a competitive inhibitor of P}\text{ i transport. Although cobalt(IIT) was shown to be transported via the Pit system in *E. coli* [30], the pitA mutant SJB201 did not exhibit a Co(II)-resistant phenotype: a possible explanation for this is that the mutant accumulated normal levels of Co(II) as a result of uptake via the constitutively expressed CorA system [19]. We have performed experiments to examine the effects on Zn(II) resistance of altering the concentration of inorganic phosphate in the growth medium: these were hampered, however, by the formation of a precipitate in the glycerol-glycerophosphate minimal medium [1] containing P, (instead of glycerophosphate) and ZnSO\text{ 4}\text{ results not shown}.

The observation that the pitA mutant accumulated slightly more zinc(II) than the wild-type at high, toxic, external Zn(II) concentrations (\(>2\text{ mM}\)) is unexplained, since the phenotype of the pitA mutant, the metal analyses of cells challenged with lower zinc concentrations (Fig. 3) and earlier reports of divalent metal transport by PitA (reviewed in [32]) all demonstrate that this system is an importer. One possible explanation is that high Zn concentrations lead to decline in intracellular polyphosphate pools, as demonstrated for *Klebsiella aerogenes* exposed to cadmium (reviewed in [33]). Keasing [33] proposes that the phosphate released from polyphosphate (by increased polyphosphatase activity) is transported via Pit out of the cell accompanied by cations, which may be toxic metal ions. Thus the Pit system may under certain conditions mediate Zn(II) efflux, or metal exchange [32] as demonstrated for Mg^2+\text{ in *E. coli* [30].}

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