Geobacillus stearothermophilus LV *cadA* gene mediates resistance to cadmium, lead and zinc in *zntA* mutants of *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Salmonella enterica serovar Typhimurium cells expressing the *cadA* gene of *Geobacillus stearothermophilus* LV exhibit a hypersensitive phenotype to cadmium chloride. Deletion of the ORF STM3576 from the *Salmonella* genome resulted in cadmium, lead and zinc sensitivity, confirming that this ORF is a homologue of the *zntA* gene. The observed sensitivity was reverted upon expression of the *G. stearothermophilus* LV *cadA* gene. These results indicate that the *cadA* gene product is involved in Cd, Pb and Zn resistance as a classical P-type ATPase and strongly suggest that the observed hypersensitive phenotype to these metals can be related to the function of the host *zntA* gene product.

Key terms: CadA, cadmium, lead, P-type ATPase, zinc, ZntA

INTRODUCTION

When present at appropriate amounts some heavy metals are essential elements for the cell metabolism. At higher concentrations, however, they can display severe toxic effects forcing the cell to maintain an exquisite surveillance on the intracellular levels of these ions.

A group of heavy metals with no known biological function that are considered merely toxics at any concentration has also been reported (Silver, 1998). These metals include cadmium, lead and mercury, behave as weak Lewis acids, and can react with thiolate and/or imidazol groups of cellular proteins affecting their biological activities (Lebrun et al., 1994). The widespread distribution of Cd, Hg and Pb in the environment has pushed the microbial populations to develop specific resistance systems. Preventing ion accumulation by

active cation efflux and sequestering by small binding proteins analogous to eukaryotic metallothioneins are some of the mechanisms that prokaryotes have developed to tolerate high concentrations of these elements (Naz et al., 2005; Rensing et al., 1998).

The main translocating proteins involved in metal homeostasis belong to the P-type ATPase family. These proteins translocate ions from the cytoplasm by active transport using ATP-driven energy. Bacterial representative members of this family include CadA, CopA, CopB and CtaA (Apell, 2004; Nucifora et al., 1989; Rosen, 2002).

The most characterized Cd (and Pb) resistance determinants are the CadA ATPase from the *Staphylococcus aureus* plasmid pI258 (Nucifora et al., 1989) and the *zntA* gene of *E. coli* (Rensing et al., 1997).

We have been interested in studying bacterial heavy metal resistance in Gram-

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positive bacteria from the soil. *Geobacillus stearothermophilus* LV is a thermophilic, plasmidless, sporeforming microorganism capable of growing at elevated concentrations of heavy metals (Lobos and Vásquez, 1993). We have previously isolated a DNA fragment from *G. stearothermophilus* LV that encodes three open reading frames (ORFs), two of them exhibiting high sequence homology and gene organization features similar to the *cadA* and *cadC* genes encoded by the *S. aureus* pI258 plasmid (Nerey et al., 2002). We also communicated that overexpression of the *G. stearothermophilus* LV *cadA* gene caused a hypersensitive phenotype to Cd in *E. coli* (Nerey et al., 2002).

Here we report that the hypersensitive phenotype previously described in cells of *E. coli* overexpressing the CadA protein is also observed in *Salmonella enterica* serovar Typhimurium. Using genetic and *in silico* analysis we found that ORF STM3576 from *S. Typhimurium* 14028s encodes a gene that is homologous to the *Escherichia coli* *zntA* gene which has been associated with Cd, Pb and Zn resistance (Rensing et al., 1997). We hypothesized that the *Salmonella* *zntA* gene could be related to the hypersensitivity phenotype generated by CadA overexpression as we did not observe the classical sensitivity to Cd, Pb or Zn in *S. Typhimurium* *zntA* mutants overexpressing the *G. stearothermophilus* LV *cadA* gene. In fact, the observed phenotype was rather an increase in the level of resistance of *Salmonella* to Cd, Pb and Zn. These results confirm that the CadA protein of *G. stearothermophilus* LV participates in Cd, Pb and Zn resistance and may also suggest that the hypersensitivity phenotype to these metals observed in wild type cells could be due to an increase in the concentration of the membrane transporters CadA and/or ZntA.

MATERIALS AND METHODS

Growth conditions

Bacteria were grown in LB broth (Sambrook et al., 1989) at 37 °C with aeration and ampicillin (50 µg/ml) or

chloramphenicol (25 µg/ml) was added to the medium as required.

For lead resistance assays bacteria were grown in PTY100B medium (0.01 % yeast extract, 0.01 % peptone, 0.01 % tryptone and 10 mM MES, pH 6.5) amended with the corresponding antibiotics at 37 °C. When cells reached the exponential phase of growth, arabinose or glucose (0.2 %) was added to the medium and cells were incubated for an additional 1 h before plating. Solid media contained 2 % agar.

Genetic manipulations

The elimination of ORF STM3576 from the genome of *S. Typhimurium* 14028s was carried out as described (Datsenko and Wanner, 2000). Nucleotide primers (44 mer) overlapping the start and stop codons (bold) matching the ends of the ORF STM3576 were synthesized. These primers were *zntA*1, GGAGGACGTTATGTCGAC CCCTGATACCGATGGCAAAAA ATGTGTAGGCTGGAGCTGCTTCGAA and *zntA*2, GGGAGAGAGATTAGTTT TTACGCAGCAAACGTAGCGCGTTGCA TATGAATATCCTCCTTAGTTCCT. The 20 bases to the 3' ends of each primer (italicized) were annealed to the 5' and 3' ends of a chloramphenicol resistance cassette flanked by the FRT sites of plasmid pKD3 (Datsenko and Wanner, 2000).

S. Typhimurium carrying pKD46 (Datsenko and Wanner, 2000) was grown at 30 °C in LB-ampicillin medium containing 100 mM arabinose to an OD₆₀₀ of 0.4, made electrocompetent and transformed with ~ 400 ng of the PCR product. Deletions of the ORF STM3576 were confirmed by PCR using primers *zntAF*, GCTGTTCA TCGGCAATATCGTCTGG and *zntAR*, CACGCTATTTCTGATTAA TGGTTTG that were complementary to flanking regions of the deleted gene.

Plasmid pBAD/CadA was constructed by insertion of the PCR-amplified *cadA* gene from *G. stearothermophilus* LV into the expression vector pBAD-TOPO (Invitrogen). Briefly, the *cadA* gene was amplified using the vector pET/CadA as template and the primers previously described (Nerey et al., 2002). The PCR

product was ligated to pBAD-TOPO and cloned into *E. coli* Top10 as recommended by the vendor. The identity and orientation of the *cadA* gene inserted into the recombinant plasmid was confirmed by DNA sequencing and also by *NcoI* restriction endonuclease analysis (not shown). The purified plasmid was introduced separately into electrocompetent *S. Typhimurium* 14028s and *S. Typhimurium* 14028s *DzntA*.

Assay for metal sensitivity

Cells to be tested for cadmium, zinc, lead or mercury sensitivity were grown overnight and then diluted 100-fold with fresh LB medium. 100 ml aliquots of these dilutions were spread over LB (Cd, Zn and Hg) or PYT100B agar plates (Pb). Plates were briefly air-dried and then 15 μ l aliquots of 1 M CdCl₂, ZnSO₄, HgCl₂ or PbCl₂ were spotted onto sterile disks of filter paper that had been previously placed in the center of the plates. Plates were incubated for 18 h at 37 °C and the area of growth inhibition zones (cm²) was determined as described (Bauer et al., 1966).

RESULTS AND DISCUSSION

Expression of G. stearothersophilus LV cadA gene results in hypersensitivity to Cd, Pb and Zn in wild type S. Typhimurium

To determine whether the *G. stearothersophilus* LV *cadA* gene is involved in cadmium resistance in *S. Typhimurium* we cloned this gene into the arabinose-inducible pBAD expression vector. The recombinant plasmid was introduced by electroporation into *S. Typhimurium*. CdCl₂ resistance assays were carried out in the presence of arabinose or glucose (repressor).

Figure 1 shows that expression of the *G. stearothersophilus* LV *cadA* gene generates a hypersensitive phenotype to Cd in *Salmonella*. This phenotype is similar to that previously observed in *E. coli* expressing the plasmid pET/CadA (Nerey et al., 2002) and in *Saccharomyces cerevisiae*

expressing the *Listeria monocytogenes cadA* gene (Wu et al., 2004).

In the presence of glucose cells carrying pBAD or pBAD/CadA showed about the same resistance to CdCl₂. These results confirm the direct relationship that exists between *cadA* overexpression and the cadmium hypersensitive phenotype (Nerey et al., 2002).

Because the *G. stearothersophilus* LV CadA protein shares a high sequence identity with the CadA protein from *S. aureus* (Nucifora et al., 1989; Rensing et al., 1998) we investigated whether the *G. stearothersophilus* LV CadA was also responsible for lead, zinc or mercury hypersensitivity in *S. Typhimurium*.

Upon induction, *S. Typhimurium* 14028s carrying pBAD/CadA display an important increase in sensitivity to PbCl₂ and ZnSO₄ while no effect is observed with HgCl₂ (Fig. 1). Conversely, when glucose was present, no sensitivity for any of these metals was observed in cells carrying pBAD/CadA. These results indicate that the observed hypersensitive phenotypes in *Salmonella* are a consequence of the expression of the *G. stearothersophilus* LV *cadA* gene supporting the idea that apart from Cd, CadA is also involved in Pb and Zn transport as it has been shown for other CadA translocases (Lebrun et al., 1994; Nucifora et al., 1989).

To our knowledge, mercury ions are not transported by CadA. The fact that *G. stearothersophilus* LV *cadA* expression had no effect on Hg resistance in *Salmonella* favors the idea that the toxic effect produced by the overexpression of *cadA* in *Salmonella* is due mostly to an increase of metal transport and is not a consequence of a membrane instability effect. This last possibility would have probably involved a generalized hypersensitivity effect to several metals including mercury.

The S. Typhimurium genome encodes a putative zntA gene

To evaluate if the hypersensitive phenotype generated by *cadA* expression is related to an increase in the concentration of general

Figure 1: Expression of *cadA* gene from *G. stearotherophilus* LV generates sensitivity to Cd²⁺, Pb²⁺ and to Zn²⁺ in *Salmonella*. The growth inhibition zones (area, cm²) generated by Cd²⁺, Pb²⁺, Zn²⁺ and Hg²⁺ to *S. Typhimurium* 14028s harboring pBAD and pBAD/CadA are shown. Cells were grown in the corresponding medium plus 0.2 % glucose or arabinose as required. Bars are the standard deviation (n = 4).

Cd transporters we looked for *G. stearothermophilus* LV *cadA* homolog genes in the *S. Typhimurium* genome. Using BlastN and BlastP softwares (<http://www.ncbi.nih.gov/BLAST/>) we found that *S. Typhimurium* ORF STM3576 shares 40 % identity (58 % similitude) with the *cadA* gene of *G. stearothermophilus* LV. ORF STM3576 also exhibits 76 % identity with the *E. coli* *zntA* gene, a well-characterized P-type ATPase involved in Cd, Pb and Zn transport (Hou and Mitra, 2003).

S. aureus CadA and *E. coli* ZntA proteins exhibit three highly conserved functional domains consistently present in metal-translocating P-type ATPases. A heavy metal-associated domain (HMA) is found at the amino terminal end while an E1-E2 ATPase motif is generally found between amino acids 199 and 400. The carboxy terminal end contains the hydrolase motif (Fig. 2).

A more in depth analysis of the deduced amino acid sequences revealed that all of these motifs are highly conserved in *G. stearothermophilus* LV CadA and in *S. Typhimurium* ORF STM3576 (Fig. 2).

A more detailed comparison of the three referred domains present in *E. coli* ZntA and in *S. Typhimurium* ORF STM3576 showed that they are about 90 % identical. This high motif conservation is noteworthy since the overall identity of both proteins is only about 75 %.

ORF STM3576 from *S. Typhimurium* mediates Cd, Pb and Zn resistance

To assess the effect of P-type ATPase concentration in CadA-dependent cadmium hypersensitivity, ORF STM3576 was deleted from the *S. Typhimurium* genome as described in Methods. The mutant strain was analyzed for Cd, Pb and Zn resistance, metals that are known to be transported by ZntA of *E. coli* (Binet and Poole, 2000; Hou and Mitra, 2003) and CadA of *S. aureus* (Nucifora et al., 1989). Because *E. coli* ZntA exhibits ATPase activity in the presence of mercury (Sharma and Mitra, 2000; Mitra and Sharma, 2001) we also investigated the participation of ORF STM3576 in mercury resistance.

As shown in Figure 3, deletion of ORF STM3576 from the *Salmonella* genome generates a hypersensitive phenotype to Cd, Pb and Zn. These results coincide with those found during the analysis of sequence of the conserved domains (Fig. 2) and support the idea that the ORF STM3576 encodes a true P-type ATPase that can be associated to the transport of these metals in *Salmonella*. No effect was observed with mercury chloride (Fig. 3D). These findings are in apparent contradiction with the putative role assigned to the *Salmonella* ZntA concerning the transport of mercury ions (NCBI accession number NP_462477).

Figure 2: Comparison of HMA, ATPase and hydrolase motifs in CadA and ZntA proteins. Solid bars represent CadA from *S. aureus* (A) and *G. stearothermophilus* LV (B), ZntA from *E. coli* (C) and ORF STM3576 from *S. Typhimurium* (D). Amino acid numbers are indicated. Boxes indicate the % of identity of each protein's domain compared to those of *S. aureus* CadA.

Figure 3: Deletion of ORF STM3576 generates sensitivity to Cd²⁺, Pb²⁺ and Zn²⁺ in *S. Typhimurium*. Growth inhibition zones (area, cm²) generated by Cd²⁺, Pb²⁺, Zn²⁺ and Hg²⁺ to *S. Typhimurium* 14028s (wt) and *S. Typhimurium* 14028s Δ *zntA* (Kan::*zntA*). Bars represent standard deviation (n = 4).

Expression of G. stearothermophilus LV cadA gene restores Cd, Pb and Zn resistance in S. Typhimurium 14028s Δ zntA

To analyze the effect of *G. stearothermophilus* LV *cadA* expression in resistance to Cd, Pb, Zn and Hg, *S. Typhimurium* Δ *zntA* cells were transformed with plasmid pBAD/CadA. As seen in Figure 4 upon CadA induction there is a complementation of the hypersensitive phenotype observed for the Kan::*zntA* mutant. The most obvious effect in resistance recovery was seen in Cd-treated cells. The effect on Pb or Zn resistance, although significant, was lower than that

observed for Cd. These results confirm that the *cadA* gene from *G. stearothermophilus* LV codes for a protein that is involved in transport of cadmium, zinc and lead as suggested from sequence comparison studies.

Several P-type ATPases have been described as result of genome sequencing. However, very little limited information is currently available about the characterization of these proteins and their participation in metal resistance. To our knowledge, there is only one report of a thermophilic microorganism P-type ATPase involved in Cd and Zn resistance (Schirawski et al., 2002). Our results also

Figure 4: Expression of *cadA* gene from *G. stearrowthermophilus* LV mediates resistance to Pb²⁺ and to Zn²⁺ in *S. Typhimurium* $\Delta zntA$. Growth inhibition zones (area, cm²) generated by Cd²⁺, Pb²⁺, Zn²⁺ and Hg²⁺ to *Salmonella* Typhimurium 14028s $\Delta zntA$ (Kan::zntA) and *Salmonella* Typhimurium 14028s $\Delta zntA$ pBAD/CadA. Cells were grown in the corresponding medium plus 0.2 % glucose or arabinose. Bars represent standard deviation (n =4).

show that the translocase from *G. stearothermophilus* LV can work with PbCl_2 as well.

These findings suggest that the CadA-mediated Cd sensitivity observed in *Salmonella* is most probably related to the expression of the heavy metal translocase encoded by ORF STM3576. Since the availability of ATP is essential for heavy metal resistance our speculation is that the observed toxicity is likely a consequence of a high local concentration of heavy metal ATP-dependent translocases generated in response to either toxic ions (ZntA) or by CadA overexpression in the *Salmonella* cell membrane.

As occurs in *E. coli*, probably also in *Salmonella* a cadmium-mediated stress would trigger a switch of aerobic to anaerobic metabolism, forcing the cell to exert a fine control on the level of ATP (Wang and Crowley, 2005). In this context, an elevated expression and/or an increased activity of ATPases, like CadA, would explain the appearance of metal hypersensitive phenotypes because of the increase in ATP consumption.

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