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Serena Ammendola, Mauro Cerasi & Andrea Battistoni

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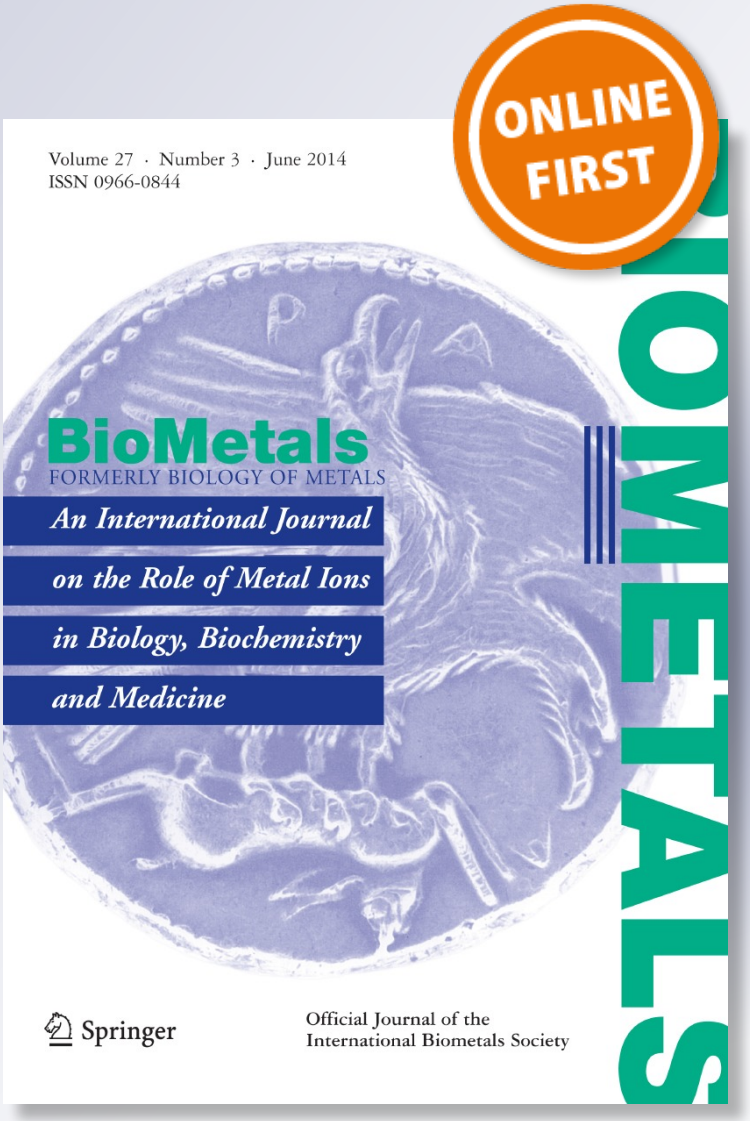

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Deregulation of transition metals homeostasis is a key feature of cadmium toxicity in *Salmonella*

Serena Ammendola · Mauro Cerasi · Andrea Battistoni

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Abstract Cadmium is a highly toxic metal whose presence in the environment represents a challenge for all forms of life. To improve our knowledge on cadmium toxicity, we have explored *Salmonella* Typhimurium responses to this metal. We have found that cadmium induces the concomitant expression of the cation efflux pump ZntA and of the high affinity zinc import system ZnuABC. This observation suggests that cadmium accumulation within the cell induces a condition of apparent zinc starvation, possibly due to the ability of this metal to compete with zinc for the metal binding site of proteins. This hypothesis is supported by the finding that strains lacking ZntA or ZnuABC are hyper-susceptible to cadmium and that the cadmium-induced growth defect of a *znuABC* mutant strain is largely relieved by zinc supplementation. A similar growth defect was observed for a mutant with impaired ability to acquire iron, whereas cadmium does not affect growth of a strain defective in manganese import. Cadmium also influences the expression and activity of the two cytoplasmic superoxide dismutases FeSOD and MnSOD, which are required to control cadmium-mediate oxidative stress. Exposure to cadmium causes a reduction of FeSOD activity in *Salmonella* wild type and the complete abrogation of its expression in the strain defective in iron import. In contrast, although MnSOD intracellular levels increase in response to cadmium, we observed discrepancies between protein levels and enzymatic activity which are suggestive of incorporation of non-catalytic metals in the active site or to cadmium-mediated inhibition of manganese import. Our results indicate that cadmium interferes with the ability of cells to manage transition metals and highlight the close interconnections between the homeostatic mechanisms regulating the intracellular levels of different metals.

Keywords Cadmium toxicity · *Salmonella* · Metal homeostasis · Zinc transport · Superoxide dismutases

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S. Ammendola (✉) · M. Cerasi · A. Battistoni
Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy
e-mail: serena.ammendola@uniroma2.it

A. Battistoni
Consorzio Interuniversitario "Istituto Nazionale Biostrutture e Biosistemi" (I.N.B.B.), Viale delle Medaglie d'Oro, 305, 00136 Rome, Italy

Introduction

Cadmium is a transition metal exhibiting high toxicity toward most living organisms. With the noticeable exception of a carbonic anhydrase from a marine diatom, where cadmium can replace the usual zinc cofactor (Lane et al. 2005), no other biological

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functions are known for this metal. Cadmium concentration in the earth crust is around 0.1 ppm (Wedepohl 1995), but it may accumulate in specific environments as a result of industrial practices, thus representing a significant risk for human health and for the whole ecosystem.

The effects of cadmium exposure on higher organisms have been studied for a long time and recently reviewed elsewhere (Thevenod and Lee 2013; Hartwig 2013; Nair et al. 2013; Andresen and Kupper 2013). Overall, cellular damage by cadmium appears to be tightly related to its ability to interfere with the homeostasis of essential metals, such as zinc, copper, manganese and iron.

Cadmium uptake by cells is mediated by protein channels which are physiologically involved in divalent cations import, exploiting the so-called "molecular and ionic mimicry" (Bridges and Zalups 2005) which take advantage of the similar chemical properties between toxic and essential metals. In fact, evidence has been provided for the ability of cadmium to enter in bacterial cells through metal importers involved in the uptake of magnesium, manganese and zinc (Nies and Silver 1989; Grass et al. 2002). Interestingly, similar mechanisms are known to facilitate cadmium entry also in mammalian cells (Okubo et al. 2003; Fujishiro et al. 2012).

It is generally assumed that a major reason for cadmium toxicity is that, once inside the cell, it can replace native metals from enzymes and other proteins, thus priming a wide range of cellular dysfunctions, including protein unfolding, apoptosis and carcinogenesis (Kitamura and Hiramatsu 2010; Cuypers et al. 2010; Hartwig 2010). Moreover, although cadmium is not a redox active metal, it can trigger ROS formation by displacing iron or copper from storage proteins or by inhibition of the activity of antioxidant enzymes (i.e. SOD and catalase) and GSH depletion (Henkler et al. 2010). However, it should be noted that despite there is a wealth of literature data showing that cadmium can easily substitute other metals in purified proteins (Predki and Sarkar 1994; DalleDonne et al. 1997; Freisinger and Vasak 2013), in vivo evidence for this phenomenon are still scarce.

Cellular defenses against cadmium exposure are essentially associated to the activation of metal efflux systems. These systems belong to the multidrug resistance ABC transporter family (MDR channels) (Broeks et al. 1996; Kim et al. 2007) and to the P-type ATPases family involved in transport of essential metals like zinc or copper (Verret et al. 2004). Metal exporters of these classes are responsible for the remarkable resistance to cadmium exhibited by several heavy-metal tolerant bacteria (Tsai et al. 1992; Legatzki et al. 2003; Schwager et al. 2012).

In enterobacteria, cadmium resistance is mediated by the P-type ATPase ZntA, initially characterized in *Escherichia coli* (Rensing et al. 1997). The *zntA* gene is under the control of the transcriptional activator ZntR, which is a member of the MerR-like proteins (Brocklehurst et al. 1999) that senses in vivo nanomolar variation of intracellular free zinc (Wang et al. 2012) and can also bind cadmium and lead (Binet and Poole 2000). *E. coli* strains lacking ZntA are hypersensitive to both zinc and cadmium (Rensing et al. 1997).

Besides inducing the upregulation of the ZntA detoxification system, several observations indicate that cadmium enhances transcription of genes involved in the response to zinc deficiency (Wang and Crowley 2005; Joe et al. 2011; Lagorce et al. 2012; Maynaud et al. 2013). This response is controlled by Zur, a zinc-binding protein which regulates the transcription of a small number of genes encoding for proteins enabling bacteria to respond to zinc starvation, including the high affinity zinc importer ZnuABC (Patzer and Hantke 1998). For example, studies carried out in *E. coli* have identified the periplasmic ZinT protein as a member of the cadmium stress stimulon (Ferianc et al. 1998), suggesting that it could be involved in cadmium detoxification. However, subsequent studies have shown that ZinT does not enhance bacterial resistance to cadmium, but contributes to ZnuABC-mediated zinc acquisition (Petrarca et al. 2010). These observations not only indicate interplay between cadmium exposure and zinc homeostasis in bacteria, but also suggest that enhanced zinc uptake is required for bacterial resistance to this toxic metal.

To better investigate the cross-talks between toxic and essential metals we have analyzed the effects of cadmium on *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) or in mutant strains with impaired ability to acquire essential metals, such as zinc, iron and manganese. Here we show that *Salmonella* resistance to cadmium requires

Table 1 *Salmonella* Typhimurium strains used in this work

Strain	Modification	Source or reference
ATCC14028	None	Lab collection
SA139	*zntA*-3XFLAG-*kan*	This work
SA186	*znuABC::*scar	Pasquali et al. (2008)
SA211	*sodA*-3XFLAG-*kan*	Pacello et al. (2012)
SA315	*mntH::kan*	This work
SA319	*sitABCD::kan*	This work
SA330	*fepA/entF::kan*	This work
SA323	*feoB::kan*	This work
SA336	*sitABCD::kan mntH::*scar	This work
SA337	*feoB::*scar *fepA/entF::kan*	This work
SA341	*sodA::*3XFLAG-*kan mntH::*scar *sitABCD::*scar	This work
SA395	*zntA::kan*	This work
SA399	*zntA*-3XFLAG-*kan znuA*-3XFLAG-scar *ilvI::Tn10dTac-cat*-3XFLAG-scar	This work
MC116	*sodA::kan*	This work
MC120	*sodB*-3XFLAG-*kan*	This work
MC122	*sodB::*3XFLAG-*kan mntH::*scar *sitABCD::*scar	This work
MC123	*sodB::cam*	This work
MC125	*sodA::kan sodB::cam*	This work
MC153	*sodA*-3XFLAG-scar *feoB::*scar *fepA/entF::kan*	This work
MC154	*sodB*-3XFLAG-*kan feoB::*scar *fepA/entF::*scar	This work

functional high affinity zinc and iron import systems and that impairment of the uptake of such metals modulates the expression and activity of metal-dependent enzymes contributing to the antioxidant defense system.

Materials and methods

Bacterial strains and growth conditions

All the *S*. Typhimurium strains used in this study are derivative of the ATCC14028 strain and are listed in Table 1. Bacteria were routinely grown in Luria–Bertani (LB) medium at 37 °C with aeration and metals were added as indicated. Cadmium acetate (Cd $(CH_3CO_2)_2$) and zinc sulphate ($ZnSO_4$) were prepared from commercial ultra-pure powders purchased from Sigma Aldrich or BDH Laboratory Supplies by solubilizing in ultra-pure water as 0.5 M stock solutions. Vogel-Bonner Medium [anhydrous $MgSO_4$ (0.04 g/l), citric acid (2 g/l), anhydrous K_2HPO_4 (10 g/l), NaH_4PO_4 (3.5 g/l), glucose (2 g/l)], hereafter referred to as Minimal Medium, was prepared under conditions minimizing zinc contaminations, avoiding use of glassware and plastic pipettes and solubilizing all components in ultra-pure water. Antibiotics were used at the following concentrations: kanamycin 50 mg/l, chloramphenicol 30 mg/ml, ampicillin 100 mg/l.

Growth curves

Strains were grown overnight in LB medium at 37 °C and then diluted 1:500 in 10 ml LB medium supplemented with metals when needed. Growth curves were performed in 50 ml Falcon tubes at 37 °C with aeration and the absorbance at 600 nm was recorded every hour for 8 h using a Perkin Elmer Lambda 9 spectrophotometer. Growth curves in Minimal Medium were performed in 96-well microtiter plates (0.1 ml/well) from preinocula grown 5 h in LB and then diluted 1:500 in Minimal Medium supplemented with metals where indicated. Microplates were incubated for 20 h at 37° in a Sunrise microplate reader (Tecan) and optical density at 595 nm was recorded every hour.

Mutants and epitope tagged strains construction

Deletions of chromosomal genes (*zntA*; *sodA*; *sodB*; *fepA/entF*; *feoB*; *mntH*; *sitABCD*) were achieved following the one step inactivation protocol (Datsenko and Wanner 2000), with slight modifications. Oligonucleotides and plasmids used for each mutant are listed in Table S1 and Table S2 (Supplementary Information). PCR fragments amplified on plasmids pKD4 or pKD3 were purified with DNA Clean and Concentration™ columns (Zymo Research) and electroporated in wild type *S*. Typhimurium expressing the Lambda Red recombinase system from plasmid pKD46. Recombinants were selected on kanamycin or chloramphenicol plates and disruption of the target coding sequence was checked by PCR using a forward

primer annealing upstream the start codon of the gene and a reverse primer annealing inside the inserted antibiotic resistance cassette. The mutations were then transduced into clean backgrounds by phage P22 HT 105/1 *int-201* (Maloy et al. 1996) and in some cases the resistance cassette was removed by the FLP recombinase system expressed from plasmid pCP20.

Double deletion mutants (*sodA sodB*; *mntH sitABCD*; *fepA/entF feoB*) were obtained by P22 transduction of one mutated allele into the strain carrying the other mutation.

Chromosomal tagging of *zntA* and *sodB* with the 3XFLAG epitope was obtained as described previously (Uzzau et al. 2001), by electroporating a PCR fragment (amplified from plasmid pSUB11 with primers oli-115/oli-116 for *zntA* and primers oli-231/oli232 for *sodB*) in ATCC14028 pKD46. Epitope tagged colonies were selected on kanamycin plates and the *zntA*-3XFLAG-*kan* allele or *sodB*-3XFLAG-*kan* allele was P22 transduced in a clean wild type background obtaining respectively strain SA139 or MC120.

To generate strains impaired in iron or manganese import and carrying MnSOD or FeSOD tagged enzymes, the *sodA*-3XFLAG or *sodB*-3XFLAG allele was moved into the desired mutant by P22 transduction, obtaining strains SA341, MC122, MC153 and MC154 (Table 1).

The multiple chromosomal tagged strain (ZnuA-3XFLAG ZntA-3XFLAG Cat-3XFLAG) was obtained in a three step protocol: (1) removal of the kanamycin resistance cassette from strain SA140 (*znuA*-3XFLAG-*kan cat*-3XFLAG-*kan*) by electroporating the termosensitive plasmid pCP20 and selecting the kanamycin sensitive (ampicillin resistant) colonies at 30 °C; (2) loss of pCP20 plasmid by switching at non-permissive temperature (ampicillin sensitive colonies selection); (3) P22 transduction into the obtained strain of the *zntA*-3XFLAG-*kan* allele from strain SA139 and selection of kanamycin resistant colonies. The resulting multiple tagged strain was named SA399.

SDS-PAGE and Western blot analysis

To analyze the accumulation of epitope tagged proteins, aliquots of bacterial cultures (approximately 5×10^8 cells) were harvested, lysed in sample buffer containing sodium dodecyl sulphate (SDS) and β-mercaptoethanol, and boiled for 8 min at 100 °C. Proteins were separated on 12 % SDS-page gels, blotted onto a nitrocellulose membrane (Hybond ECL; Amersham) and revealed by mouse anti-FLAG antibody (dilution 1:10000, Sigma Aldrich) as the primary antibody and goat anti-mouse horseradish peroxidase-conjugated antibody (dilution 1:100,000, Bio-Rad), followed by the enhanced chemiluminescence reaction (GE-Healthcare).

Superoxide dismutase activity assay

Activity of cytoplasmic superoxide dismutases (MnSOD and FeSOD) was analyzed in bacterial extract prepared as follows: aliquots corresponding approximately to 2×10^{10} cells from liquid cultures were harvested and resuspended in 1 ml of Lysis Buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) and kept on ice. Suspensions were then lysed with a Branson SLPe sonicator (microtip at 30 % amplitude, 30 s on/off for three times) and centrifugated at $17{,}000 \times g$ for 20 min at 4 °C. Protein contents of supernatants were quantified by the DC Protein Assay (Biorad) and 50 micrograms per sample were run on 10 % polyacrylamide native gels (without SDS and reducing agents). The gels were stained for superoxide dismutase activity as described (Beauchamp and Fridovich 1971).

Results

Cadmium induces the expression of proteins involved in zinc homeostasis

To evaluate the effects of cadmium exposure on *Salmonella* genes involved in zinc homeostasis, we have initially analyzed the accumulation profiles of two major proteins involved in zinc import (ZnuA) and extrusion (ZntA), under different conditions. To this aim we have constructed a multiple chromosomally tagged strain which carries 3XFLAG epitopes fused to 3′-terminus of *znuA* and of *zntA*. This strain (SA399) was grown for 18 h in LB medium supplemented with either the metal chelator EDTA or metals (cadmium, zinc or both). The accumulation pattern of the tagged proteins was visualized by SDS-PAGE and Western blot. As shown in Fig. 1, a reduced availability of free zinc in the culture medium due to the presence of

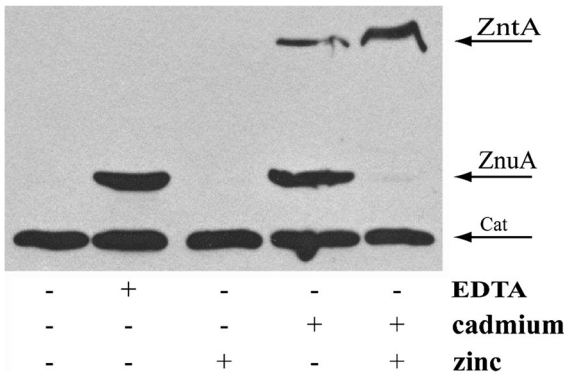

Fig. 1 Accumulation pattern of ZnuA and ZntA in response to metal chelation, cadmium or zinc exposure. Western blot of bacterial lysate (approximately 5×10^8 bacteria/sample) of strain SA399 (*znTA-3Xflag znuA-3Xflag ilvI::Tn10dTac-cat-3Xflag*) grown overnight in LB supplemented with EDTA 0.5 mM, cadmium 1 mM and zinc 1 mM as indicated. Cat protein is used as an internal standard

EDTA induces the *znuABC* operon (Petrarca et al. 2010; Ammendola et al. 2007), but not the divalent cation exporter ZntA. Cadmium supplementation leads to an accumulation of ZnuA comparable to that induced by the metal chelator EDTA and to a simultaneous induction of ZntA. ZntA accumulation is even enhanced when bacteria are exposed at the same time to cadmium and zinc, in line with the expected role of this metal exporter in extrusion of both metals. Moreover, zinc supplementation to the cadmium containing medium triggers *znuA* repression, although it does not completely abolish its expression.

Strains with mutations in zinc uptake or extrusion are hyper-susceptible to cadmium

As ZntA was previously described as a Zn(II)-translocating P-type ATPase that confers zinc and cadmium resistance to *E. coli* (Rensing et al. 1997), we have evaluated the growth of a *Salmonella* strain lacking *zntA* (SA395) in presence of variable amounts of these metal ions.

Sensitivity of this strain toward metals was initially analyzed on LB agar plates supplemented with zinc 1 mM or cadmium 0.5 mM, in comparison with the wild type strain. We have found that the *zntA* mutant, but not the wild type strain, fails to grow in presence of the metals, likely due to metal accumulation in the cytoplasm (data not shown).

To better analyze the different sensitivity of these strains toward cadmium and zinc, wild type and *zntA*

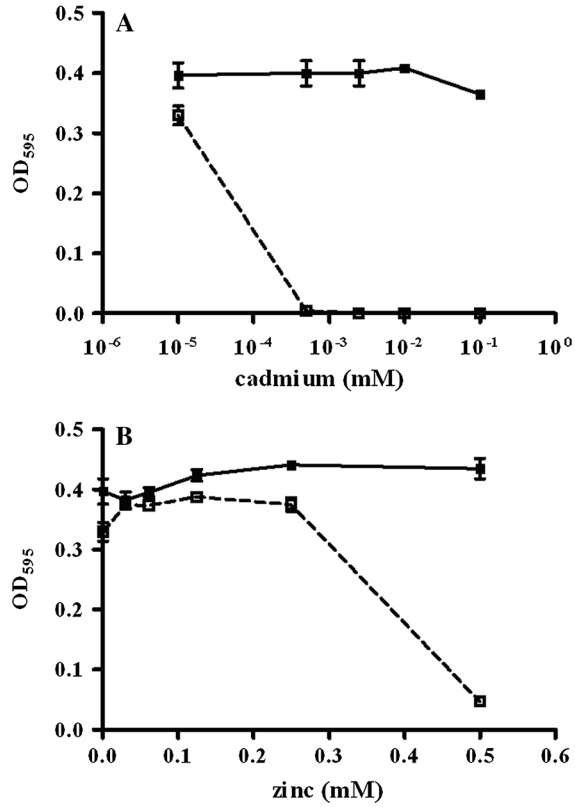

Fig. 2 Cadmium and zinc sensitivity of the *zntA* mutant. *S.* Typhimurium wild type (*continuous line*) and SA395 (*zntA*, *dashed line*) and were incubated at 37 °C in Minimal Medium supplemented with increasing concentration of cadmium (panel **A**) or zinc (panel **B**) as indicated. Optical densities at 595 nm (OD_{595}) were recorded after 16 h

strains were grown in LB medium supplemented with increasing concentration of cadmium (from 0.5×10^{-3} to 0.1 mM) or zinc (from 0.05 to 0.5 mM) and the bacterial density was recorded after 18 h incubation at 37 °C with aeration. The *zntA* strain is hyper susceptible to micromolar cadmium concentrations and it completely fails to grow in media containing as low as 5×10^{-3} mM cadmium, while the wild type strain is only moderately affected by a 100 fold higher metal concentration (Fig. 2, panel A). The *zntA* strain is also more sensitive to zinc than the wild type strain (Fig. 2, panel B).

As cadmium induces accumulation of the periplasmic component of the ZnuABC high affinity zinc importer (ZnuA), we have hypothesized that inactivation of this transporter could alter *Salmonella* sensitivity towards cadmium. We have therefore examined the growth of a *znuABC* strain

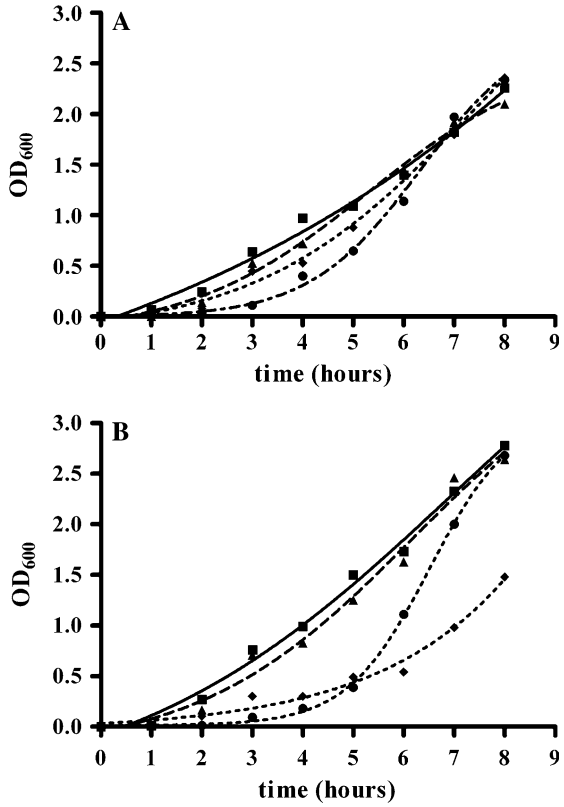

Fig. 3 Growth curves of *S.* Typhimurium wild type and *znuABC* in rich medium supplemented with metals. Wild type (panel **A**) and SA186 (*znuABC*) (panel **B**) were incubated at 37 °C with aeration in LB (*squares*) or LB supplemented with zinc 0.5 mM (*triangles*), cadmium 0.5 mM (*diamonds*) or both (*circles*). Optical densities at 600 nm (OD_{600}) were recorded every hour for 8 h

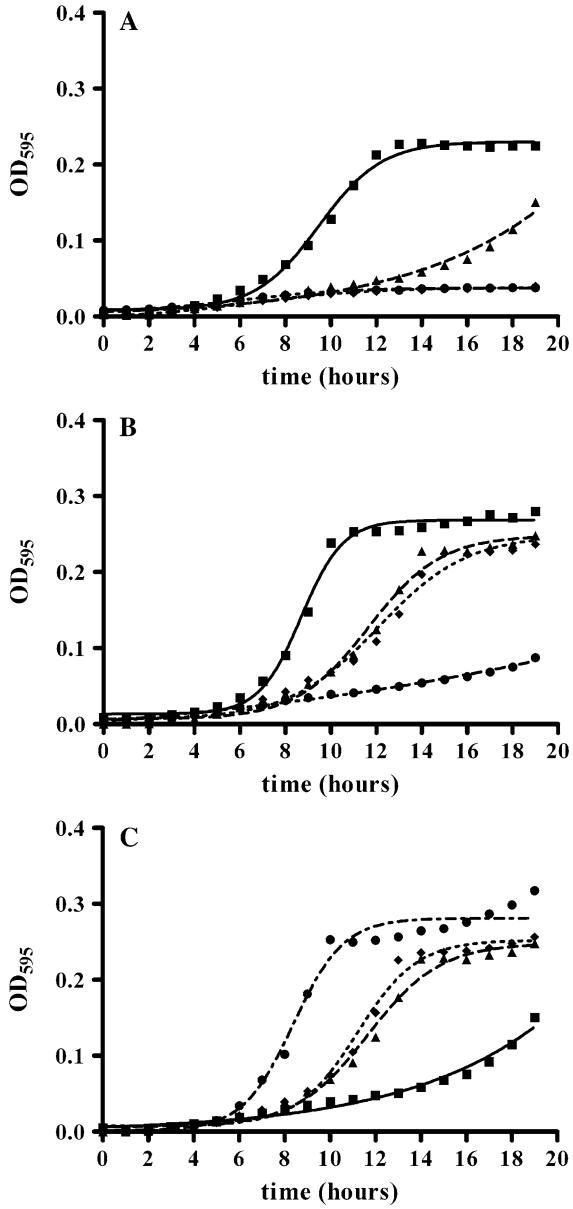

Fig. 4 Cadmium tolerance of *znuABC* mutant. Panel **A**. Strain SA186 (*znuABC*) was grown in Minimal Medium (*squares*) supplemented with cadmium 0.005 mM (*triangles*), 0.010 mM (*diamonds*) and 0.100 mM (*circles*). Panel **B**. Strain SA186 (*znuABC*) Minimal Medium containing zinc 0.005 mM (*squares*) and supplemented with cadmium 0.005 mM (*triangles*), 0.010 mM (*diamonds*) and 0.100 mM (*circles*). Panel **C**. Strain SA186 (*znuABC*) was grown in Minimal Medium containing cadmium 0.005 mM (*squares*) and supplemented with zinc 0.005 mM (*triangles*), 0.010 mM (*diamonds*) and 0.100 mM (*circles*). Optical densities at 595 nm (OD_{595}) were recorded every hour

(SA186) in LB medium, supplemented or not with zinc, cadmium or both the metals (Fig. 3). When compared to the wild type, whose growth is only slightly modified in presence of metals (panel A), the *znuABC* strain displays a significant reduction in growth rate when the medium is supplemented with cadmium (panel B). This cadmium-dependent growth defect is largely rescued by the addition of an equimolar concentration of zinc, although under this condition the mutant exhibit a remarkable elongation of the lag phase.

Cadmium tolerance of wild type, *zntA* and *znuABC* strains was further analyzed growing bacteria in a chemical defined medium (Minimal Medium) supplemented with metals (Fig. 4). The growth rate of

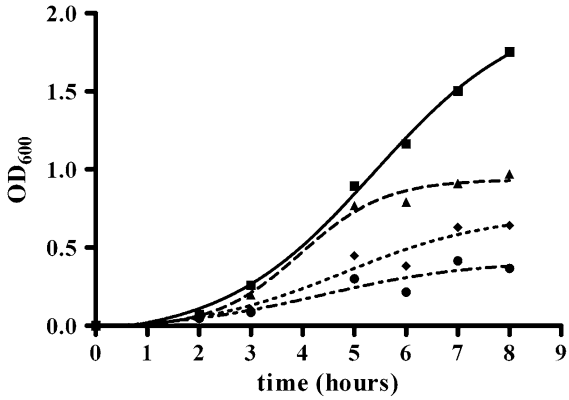

Fig. 5 Growth curves of *S* Typhimurium strains with impaired iron uptake. Strain SA337 (*fepA/entFfeoB*) was incubated at 37 °C with aeration in LB (*squares*) or LB supplemented with zinc 0.5 mM (*triangles*), cadmium 0.5 mM (*diamonds*) or both (*circles*). Optical densities at 600 nm (OD_{600}) were recorded every hour

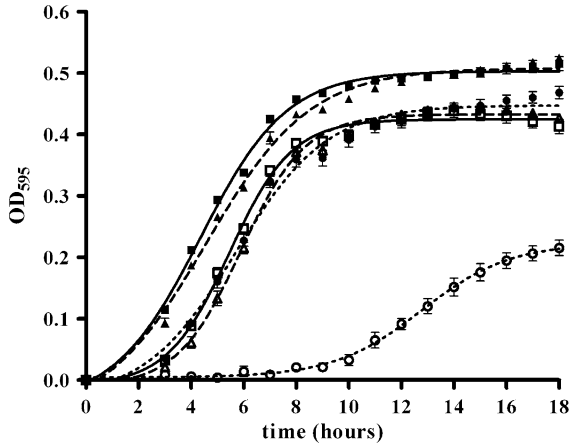

Fig. 6 Cadmium tolerance of *S.* Typhimurium strains lacking cytoplasmic superoxide dismutases. Strains MC116 (*sodA*, *squares*), MC123 (*sodB*, *triangles*) and MC125 (*sodAsodB*, *circles*) were grown in LB alone (*filled symbols*) or supplemented with cadmium 0.5 mM (*empty symbols*). Optical densities at 595 nm (OD_{595}) were registered every hour

Salmonella wild type is unaffected under the conditions tested, whereas cadmium (from 0.005 to 0.100 mM) causes the complete growth inhibition of the *zntA* strain (data not shown). The growth rate of *znuABC* strain is significantly decreased in presence of cadmium 0.005 mM and is almost inhibited at cadmium concentrations higher than 0.010 mM (panel A). In contrast, in a Zn-replete Minimal Medium the *znuABC* strain shows increased tolerance toward cadmium (panel B). Interestingly, a high zinc concentration (0.100 mM) can completely complement the inhibitory effect of 0.005 mM cadmium on *Salmonella* growth (panel C), which becomes comparable to that of the wild type (not shown).

Mutations in the iron import apparatus increases sensitivity to cadmium

Cadmium toxicity has been correlated to interferences with the homeostasis of transition metal ions other than zinc, i.e. iron or manganese (Moulis and Thevenod 2010; Moulis 2010). We have thus evaluated the effect of cadmium exposure and zinc supplementation on the growth of *Salmonella* strains with impaired ability to efficiently acquire either iron (SA337) or manganese (SA336). The former strain carries deletions in the *fepA/entF* region, coding for genes involved in biosynthesis, modification and uptake of enterobactin and in the *feoB* gene, which encodes a ferrous iron transporter. The latter strain is inactivated in the two major manganese import systems, the *sitABCD* operon encoding a Mn/Fe ABC-type transporter and *mntH*, codifying for a manganese transport protein belonging to NRAMP family.

Bacteria were grown in LB supplemented or not with zinc, cadmium or both metals and growth was monitored over time. We have found that the growth of the strain lacking SitABCD and MntH is not affected by the presence of metals (not shown). In contrast, the mutant strain defective in iron uptake (*fepA/entF feoB*) displays increased sensitivity towards both cadmium and zinc (Fig. 5). Moreover, unlike the case of the *znuABC* mutant, we have found that zinc addition to the cadmium containing medium worsen, rather than improve, growth of the *fepA/entF feoB* strain.

Cadmium effects on manganese- and iron-cofactored superoxide dismutases

It is known that cadmium exposure induces oxidative stress (Henkler et al. 2010) and that the activity of the two cytoplasmic superoxide dismutases (the manganese containing MnSOD, encoded by *sodA* and the iron containing FeSOD, encoded by *sodB*) contribute to the ability of *E. coli* to withstand cadmium toxicity (Geslin et al. 2001). We have thus analyzed the growth of *Salmonella* strains lacking one (*sodA*, MC116 or *sodB*, MC123) or both SOD enzymes (*sodA sodB*,

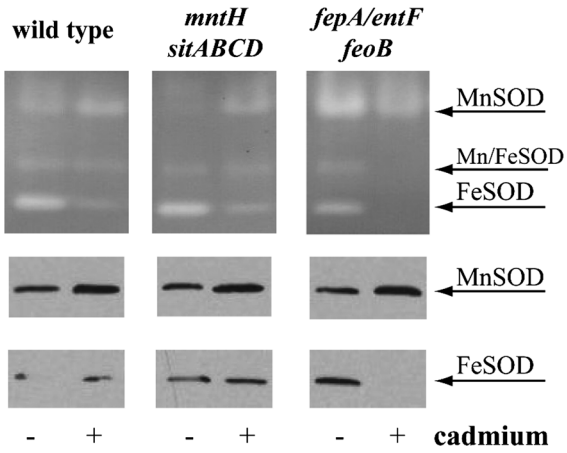

Fig. 7 Cadmium dependent modulation of SOD enzymes. *Upper panels S. typhimurium* wild type, SA336 (*mntH sitABCD*) and SA337 (*fepA/entF feoB*) were grown in LB supplemented or not with cadmium 0.5 mM and bacterial lysates were loaded on native gel and than stained for SOD activity. *Middle* and *lower panels S. typhimurium* SA211 (*sodA*-3XFLAG), SA341 (*sodA*-3XFLAG *mntH sitABCD*), MC153 (*sodA*-3XFLAG *fepA/entF feoB*), MC120 (*sodB*-3XFLAG), MC122 (*sodB*-3XFLAG *mntH sitABCD*) and MC154 (*sodB*-3XFLAG *fepA/entF feoB*) were grown in LB supplemented or not with cadmium 0.5 mM and epitope tagged MnSOD (*middle panels*) and FeSOD (*lower panels*) were revealed as described in "Materials and methods" section

MC125) in LB supplemented or not with cadmium 0.5 mM. As shown in Fig. 6, cadmium exposure slightly modifies the growth curve of *sodA* and *sodB* deleted strains, but significantly affects growth of a mutant *Salmonella* strain lacking *sodA sodB*.

In the light of the results obtained, we have analyzed the effects of this metal on the two cytoplasmic superoxide dismutases (MnSOD and FeSOD). We were particularly interested in these enzymes because their activity depends on the incorporation of the correct metal cofactor and their expression is modulated by the iron responsive transcription factor Fur (Niederhoffer et al. 1990).

Thus, bacterial lysates of wild type and mutant strains with impaired ability to efficiently import manganese (*mntH sitABCD*) or iron (*fepA/entF feoB*) were separated on native polyacrylamide gels and stained for superoxide dismutase activity. Figure 7 (upper panel) shows that exposure to cadmium enhances MnSOD activity in the wild type and *mntH sitABCD* strains but not in the *fepA/entF feoB* strain, where cadmium slightly decreases the activity of MnSOD. Moreover, cadmium reduces FeSOD activity in the wild type and *mntH sitABCD* strains and causes its complete inhibition in the *fepA/entF feoB* strain. As shown in middle and lower panels of Fig. 7 cadmium induces an increase in MnSOD intracellular content in all the strains and the complete inhibition of FeSOD accumulation in the *fepA/entF feoB* strain. It should be noted that despite the MnSOD protein levels are comparable in all the strains, in the cadmium-devoid LB medium the *fepA/entF feoB* strain shows higher MnSOD activity than the other strain (compare upper and middle panels).

Discussion

We have previously reported that in *S*. Typhimurium cadmium induces expression of several Zur-regulated proteins, such as the periplasmic and transmembrane components of the high affinity zinc uptake system (respectively ZnuA and ZnuB) and the auxiliary periplasmic zinc chaperone ZinT (Petrarca et al. 2010). Moreover, we have shown that the disruption of the *znuABC* operon, but not of *zinT*, causes a growth defect on a medium supplemented with sublethal doses of cadmium (Petrarca et al. 2010). To investigate the functional meaning of the induction of Zur-regulated genes in response to cadmium, we have started the present study by analyzing the accumulation pattern of two major inducible proteins involved in zinc influx and efflux, ZnuA and ZntA, in response to cadmium and/or zinc exposure. ZnuA is usually induced by bacteria to ensure zinc recruitment under conditions of metal starvation, whereas the ZntA exporter is expressed in condition of metal overload and confers resistance to both zinc and cadmium (Rosen 2002; Rensing et al. 1997). In line with this general scheme, in wild type *Salmonella* growing in LB (which is a zinc replete medium) neither ZnuA nor ZntA are expressed, while, in presence of a metal chelator, bacteria ensure an efficient zinc uptake by expressing the high affinity importer (Fig. 1). Upon zinc supplementation (1 mM) ZnuA accumulation is abolished (Ammendola et al. 2007; Petrarca et al. 2010), but ZntA is yet undetectable, possibly because the concentration of intracellular free zinc is below the activation threshold of ZntR, the transcriptional regulator of *zntA* which responds to nanomolar concentration of intracellular free zinc in vivo (Wang et al. 2012), or because it accumulates only to levels which

are below the threshold of immunodetection. In contrast, when bacteria are exposed to cadmium, it is possible to observe the simultaneous accumulation of ZnuA and ZntA, a paradoxical condition that is never observed in the absence of this toxic metal, because the two systems are normally activated at different zinc concentrations to guarantee the maintaining of adequate intracellular zinc levels (Wang et al. 2011, 2012). The exposure to both cadmium and zinc causes a significant down-regulation of the high affinity zinc importer and a strong induction of the detoxification pump, indicating the primary role for ZntA in extrusion of both metals.

The accumulation of proteins involved in zinc recruitment during cadmium exposure in bacteria growing in a medium (LB) where the Zur-dependent genes are usually repressed, suggests that this toxic metal induces a condition of zinc starvation. This condition could be induced either by cadmium inhibition of low affinity zinc importers or by the binding of cadmium to the metal binding site of intracellular zinc-containing proteins, with a consequent alteration of their function. To confirm this hypothesis we have therefore investigated if the deletion of ZnuABC or ZntA could affect *Salmonella* response to cadmium and/or to zinc. The results obtained both in semi-solid and in liquid media (Fig. 2A) have shown that while the wild type strain is able to tolerate up to 10^{-2} mM cadmium without evident changes in its growth, a *zntA* strain completely fails to grow at concentrations below 1 μM cadmium, indicating that this pump is necessary to reduce intracellular cadmium levels. In line with previous studies carried out in *E. coli* and other enterobacteria (Chien et al. 2013; Wang et al. 2012; Rensing et al. 1997), this strain is much more sensitive than the wild type also toward zinc (Fig. 2B). Moreover, we have observed that the growth of a *znuABC* strain is significantly impaired by cadmium with respect to the wild type strain and that this growth defect can be largely restored by increasing the zinc concentration in the culture medium (Fig. 3). These findings indicate that induction of ZnuABC is required to increase zinc uptake in response to cadmium intoxication. The protective effect of zinc against cadmium toxicity has long been documented in eukaryotic systems, where it was observed that zinc supplementation can lower the deleterious effects of cadmium exposure (Claverie et al. 2000; Brzoska et al. 2007; Galazyn-Sidorczuk et al. 2012), and that a low dietary zinc intake can be associated with a higher risk of cadmium-induced cancer in humans (Lin et al. 2013). Our study on *Salmonella* and other studies on prokaryotes (Maynaud et al. 2013; Zeng et al. 2012) support the idea that a proper zinc intake is necessary to face cadmium-induced stress, because cadmium triggers signals of zinc shortage. Even if the growth curves of the *znuABC* strain in Zn- or Cd-containing minimal medium supplemented with increasing concentration of cadmium or zinc, respectively, could be explained by a reduction of zinc uptake due to competition between the two metals for low affinity transporters (Fig. 4, panels B and C), the experiments with the *znuABC* mutant strain indicate that cadmium does not cause irreversible inactivation of these zinc importers, since zinc administration restores bacterial growth. Therefore, the more plausible explanation for the protective effect of zinc is that high intracellular levels of this metal prevent the incorporation of cadmium in Zn-containing proteins. For example, we hypothesize that cadmium could induce the *znuABC* operon by replacing zinc in metal-binding sites of Zur, thereby affecting its transcriptional activity. In fact, it has already shown that metal replacement in DNA-binding proteins can alter their substrate specificity (Predki and Sarkar 1994) and that the substitution of zinc with cobalt in Zur is associated to a change in the metal coordination environment (Outten et al. 2001).

Since cadmium can interfere also with the homeostasis of other divalent metals (Lagorce et al. 2012; Moulis 2010), we have investigated if an impairment in the ability to import iron or manganese could alter *Salmonella* sensitivity towards cadmium. To this aim we have analyzed the growth curve profiles of mutant strains with significantly altered capability to import iron or manganese (*fepA/entF feoB* or *sitABCD mntH*) in presence or absence of cadmium. The inactivation of the two major manganese uptake systems does not induce growth defects in bacteria growing in presence of cadmium (not shown), possibly due to the use of manganese as a metal cofactor in a rather limited number of bacterial proteins (Papp-Wallace and Maguire 2006).

In contrast, the strain impaired in iron uptake is hypersensitive to cadmium and, to a lesser extent, also to zinc (Fig. 5). Moreover, unlike the case of the *znuABC* strain, the cadmium-induced growth defect of the *fepA/entF feoB* strain can not be reversed by zinc supplementation, that, rather, worsens its growth. Since iron is used as cofactor by several enzymes

involved in the oxidative stress response, it is likely that the high susceptibility of the *fepA/entF feoB* to cadmium may involve different overlapping mechanisms. However, the observation that a reduced iron influx is associated to high susceptibility to either cadmium or zinc strongly suggest that the toxicity of these divalent metals involves their erroneous incorporation in iron-containing enzymes or iron-regulated proteins.

To deepen our investigation on cadmium interference with metal homeostasis and to explore whether cadmium exposure could trigger oxidative stress, we have monitored the growth of *Salmonella* mutant strains lacking one or both the cytoplasmic superoxide dismutases, in presence of sublethal doses of cadmium (Fig. 6). We have found that cadmium has a marked effect on the *sodA sodB* double mutant which takes several hours to enter into the exponential phase of growth and is not able to reach the same cell density of the wild type strain or of the *sodA* or *sodB* single mutants (Fig. 6 and data not shown). These results confirm that cadmium exposure induces oxidative stress in *Salmonella* and that the enzymes able to detoxify the superoxide anion contribute to cadmium resistance.

To identify possible links between cadmium toxicity, superoxide dismutase activity and availability of metals we have analyzed MnSOD and FeSOD activity and accumulation as a function of cadmium exposure in the wild type and in the *fepA/entF feoB* and *sitABCD mntH* mutant strains. In fact, these metal-cofactored enzymes may be considered interesting markers of intracellular iron or manganese availability during cadmium exposure. It should be also reminded that MnSOD is transcriptionally repressed by Fur (which senses intracellular iron content) while FeSOD synthesis is positively regulated by Fur via small RNAs and Hfq (Niederhoffer et al. 1990; Hassan and Schrum 1994; Troxell et al. 2011).

We have found (Fig. 7, upper panels) that in the wild type and in the *sitABCD mntH* strains cadmium enhances the activity of MnSOD and simultaneously decreases that of FeSOD. This increase in activity is paralleled by higher MnSOD accumulation. In contrast cadmium decreases the activity of both the FeSOD and MnSOD in the strain defective in iron uptake. It should be noted that for equal levels of MnSOD protein, different levels of activity are observed in the different strains grown in standard LB. The *sitABCD mntH* shows lower level of MnSOD activity with respect to the wild type strain, likely due to reduce intake of manganese. On the contrary, MnSOD activity is very high in the *fepA/entF feoB strain.* Previous studies have established that in bacteria growing in rich media a quota of MnSOD is inactive due to incorporation of iron in the active site (Beyer and Fridovich 1991; Privalle and Fridovich 1992; Whittaker 2003). The higher MnSOD activity observed in the *fepA/entF feoB* strain may be therefore explained by an increased ability to incorporate the metal conferring activity, and/or by the activation of manganese import under oxidative stress (Anjem et al. 2009). In this strain cadmium produces a clear reduction of MnSOD activity in spite of a strong elevation of its intracellular level, suggesting that cadmium may interfere with manganese acquisition (Counago et al. 2014; McDevitt et al. 2011) or that it may directly bind to the enzyme. This last possibility is supported by different studies which have demonstrated that cytoplasmic SODs may accommodate different metals in the active site when reconstituted in vitro (Ose and Fridovich 1979; Yamakura and Suzuki 1980). Moreover, we observed that in this mutant there is a total shut down of FeSOD activity and accumulation in presence of cadmium, which is indicative of a drastic reduction of iron binding to Fur (Taylor et al. 2009).

On the whole, this study provides new clues to understand the mechanisms of cadmium poisoning in bacteria, highlighting its ability to interfere with the homeostasis of transition metals. Moreover it underlines the critical importance of carefully controlling the intracellular levels of zinc and iron to resist to cadmium toxic effects and the interconnections between the mechanisms regulating the intracellular concentration of different metals.

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