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ORIGINAL PAPER

Role of respiration and glutathione in cadmium-induced oxidative stress in *Escherichia coli* K-12

Catarina C. Pacheco · João F. Passos · A. Rita Castro · Pedro Moradas-Ferreira · Paolo De Marco

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Abstract Cadmium is a widespread pollutant that has been associated with oxidative stress, but the mechanism behind this effect in prokaryotes is still unclear. In this work, we exposed two glutathione deficient mutants (Δ*gshA* and Δ*gshB*) and one respiration deficient mutant (Δ*ubiE*) to a sublethal concentration of cadmium. The glutathione mutants show a similar increase in reactive oxygen species as the wild type. Experiments performed using the Δ*ubiE* strain showed that this mutant is more resistant to cadmium ions and that Cd-induced reactive oxygen species levels were not altered. In the light of these facts, we conclude that the interference of cadmium with the respiratory chain is the cause of the oxidative stress induced by this metal and that, contrary to previously proposed models, the reactive oxygen species increase is not due to glutathione depletion, although this peptide is crucial for cadmium detoxification.

Keywords *Escherichia coli* · Cadmium toxicity · Oxidative stress · Glutathione · Respiratory chain

Abbreviations

BHT	2,6-Di-*tert*-Butyl-*p*-cresol
DHR	Dihydrorhodamine 123
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5′-Dithiobis (2-nitrobenzoic acid)
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MDA	Malondialdehyde
MOPS	3-(N-morpholino)propanesulfonic acid
ROS	Reactive oxygen species
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

Cadmium is a relatively abundant heavy metal that in the last decades has been utilized in large scale and has been listed as a priority pollutant by the US Environmental Protection Agency (Waisberg et al. 2003). Among its biological effects are inhibition of DNA repair, interference with the cellular antioxidant system, inhibition of DNA methylation, disruption of cell adhesion and induction of apoptosis (Stohs and Bagchi 1995; Waisberg et al. 2003).

Cadmium induces specific alterations in mitochondria, which are the main source of reactive oxygen species (ROS) in eukaryotic cells. In guinea pig liver cells, it interferes with the electron transport chain leading to the

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C. C. Pacheco · J. F. Passos · A. R. Castro · P. Moradas-Ferreira · P. De Marco (✉)
Grupo de Microbiologia Celular e Aplicada (MCA),
IBMC, R. Campo Alegre, 823, 4150-180 Porto, Portugal
e-mail: pmarco@ibmc.up.pt

C. C. Pacheco · P. Moradas-Ferreira
Departamento de Biologia Molecular,
ICBAS, Universidade do Porto, Lgo. Abel Salazar,
2, 4099-003 Porto, Portugal

Present Address:
J. F. Passos
Henry Wellcome Laboratory for Biogerontology Research,
Institute for Ageing and Health, University of Newcastle,
Newcastle upon Tyne, UK

Present Address:
A. R. Castro
Centro de Biologia Celular, Universidade de Aveiro,
3810-196 Aveiro, Portugal

accumulation of semiubiquinones. These molecules are unstable and prone to donating electrons directly to molecular oxygen, generating superoxide radicals (Wang et al. 2004).

Cadmium-induced ROS lead to the oxidation of lipids, which results in the permeabilization of the plasma membrane (Gadd 1993; Howlett and Avery 1997). Increased lipid peroxidation in the presence of Cd^{2+} was indeed detected in *Saccharomyces cerevisiae* (Vido et al. 2001) and in several rat tissues (Manca et al. 1991; Stohs and Bagchi 1995).

It is known that Cd^{2+} interacts with thiol groups of proteins resulting in structural modification and/or in their inactivation (Chrestensen et al. 2000; Thévenod and Friedmann 1999).

Complex formation between Cd^{2+} and glutathione or phytochelatins and sequestration by metallothioneins are the general mechanisms by which eukaryotes detoxify this metal (Adamis et al. 2004; Hatcher et al. 1995; Li et al. 1997; Perego and Howell 1997). The formation of the complex with GSH leads to a depletion of the cytoplasmatic concentration of this molecule. In *S. cerevisiae*, the cadmium–glutathione complex is transported to the vacuole and thus removed from the cytoplasm (Li et al. 1996, 1997).

Since cadmium is not a redox-active metal, it was hypothesized that it could induce oxidative stress indirectly by displacement of redox-active metals, by the depletion of endogenous radical scavengers (e.g., GSH) or by affecting the activity of antioxidant enzymes. Glutathione depletion is pointed out to be the cause of generation of ROS and oxidative stress (Almazan et al. 2000; Avery 2001; Ercal et al. 2001; Liu et al. 2005; Rikans and Yamano 2000; Stohs et al. 2001; Wolf and Baynes 2007).

Most of the works on the effects of heavy metals in prokaryotes published to date have focused on long-term resistance mechanisms and still relatively few on stress response (Ackerley et al. 2006; Banjerdkij et al. 2003, 2005; Ferianc et al. 1998; Hu et al. 2005; Kershaw et al. 2005; Lee et al. 2005; Puškárová et al. 2002; Wang and Crowley 2005).

Previous works, on *E. coli*, reported that exposure to low Cd^{2+} concentrations (3 μM) induced a temporary growth stasis. During this phase, Cd-induced damage is repaired and cell physiology is adjusted to limit the distribution of the ion within the cell (Mitra et al. 1975; Mitra and Bernstein 1977). It was also observed that, during stasis, there was loss of cell viability, although synthesis of some specific proteins was increased (Ferianc et al. 1998; Khazaeli and Mitra 1981; Shapiro and Keasling 1996) and the repair of DNA damage was concomitant with recovery of viability (Mitra 1984; Mitra and Bernstein 1978).

Proteomic analysis in *E. coli* (Ferianc et al. 1998; VanBogelen et al. 1987) confirmed the activation of the SOS, heat shock and oxidative stress regulons, although they were only a minor part of the response to cadmium.

Copper is a metal with important biological functions, but, being a redox-active metal, when present in excess it can react with H_2O_2 generating hydroxyl radicals and cellular damage (Rensing and Grass 2003). Less is known on the mechanisms of toxicity of Ag^{+}, Hg^{2+} and Zn^{2+}, but data point to their interaction with the respiratory chain (Bragg and Hou 1968; Bragg and Rainnie 1974; Kasahara and Anraku 1972; Kim and Bragg 1971; Poole et al. 1989).

This study was designed to understand the mechanisms involved in cadmium toxicity in *E. coli* and assess the roles of respiration, GSH and oxidative stress in the process.

Materials and methods

Strains and culture conditions

Escherichia coli K-12 BW25113, the deletion mutants derived from this strain Δ*gshA*, Δ*gshB*, Δ*ubiE* (obtained from the Keio Collection, Keio University, National BioResource Project, NIG, Japan; Baba et al. 2006) and *Escherichia coli* K-12 MG1655 (obtained from the *E. coli* Genetic Stock Center, University of Yale, USA) were used throughout this study.

The minimal medium was constituted of: NaCl 0.5 g l^{-1}, NH_4Cl 1.0 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 49.2 mg l^{-1}, K_2SO_4 48 mg l^{-1}, $K_2HPO_4{\cdot}3{\cdot}H_2O$ 46 mg l^{-1} (to give a final concentration of 200 μM of phosphate), micronutrient solution (Tuovinen and Kelly 1973) 2 ml l^{-1}, 3-(N-Morpholino)propanesulfonic acid (MOPS) 40 mM pH 7.4 and glucose 20 mM. The concentration of phosphate in the medium was minimized to avoid cadmium precipitation as Cd phosphate.

Cultures in aerobic conditions were inoculated from overnight pre-cultures and grown in an orbital shaker (130 rpm) at 37°C to $OD_{420} = 0.5$ (UVmini-1240, Shimadzu). For cultures grown in fermentative conditions, Erlenmeyer flasks with a magnetic bar were filled almost to the top with sterile medium that was stripped of oxygen by bubbling with a nitrogen stream. To avoid contact with air, sterile SubaSeal® rubber stoppers were used.

Kanamycin (25 mg l^{-1}) was added to the mutants pre-cultures.

Effect of Cd on cell growth and culturability

To assess the effect of Cd on cell growth, OD_{420} was monitored in control cells (untreated) and cultures treated with 30 μg ml^{-1} Cd^{2+} (273 μM $CdCl_2$), a concentration used in previous works on cadmium stress in *E. coli* K-12 (Ferianc et al. 1998). To test the effect of glutathione on Cd-induced

growth arrest, exogenous GSH and GSSG were added to a final concentration of 4 mM, 5 min prior to Cd exposure.

Culturability assays were performed by drawing aliquots at different time points (0, 30 and 60 min after cadmium was added), serial diluting sterile saline solution and plating onto LB agar. Culturability was expressed as the percentage of colony-forming units (CFU).

Assessment of oxidative damage

Protein oxidation was assessed by immunodetection of protein carbonyls. Control and Cd-treated cells were collected by centrifugation for 10 min at 20,000×*g* at 4°C, then washed twice in cold potassium phosphate buffer 50 mM pH 7.0 and used immediately or stored at −80°C for later usage.

The pellets were resuspended in potassium phosphate buffer (at) 50 mM, pH 7.0, containing protease inhibitors (Complete™ Mini EDTA-free Protease cocktail inhibitor, Roche). The cells were then disrupted by sonication on ice with a Branson Sonifier 250 using two cycles of 15 s (50% duty cycle, output 3) intercalated with one cycle of 1 min off duty and centrifuged for 10 min at 16,000×*g* at 4°C, keeping the supernatants. Protein concentration was measured by the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) as described by Levine et al. (1990), separated by SDS-PAGE and stained with Coomassie blue or blotted onto a Hybond-ECL membrane (GE Healthcare, UK). Immunodetection was performed using a rabbit anti-dinitrophenyl (DNP) IgG (Dako Cytomation, Glostrup, Denmark) at 1:5,000 dilution as primary antibody and goat anti-rabbit IgG peroxidase conjugate (Sigma, St Louis, USA), at 1:5,000 dilution as secondary antibody. Detection was performed by chemiluminescence using the kit ECL Western blotting (GE Healthcare, UK). Analysis of SDS-PAGE gels and carbonyl immunodetection films was performed using the Quantity One® programme version 4.5 (Bio-Rad).

The determination of lipid peroxidation was performed according to Steels et al. (1994) with adaptations: *E. coli* cell pellets obtained from 50 ml cultures were resuspended in 250 µl potassium phosphate buffer 50 mM pH 7.0 and sonicated as described above. To each sample, 28 µl of trichloroacetic acid (TCA) was added and the mixture was vortexed at a maximum speed for 2 min. Extracts were then centrifuged for 15 min at 2,000×*g* at 4°C and to 100 µl supernatant, 100 µl EDTA 0.1 M plus 600 µl of a solution of 2-thiobarbituric acid (TBA) 1% (w/v), NaOH 50 mM and 2,6-di-*tert*-Butyl-*p*-cresol (BHT) 0.025% (w/v) were added. Samples were kept in boiling water for 15 min and, after cooling, the A_{532} was measured.

Catalase activity and glutathione measurement

For the determination of catalase activity, *E. coli* extracts were prepared as described in "Assessment of oxidative damage". Samples were dialyzed overnight against cold potassium phosphate buffer at 50 mM, pH 7.8, EDTA 0.1 mM. Protein concentration was determined as described in "Assessment of oxidative damage". Catalase activity was measured as described in Beers and Sizer (1952) following the decrease of A_{240} due to H_2O_2 disappearance. One unit of catalase is defined as the quantity of enzyme needed to degrade 1 µmol of H_2O_2 per minute at 25°C.

For glutathione determination, cells were collected by centrifugation and washed as described in "Assessment of oxidative damage". The cell pellets were resuspended in 200 µl phosphate buffer at 100 mM, EDTA at 2 mM, pH 7.4, and 200 µl HClO4 at 2 M was added. Then they were sonicated as described in "Assessment of oxidative damage" and centrifuged for 10 min at 16,000×*g* at 4°C. The extracts were neutralized to pH 6–7 using 200 µl KOH at 2 M and MOPS at 0.3 M and spun at 16,000×*g* at 4°C for 2 min. Total intracellular glutathione was determined by the DTNB-GSSG reductase recycling method as described by Akerboom and Sies (1981). The levels of extracellular glutathione were determined in culture supernatants. For this purpose, 20 ml aliquots of the supernatants were collected, frozen to −80°C and lyophilized to dryness (Owens and Hartman 1986). The lyophilized supernatants were resuspended in phosphate buffer at 100 mM, EDTA at 2 mM, pH 7.4, and $HClO_4$ at 2 M (in equal volumes) and neutralized to pH 6–7 as described above. Total glutathione was determined using the same method as described for the cell pellets.

Respiration measurement

Cells were grown to an $OD_{420} = 0.5–0.7$ and harvested at 4,500×*g* at 4°C, washed twice in 5 ml MOPS buffer at 40 mM, pH 7.4, and resuspended in the same buffer. Respiration rates were determined polarographically for suspensions with final $OD_{420} = 2$ in 1.5 ml working volume in a Clark-type oxygen electrode (Oxygraph, Hansatech Instruments, UK) after the addition of either glucose, glycerol, succinate, lactate, fumarate or pyruvate at 2 mM final concentration. In inhibition tests, Cd^{2+} 30 µg ml^{-1} was added in the absence or presence of GSH at 2 mM or GSSG at 2 mM. The results were normalized with respect to dry weight.

Quantification of intracellular ROS

E. coli suspensions were incubated with dihydrorhodamine-123 (DHR), 0.025 µg $µl^{-1}$ (Molecular Probes, Eugene,

Oregon, USA) at 37°C shaking for 2 h in the dark using 500 µl of culture in 2 ml microtubes. The treated samples received Cd^{2+} 30 µg ml^{-1}, 1 h after the addition of DHR, so that they were exposed to Cd^{2+} for 1 h. Control samples received an equivalent volume of sterile distilled water. The samples were then diluted 1:20 in PBS (NaCl 8% (w/v); KCl 0.02% (w/v); Na_2HPO_4 0.18% (w/v) pH 7.4). Cell-associated fluorescence was measured by flow-cytometry with a Becton Dickinson FACSCalibur (San Jose, California, USA). For each sample, 10,000 cells were acquired and results were analyzed using the CellQuest programme version 3.3.

Statistical data treatment

All results in this study are expressed as means of at least three independent replicates with the associated standard deviation. Differences between treatments were considered statistically significant when Student's *t*-test was <0.05.

Results

Aiming to assess the effect of cadmium on *E. coli* cells, cultures were grown to early logarithmic phase and exposed to 30 µg ml^{-1} of cadmium (273 µM of $CdCl_2$), a sublethal concentration able to induce growth arrest The results show that cadmium treatment induced a stasis in the growth of aerobic cultures (Fig. 1a, b) accompanied by a decrease in cell viability (CFUs; Fig. 2), as expected. On the other hand, the effect of Cd^{2+} on growth and culturability was much less pronounced in cultures grown by fermentation. These results are consistent with published data suggesting that ROS associated with aerobic metabolism contribute to Cd-induced cell death in *S. cerevisiae* (Brennan and Schiestl 1996).

Since glutathione depletion has been pointed out to be the cause of oxidative stress in Cd-exposed cells, we analyzed the effect of its absence on toxicity by using glutathione mutants. GshA catalyzes the first step in glutathione biosynthesis, whereas GshB converts gamma-glutamylcysteine into glutathione. The growth stasis of the mutants Δ*gshA* and Δ*gshB* was similar to that seen in the wild-type strain (Fig. 1a). However, GshA deficiency significantly decreased the culturability of cadmium-treated cells: the Δ*gshA* and Δ*gshB* strains showed 33 and 54% culturability, respectively, compared to 63% in the wild-type strain (Fig. 2). These results are in agreement with a very important role of glutathione in cadmium detoxification.

In previous works, the depletion of glutathione has been pointed out as the direct cause for the rise of ROS levels in cadmium-exposed cells. In *E. coli* MG1655 cultures treated with cadmium, we observed a 72% depletion of intracellular

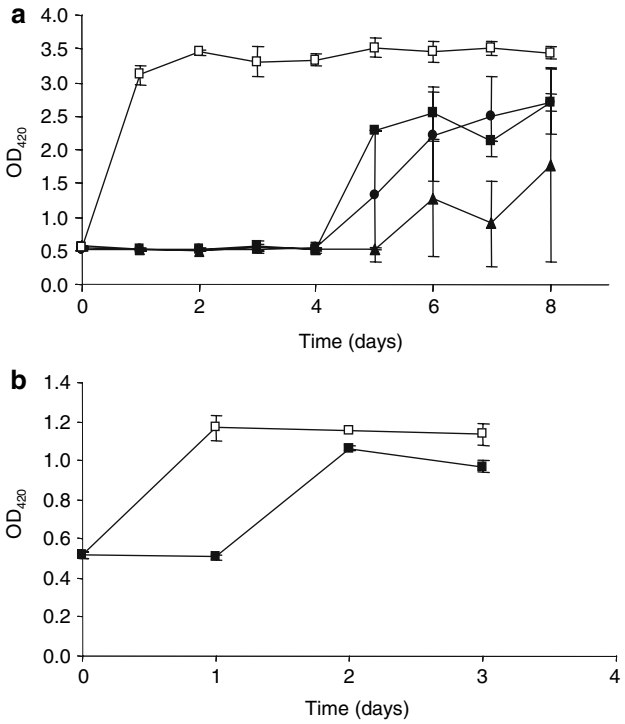

Fig. 1 Effect of 30 µg ml^{-1} Cd^{2+} on cell growth. **a** Untreated culture (*open square*), wild-type + Cd (*filled square*), Δ*gshA* + Cd (*filled triangle*), Δ*gshB* + Cd (*filled circle*). The untreated culture growth curve shown is representative of the curves obtained for all the untreated cultures of the strains used in the experiments: wild-type BW25113, Δ*gshA* and Δ*gshB*. **b** Δ*ubiE* untreated (*open square*), Δ*ubiE* + Cd (*filled square*)

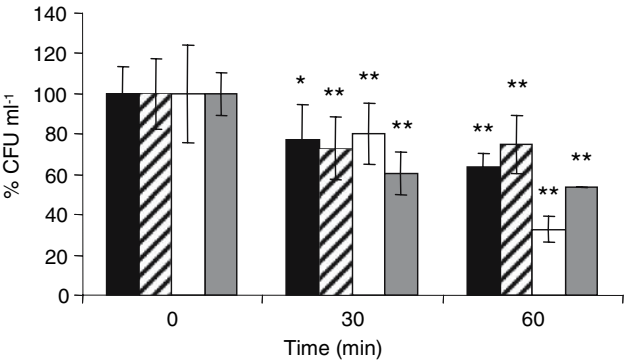

Fig. 2 Effect of 30 µg ml^{-1} Cd^{2+} on culturability: wild type (*filled bar*), Δ*ubiE* (*diagonal stripe bar*), Δ*gshA* (*open bar*), Δ*gshB* (*gray bar*). The absolute values (CFU ml^{-1}) corresponding to 100% (T_0) are: wild type = 1.41E + 08 ± 1.90E + 07, Δ*ubiE* = 1.74E + 08 ± 3.03E + 07, Δ*gshA* = 1.53E + 08 ± 3.67E + 07 and Δ*gshB* = 1.86E + 08 ± 2.01E + 07. Statistically significant differences between Cd-treated and untreated cultures are identified: * ($P < 0.05$) and ** ($P < 0.01$)

glutathione, which was accompanied by a rise in the extracellular concentration of this molecule (3.4 times; Fig. 3). Extracellular glutathione has a protective role as the Cd-dependent growth-arrest effect was not observed when exogenous GSH (but not GSSG) was added to the growth

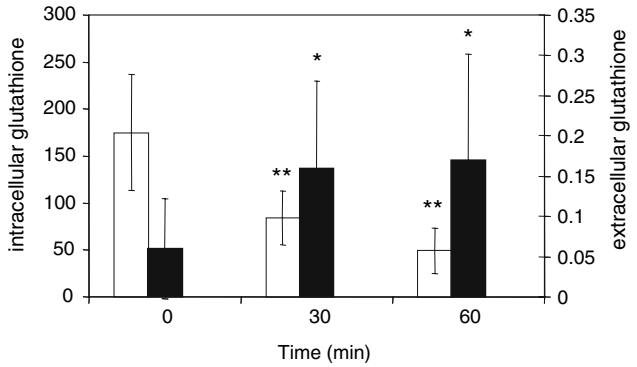

Fig. 3 Effect of 30 μg ml^{-1} Cd^{2+} on total glutathione: intracellular concentration (nmol (GSH + 2GSSG) mg^{-1} protein; *open bar*), extracellular concentration (μM (GSH + 2GSSG); *filled bar*). * ($P < 0.05$) and ** ($P < 0.01$) express significant differences between Cd-treated (30 and 60 min) and untreated (0 min) samples

medium (data not shown). To investigate if glutathione deficiency increased Cd-induced production of ROS, we used DHR 123 to assess the amount of peroxides formed in the different strains. As shown in Fig. 4, exposure of the wild-type cells to cadmium increased by twofold the levels of ROS. Accordingly, we detected a 100% increase in catalase activity in Cd-treated cultures (data not shown). When Δ*gshA* and Δ*gshB* mutant cells were exposed to the heavy metal, the levels of ROS increased by about 40%. This result clearly indicates that intracellular glutathione depletion cannot be the cause of the Cd-induced production of ROS.

It has been suggested that the toxic effect of Cd and other metals such as zinc, silver and mercury involves interference with the respiratory chain. To test this hypothesis, we analyzed the effect of cadmium on oxygen consumption

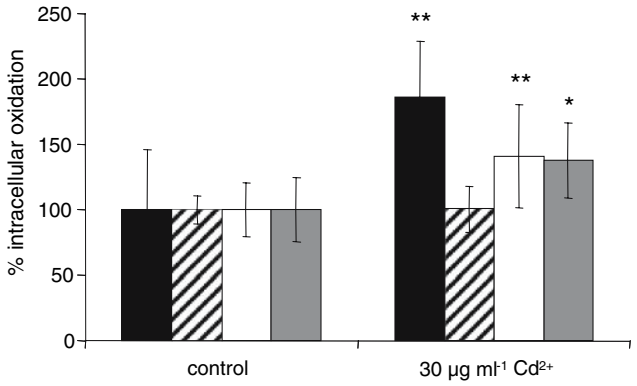

Fig. 4 ROS detection by flow-cytometry: wild type (*filled bar*), Δ*ubiE* (*diagonal stripe bar*), Δ*gshA* (*open bar*), Δ*gshB* (*gray bar*). The absolute values (arbitrary fluorescence units) corresponding to 100% (T_0) are: wild type = 14.99 ± 6.85, Δ*ubiE* = 27.09 ± 2.92, Δ*gshA* = 4.82 ± 1, Δ*gshB* = 8.08 ± 2.01. * ($P < 0.05$) and ** ($P < 0.01$) mean that there is a significant difference between the control and treated samples

and Cd toxicity in Δ*ubiE* mutant cells that have a deficient respiratory chain due to lack of ubiquinone and menaquinone (Gennis and Valley 1987). In wild-type cells of MG1655 strain, cadmium inhibited oxygen consumption and this effect was reverted by the exogenous addition of GSH, but not GSSG (Fig. 5). In Δ*ubiE* mutant cells, oxygen consumption was 38% of that observed in its isogenic wild-type strain (data not shown). Notably, this reduction in aerobic metabolism significantly decreased the growth stasis induced by cadmium. Indeed, cell growth was resumed after just 2 days of Cd exposure (vs. 5 days in the wild type; Fig. 1b). In addition, the culturability of Cd-treated Δ*ubiE* cells was higher compared to the wild type (Fig. 2). Oxidative stress markers were analyzed in the Δ*ubiE* mutant cells to test the link between the toxicity of cadmium and the respiratory chain. The constitutive levels of ROS were higher in this strain than in the wild-type: this fact can be justified by the deficient flow of electrons in the respiratory chain, which probably leads to the accumulation of unstable reduced intermediates of the chain. However, Cd-induced ROS production was suppressed in this mutant (Fig. 4), whereas the direct exposure to 10 mM H_2O_2 still increased intracellular oxidation (data not shown).

ROS production is usually associated with oxidative damage in proteins and lipids. For this reason, we analyzed the levels of protein carbonylation and lipid peroxidation in the wild-type and in the Δ*ubiE* mutant. In wild-type cells, the exposure to cadmium increased protein oxidation (Fig. 6a) and lipid peroxidation (Fig. 6b). In the Δ*ubiE* strain, the constitutive levels of protein and lipid oxidation were higher, compared to those observed in the untreated wild-type cells, which is in accordance with the higher constitutive ROS levels detected in this mutant. Notably, Cd-induced protein carbonylation was significantly reduced: the increase in protein carbonyl content was 278% in the wild-type cells and just 70% in the Δ*ubiE* mutant cells

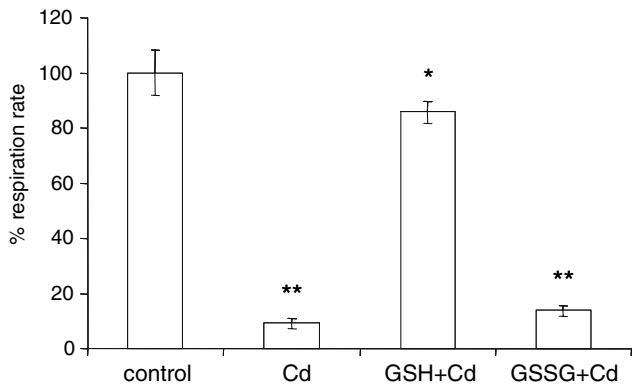

Fig. 5 Effect of 30 μg ml^{-1} Cd^{2+} on respiration rate. The absolute value (nmol O_2 min^{-1}) corresponding to 100% is 98 ± 7.9. Significant differences between the control and the addition of Cd or GSH + Cd or GSSG + Cd are represented: * ($P < 0.05$) and ** ($P < 0.01$)

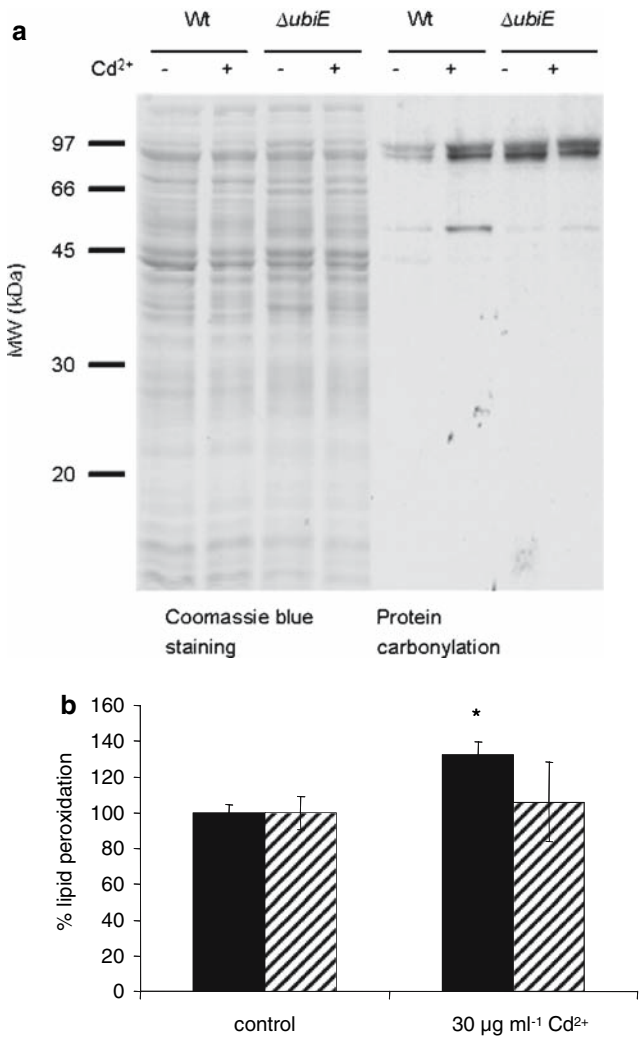

Fig. 6 Effect of 30 μg ml^{-1} Cd^{2+}: **a** on protein carbonyl levels. SDS-PAGE is shown as loading control. b) on lipid peroxidation levels: wild type (*solid bar*), Δ*ubiE* (*diagonal stripe bar*). The absolute values (μmol MDA mg^{-1} prot) corresponding to 100% are: wild type = 6.60 ± 0.29, Δ*ubiE* = 10.68 ± 0.99. In the wild type there is significant difference between the control and treated samples * $P < 0.05$)

(Fig. 6a). In addition, no increase in lipid peroxidation was detected in Δ*ubiE* cells treated with cadmium (Fig. 6b).

All these results support the hypothesis that Cd-induced ROS production is associated with the activity of the respiratory chain.

Discussion

Cadmium is a relatively abundant heavy metal and its toxic effects have been associated with the induction of ROS production and mutagenesis (Clark and Kunkel 2004; Pathak and Khandelwal 2006). Although the effects of cadmium have been widely described, the molecular mechanisms underlying its toxicity are still unclear. In order to investigate these mechanisms and their relationship with oxidative stress in *E. coli*, K-12 strains were submitted to a sublethal concentration of the metal that is able to induce growth arrest. Similar to observations from previous reports, growth inhibition was accompanied by a decrease in culturability and by an increase in oxidative stress markers. Indeed, cadmium increased ROS production and induced catalase activity. Concomitantly, there was a Cd-dependent depletion of intracellular glutathione and a rise in its external concentration. These observations are in accordance with the general mechanism by which cadmium is detoxified in *E. coli*, whereby the bis(glutathionato)cadmium complex is formed and presented to the ZntA pump that excretes the metal from the cell (Blencowe et al. 1997; Li et al. 1997; Rensing et al. 1997; Sharma et al. 2000). The rise in the extracellular concentration of glutathione can constitute a further defence mechanism of the cell to prevent the entrance of the metal. Owens and Hartman (1986) reported GSH export in *E. coli* and some years later a bacterial transporter of reduced glutathione was identified (Pittman et al. 2005).

The depletion of glutathione by cadmium has been pointed out as the cause of the oxidative stress status in the presence of this metal. To clarify the contribution of glutathione depletion in cadmium toxicity in *E. coli*, we used Δ*gshA* and Δ*gshB* mutants that lack glutathione. The results obtained reveal that the effects of cadmium exposure in these mutants were similar to those observed in the wild type because a rise of ROS is detected whether GSH is present in the cells or not. Our data clearly show that the generation of ROS in cadmium-exposed cells is completely independent of the presence of glutathione. Despite this fact, glutathione mutants seem to recover more slowly from cadmium stress, which confirms the importance of GSH in cadmium detoxification. The Δ*gshB* strain was probably less affected by Cd because the first step in GSH biosynthesis still occurs in this mutant and the dipeptide gamma-glutamylcysteine may result in partial protection against cadmium (Cruz-Vásquez et al. 2002).

We also observed that cadmium inhibits oxygen consumption and that fermenting cultures are less sensitive to this metal. A reduction in cadmium toxicity has also been observed in fermenting *S. cerevisiae* (Brennan and Schiestl 1996; Vido et al. 2001). Our results indicate that cadmium toxicity is correlated with ROS production by the respiratory chain. In agreement with this hypothesis, the detrimental effects of cadmium on growth and culturability were significantly reduced in Δ*ubiE* cells that show diminished respiration rates. Furthermore, although ROS levels were constitutively higher in Δ*ubiE* cells, no increase was detected after exposure to the heavy metal.

Other studies have shown an interaction of cadmium with mitochondrial respiration (Wang et al. 2004) and of

other heavy metal ions (Ag^{+}, Hg^{2+} and Zn^{2+}) with the bacterial respiratory chain (Bragg and Rainnie 1974; Kasahara and Anraku 1972; Kim and Bragg 1971).

Wang and Crowley (2005) reported in their transcriptome study on *E. coli* K-12 that cadmium affects the expression of genes associated with protein synthesis, energy metabolism and cell rescue. The up-regulation of genes associated with anaerobic metabolism and the shutdown of all high-energy consumption processes such as the biosynthesis of amino acids suggests that, when exposed to cadmium, cells switch to an energy conservation mode. Our hypothesis whereby cadmium poisons the respiratory chain is in accordance with these observations. These authors conclude that ROS are not a direct cause of Cd^{2+} toxicity, because the base excision DNA repair system and OxyR were not induced and because the genes affected by cadmium are quite a different subset from those affected by superoxide. However, it should be noted that the concentration of cadmium used in their study was very low, 1 μg ml^{-1}, a level at which we were not able to detect any effect on growth kinetics.

We propose the following model for Cd toxicity in respiring *E. coli* cells: cadmium enters the cell through one of the essential metal transporters, like ZupT, a zinc transporter (Grass et al. 2002). Here it poisons the respiratory chain leading to the accumulation of unstable reduced intermediates (Messner and Imlay 1999) that reduce molecular oxygen to ROS. Although the cell defenses are activated to eliminate the ROS, they are not enough to completely prevent oxidative damage. The poisoning of the respiratory chain leads to a depletion of the ATP stock and to growth arrest. Cadmium is detoxified through the excretion from the cell by the ZntA pump (Blencowe et al. 1997; Rensing et al. 1997; Sharma et al. 2000) and through the formation of a complex with the GSH excreted. Although glutathione plays a crucial role in detoxifying the heavy metal ion, clearly its depletion is not the cause of the ROS burst resulting from exposure to cadmium.

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