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Adaptive response of *Escherichia coli* K-12 strains to cadmium-induced oxidative stress

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Publications

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Kalyuzhnaya MG, De Marco P, Bowerman S, **Pacheco CC**, Lara JC, Lidstrom ME, Chistoserdova L (2006) *Methyloversatilis universalis* gen. nov., sp. nov., a novel taxon within the Betaproteobacteria represented by three methylotrophic isolates. Int J Syst Evol Microbiol 56:2517-2522

Jamshad M, De Marco P, **Pacheco CC**, Hanczar T, Murrell JC (2006) Identification, mutagenesis, and transcriptional analysis of the methanesulfonate transport operon of *Methylosulfonomonas methylovora*. Appl Environ Microbiol 72:276-283

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Franco AR, Calheiros CS, **Pacheco CC**, De Marco P, Manaia CM, Castro PM (2005) Isolation and characterization of polymeric galloyl-ester-degrading bacteria from a tannery discharge place. Microb Ecol 50:550-556

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Resumo

O cádmio é um elemento ubíquo e que se tornou uma preocupação crescente devido à sua elevada toxicidade a baixas concentrações. Em estudos publicados sobre os efeitos crónicos da exposição ao cádmio é sugerido que este metal está implicado em doenças como osteoporose, enfisema, anemia, em danos renais crónicos e diferentes tipos de cancro. Embora os efeitos do cádmio tenham sido exaustivamente descritos, os mecanismos moleculares por trás da sua toxicidade em procariotas são ainda desconhecidos. Uma vez que o cádmio não é um metal redox-activo, foi levantada a hipótese de que este pode provocar stresse oxidativo através de mecanismos indirectos, tais como: substituição de metais redox-activos, depleção dos captadores de metais endógenos (p. ex. glutationa) ou afectando a actividade das defesas antioxidantes. Este facto levou-nos a investigar os mecanismos moleculares envolvidos na toxicidade do cádmio e a resposta adaptativa despoletada em *Escherichia coli* quando na presença deste metal. Para além disso, pretendíamos ainda determinar a relevância dos mecanismos de toxicidade previamente propostos em eucariotas, nomeadamente, a depleção de glutationa e a inibição da respiração.

Neste trabalho mostrámos que a toxicidade do cádmio está, de facto, associada ao stresse oxidativo conduzindo ao aumento da produção de espécies reactivas de oxigénio (EROS). Na presença do cádmio observou-se a paragem do crescimento, perda de culturabilidade e ainda o aumento dos marcadores de stresse oxidativo. Em células a fermentar, cultivadas em ausência de oxigénio ou nitrato, verificou-se uma redução dos efeitos tóxicos deste metal. Além disso, verificou-se que o consumo de oxigénio é inibido na presença do cádmio e que esta inibição é revertida pela adição de glutationa reduzida (GSH). A análise dos níveis de glutationa revelaram que, quando as células são expostas a cádmio, há um decréscimo da concentração intracelular e esta redução é acompanhada pelo aumento da concentração extracelular deste tiol. Embora pareça evidente que a GSH constitui uma defesa de primeira linha na destoxificação do cádmio, estudos realizados usando mutantes deficientes em GSH mostraram que este tiol não é essencial para a sobrevivência das células. A depleção de glutationa pelo cádmio é geralmente considerada como estando na origem do stresse oxidativo observado na presença do metal. Esta hipótese foi refutada pela observação que o aumento da produção de EROS é independente da GSH.

Um aumento na actividade da catalase foi verificado em todas as condições em que as bactérias foram expostas ao cádmio, mesmo em situações em que a toxicidade deste metal era claramente reduzida. Estes dados sugerem que há um mecanismo de indução da catalase pelo cádmio que é independente da geração de EROS. A análise da toxicidade do cádmio em mutantes deficientes na catalase demostrou que, tal como para os mutantes deficientes em GSH, esta enzima antioxidante não é essencial no combate ao cádmio.

Finalmente, a análise de mutantes com função respiratória comprometida mostrou que o mecanismo de toxicidade do cádmio está associado a uma interferência com a cadeia de transporte de electrões (CTE). Embora tenham sido levados a cabo estudos utilizando diversos mutantes na CTE, não foi possível identificar quais os alvos do cádmio nesta cadeia. Contudo, os dados reunidos parecem sugerir que o cádmio afecta a CTE ao nível das NADH desidrogenases e das quinonas.

Globalmente os resultados indicam que a toxicidade do cádmio é um processo multifactorial e que está associado ao stresse oxidativo. Em células de *E. coli* a respirar, a interferência do cádmio com a CTE é o maior contribuinte para o stresse oxidativo induzido na presença deste metal. A GSH tem um papel crucial na destoxificação do cádmio mas, contrariamente ao que foi proposto, a depleção deste tiol não está na origem do stresse oxidativo observado em células expostas a cádmio.

Abstract

Cadmium is a widespread metal that became an increasing concern due to its high toxicity at low concentrations. Studies on the effects of chronic exposure to cadmium have been published implicating this metal in several diseases, such as osteoporosis, emphysema, anaemia, irreversible renal tubular injury and different types of cancer. Although the toxic effects of cadmium have been comprehensively described, the molecular mechanisms underlying its toxicity in prokaryotes are still unclear. Since cadmium is not a redox-active metal, it was hypothesized that it could induce oxidative stress indirectly by displacement of redox-active metals, depletion of endogenous radical scavengers (e.g. glutathione) or affecting the activity of antioxidant defences. This led us to investigate the molecular mechanisms behind cadmium toxicity and the adaptive response triggered by this metal in *Escherichia coli*. Moreover, we wanted to assess the relevance of the toxicity mechanisms proposed for eukaryotes namely the depletion of glutathione and inhibition of respiration.

In this work, we have shown that cadmium toxicity is indeed associated with oxidative stress leading to an increase of the reactive oxygen species (ROS) production. Cell growth arrest and loss of culturability were observed, and also the increase of the oxidative stress markers was registered. These effects were clearly reduced in fermenting cells grown in the absence of oxygen or nitrate. Furthermore, oxygen consumption was shown to be inhibited in the presence of cadmium and this inhibition was abolished upon addition of reduced glutathione (GSH). Analysis of the levels of glutathione revealed that in the presence of cadmium the intracellular levels of this thiol decrease, and this is accompanied by an increase of the extracellular concentration. Even though it is clear that GSH constitutes a primary line of defence against cadmium, the studies carried out using GSH-deficient mutants showed that it is not essential for cell survival. The depletion of glutathione by cadmium is generally accepted as the cause for the oxidative stress associated with this metal. The observation that cadmium-induced ROS is independent of GSH shows that this model is not sustained.

Catalase activity was always induced by cadmium, even when toxicity was clearly reduced, suggesting that there is a mechanism of activation of this enzyme that is independent of ROS formation. Analysis of the cadmium toxicity in

catalase-deficient mutants showed that, like for GSH, this antioxidant enzyme is not essential in the response against this metal.

Finally, analysis of mutants with impaired respiratory function showed that the mechanism of cadmium toxicity is associated with an interference with the electron transport chain (ETC). Despite the use of several mutant strains, we were not able to indentify the targets of this metal within the ETC. The data collected suggest that cadmium affects the electron transport at the NADH-dehydrogenase and quinone levels.

The overall results indicate that cadmium toxicity is a multifactorial process associated with oxidative stress. In *E. coli* respiring cells, interference of this metal with the ETC is clearly the major contributor for the cadmium-induced oxidative stress. The role of GSH in the detoxification of cadmium is crucial but contrary to what was previously proposed, the depletion of this thiol is not the cause for oxidative stress experienced by cells exposed to this metal.

Résumé

Le cadmium est un métal couramment répandu qui est devenu une préoccupation croissante due à sa toxicité élevée à de basses concentrations. Des études sur les effets de l'exposition chronique au cadmium ont été publiées, impliquant ce métal dans plusieurs maladies, telles que l'ostéoporose, l'emphysème, l'anémie, ainsi que dans des dommages tubulaires rénaux irréversibles et dans différents types de cancer. Bien que les effets toxiques du cadmium aient été largement décrits, les mécanismes moléculaires étant à la base de sa toxicité dans les procaryotes ne sont pas encore très clairs. Vu que le cadmium n'est pas un métal redox-actif, l'hypothèse qu'il pourrait induire du stress oxydant indirectement par le déplacement des métaux redox-actifs, l'épuisement des extracteurs de radicaux endogènes (par exemple, glutathion) ou d'affecter l'activité des défenses antioxydantes a été soulevée. Ceci nous a menés l'étude des mécanismes moléculaires impliqués dans la toxicité du cadmium et la réponse adaptative déclenchée par ce métal dans *Escherichia coli*. Ensuite, nous prétendions évaluer la pertinence des mécanismes de toxicité préalablement proposés pour des eucaryotes, notamment l'épuisement du glutathion et l'inhibition de la respiration.

Nous avons montré par ce travail que la toxicité du cadmium est en effet associée au stress oxydant menant à une augmentation de la production d'espèces réactives de l'oxygène (ERO). La perte de culturabilité et l'arrêt de la croissance en présence du cadmium a été observé, ainsi que l'augmentation des marqueurs du stress oxydant. Ces effets ont été clairement réduits dans les cellules fermentées et en croissance sans la présence d'oxygène ou de nitrate. En outre, il s'est vérifié que la consommation d'oxygène est inhibée en présence du cadmium et que cette inhibition est abolie par l'addition de glutathion dans sa forme réduite (GSH). L'analyse des niveaux du glutathion a indiqué qu'en présence du cadmium il y a une diminution de la concentration intracellulaire de ce thiol et que cette réduction est accompagnée par l'augmentation de la concentration extracellulaire. Même s'il est clair que le glutathion constitue une défense de première ligne contre le cadmium, les études réalisées en utilisant des mutants déficients en GSH ont montré que ce thiol n'est pas essentiel pour la survie des cellules. L'épuisement du glutathion par le cadmium est, en règle

générale, accepté comme cause du stress oxydant imposée par ce métal. Ceci a été réfutée par l'observation que l'augmentation de la production de ERO est indépendante du glutathion.

L'activité de la catalase a toujours été induite dans les bactéries par le cadmium, même dans des situations où la toxicité de ce métal était clairement réduite. Ceci suggère qu'il y a un mécanisme d'induction de cette enzyme par le cadmium qui est indépendant de la formation d'ERO. L'analyse de la toxicité de cadmium dans les mutants manquant la catalase a demontré que, telle que pour les mutants manquant le glutathion, cette enzyme antioxydante n'est pas essentielle dans la réponse contre le cadmium.

Finalement, l'analyse des mutants ayant leur fonction respiratoire altérée a montré que le mécanisme de la toxicité du cadmium est associé à une interférence avec la chaîne de transport d'électron (CTE). Bien que plusieurs études aient été faites dans divers mutants, il ne nous a pas été possible d'identifier les cibles du cadmium dans cette chaîne. Les données rassemblées semblent suggérer que le cadmium peut affecter le transport d'électrons au niveau de la NADH-déshydrogénase et de la quinone.

Les résultats de ce travail indiquent que la toxicité du cadmium est un processus multifactoriel qui est associé au stress oxydant. Dans les cellules en respiration d'Escherichia coli, l'interférence de ce métal avec la CTE est clairement le principal contribuant pour le stress oxydant induit par le cadmium. Le rôle du glutathion dans la détoxication du cadmium est fondamental mais contraire à ce qui a été précédemment proposé, l'épuisement de ce thiol n'est pas la cause du stress oxydant observée dans des cellules exposées au cadmium.

List of abbreviations

A	absorbance
ATP	adenosine-5'-triphosphate
ATPase	ATP synthase
BHT	2,6-di-*tert*-Butyl-*p*-cresol
bp	base pairs
cDNA	complementar deoxyribonucleic acid
CFU	colony forming units
DHR	dihydrorhodamine 123
DMK	demethylmenaquinone
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
dNTP	deoxynucleotides triphosphate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetracetic acid
ETC	electron transport chain
g	acceleration = 9.8 $m \cdot s^{-2}$
GPx	glutathione peroxidase
GR	glutathione reductase
$GS^{\cdot}$	glutathione radical
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
HP I	hydroperoxidase I
HP II	hydroperoxidase II
Km	kanamycin
Km^R	kanamycin resistance
MDA	malondialdehyde
MeHg	methylmercury
MetSOX	methionine sulfoxide
MK	menaquinone
MOPS	3-(N-morpholino)propanesulfonic acid
MW	molecular weight
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate

OD	optical density
OMP	outer membrane protein
o.n.	over night
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	isoelectric point
pmf	proton-motive force
PMF	peptide mass fingerprint
PTS	phosphotransferase system
RNA	ribonucleic acid
rRNA	ribosomic ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
sRNA	small ribonucleic acid
TBA	2-thiobarbituric acid
TCA	tricarboxylic acid cycle
Tet	tetracycline
Tet^R	tetracyclin resistance
Tm	melting temperature
UQ	ubiquinone
w/v	weight per volume

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1. General introduction

1.1. Oxidative stress

Cells face oxidative stress when the prooxidant-antioxidant balance is in favour of the prooxidants. Environmental conditions that lead to the increase the of reactive oxygen species (ROS) or to the depletion of antioxidant molecules or enzymes can induce an oxidative stress situation (Storz and Zheng 2000). This can be observed when cells are exposed to reactive oxygen intermediates, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$), and as a consequence proteins, nucleic acids and cell membrane lipids are oxidatively damaged (Storz and Imlay 1999). The generation of ROS is the result of electron leakage from the respiratory chain that instead of reducing oxygen to water produces $O_2^{\cdot-}$. Oxygen possesses two unpaired electrons in the same spin state, which makes it a paramagnetic element. For this reason it is impossible for O_2 to receive a pair of electrons at one time (Fridovich 1998), and upon acceptance of one, two or three electrons oxygen intermediates are formed (Figure 1.1).

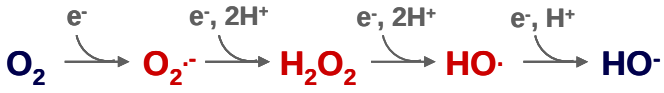


Figure 1.1 The redox states of oxygen.

These species are inevitable by-products of aerobic metabolism and they can damage the cell unless they are scavenged or destroyed. The generation of distinct types of damage within the cell is due to the different chemical reactivities of ROS. Superoxide is capable of oxidizing thiols, ascorbate, tocopherol; and also proteins containing Fe-S clusters are highly susceptible to the attack by this radical. In *Escherichia coli* (*E. coli*), when electrons are migrating through the respiratory chain at the ubiquinone and NADH dehydrogenase the leakage of electrons is associated with the generation of superoxide anion, similar to the eukaryotic mitochondria (González-Flecha and Demple 1995). Subsequently, superoxide originates hydrogen peroxide by dismutation and is also capable of reducing transition metals (e.g. Cu^{2+} and Fe^{3+}) and metal complexes (Farr and Kogoma 1991; Storz and Imlay 1999). The auto-oxidation of enzymes, like NADH dehydrogenase, succinate dehydrogenase and D-lactate dehydrogenase has been identified as major source of $O_2^{\cdot-}$ (Farr and Kogoma

1991; Imlay 1995). Also the auto-oxidation of several non-enzymatic cellular components (e.g. ubiquinols, cathecols, thiols and flavins) gives rise to this radical (Farr and Kogoma 1991).

As far as hydrogen peroxide is concerned, this species can act as a weak oxidizing agent attacking thiol groups of proteins (thus likely leading to their inactivation) and reduced glutathione (GSH). The participation in the Fenton reaction is worth of notice (Equation 1), since in the presence of a reduced metal (e.g. Cu^{+} or Fe^{2+}), hydrogen peroxide reacts generating the highly reactive hydroxyl radicals (Farr and Kogoma 1991; Storz and Imlay 1999).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \qquad \text{Equation 1}$$

Finally, the hydroxyl radical due to its high standard reduction potential will react with most biomolecules within the cell, being DNA a particular target of this radical (Farr and Kogoma 1991). Just like the targets of hydroxyl radical are manifold, so are the sources, being the Fenton reaction one of the most relevant (Farr and Kogoma 1991).

1.2. The redox sensors and induced responses

If the cell is performing aerobic metabolism, naturally it will witness the formation of reactive oxygen intermediates. Since these intermediates constitute a serious threat, cells have developed defence systems to keep them at tolerable levels. The antioxidant defence system in *E. coli* includes superoxide dismutases (Mn, Fe and Cu,Zn containing enzymes), two catalases (hydroperoxidase I and II) and the alkylhydroperoxide reductase (AhpCF) that is an additional defence against organic hydroperoxides. This bacterium also contains the glutathione and thioredoxin-dependent reduction systems that are also responsible for the maintenance of the redox balance (Carmel-Harel and Storz 2000).

In certain stress conditions, bacteria respond activating the so-called stimulons, which are sets of globally stimulated genes. In the case of oxidative stress, one or two multigene systems can be activated: the *oxyR* and/or the *soxRS* regulons (Farr and Kogoma 1991).

1.2.1. The peroxide stress response

When *E. coli* is treated with hydrogen peroxide, the synthesis of at least 30 proteins is observed. This results from the induction of specific genes that are under positive regulation of the OxyR protein thus constituting the *oxyR* regulon (Table 1.1).

Table 1.1 Members of the H_2O_2- stress regulon*

Role	OxyR response
H_2O_2 scavenging	Alkylhydroperoxide reductase (*ahpCF*)
	Hydroperoxidase I (*katG*)
Heme synthesis	Ferrochetalase (*hemH*)
Fe-S assembly	Suf operon (*sufABCDE*)
Iron scavenging	Ferretin homolog (*dps*)
Iron-import control	Ferric uptake regulator (*fur*)
Divalent cation import	Manganese transporter (*mntH*)
Disulfide reduction	Thioredoxin 2 (*trxC*)
	Glutaredoxin 1 (*grxA*)
	Glutathione reductase (*gorA*)
	Periplasmic reductase (*dsbG*)
Regulatory RNA	OxyS (*oxyS*)
Unknown Function	Several

*Source: Imlay (2008).

The Dps protein and regulatory RNA OxyS, which seem to be involved in protection against mutagenesis, are OxyR-regulated (Storz and Imlay 1999). Additionally, Dps was found to be a ferretin homolog suggesting that it might be involved in the sequestration of iron thus protecting DNA and other biomolecules from damage mediated by this metal (Grant et al. 1998).

The activation of the *oxyR* regulon is carried out by the OxyR protein that exists in the oxidized or reduced form, but only the first is able to activate the regulon transcription. The direct oxidation of OxyR by H_2O_2 is the mechanism whereby cells sense oxidative stress and induce the regulon (Storz and Imlay 1999). The enzyme glutaredoxin 1 reduces back OxyR deactivating the protein constituting an autoregulated response, since the expression of *grxA* is regulated by OxyR (Aslund et al. 1999).

1.2.2. The superoxide stress response

Elevated levels of superoxide trigger a cell response inducing a stimulon that is different from the one induced by hydrogen peroxide. More than 30 proteins were observed to be induced by superoxide and most of them differ from the ones involved in the hydrogen peroxide stress response. The genes that code for the superoxide responsive proteins are under positive regulation of SoxR and SoxS proteins constituting the *soxRS* regulon (Farr and Kogoma 1991).

Table 1.2 Members of the $O_2^{\cdot-}$-stress regulon*

Role	SoxRS response
Oxidant resistant dehydratase isozymes	Fumarase C (*fumC*)
	Aconitase A (*acnA*)
Suspected cluster repair	*yggX*
	Glucose-6P dehydrogenase (*zwf*)
	NADPH:flavodoxin (*fpr*)
	Flavodoxin A (*fldA*)
	Flavodoxin B (*fldB*)
Drug efflux/resistance	drug efflux pump (*acrAB*)
	OMP component of drug efflux pump (*tolC*)
	OmpF antisense sRNA (*micF*)
	multiple antibiotic resistance operon (*marAB*)
	Nitroreductase (*nfnB*)
	modification of ribosomal protein S6 (*rimK*)
Other	Endonuclease IV (*nfo*)
	Iron-uptake regulatory protein (*fur*)
	MnSOD (*sodA*)
	GTP cyclohydrolase (*ribA*)

*Source: Imlay (2008).

Among the SoxRS-inducible activities are: the manganese SOD (encoded by *sodA*), the DNA repair enzyme endonucleases IV (*nfo*), and the superoxide resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*). The activation of this regulon also leads to increased levels of glucose-6-phosphate dehydrogenase (*zwf*) that generates reducing power, and to elevated levels of the Fur (Ferric Uptake regulator encoded by *fur*) which may decrease the iron levels and subsequently the formation of hydroxyl radical. In agreement, the regulation of

genes related to the maintenance of the reduced state of the Fe-S clusters was also reported (Storz and Imlay 1999).

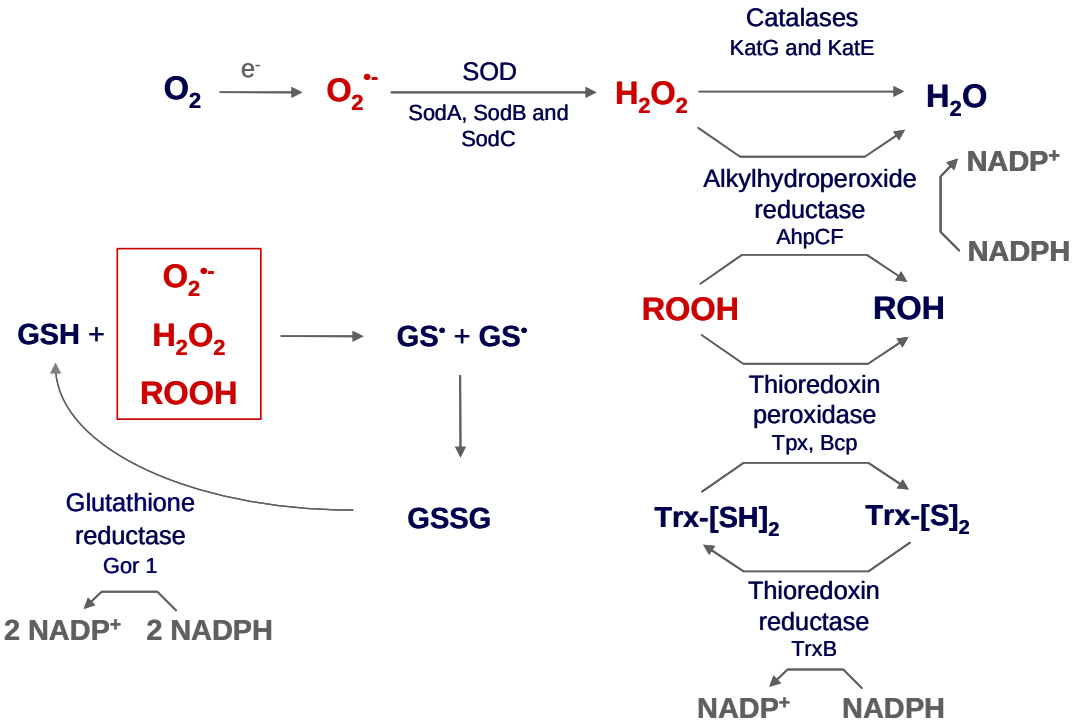
The regulation of the *soxRS* regulon occurs by a two-stage process: the SoxR protein is converted to an active form that enhances the *soxS* transcription, and the increased level of SoxS in turn activates the expression of the regulon. The SoxR protein is a homodimer that contains a $[2Fe\text{-}2S]^{+}$ cluster per subunit, and the protein activation is achieved by oxidation of the cluster to a 2+ state but the nature of the oxidant is still under debate (Storz and Imlay 1999; Imlay 2008).

1.3. Oxidative stress – cellular responses

Cells facing a sub-lethal challenge with an oxidant induce defences to scavenge or destroy ROS (primary defences) and defences that can repair damages caused by ROS (secondary defences).

1.3.1. Prevention of oxidative damage

This class of defences includes enzymatic and non-enzymatic components; the former may act directly in the destruction of ROS (Figure 1.2) or in the production of the non-enzymatic components.



Legend: ROOH – alkylhydroperoxides, GS• – glutathione radical, GSH – reduced glutathione, GSSG – oxidized glutathione, Trx-$[SH]_2$ – reduced thioredoxin, Trx-$[S]_2$ –oxidized thioredoxin

Figure 1.2 *Escherichia coli* primary defences.

Enzymatic defences are common among aerobic microorganisms and, in *E. coli*, the enzymes that provide the bulk of protection are: superoxide dismutases (encoded by *sodA*, *sodB* and *sodC*), catalases (*katG* and *katE*), alkylhydroperoxide reductase (*ahpCF*), γ-glutamylcysteine synthetase (*gshA*), glutathione synthetase (*gshB*), glutaredoxins (*grxA*, *grxB* and *grxC*), glutathione reductase (*gorA*), thioredoxins (*trxA* and *trxC*), thioredoxin reductase (*trxB*) and thioredoxin peroxidases (*tpx* and *bcp*) (Farr and Kogoma 1991; Carmel-Harel and Storz 2000).

1.3.1.1. Superoxide Dismutase (SOD)

Based on the type of ligands, different types of SODs have been described: MnSOD (*sodA*), FeSOD (*sodB*), NiSOD (*sodN*), Cu,ZnSOD (*sodC*) and the Fe,ZnSOD (*sodF*). The presence in all SODs of a transition metal is explained by its role in electron transfer during superoxide dismutation reaction (Equation 2).

$$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{Equation 2}$$

The FeSOD can be found in prokaryotes while the Cu,ZnSOD is generally not found in bacteria and the MnSOD (*sodB*) can be found both in prokaryotes and eukaryotes (Farr and Kogoma 1991). The NiSOD and FeZnSOD have been described in *Streptomyces* strains (Eun-Ja et al. 1996; Dupont et al. 2008).

In *E. coli*, the steady-state concentration of SOD is 10^{-5} M and the steady-state concentration of $O_2^{\cdot-}$ is about 10^{-9} to 10^{-10} M (in a wild-type strain in aerobic conditions) (Fridovich 1983). In a SOD mutant, the concentration of superoxide has been seen to rise to a value of about 10^{-6} M which means that SOD reduces the steady-state concentration of this radical in three orders of magnitude (Imlay et al. 1988). Hydrogen peroxide is one of the products of superoxide dismutation thus, the reaction catalyzed by SODs cannot be beneficial to the cell unless H_2O_2 is less toxic than $O_2^{\cdot-}$, or H_2O_2 is disproportionated more rapidly than $O_2^{\cdot-}$ or SOD avoids the clearance of $O_2^{\cdot-}$ through other more dangerous routes, such as the reaction with glutathione (that gives rise to the toxic glutathione radical - $GS^{\cdot}$). As to the toxicity of either oxygen intermediates it is difficult to conclude which one is more detrimental to the cell.

1.3.1.2. Catalase

These enzymes defend the cell against H_2O_2 (Equation 3), even if it is energy-depleted because the disproportion reaction does not need energy or reducing power (H_2O_2 is the source of electrons). There are two catalases in *E. coli*, the hydroperoxidase I (HP I) and the hydroperoxidase II (HP II) that are found in the periplasm and the cytoplasm, respectively (Heimberger and Eisenstark 1988). The location and induction patterns of the two enzymes suggest that there are different hydrogen peroxide sources depending on the cell growth phase.

Peroxidases also catalyze a reaction that leads to H_2O_2 destruction (Equation 4) but they require NADH or NADPH, therefore the role of these enzymes is dependent of the redox state of the cell. Another enzyme that may play a role in the cellular antioxidant defence system is the alkylhydroperoxide reductase that is capable of catalyzing the reduction of organic hydroperoxides. Moreover, this enzyme was suggested to be the primary scavenger of endogenous H_2O_2 in *E. coli* (Seaver and Imlay 2001).

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O \qquad \text{Equation 3}$$

$$RH_2 + H_2O_2 \rightarrow R + 2H_2O \qquad \text{Equation 4}$$

1.3.1.3. Glutathione

This molecule (Glu-Cys-Gly) is the major non-protein thiol in the cell and is an important non-enzymatic component of the antioxidant defence system. Reduced glutathione is produced in two reactions that are catalyzed by γ-glutamylcysteine synthetase GshA and the glutathione synthetase GshB (Toledano et al. 2007). Glutathione plays a key role in the intracellular redox balance as it is present in reduced form that is reversibly converted to an oxidized form. The fact that glutathione harbours a thiol group leads to the establishment of thio-ester bounds with cysteine groups of proteins. This thiolation reaction may serve as a protection mechanism, since thiolated proteins are resistant to oxidative damage (Kiley and Storz 2004). Glutathione also helps maintaining the reducing environment within the cell by reacting with H_2O_2, $O_2^{\cdot-}$ and other radicals originating a $GS^{\cdot}$. The formed radicals will dimerize to form the oxidized molecule

of glutathione (GSSG) that will be reduced back to GSH by the enzyme glutathione reductase (GR) (Meister and Anderson 1983). Another function of GSH is to reduce back the disulfide bonds introduced by oxidative damage in proteins. Although the formation of disulfide bonds is easily reversed, their effect is quite drastic because their presence can alter the protein function (Levine et al. 1981).

Even though glutathione plays a relevant biochemical role, its function can be replaced by other molecules, such as low molecular weight thiols like thioredoxin (Farr and Kogoma 1991; Toledano et al. 2007). Other non-enzymatic components of the antioxidant defence system in *E. coli* are ubiquinone (UQ) and menaquinone (MK) that can function as membrane-associated antioxidants.

1.3.2. Oxidative damage and repair

ROS are able to damage cell components and the accumulation of these damages may lead to cell death. To repair, degrade or replace the damaged molecules, the cell must utilize energy and resources and thus undergoes a reprogramming process to survive.

1.3.2.1. DNA damage

Cell exposure to $O_2^{\cdot-}$, H_2O_2, ionizing radiation or organic hydroperoxides can lead to numerous types of DNA lesions; more than 100 products of oxidation of this macromolecule have been identified. ROS-induced DNA damage involves single- and double-stranded DNA breaks, purine, pyrimidine or deoxyribose modifications and DNA cross-links (Farr and Kogoma 1991). DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability (Valko et al. 2006). Strand breaks and lesions that block replication are more likely to contribute to lethality than base damage that does not hinder replication, although it may contribute significantly to mutagenesis.

The hydroxyl radical attacks the sugar moiety of DNA leading to sugar fragmentation and production of strand breaks (Farr and Kogoma 1991). Also permanent modification of purines and pyrimidines by this radical represents the first step involved in mutagenesis, ageing and carcinogenesis (Valko et al. 2006). Besides direct damage caused by ROS, organic radicals formed in the lipid peroxidation process can react with DNA. These radicals may inflict damage to purines by base-alkylation or formation of intra- or interstrand cross-links.

In prokaryotes, the enzymes exonuclease III (ExoIII), endonulcease IV (EndoIV) and excinuclease (A)BC have an important role in repairing the DNA damage provoked by oxidative stress by removing the damaged bases. DNA polymerases I and III are also important to repair the single-strand breaks that occur in presence of hydrogen peroxide (Farr and Kogoma 1991).

1.3.2.2. Membrane damage

Membranes are affected by ROS either by damage to lipids or proteins. Reactive oxygen species, such as the hydroxyl radical can lead to lipid peroxidation, this damage process entails three steps: initiation, propagation and termination (Figure 1.3). Initiation takes place when a lipid radical ($L^{\cdot}$) is formed by abstraction of hydrogen from an unsaturated fatty acid (LH). Then the lipid radical reacts with molecular oxygen to form a lipid peroxydienyl radical ($LOO^{\cdot}$) that in turn attacks another unsaturated fatty acid forming a lipid hydroperoxide (LOOH). The hydroperoxides can break down in presence of superoxide or reduced transition metals forming lipid peroxydienyl radicals ($LOO^{\cdot}$) or lipid alkoxydienyl radical ($LO^{\cdot}$), both capable of initiating new rounds of peroxidation (Farr and Kogoma 1991; Spiteller 2006).

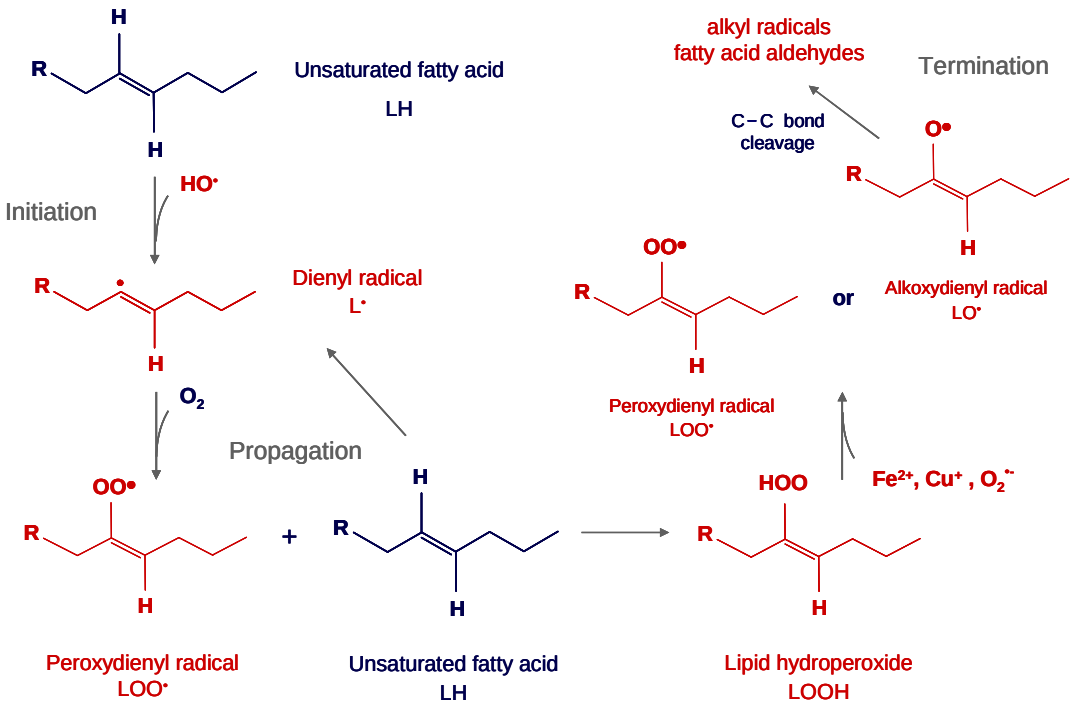


Figure 1.3 Lipid peroxidation process.

Lipid peroxidation generates products which are shorter than the initial lipid and subsequently, when fatty acids become shorter or gain charge, the membrane structure becomes more fluid. This increase in fluidity results in a loss of structural integrity compromising the transport of nutrients, ATP synthase (ATPase) activity and prevention of osmotic imbalance. The permeabilization of the membrane destroys the proton gradient leading to a lower internal pH which can result in further oxidative damage (Farr et al. 1988; Farr and Kogoma 1991). Another consequence of peroxidation is mutagenesis due to the formation of toxic intermediates and end products, such as alkanes, ketones, epoxides and aldehydes. These products can react with DNA and proteins leading to inter-strand (DNA-DNA) and DNA-protein cross-links (Chio and Tappel 1969; Segerback 1983; Summerfield and Tappel 1983).

Bacterial membranes contain mainly saturated and monounsaturated lipids thus decreasing the occurrence of lipid peroxidation, as the rate of fatty acid peroxidation is directly proportional to the number of unsatured (C=C) bonds (Farr and Kogoma 1991).

1.3.2.3. Protein damage

Oxygen radicals are capable of reacting with several protein residues leading to the inactivation of enzymes and, in some cases to their targeted degradation (Costa et al. 2007). Several studies demonstrated that the modification of proteins is mainly initiated by reaction with $HO^{\cdot}$. However, the course of the oxidation process is determined by the availability of O_2 and $O_2^{\cdot-}$ (Garrison et al. 1962; Schuessler and Schilling 1984; Garrison 1987). ROS can lead to the oxidation of amino acid side residue chains, formation of protein-protein cross-links or oxidation of the protein backbone that will lead to protein fragmentation (Berlett and Stadtman 1997). It was also shown that other forms of reactive species, besides $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$, may yield similar effects and that transition metal ions can substitute $HO^{\cdot}$ and $O_2^{\cdot-}$ in some of the reactions (Garrison 1987).

All amino acid residues are susceptible to oxidation by the hydroxyl radical, but cysteine and methionine are particularly sensitive to oxidation by all forms of ROS. Even under mild conditions, cysteine residues can be converted to disulfides and methionine residues are converted to methionine sulfoxide (MetSOX) residues. These are the only oxidative modifications that can be repaired, which

is performed by the disulfide and MetSOX reductases (Berlett and Stadtman 1997) (Figure 1.4).

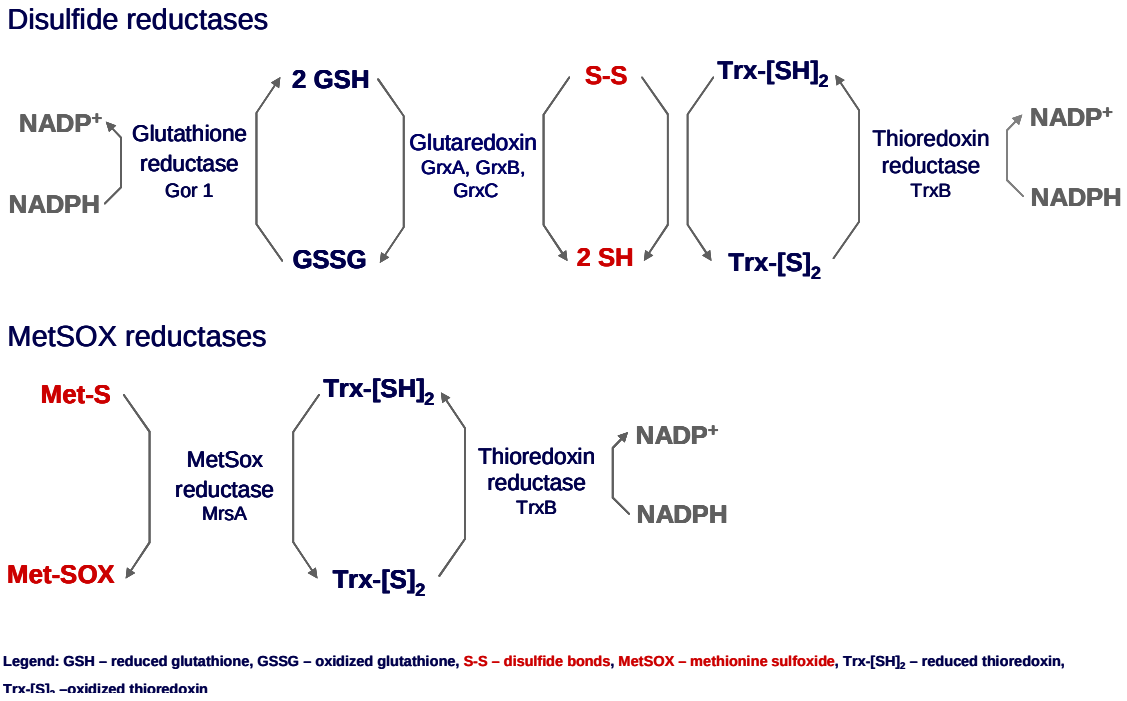


Figure 1.4 Protein damage repair systems.

The formation of carbonyl groups in proteins may arise from: i) the direct oxidation of different classes of amino acids, ii) reaction of proteins with by-products of lipid peroxidation or reactive carbonyl derivatives or iii) reaction of reducing sugars or their products with lysine residues. The presence of carbonyl groups in proteins has therefore been used as a marker for ROS-mediated protein oxidation (Berlett and Stadtman 1997; Costa et al. 2002).

The Fe-S clusters of dehydratases are vulnerable to oxidation by any small univalent oxidants that can penetrate into the active site and among these oxidants we can find $O_2^{\cdot-}$, H_2O_2 and peroxynitrite ($ONOO^-$) (Keyer and Imlay 1997; Djaman et al. 2004). These clusters appear to be primary targets during oxidative stress; when exposed to ROS, the clusters are converted to unstable forms that quickly decompose. This damage can result in protein inactivation and biochemical pathway failure. Furthermore, the destruction of Fe-S clusters leads to the release of iron ions that can participate in the Fenton reaction (Imlay 2006).

The intracellular level of oxidized proteins results from the rate of protein oxidation and the rate of oxidized protein degradation. This balance is achieved by a complex net of factors that lead to the formation of ROS and of the factors that determine the concentration/activity of the proteases responsible for the degradation of oxidized proteins (Berlett and Stadtman 1997).

1.4. Heavy metal – Definition

The term "heavy metal" has never been defined by any authoritative body such as IUPAC (International Union of Pure and Applied Chemistry). It is used as a group name for metals and semimetals (metalloids) that are associated with contamination and potential toxicity or ecotoxicity (Duffus 2002). Thus, it has been employed without an accurate definition although several definitions have been proposed. No relationship has been found between density (specific gravity) or other physicochemical concepts that might define heavy metals and the toxicity or ecotoxicity attributed to this group (Duffus 2002). In this work, the "heavy metal" definition employed is associated with metal toxicity.

1.5. Heavy metal pollution

Industrialization is a hallmark of civilization however, as a result of the industrial development, soil and waters became contaminated due to the generation of effluents containing toxics and heavy metals. The negative impact of pollution on society is growing and is a major concern worldwide and for this reason bioremediation of waters and soils is a main topic of research.

The heavy metals that are most commonly associated with anthropogenic activities (such as disposal of wastes in landfills, generation of chemical waste leachates and sludges) include Cd, Cr, Cu, Fe, Pb, Hg, Ni and Zn (Mulligan 2005). Regarding health issues, Cd, Pb, Hg and As (that is in fact a metalloid) have been identified as the most toxic. Cadmium is present in a wide number of equipments and consume goods; it is commonly used in re-chargeable batteries however, the most significant intake is by cigarette smoke and food. Mercury is also present in the food chain, being fish the main source of contact. The use of leaded gasoline has contributed to atmospheric emissions of Pb though exposure through food should not be underestimated. As to arsenic, the main source of exposure is

contaminated water and food (Järup 2003). At present, it is quite clear that metals are accumulated in the food chain where their concentrations get "biomagnified" and for this reason food constitutes the main source of heavy metal exposure/intake.

In the last years, the emissions of Cd, Pb and Hg have declined in European countries. Between 1990 and 2005 the trend of total anthropogenic emissions, in this region, decreased: 87% for lead, 50% for cadmium and 48% for mercury (Ilyin et al. 2007). The policy of decreasing heavy metal emissions is being followed by developed countries, while in the less developed countries it is a growing environmental risk.

1.6. Bacteria and heavy metals – resistance and bioremediation

From the physiological point of view, metals can be grouped into three main categories: (i) essential and basically non-toxic (e.g. Ca, K, Mg and Na), (ii) essential but harmful at high concentrations (e.g. Fe, Mn, Zn, Cu, Co, Ni and Mo), and (iii) toxic (e.g. Hg or Cd) (Valls and Lorenzo 2002). Both eukaryotic and prokaryotic cells require some metals in minute amounts for normal metabolism, as they are components of enzymes and their activity is often dependent on the presence of the metal. Other metals that can be present in cells serve no biological relevant function such as cadmium. However, it was recently found that cadmium can replace zinc in the active centre of the carbonic anhydrase in marine diatoms (Xu et al. 2008).

Cells have developed different mechanisms of interaction that allow them to keep or eliminate different metals. Regarding toxic metals, the question may arise whether the resistance mechanisms displayed by microbes are a response to pollution or whether they already existed. In reality, this question has a simple answer. The fact that these mechanisms are abundant and ubiquitous among bacteria that are present from pristine to heavily polluted sites is a clear argument in favour of the latter option (Silver and Phung 2005). The largest group of resistance systems functions by energy-dependent efflux of toxic ions (Figure 1.5) while fewer involve enzymatic transformations or metal-binding proteins (Silver and Phung 2005).

The transport of metals across the cell membranes is mediated by proteins belonging to different protein families. Some metals probably diffuse across the outer membrane through porins, such as the outer membrane protein F (OmpF).

At the cytoplasmic membrane a wide diversity of metal transporters, some for import and others for export, can be found: ABC (ATP-binding cassette)-type ATPases, P_1-type ATPases, RND (resistance and nodulation) proteins, cation diffusion facilitator (CDF) proteins, NiCoT (Ni^{2+} and Co^{2+} transporter) proteins, CorA (Co resistance), NRAMP (natural resistance associated with macrophage protein), and ZIP (Zrt-Irt-like protein)-family transporters (Waldron and Robinson 2009). The ABC-type ATPases selectively import Mn^{2+}, Zn^{2+}, Ni^{2+} or Fe^{2+} across the membrane aided by substrate binding proteins that may be located in the periplasm (Gram-negative bacteria) or associated with the plasma membrane (Gram-positive bacteria). The P_1-type ATPases are mainly metal exporters that possess a complex structure. CDF proteins drive the efflux of surplus transition metals, probably through proton antiport. RND proteins mediate the efflux of Co^{2+}, Zn^{2+}, Ni^{2+} or Cu^{+} across the outer membrane of Gram-negative bacteria (Waldron and Robinson 2009).

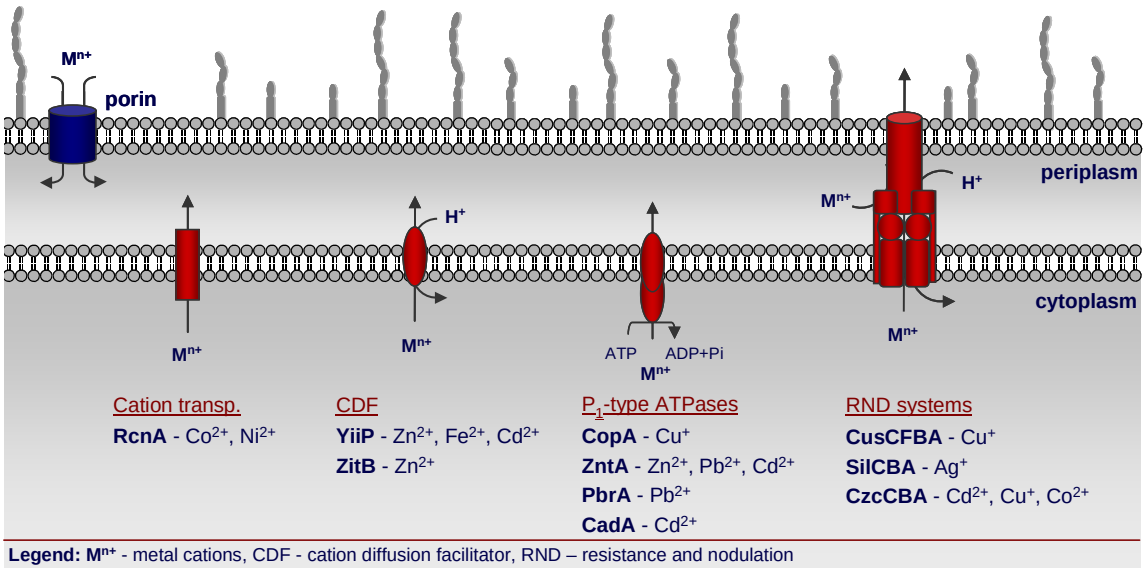


Figure 1.5 Prokaryotic efflux systems.

Resistance to Hg^{2+} and to organomercurials involves metal-binding and membrane transport proteins, as well as the mercuric reductase (MerA) and organomercurial lyase (MerB) that convert their substrates to less toxic forms. Arsenic resistance is mediated by the *ars* operon that is responsible for the metalloid efflux, the periplasmic arsenate reductase (terminal electron acceptor in anaerobic respiration) and the periplasmic arsenite oxidase (initial electron donor

in aerobic respiration). Metal resistance mediated by the metallothionein SmtA, chaperone CopZ and periplasmic silver binding protein SilE was also reported (Silver and Phung 2005).

Bacterial resistance has received much attention in the last decades and the knowledge of these mechanisms has contributed to understand the basis of biogeochemical cycles of several elements. Besides, it provided the possibility of using resistant microorganisms in biotechnological applications including bioremediation of soils and water contaminated with metals and/or organic pollutants. Bioremediation is the exploitation of biological activities for mitigation or elimination of the noxious effects caused by environmental pollutants (Valls and Lorenzo 2002) and can be generally classified as *in situ* or *ex situ*. The first involves treating the contaminated material at the site while the second involves the removal of this material. While biodegradation deals with the biological basis of the metabolism of the pollutants, bioremediation is about intervention with the aim to alleviate pollution and therefore is in the field of biotechnology. Depending on the degree of intervention, bioremediation includes natural attenuation, bio-stimulation or bio-augmentation. Natural attenuation is a strategy that requires little or no human action whereas bio-stimulation involves the addition of nutrients or electron donors/acceptors to promote the growth of microorganisms. At last, in bio-augmentation strategies natural or genetic engineered microorganisms with desired catalytic capabilities are deliberately added to the process (Valls and Lorenzo 2002).

Conventional decontamination strategies of organic pollutant contaminated sites (e.g. land-filling, recycling, pyrolysis and incineration) are inefficient, costly and can lead to the formation of toxic intermediates. The advantage of the biological methods is that microorganisms can degrade numerous pollutants without producing toxic intermediates (Paul et al. 2005). Biodegradation can be described as a process by which a toxic compound is transformed into a non-toxic one. Though many achievements have been reported in the basic and applied aspects of the biodegradative pathways and in the characterization of the enzymes involved, the development of bioremediation applications has not yet been fully accomplished (Parales and Haddock 2004). New molecular ecology techniques have enabled culture-independent approaches to analyze and assess biodegradation processes *in situ* and to characterize natural and engineered pollutant-degrading microbial associations (Paul et al. 2005; Deutschbauer et al. 2006; Singh 2006). Also genome sequencing of biodegradation-relevant

microorganisms, such as *Pseudomonas putida* KT2440, *Geobacter sulfurreducens*, *Rhodopseudomonas palustris* CG009 and *Shewanella oneidensis* MR-1, has provided the first whole-genome insight into the full genetic background of the metabolic capability and biodegradation versatility of these organisms (Heidelberg et al. 2002; Jimenez et al. 2002; Nelson et al. 2002; Methe et al. 2003; Larimer et al. 2004). Systems biology approaches are becoming increasingly helpful to unravel, predict and quantify metabolic activities in a particular organism or within microbial consortia (Pieper et al. 2004). The knowledge provided by these new approaches will certainly step up the development of bioremediation technologies and biotransformation processes.

Many contaminated sites are co-contaminated with organic and metal pollutants and, the latter may inhibit organic biodegradation by having an impact on the physiology of the degrading organisms and on the structure of microbial communities (Sandrin and Maier 2003). Treatment of co-contaminated sites is a complex problem, the remediation of toxic metals requires appropriate methods for their removal because, compared to organic pollutants, metals cannot be decomposed (Sandrin and Maier 2003). The most common physicochemical processes for removal of heavy metals from the environment such as oxidation, reduction, chemical precipitation, filtration, electrochemical treatment, evaporation, solubilisation, complexation, ion-exchange or reverse osmosis tend to be very expensive (Malik 2004; Lebeau et al. 2007). Some heavy metals are key components in different industrial applications thus, their recovery and recycling is a significant factor to be considered in the decrease of remediation costs (Malik 2004). Also advanced environmental regulations now compel industry to shift to cleaner production methods demanding the development of environmentally friendly, low-cost and efficient treatment for metal rich effluents.

Microorganisms have evolved various mechanisms to respond to heavy-metal stress, such as transport across the membrane, biosorption to cell walls, entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Malik 2004). Metal biosorption allows metal recovery through the use of biomass (not only from bacteria but also from algae and plants) that uptake or bind the heavy metals to cellular components. The concept of biosorption incorporates the use of both live and dead biomass and also biopolymers (Diels et al. 1999; Gadd 2000; Gutnick and Bach 2000). Although, the usage of synthetic resins is well established, biosorption methods are more effective in removing metals from solutions where they are usually present at low

concentrations. Another advantage of using the biological systems is the possibility of genetic modification to increase specificity towards certain metals or bioaccumulation yields (Valls and Lorenzo 2002).

Metal precipitation is another mechanism that microorganisms use to immobilise metals; by reducing these elements to a lower redox state, they may produce less reactive species (e.g. Fe, Co, U, Se, Te, Au and Cr). Chemical reduction is widespread among bacteria and is either the result of dissimilatory metabolism (e.g. U(VI), Cr(VI), Fe(III) and Mn(IV) reduction catalysed by *c*-type cytochromes or associated enzymes and by arsenate respiration) or a secondary consequence of diverse metabolic processes not directly related to the transformed metal. This indirect reduction takes place, for example, in the formation of metal sulfides (White et al. 1998; White and Gadd 2000). Another type of immobilisation mechanism is the precipitation of metal phosphates, which is a strategy that has received most interest (Keasling et al. 1998; Keasling et al. 2000; Macaskie et al. 2000). Bacteria are also able to transform certain metals species (e.g. Hg, As, Se and Te) through oxidation, reduction, methylation and alkylation yielding less toxic species (Tamaki and Frankenberger 1992; Michalke et al. 2000; Gihring and Banfield 2001).

1.7. Heavy metal-induced oxidative stress

Metal-induced toxicity involves multifactor mechanisms, such as the metal-induced reactive oxygen species mechanism. The Fenton-like reactions appear to play a major role in the oxidative stress observed in redox-active metal toxicity. It has also been reported that non-redox-active metals are able to induce oxidative stress, however, the mechanisms associated are still unclear. The hypothesis suggests that the depletion of the sulphydryl reserves of the cell is an important indirect mechanism for the stress induced by these metals (Ercal et al. 2001).

Transition metals act as catalysts in the oxidative deterioration of biological macromolecules and at least part of the toxicity associated to these metals is due to oxidative damage. It has been shown that metals like Fe, Cu, Cd, Cr, Pb, Hg, Ni and Va are able to induce the generation of reactive oxygen species (Stohs and Bagchi 1995). The mechanisms behind metal-induced oxidative stress will be briefly mentioned for some of the most significant metals.

1.7.1. Iron

Iron is an abundant metal, in fact it is the fourth most plentiful element in the Earth's crust. Iron can adopt different spin states both in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms, which makes it an extremely versatile prosthetic component for incorporation into proteins as a biocatalyst or electron carrier. Although there are few exceptions, iron is absolutely required for life, participating in many major biological processes such as: photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al. 2003). Together with copper, iron is the most studied transition metal.

When iron is bound to chelators by oxygen atoms (e.g. citrate) there is a decrease in the reduction potential of the metal. Therefore chelators with oxygen ligands promote oxidation of Fe(II) to Fe(III) with concomitant reduction of molecular oxygen to partially reduced oxygen species (Welch et al. 2002).

The involvement of iron in the Fenton reaction *in vivo* is controversial because the metal must be in a free or catalytically active form. Actually, little free iron exists in healthy cells due to the fact that intracellular iron can be oxidized and stored in iron storing proteins (e.g. ferritin) or associated with transport proteins (e.g. transferrin). The release of iron from ferritin by several xenobiotics has been demonstrated (Ryan and Aust 1992) therefore, compounds like paraquat and nitrofurantoin enhance the formation of ROS through an indirect mechanism that involves the release of iron from cell storages (Welch et al. 2002). There are also indications that iron complexed with nitrilotriacetate is involved, *in vitro*, in the production of single- and double-strand breaks in DNA (Toyokuni and Sangripanti 1993).

1.7.2. Copper

This metal is widespread in nature and is essential for life; it may function as a cofactor and is required for structural and catalytic properties of several key enzymes (Gaetke and Chow 2003). On the other hand, like iron, copper is a well known prooxidant and may participate in the Fenton reaction and in metal-catalyzed peroxidation of lipids (Valko et al. 2006). Similar to iron, copper is capable of originating ROS leading to DNA strand breaks and base oxidation (Kawanishi et al. 1989). It has been shown that physiological amounts of copper are essential for antioxidant defence capacity and there is an increased cellular

susceptibility to oxidative stress in Cu-deficiency situations (Gaetke and Chow 2003).

1.7.3. Lead

Lead is not a redox-active metal and, for this reason, the mechanisms through which it leads to oxidative stress are not clear. Several works report a direct correlation between the concentration of lead and lipid peroxidation (Rehman 1984; Rehman et al. 1995; Sandhir and Gill 1995; Yiin and Lin 1995). Based on these observations, several models were proposed for the prooxidant activity of this metal: i) direct effect on cell membranes, ii) interaction with haemoglobin, iii) δ-aminolevulinic acid (δ-ALA)-induced generation of ROS and iv) effect on the antioxidant defence system (Ercal et al. 2001).

The toxic effect of lead on membrane components is directly correlated to the composition of polyunsaturated fatty acids in this structure suggesting a possible association with a peroxidation process (Gurer and Ercal 2000). Also the incubation of lead with different polyunsaturated fatty acids (linoic, linolenic and arachidonic acid) resulted in enhanced malondialdehyde (MDA) concentration, one of the lipid peroxidation products (Yiin and Lin 1995). These data suggest that lead alters the membrane lipid composition resulting in compromised integrity and permeability making membranes more prone to damage (Gurer and Ercal 2000; Ercal et al. 2001).

As to the effect of lead on the antioxidant defence system of the cell, it was found that this metal interferes in GSH balance by inhibiting the GR. The inhibition of this enzyme prevents GSSG reduction, leaving cells more susceptible to oxidative damage (Schrauzer 1987; Sandhir and Gill 1995). Other enzymes like catalase, SOD and glutathione peroxidase (GPx) are also inhibited because lead interacts with the metals present in their structure (Howard 1974; Schrauzer 1987; Adler et al. 1993; Ariza et al. 1998).

1.7.4. Mercury

The two major absorbed species of mercury are elemental mercury (Hg^0) and methyl mercury (MeHg), the latter is commonly found in fish and shellfish while the former is the main component of dental amalgams. Elemental mercury is absorbed and once inside the cell it can be oxidized by catalase originating the highly reactive Hg^{2+} while MeHg due to its lipid soluble nature can easily cross membranes (Crinnion 2000). Mercury has a low clearance rate and accumulates in

the kidneys, nervous tissue and in the liver. The characteristics and biological reactivity of this metal suggest that oxidative stress is involved in its toxicity (Ercal et al. 2001). One of the most important mechanisms for oxidative stress induced by mercury is its reactivity with sulphydryls. Kidney GSH depletion was shown in rats exposed to mercury (Gstraunthaler et al. 1983), suggesting that this thiol constitutes a primary cell defence against this metal (Quig 1998). As a result from mercury exposure, increased lipid peroxidation has been reported (Gstraunthaler et al. 1983). Interference of this metal in oxidative phosphorylation has also been proposed, since it is known that MeHg can act as an uncoupling agent (Verity et al. 1975). In rat mitochondria incubated *in vitro* with mercuric ion, an increased production of H_2O_2 was detected and was accompanied by an increase in lipid peroxidation (Lund et al. 1991). The same authors confirmed the increased H_2O_2 production *in vivo*, and reported an alteration in mitochondrial calcium homeostasis (Lund et al. 1993). Methyl mercury was also shown to interfere with calcium homeostasis by accelerating the influx of the cation from the extracellular medium and by mobilizing intracellular calcium stores (Tan et al. 1993). The increased cytoplasmic concentration of calcium leads to the activation of hydrolytic enzymes, such as proteases, endonucleases and phospholipases. Activation of the phospholipase A_2 (PLA_2) by mercury was reported, this enzyme catalyzes the hydrolysis of membrane phospholipids resulting in an increased concentration of arachidonic acid (Mazerik et al. 2007). This unsaturated fatty acid is the preferred substrate for lipoxygenases and ciclooxygenases that are known sources of superoxide (Ercal et al. 2001). Another consequence of the increased concentration of calcium is the conversion of xanthine dehydrogenase to xanthine oxidase that leads to increased production of hydrogen peroxide and superoxide, that are by-products of the reaction catalyzed by this enzyme (Ariza et al. 1998). The displacement of iron and copper by mercury has been suggested to contribute to Fenton-mediated ROS formation (Verity et al. 1975; Huang et al. 1996). The effects of mercury on the antioxidant defences, like for other metals, are tissue-dependent. Besides interfering with the thiol pool of the cell, this metal interferes with enzymes like SOD, catalase, GPx and GR (Gstraunthaler et al. 1983; Ariza et al. 1998; Hussain et al. 1999).

1.7.5. Arsenic

Arsenic is a metalloid but due to its high toxicity, the elucidation of its mechanisms of action is mandatory. Arsenic has been implicated in several diseases including diabetes, hypertension and several types of tumours (Chen and Wang 1990; Lai et al. 1994; Chen et al. 1995). The generation of ROS and the interference with the antioxidant defence systems are included in the mechanisms of toxicity proposed for this metalloid. Arsenate (AsO_4^{3-}) is a molecular analogue of phosphate and enters the cell through phosphate transporters inhibiting oxidative phosphorylation. As to arsenite (AsO_2^-), this form of arsenic is generally more toxic because it can bind to sulphydryl groups impairing the function of proteins, such as pyruvate dehydrogenase and 2-oxo-glutarate dehydrogenase that are involved in respiration (Oremland and Stolz 2003). It was also shown that methylated organoarsenicals (e.g. dimethylarsinic acid and dimethylarsine) can undergo a series of reactions that originate oxygen and arsenic radicals (dimethylarsenic radical and demethylarsenic peroxyl radical). The involvement of cellular iron and other transition metals in this process was also suggested (Yamanaka et al. 1989; Yamanaka et al. 1990; Ercal et al. 2001). More recently, the production of H_2O_2 involving the oxidation of As (III) to As (V) was proposed (Valko et al. 2006).

Arsenic compounds have been shown to interfere with the antioxidant defence system but their effects are tissue-dependent. Interference with the GSH metabolism enzymes such as GPx, GR and glutathione transferase (GST) and also with other enzymes of the antioxidant defence system like catalase and SOD was observed (Ercal et al. 2001).

1.7.6. Chromium

The use of chromium in industry is widespread (as a constituent of alloys, leather tanning, pigments and electroplating) and for this reason, this metal has become a serious pollutant present in air, soil and water. Although it is able to exist in several oxidation states, the most stable and common forms are the trivalent Cr(III) and hexavalent Cr(VI) species (Cervantes et al. 2001). The hexavalent species is considered the most toxic form of Cr and is usually present in the chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ionic forms. Transport of Cr (VI) through the sulphate transport system was shown for different bacteria but, in the yeast *S. cerevisiae* this metal may enter the cell through a non-specific carrier such as the permease system that transports sulphate and phosphate (Cervantes

et al. 2001). Once inside the cell, Cr(VI) can undergo a reductive metabolism to Cr (III). The latter form is usually present in the form of oxides, hydroxides or sulphates and is found mainly bound to organic matter (Cervantes et al. 2001). Trivalent chromium is probably uptaken through an iron transporter (Ramana and Sastry 1994).

Regarding the biological effects of chromium, the toxicity of this metal seems to be related to the process of reduction of Cr(VI) to lower oxidation states (Equations 5-9) in which oxygen radicals are generated (Kawanishi et al. 1986; Cervantes et al. 2001; Liu and Shi 2001). For instance, hexavalent chromium can be reduced *in vivo* by ascorbate or GSH leading to the transient formation of Cr(V) complexes (Equation 5),which in turn react with H_2O_2 to generate $HO^{\cdot}$ (Equation 6) (Shi and Dalal 1990).

$$Cr(VI) + GSH \rightarrow Cr(V) + GS^{\cdot} \qquad \text{Equation 5}$$

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + HO^{\cdot} + HO^{-} \qquad \text{Equation 6}$$

$$Cr(V) + GSH \rightarrow Cr(IV) + GS^{\cdot} \qquad \text{Equation 7}$$

$$Cr(IV) + H_2O_2 \rightarrow Cr(V) + HO^{\cdot} + HO^{-} \qquad \text{Equation 8}$$

$$Cr(IV) + GSH \rightarrow Cr(III) + GS^{\cdot} \qquad \text{Equation 9}$$

The trivalent form may be sequestered by DNA phosphate groups affecting replication, transcription and causing mutagenesis (Kortenkamp et al. 1991; Bridgewater et al. 1994). In this form chromium may also react with carboxyl and sulphydryl groups of enzymes leading to alterations in their structure and activity (Cervantes et al. 2001).

1.7.7. Nickel

Small amounts of this metal have been shown to be essential for normal growth and reproduction in some animals, and may also be essential to humans (Valko et al. 2006). On the other hand, nickel dermatitis is the most common form of allergic contact dermatitis. This metal is a known human carcinogen that alters gene expression by enhancing DNA methylation and packaging rather than through mutagenic mechanisms (Lee et al. 1995). In addition, nickel may interfere with DNA repair systems and in high doses may induce lipid peroxidation and protein carbonylation (Valko et al. 2006). In rats it was observed

that exposure to nickel results in enhanced lipid peroxidation, decrease of GPx activity and increased iron levels (Athar et al. 1987). Another potential nickel toxicity mechanism involving cellular antioxidants like GSH was suggested (Shi et al. 1992). Stohs and Bagchi (1995) proposed that nickel detoxification by GSH will result in the production of glutathione radicals that will lead to the generation of superoxide. Therefore, cellular reductants may facilitate nickel-mediated free radical generation *via* the "radical sink" pathway that proposes that superoxide acts as a sink for intracellular generated radicals (Winterbourn 1993). Concerning nickel toxicity, incubation of this metal with oligopeptides considered to be antioxidants (glutathione, carnosine and homocarnosine) resulted in formation of free radicals from lipid hydroperoxides (Shi et al. 1992). The nickel-induced GSH depletion may be another toxicity mechanism for this metal (Li et al. 1993).

1.8. Metal-induced oxidative stress in Escherichia coli

Most of the works on the effects of heavy metals in prokaryotes published to date have focused on long-term resistance while only few addressed stress response (Ferianc et al. 1998; Puskárová et al. 2002). This section will focus just on the literature on heavy metal stress in *Escherichia coli*, particularly on cadmium.

1.8.1. Cadmium

Cadmium is a toxic metal that can be found in food, water and tobacco smoke and thus can be uptaken by humans through ingestion or inhalation. This metal is also released by treatment of urban waste containing Li-Cd batteries (now discontinued or treated separately) and as a by-product of zinc and lead mining and smelting (Stohs et al. 2000).

Cadmium, like lead, is a non-redox metal and therefore its link with oxidative stress is not straightforward. This metal accumulates in soft tissues especially in liver and kidneys: its half-life in the human body is long because it is excreted very slowly (Jones and Cherian 1990).

Although the effects and response to short-term cadmium exposure have been studied, the mechanisms of cadmium toxicity and cell defences remain unclear.

Like for other toxic metals, cadmium enters the cell gratuitously through essential metal transport systems. First Laddaga and Silver (1985) reported that

cadmium uptake in *E. coli* occurred by an active transport system and later it was suggested that cadmium uptake is mediated by a zinc transporter, ZupT (Grass et al. 2002). Early works reported that cell exposure to low Cd^{2+} concentrations (3 μM) induced temporary cell growth stasis. During this phase, Cd-induced DNA damage was repaired and cell physiology adjusted to limit the distribution of the ion within the cell (Mitra et al. 1975). It was also reported that there was an increase in single-strand DNA breakage, related to the inhibition of enzymes involved in DNA replication and not to direct interaction of the metal with the genetic material (Mitra and Bernstein 1977; Mitra and Bernstein 1978).

A loss of cell viability concomitant to an increased synthesis of specific proteins was observed (Khazaeli and Mitra 1981; Mitra 1984; Morozzi et al. 1993). Indeed, the induction of the *recA* gene was observed and this lead to the activation of the SOS response and also of DNA-repairing enzymes (VanBogelen et al. 1987; Shapiro and Keasling 1996; Ferianc et al. 1998), thus cell viability was recovered (Mitra and Bernstein 1978; Mitra 1984).

Proteomic studies in *E. coli* (VanBogelen et al. 1987; Ferianc et al. 1998) confirmed the activation of the stringent response, SOS, heat shock and oxidative stress regulons. Their activation is only a part of the response to cadmium because more than 20 proteins are induced outside these regulons (VanBogelen et al. 1987). It was also reported that the *oxyR* regulon was induced during the first hour of Cd-exposure decreasing to normal levels after recovery of growth, suggesting that the cadmium-induced oxidative stress was higher in the initial phase and was not sustained during the growth period (Ferianc et al. 1998).

Efflux systems constitute an effective mechanism for the elimination of heavy metals from the cell. In *E. coli* the overexpression of the *zntA* gene enconding a P-type ATPase was found to increase tolerance against Zn and Cd while the mutant exhibited hypersensitivity to the two metals (Blencowe et al. 1997; Rensing et al. 1997). It was also reported that the highest activity for the ZntA pump was obtained when the metals were present as thiolate complexes of cysteine or glutathione suggesting that the metal-thiolate complexes are the substrate for the pump *in vivo* (Sharma et al. 2000). This observation is in agreement with the fact that glutathione has an important role in the detoxification of heavy metals.

More recently, a microarray study was published in which the transcriptional response to cadmium (Wang and Crowley 2005) was analysed. In this work it was observed that when cells were exposed to the metal there was a shift from aerobic respiration to the anaerobic metabolism. At the same time, there was an

activation of the low-energy-requiring transport systems for the import of amino acids and carbon sources while high-energy systems were shut down. A down-regulation of the genes involved in DNA replication, amino acids biosynthesis and protein translation was also observed suggesting an arrest in DNA and protein synthesis. According to previous observations, induction of a complex network of regulatory systems was observed, including genes for DNA repair, heat shock, oxidative stress, cold shock, osmotic stress as well as efflux systems for heavy metals. The authors also report that there was no induction of the *oxyR* regulon which prompted them to suggest that damage caused by cadmium was not directly associated with ROS. However, it has to be noticed that the concentration of $CdCl_2$ used in this study was very low, 1 $mg \cdot L^{-1}$.

1.8.2. Other metals

Early works focusing on the toxicity of metals such as zinc, cobalt and silver reported an interference of these metals with the respiratory chain however, the mechanisms and targets need to be further elucidated. Zinc is a known inhibitor of respiration by preventing electron transfer at the level of the succinate dehydrogenase and of the two terminal oxidase complexes (cytochromes *bd* and bo_3) (Kasahara and Anraku 1972; Kita et al. 1984; Poole et al. 1989). Inhibition of the terminal oxidases by cobalt and cadmium was also reported (Kita et al. 1984; Beard et al. 1995). The NADH dehydrogenase complexes have also been identified as possible inhibition sites by silver and zinc (Nicholls and Malviya 1968; Bragg and Rainnie 1974).

Recently, the *E. coli* stress responses induced by copper (Kershaw et al. 2005), zinc (Lee et al. 2005), chromium (Ackerley et al. 2006) and cobalt (Ranquet et al. 2007) were published.

Kershaw et al. (2005) analysed the transcriptional responses to a minimal (no copper added), optimal (750 µM) and excess (2 mM) copper concentrations. The excess copper concentration resulted in increased transcription of the superoxide stress response, iron homeostasis and envelope stress. Up-regulation of the copper-efflux systems and increased expression of the *cpx* regulon, the last possibly due to accumulation of misfolded proteins, was also observed. The production of ROS resulting from redox cycling of copper is most likely at the origin of the abnormal proteins. Previous works reported that exposure to copper caused superoxide-induced stress in *E. coli* (Kimura and Nishioka 1997), which

matches with the induction of the *soxRS* regulon. The *znuABC* genes were down-regulated under high copper, since these genes encode a zinc ABC transporter, this suggests that this pump may function also as a copper-import system. This is an interesting observation since a specific copper-uptake system has not yet been identified in this bacterium.

The transcriptome profile for zinc stress was analysed by Lee and colleagues (2005), but most of the genes whose transcription was altered by this metal have unclassified or unknown function. Genes involved in the maintenance of cell membrane structure, membrane transport, sensing and regulation were highly represented among those up-regulated. The well known zinc efflux systems (*zntA* and *cus* operon) and an antibiotic resistance system (the *mtdBC* operon) were induced. The two-component systems BaeRS and BasRS were also increased; the former regulates the *mtd* operon while the latter together with the Rcs signalling system is involved in the regulation of synthesis and modification of lipopolysaccharides. The down-regulated group included genes that are known to be induced in the stationary phase and encode proteins involved in detoxification and resistance mechanisms. It was also observed that genes encoding proteins that provide protection against acid stress had reduced transcription under zinc conditions. Two of these genes, *gadB* and *gadC* code for proteins proposed to function together to help maintain the intracellular pH when cells are exposed to extremely acidic conditions.

Transcriptomics works, like the ones referred above, generate a bulk of results and, most of the times, they report the up- and down-regulation of genes giving little insight to mechanisms of toxicity and the physiological status of the cell.

In the work by Ackerley et al. (2006) the effect of chromate stress in *E. coli* was addressed. When the chromate was added to the cultures there was growth stasis and cells exhibited aberrant filamentous morphology. There was chromate-induced ROS generation that was accompanied by a decline in the levels of glutathione. Although the antioxidant capabilities of GSH are well established, as already reported for other stress defences, this molecule was not indispensable for protection against chromate. In fact some speculation on the participation of GSH leading to negative effects, such as redox-cycling of chromium (Shi and Dalal 1988) and the formation of GSH-Cr(III)-DNA adducts has been reported (Voitkun et al. 1998). Ackerley and colleagues (2006) conclude that oxidative stress plays a major role in chromate toxicity and the cell defence mechanism involves the activation of antioxidant mechanisms. Enzymes with potential antioxidant role

were induced including the FeSOD (SodB) and two other enzymes that contribute to cysteine biosynthesis (CysN and CysK). Activation of the SOS response was also observed when cells were exposed to chromate.

The work focusing on cobalt stress (Ranquet et al. 2007) revealed that exposure to this metal leads to inactivation of Fe-S enzymes and to activation of the *suf* operon, that is involved in Fe-S cluster repair and assembly under stress conditions (e.g. oxidative stress and iron limitation). Cobalt inactivation of the Fe-S proteins does not result from the direct interaction of the metal with the clusters; instead it interferes with the scaffold proteins during cluster assembly. This interference results in the incorporation of cobalt in the clusters that leads to the inactivation of Fe-S proteins. Clearly cobalt toxicity results in changes in iron homeostasis leading to iron depletion, which had already been reported for *Neurospora crassa* (Venkateswerlu and Sastry 1970). According to this, increased expression of iron uptake genes was observed. It was also suggested that there is a yet to be explained link between cobalt toxicity and oxidative stress, since cells grown in aerobic conditions are more sensitive to this metal than cells grown in an anoxic environment (Wu et al. 1994; Ranquet et al. 2007).

1.9. Cadmium and other prokaryotes

In the Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*) and *Lactobacillus plantarum* the uptake of cadmium is mediated by the Mn^{2+} active transport system (Weiss et al. 1978; Tynecka et al. 1981; Perry and Silver 1982; Archibald and Duong 1984; Laddaga et al. 1985).

Inside the cell, numerous toxic effects of this metal have been observed: interference in cell growth, protein inactivation due to binding to sulphydryl groups and substitution for other metals in protein active centres (Babich and Stotzky 1977; Aiking et al. 1982; Trevors et al. 1986; Cunningham and Lundie 1993). In cultures of *B. subtilis* exposed to cadmium there was a decrease in cell viability and also an inhibition in the oxygen consumption rates (Surowitz et al. 1984). The accumulation of cadmium in *S. aureus* also lead to the inhibition of respiration and ATP synthesis due to loss of membrane potential (Tynecka et al. 1990). A similar mechanism of interference was proposed for zinc in *Azotobacter vinelandii* and *Salmonella typhimurium* (Singh and Bragg 1974; Kleiner 1978). Induction of high molecular weight proteins was reported for *Pseudomonas putida*, *Vibrio alginoliticus* and *Rhizobium leguminosarum* (Higham et al. 1984; Harwood-Sears and Gordon 1990; Pereira et al. 2006), in the presence of

cadmium or copper. In *Xanthomonas campestris* cadmium was shown to induce peroxide protective enzymes, such as the alkylhydroperoxide reductase, catalase and the organic hydroperoxide resistance protein. It was also reported that an increase in the hydroperoxide levels was responsible for cadmium toxicity in this bacterium (Banjerdkij et al. 2005). In *Caulobacter crescentus* activation of several enzymes like SOD and enzymes related to GSH metabolism and DNA repair was observed in the presence of cadmium (Hu et al. 2005).

1.10. Cadmium and Saccharomyces cerevisiae

The proteomic analysis of the cadmium response in baker's yeast revealed that enzymes of the cysteine and GSH biosynthesis pathways and other proteins with antioxidant properties were highly induced (Vido et al. 2001). The analysis of the transcriptome confirmed the increased expression of the corresponding genes (Momose and Iwahashi 2001) confirming that the thioredoxin- and glutathione-dependent redox systems are essential for defence against cadmium.

In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) proteomic analysis of the cadmium response revealed the increased expression of genes related to the detoxification of ROS and repair of the damage induced by them. Although the cadmium detoxification mechanisms in this yeast differ from those in *S. cerevisiae*, the genes involved in the thiol redox systems were also up-regulated in this organism (Bae and Chen 2004).

In *S. cerevisiae*, the uptake of Cd^{2+} is mediated by Zrt1 (Gomes et al. 2002) and Fet4 (Liu et al. 1997). Inside the cell, cadmium is detoxified by glutathione or phytochelatins and sequestered by metallothioneins, the general mechanisms by which eukaryotes detoxify cadmium (Hatcher et al. 1995; Li et al. 1996; Perego and Howell 1997; Adamis et al. 2004). The cadmium-glutathione complex is transported to the vacuole by the Ycf1 pump and thus removed from the cytoplasm (Li et al. 1996; Li et al. 1997). Brennan and Schiestl (1996) reported that cadmium is an inducer of oxidative stress in *S. cerevisiae* and its toxicity resulted in decreased cell viability. The transcription factor Yap1p that regulates the response to oxidative stress was also induced (Vido et al. 2001). Strains deficient in the antioxidant defence enzymes were hypersensitive to cadmium thus decreasing the cytosolic pool of glutathione is probably the cause for the oxidative stress that follows cadmium treatment. In the case of cadmium, it was proposed that the depletion of GSH would lead to the accumulation of ROS that

would then provoke cell damage. Indeed, in yeast and higher fungi increased lipid peroxidation was detected in presence of this metal (Gadd 1993; Vido et al. 2001), leading to the permeabilization of the plasma membrane and K^+ efflux (Gadd 1993; Howlett and Avery 1997). Cadmium is also considered a mutagen and in *S. cerevisiae* it was observed that the metal inhibits the DNA mismatch repair (MMR) system, *in vivo* (Jin et al. 2003). This inhibition is due to the fact the Msh2-Msh6 complex is unable to hydrolyse ATP and to bind DNA (Clark and Kunkel 2004; Banerjee and Flores-Rozas 2005), and not to the interaction of the ion with zinc-metalloproteins. Besides damage to lipids and DNA, protein damage was also observed in *S. cerevisiae*. The increased expression of the ubiquitin-dependent proteolysis pathway in cadmium-exposed conditions was observed and indeed mutants deficient in this mechanism revealed to be hypersensitive to this metal (Jungmann et al. 1993).

1.11. Cadmium and other eukaryotes

Several studies have been published on the effects of chronic exposure to cadmium which reveal that the concern about this metal is rising due to its high toxicity at low concentrations. It constitutes a serious health hazard that in humans has been implicated in several diseases namely in different types of cancer. Among its effects are: inhibition of DNA repair and methylation, interference with the cellular antioxidant system, disruption of cell adhesion and induction of apoptosis (Stohs and Bagchi 1995; Waisberg et al. 2003; Martelli et al. 2006).

As reported for other organisms, in higher eukaryotes cadmium enters the cells through the essential metal transporters, such as the proton-coupled iron transporter DMT1, the calcium channels and zinc transporters (Martelli et al. 2006). This entrance mechanism, known as ionic mimicry, refers to the ability of a cationic form of a toxic metal to mimic an essential element or its cationic species at the site of its transporter (Bridges and Zalups 2005). Molecular mimicry constitutes another entrance mechanism for toxic metals that refers to the ability of a metal ion to bind to an endogenous organic molecule to form an organic metal species that acts as a structural or functional mimic of essential molecules at the site of their transporters (Bridges and Zalups 2005). Receptor-mediated endocytosis of Cd-methallothionein, Cd-albumin and Cd-ferritin or transferrin complexes has been reported (Bridges and Zalups 2005; Martelli et al. 2006).

Cadmium can act as a mutagen, indirectly, by interfering with the DNA repair system. *In vitro* studies using human cell extracts demonstrated that cadmium inhibits the DNA mismatch repair system (MMR) activity (Jin et al. 2003). Damage induced by cadmium on lipids and proteins has also been reported. This heavy metal interacts with thiol groups of proteins resulting in structural modification and/or in their inactivation (Thévenod and Friedmann 1999; Chrestensen et al. 2000). Exposure to cadmium also promoted the accumulation of ubiquitinated proteins (Figueiredo-Pereira et al. 1997; Thévenod and Friedmann 1999), which is the signal for recognition and degradation by the proteasome complex.

Although cadmium itself is not able to generate free radicals directly, the increase of various oxygen intermediates like superoxide radical, hydroxyl radical, nitric oxide and also hydrogen peroxide has been reported (Galan et al. 2001; Watanabe et al. 2003). As a consequence of ROS production, increased levels of lipid peroxidation were observed (Manca et al. 1991; Nigam et al. 1999; Tandon et al. 2003). Cadmium was also shown to induce specific alterations in mitochondria which are the main source of ROS in eukaryotic cells. In different guinea pig tissues, this ion was shown to interfere with the electron transport chain leading to the accumulation of semiubiquinones (Wang et al. 2004). These molecules are unstable and prone to donating electrons to molecular oxygen, generating superoxide radicals. The dissipation of mitochondrial membrane potential and generation of hydroxyl radical was also reported in a different work (Bolduc et al. 2004).

To explain the induction of oxidative stress by cadmium, many hypotheses have been proposed: displacement of redox active metals, depletion of endogenous radical scavengers or interference with the activity of antioxidant enzymes. In the presence of cadmium increased iron content was detected, probably due to iron displacement from binding sites which lead to iron-mediated lipid peroxidation (Koizumi and Li 1992). The substitution of copper and iron by cadmium in various cytoplasmic and membrane proteins was proposed (Casalino et al. 1997) and the subsequent release of these redox-active metals would then lead to the production of ROS through the Fenton reaction.

Several evidences point to an interaction of cadmium with GSH: some report the increase in the levels of this molecule following cadmium exposure (Kamiyama et al. 1995; Rana and Verma 1996) while others its depletion (Shibasaki et al. 1996; Karmakar et al. 1998). Glutathione depletion has been put forward as the main cause for the indirect generation of ROS giving rise to

oxidative stress (Stohs and Bagchi 1995; Howlett and Avery 1997; Ercal et al. 2001; Banjerdkij et al. 2005; Liu et al. 2005; Wolf and Baynes 2007). Cadmium was shown to interfere with the activity of several antioxidant defence enzymes but the effects of this heavy metal on the different enzymes are tissue-dependent.

1.12. Scope of this thesis

A number of reports indicated that cadmium is a powerful oxidant thus its toxicity is associated with oxidative stress. However, cadmium is not a redox-active metal, and 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 defences. In this study, we wanted to understand the molecular mechanisms underlying cadmium toxicity in *Escherichia coli* and assess the role of oxidative stress in the process. Furthermore, we aimed to test the relative importance of previously proposed mechanisms of cadmium toxicity in eukaryotes, such as inhibition of respiration or glutathione depletion.

The work was initiated with the set up of the physiological conditions to analyze the effect of cadmium on growth and culturability, this was followed by the assessment of oxidative damage on proteins and lipids (Chapter 3). The antioxidant cell defences were also analyzed in the presence and absence of the heavy metal (Chapter 4). Subsequently, oxygen consumption and reactive oxygen species production was investigated (Chapter 5). The data gathered within this work together with the literature allowed us to propose a model for cadmium-induced toxicity in *E. coli* cells.

2. Materials and Methods

2.1. Strains used

Table 2.1 – *Escherichia coli* strains used

Strain	Genotype	Source/Reference
E.coli K-12 MG1655	F^-, λ^-, *ilvG*$^-$, *rfb*-50, *rph*-1	*E. coli* Genetic Stock Center
E. coli K-12 BW25113	F^-, λ^-, *rrnB*$_{T14}$, *rph*-1 Δ*lacZ*$_{WJ16}$, *hsdR*514, Δ*ara*BAD$_{AH33}$, Δ*rha*BAD$_{LD78}$	Baba et al. (2006)
ΔgshA	BW *gshA*::Km^R	
ΔgshB	BW *gshB*::Km^R	
ΔkatE	BW *katE*::Km^R	
ΔkatG	BW *katG*::Km^R	
Δndh	BW *ndh*::Km^R	
ΔnuoB	BW *nuoB*::Km^R	
ΔubiE	BW *ubiE*::Km^R	

2.2. Media

Luria-Bertani plates (LB agar) were prepared as described in Sambrook et al. (1989) and 15 $g \cdot L^{-1}$ of agar was added prior to sterilisation.

The minimal medium was prepared according to the following formula: NaCl 0.5 $g \cdot L^{-1}$, NH_4Cl 1.0 $g \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 49.4 $mg \cdot L^{-1}$, K_2SO_4 48 $mg \cdot L^{-1}$, $K_2HPO_4 \cdot 3H_2O$ 46 $mg \cdot L^{-1}$, micronutrient solution (Tuovinen and Kelly 1973) 2 $mL \cdot L^{-1}$, 3-(N-Morpholino)propanesulfonic acid (MOPS) 40 mM pH 7.4 and glucose 20 mM.

Antibiotics were used in plates and pre-cultures of the mutant strains, at the following final concentrations: kanamycin 25 $\mu g \cdot mL^{-1}$ and tetracycline 12 $\mu g \cdot mL^{-1}$.

2.3. Growth and maintenance of bacterial cultures

2.3.1. Maintenance of cultures

Escherichia coli strains were maintained by subculturing every week on LB agar plates, incubated at 37 ºC o.n. and then stored at room temperature.

2.3.2. Growth conditions

2.3.2.1. Aerobic growth

Cultures were grown using Erlenmeyer flasks maintaining a minimal medium to headspace proportion of 1:4. Cultures were inoculated from o.n. pre-cultures and incubated in an orbital shaker (130 rpm) at 37 ºC.

2.3.2.2. Fermentative growth

For growth in the absence of oxygen, 100 mL Erlenmeyer flasks containing a magnetic bar were filled almost to the top with sterile medium. The stripping of oxygen was achieved by bubbling the medium with a sterile nitrogen stream and to avoid contact with air, sterile SubaSeal® rubber stoppers were used. Subsequent additions (of inoculum and cadmium solution) were delivered through the stopper septa using sterile syringes and needles. Cultures were inoculated from fermentative o.n. pre-cultures and grown on a magnetic multi-stirrer (130 rpm) at 37 ºC.

2.4. Growth curves and culturability assessment

To determine the effect of cadmium on growth, the OD_{420} of cultures was followed until it reached the early logarithmic phase (OD=0.5). At this stage, cultures grown in aerobic conditions were split into control and treated cultures. The latter received 30 $mg \cdot L^{-1}$ cadmium ($CdCl_2$ 273 µM) and then growth was monitored for the following hours or days. When the effect of glutathione on growth was assessed, 2 mM GSH or GSSG was added 5 min prior to the addition of cadmium, both in control and cadmium-treated cultures.

In the case of fermentation the cultures could not be divided to maintain anoxic conditions so control and treated cultures were grown separately.

To assess the effect of cadmium on culturability, aliquots were drawn at different time-points and serial diluted in saline solution (NaCl 0.9%). Dilutions were plated on LB agar and incubated o.n. at 37 ºC; the next day the colony forming units (CFU) were counted. Culturability was expressed as the percentage of CFU per mL.

2.5. Sample collection and cell extracts preparation

Cell cultures were grown to an OD_{420}=0.5 and at this point the cultures were divided in two: one received 30 $mg \cdot L^{-1}$ $CdCl_2$ and was incubated for 1 h while the other, which served as a negative control, was immediately harvested. Cells were collected by centrifugation for 10 min at 20,000 x*g*, 4 ºC and then washed twice in cold phosphate buffer 50 mM pH 7.0. The cell pellets were used immediately or stored at -80 ºC for later usage. *E. coli* cell pellets were resuspended in 250 µL cold potassium phosphate 50 mM pH 7.0 containing protease inhibitors

(Complete™ Mini EDTA-free Protease inhibitor, Roche), that were added according to manufacturer's instructions. 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 1 min off duty and then centrifuged for 8 min at 16,000 x*g*, 4 ºC. The supernatants were kept.

2.6. Protein quantification

Protein concentration was measured using the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

2.7. Oxidative damage assessment

2.7.1. Lipid peroxidation

The determination of lipid peroxidation was performed according to Steels et al. (1994) with adaptations: 10% trichloroacetic acid was added (final concentration) to each sample and the mixture was vortexed at maximum speed for 2 min. Extracts were then centrifuged for 15 min at 2,000 x*g*, 4 ºC 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. The results were expressed as mol of MDA per mg of protein.

2.7.2. Protein carbonylation

The levels of oxidized proteins were measured by immunodetection of carbonyls. The protein samples 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-DNP IgG (Dako Cytomation, Glostrup, DK) at 1:5000 dilution as primary antibody and goat anti-rabbit IgG peroxidase conjugate (Sigma, St. Louis, USA) at 1:5000 dilution as secondary antibody. Detection was performed using the ECL Western Blotting kit (GE Healthcare, UK). Acquisition and analysis of SDS-PAGE gels and carbonyl immunodetection films was performed using the Quantity One® programme version 4.5 (Bio-Rad, Bio-Rad Laboratories).

2.7.3. Protein identification

This procedure was performed by the staff at the proteomics unit - IPATIMUP (Institute for Molecular Pathology and Immunology of the University of Porto).

The proteins for identification were obtained by excising the protein bands from the SDS-PAGE gels. The samples were tripsin-digested in gel followed by a peptide extraction from the gel. The peptide extract was prepared for identification by MALDI-TOF. A Peptide Mass Fingerprint (PMF) was performed using as internal standards the peptides resulting from the tripsin autolysis. In the PMF the experimental mass spectra obtained are compared to the mass spectra derived from theoretical tripsin digestion of all the proteins that are listed in a given database. The proteins with the spectra with higher similarity are identified as the most probable protein for each sample. The experimental spectra were compared to the theoretical spectra of the proteins in the *E. coli* SwissProt database.

2.8. Antioxidant defences

2.8.1. Catalase activity

For the determination of catalase activity, *E. coli* extracts were dialyzed o.n. against cold potassium phosphate buffer 50 mM, EDTA 0.1 mM pH 7.8. 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 ºC. Catalase activity was expressed as catalase units per mg of protein.

Catalase gel zymography was performed by running the protein samples in native polyacrilamide gel (7.5% (w/v)) at 4ºC (running buffer glycine 192 mM, Tris-HCl 25 mM, pH 8.3) and developing the gel as described in Clare et al (1984).

2.8.2. Peroxidase activity

Peroxidase gel zymography was performed by running the samples in native polyacrilamide gels (10% (w/v)) as described for catalase zymography, and staining the gel using the 4-chloro-1-naphthol (4-CN) and the *N,N*-diethylphenylenediamine monohydrochloride (DEPDA) as described by Conyers and Kidwell (1991).

2.8.3.SOD activity

Superoxide dismutase activity was quantified as described by Flohé and Ötting (1984) by the xanthine/ xanthine oxidase/ cytochrome *c* method. One SOD unit is defined as the quantity of enzyme necessary to cut by 50% the cytochrome c reduction rate at 25 ºC. The enzyme activity was expressed as SOD units per mg of protein.

SOD gel zymography was performed by running the samples in native polyacrilamide gels (10% (w/v)) as described for catalase zymography, and developing the gel according to Beauchamp and Fridovich (1971).

2.8.4.Glutathione measurement

Total and oxidized glutathione (GSSG) were determined by the DTNB-GSSG reductase recycling method as described by Akerboom et al. (1981). To perform this measurement, cell pellets were resuspended in 200 µL potassium phosphate buffer 100 mM, EDTA 2 mM pH 7.4 and 200 µL $HClO_4$ 2 M; then cell suspensions were sonicated and spun as described in section 2.5. After centrifuging, the supernatants were immediately neutralized to pH 6-7 using 200 µL KOH 2 M, MOPS 0.3 M and spun again for 2 min. Then the neutralized samples were used to determine the total intracellular glutathione. For the determination of GSSG, sample aliquots were treated with 2-vinylpiridine and mixed continuously for 1h for derivatization of GSH. Oxidized glutathione was then measured as described above for total glutathione. The glutathione concentration was expressed as function of the protein content.

Total extracellular glutathione was determined in culture supernatants. For this purpose 20 mL aliquots of the supernatants were collected, frozen to -80 ºC and lyophilised to dryness (Owens and Hartman 1986). The lyophilised supernatants were resuspended in potassium phosphate buffer 100 mM, EDTA 2 mM pH 7.4 and $HClO_4$ 2 M (in equal volumes) and neutralized as described above. Total glutathione concentration was determined using the same method as described for intracellular extracts. In this case, the glutathione concentration was normalized with respect to the original culture volume.

2.9. Intracellular ROS determination

E. coli cultures were grown to an OD_{420}=0.5 at which point 500 µL of culture were transferred to 2 mL microtubes and incubated in the dark with dihydrorhodamine-123 (DHR) 0.025 $\mu g \cdot \mu L^{-1}$ (Molecular Probes, Eugene, Oregon, USA) at 37 ºC shaking for 2 h. Treated samples received Cd^{2+} 30 $\mu g \cdot mL^{-1}$ 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 distilled water.

In the experiments performed using strains MG1655 after incubation with DHR, cells were harvested by centrifugation at 11,000 x*g* for 2 min. Then washed once in PBS (NaCl 8 % (w/v), KCl 0.02 % (w/v), Na_2HPO_4 0.18 % (w/v) pH 7.4) and finally resuspended in 2 mL PBS. Cell-associated fluorescence was measured by flow-cytometry with a Becton Dickinson FACSort (San Jose, California, USA). For each sample 100,000 cells were analyzed.

For the experiments performed using the BW25113 and the respective mutants, after DHR incubation, samples were diluted 1:20 in PBS. 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.

Analysis of results was performed using the CellQuest® programme version 3.3. The results were expressed as the percentage of the mean fluorescence of the whole population of cells acquired.

2.10. Respiration measurements

Cells were grown to an OD_{420}=0.5-0.7 and harvested at 4,500 x*g*, 4ºC, washed twice in 5 mL MOPS buffer 40 mM pH 7.4 and resuspended in the same buffer. Respiration rates were determined polarographically for suspensions with a final OD_{420}=2 in 1.5 mL working volume in a Clark-type oxygen electrode (Oxygraph, Hansatech Istruments, UK) after the addition of the carbon source. The carbon sources tested were: glucose, glycerol, succinate, lactate, fumarate and pyruvate at 2 mM final concentration. In inhibition tests, Cd^{2+} 30 $\mu g \cdot mL^{-1}$ was added in the absence or presence of GSH or GSSG 2 mM. Results were expressed as nmol of O_2 per minute, normalized with respect to dry weight.

Different uncouplers and inhibitors of the electron transport and phosphorylation systems were tested in order to determine the concentration necessary to inhibit these systems in whole cells. The inhibitory effect by rotenone, piericidin A, potassium cyanide (KCN) and carbonyl cyanide m-chloro phenyl hydrazone (CCCP) was tested.

2.11. Molecular biology techniques – real-time RT-PCR

2.11.1. Primer Design

The relative expression of the catalase encoding genes *katG* and *katE* was analyzed in a two-step real-time RT-PCR experiment. To obtain a relative quantification of the expression, a reference gene or genes must be chosen. Ideally, these genes should have a constant expression in all samples tested, and their expression should not vary under the study conditions. Based on a transcriptome study on cadmium toxicity (Wang and Crowley 2005), genes *dnaK*, *rnb* and *rrsA* (16S rRNA) were initially chosen as possible reference genes.

All primers were designed using the computer software Beacon Designer 6.0 (Premier Biosoft International, USA). Standard parameters of the SYBR® Green design mode were used and the location of the primers was defined in order to originate a PCR product of 150-200 bp, internal to the gene.

Table 2.2 Primers used for real-time RT-PCR

Oligo	Sequence (5'→3')	Length (bp)	Tm (ºC)	Target gene
dnaK F	TAACCTGCCATACATCACTGC	21	62.5	*dnaK*
dnaK R	GACCACCAACGAGGATAACG	20	64.2	843-1027
katE F	GCGAATTACGAACCGAACTC	20	63.4	*katE*
katE R	TGACTTAGCCAGAACAGACG	20	60.6	1372-1544
katG F	TGGTGTGGTTGGTGTTGAG	19	63.3	*katG*
katG R	GACTCGGTGGTGGAAACG	18	64.3	1596-1778
rnb F	CAGGCAGATAGTTGGTGAATG	21	62.1	*Rnb*
rnb R	CAGCACAGAAGATATGGATGAC	22	61.1	1170-1327
rrsA F	CACACTGGAACTGAGACACG	20	62.1	*rrsA* (16S)
rrsA R	GCTTCTTCTGCGGGTAACG	19	64.7	312-501

2.11.2. RNA extraction and analysis

E. coli cultures for RNA extraction were collected by centrifugation at 20,000 x*g*, 4 ºC for 10 min. The pellets were resuspended in RNA*later* RNA protect™ Bacteria Reagent (Qiagen) according to manufacturer's instructions and frozen at

-80 ºC or used immediately for RNA extraction with RNeasy® Mini Kit (Qiagen). Extraction was performed according to the protocol for isolation of total RNA from bacteria, including the additional step of on-column DNase digestion using the RNase-free DNase set (Qiagen).

Analysis and quantification of the RNA samples was performed with the Experion Automated Electrophoresis System (Bio-Rad) using the "Prokaryote Total RNA Std Sens Assay". This system analyzes the integrity of RNA by visualization of the 23S and 16S ribosomal bands. The 23:16 ratio is indicative of the sample degradation and/or contamination (the ideal value for this ratio is 2). RNA samples with similar ratios were used in the experiments performed (23:16= 1.3-1.5).

2.11.3. cDNA synthesis – reverse transcription reaction

One microgram of RNA was treated with the RQ1 RNase-free DNase according to the manufacturer's instructions (Promega) in a final volume of 10 µL. For each reaction, 2 µL aliquots were taken and used as a template in a control PCR to assess the absence of contaminating genomic DNA. For this purpose, the reference gene (*rrsA*) primers were used in an amplification reaction containing: 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's mix (Fermentas), 1x reaction buffer and 1 U of Taq polymerase (ABgene). The cycling conditions comprised: one step at 95 ºC for 5 min, 40 cycles at 55 ºC for 30 s and 72 ºC for 20 s and 95 ºC for 30 s and a final cycle at 55 ºC for 30 s and 72 ºC for 5 min. The PCR products were analyzed in a 2% (w/v) agarose gel electrophoresis.

For the RT (reverse transcription) reaction, the iScript Select cDNA synthesis kit (Bio-Rad) was used, according to the manufacturer's instructions. The protocol for reaction setup with random primers was followed; the volume of all the components was adjusted for a reaction with a final volume of 10 µL. Eight hundred nanogram of total RNA previously treated with RQ1 RNase-free DNase, was used as template. Synthesis of cDNA was performed for 30 min at 45 ºC followed by an enzyme inactivation step of 5 min at 85 ºC.

2.11.4. Real-time PCR

For real-time PCR, the IQ™ SYBR® Green Supermix kit (Bio-Rad) was used and the reactions were setup in 96-well plates. The total volume of each reaction was 20 µL and contained: 1x IQ SYBR Green Supermix, 200 nM of each primer, 2 µL of

cDNA template. Sterile water was used to make up the volume. A three-step PCR was performed, according to the thermal profile: one step at 95 ºC for 3 min, 40 cycles at 95 ºC for 30 s and 55 ºC for 30 s and 72 ºC for 30 s, in each cycle the fluorescence data was collected after the extension step. Finally a melting curve was performed for each PCR product, comprising 81 cycles of 15 s each, starting at 55 ºC and increasing temperature by 0.5 ºC per cycle, fluorescence data was collected after each cycle. This step assesses the specifity of the PCR reaction by analyzing the formation of non-specific products.

For each primer pair a standard dilution of the cDNA was used to check the efficiency (E) and quality of the primers. For the standard curve, cDNA S1 (wild-type, control sample) was used. The conditions studied were S2 (wild-type, cadmium treated), S3 (Δ*ubiE*, control) and S4 (Δ*ubiE*, cadmium treated). A negative control was included in all real-time PCR assays, and each experiment was performed in duplicate. The *rrsA* (16S rRNA) was used as reference gene.

This technique was performed using the iCycler iQ5 Real-Time PCR (Bio-Rad) and the iQ5 software version 2.2 was used for equipment setup and data analysis.

Relative gene expression was calculated using the Pfaffl method (Pfaffl 2001).

2.12. Statistical treatment

All results in this study are expressed as means of at least 3 independent replicates with the associated standard deviation. Differences between treatments were considered statistically significant when Student's t-test was <0.05 (P<0.05=*; P<0.01=**).

3. Effect of cadmium on growth and assessment of oxidative damage on proteins and lipids

3.1. Introduction

Bacterial cell growth involves the coordination of metabolic pathways that produce the compounds needed to form the cell. Indeed, the macromolecules are synthesized from a small group of precursors that are the intermediates of the central metabolic pathways (Holms 1996).

Glucose is the most abundant aldose in nature and is the preferred carbon and energy source for *E. coli* (Gutierrez-Ríos et al. 2007). This bacterium can utilize glucose to build the carbon skeletons for every amino acid, nucleotide, coenzyme, fatty acid or other metabolic intermediate. The metabolism of glucose is regulated at different levels, starting at its transport system that is activated when the presence of glucose in the medium is detected by the cell. This sugar is internalized and phosphorylated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in which the translocation is coupled to phosphorylation. The PTS is widespread in bacteria and is composed of soluble non sugar-specific protein components: Enzyme I (EI) and the phosphohistidine carrier protein (HPr) which relay a phosphoryl group from the glycolytic intermediate phosphoenolpyruvate (PEP) to any of the different sugar-specific enzyme II complexes. In the case of glucose, this sugar is imported by the II^{Glc} complex, composed of the soluble protein IIA^{Glc} and the integral membrane permease $IICB^{Glc}$ (Tchieu et al. 2001). Once inside the cell, glucose is targeted to the central carbon metabolism (CCM) that includes: Glycolysis (Embden-Meyerhof Pathway - EMP), the Pentose Phosphate Pathway (PPP) and Tricarboxylic Acid (TCA) cycle (Hardiman et al. 2007). The maintenance of bacterial metabolism depends on biological oxidation-reduction reactions both for the biosynthetic intermediates and for the ATP and reducing equivalents generated. The NAD^+ cofactor plays a key role in many of these redox reactions. In *E. coli*, the glycolysis and the TCA cycle are the major sources of metabolic intermediates and reduced cofactor NADH (Leonardo et al. 1996). In the presence of oxygen, reducing equivalents from NADH are transferred to the electron transport chain (ETC), generating H_2O and membrane potential, which, in turn, is used to synthesize ATP. In this way NAD^+ is regenerated for use in subsequent reactions. In anoxic conditions, an alternative electron transport chain is available if electron acceptors such as nitrate, trimethylamine *N*-oxide or fumarate are present. If these alternative oxidants are absent, the cell must recycle NADH through fermentation. To maintain the glycolytic flux, NAD^+ is regenerated by depositing the reducing equivalents on partially oxidized metabolic intermediates that are

then excreted from the cell. This process results in the loss of most of the energy available from the carbon source, but allows the bacteria to grow, although with reduced growth yields. *E. coli* converts sugars and their derivatives to a mixture of products that include: acetate, lactate, succinate, formate and ethanol (Böck and Sawers 1996).

3.2. Effect of cadmium on growth and culturability

Before testing the effect of cadmium on *E. coli* cell growth, experiments were performed to evaluate metal bioavailability and the cadmium concentration to be used throughout this work. *E. coli* can grow in the minimal medium M9, however the use of phosphate buffer leads to cadmium precipitation thus decreasing the ion availability. To overcome the precipitation in the medium, phosphate buffer was replaced by MOPS buffer as described for the growth of enterobacteria (Neidhardt et al. 1974). The concentration of phosphate was adjusted to a minimum of 200 μM that allowed the normal growth of the cultures and avoided the precipitation of cadmium. Selection of the cadmium concentration was achieved by assessing the growth rate in the presence of different concentrations of metal (data not shown). The data obtained after several experiments led to the selection of the cadmium concentration of 30 $\mu g \cdot mL^{-1}$, which lead to a temporary stasis situation from which the cells were later able to recover. Thus the biochemical parameters analyzed were obtained from cultures in growth stasis due to a stress situation and not from cultures experiencing necrosis/massive cell death.

Aiming at assessing the effect of cadmium on the growth of *E. coli* MG1655, the cultures were grown to early logarithmic phase and at this point cadmium was added to a final concentration of 30 $\mu g \cdot mL^{-1}$ (273 μM $CdCl_2$). The effect of the metal was determined both in aerobic and fermentation conditions (Figure 3.1a and 3.1b).

In aerobic conditions, after cadmium was added to the cultures, a stasis in growth was observed (Figure 3.1a). Although it is not shown, cell growth recovery was observed after ca. 40 hours, and the treated culture achieved a similar final OD to the untreated culture.

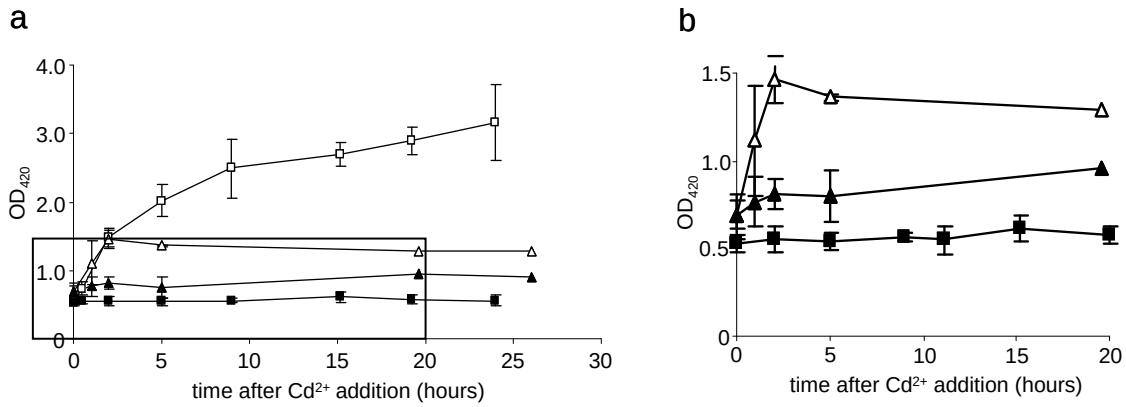


Figure 3.1 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on wild-type cell growth in aerobic and fermentative conditions: aerobic untreated (open square), aerobic Cd-treated (filled square), fermentative untreated (open triangle) and fermentative Cd-treated (filled triangle). **(b)** Enlarged area corresponding to the rectangle on the graph **(a)**.

In fermentative conditions, a decrease in the growth rate, rather than a clear growth arrest, was observed after adding the heavy metal (Figure 3.1a). This effect is evident when the treated fermenting cultures are compared to the aerobic cultures under the same conditions (Figure 3.1b); two hours after cadmium was added, fermenting cells are growing while respiring cells are under stasis.

In agreement with the literature data (Böck and Sawers 1996), a reduced growth yield was observed when cultures were grown in fermentative conditions (in the absence of oxygen or nitrate). Also, in these conditions the cell density of the treated cultures was lower than that obtained in control cultures, in contrast to the observed in the presence of oxygen (data not shown).

The effect of cadmium on the fraction of cultivatable cells was also assessed in respiring and fermenting cultures (Figure 3.2). In aerobic conditions, cadmium treatment lead to a 25% decrease in cell culturability, while in fermenting cultures the effect of the metal was reduced. Indeed after 30 min of exposure, a 13% decrease in cultivatable cells was observed but, after 60 min this cell fraction was similar to that registered for the untreated culture.

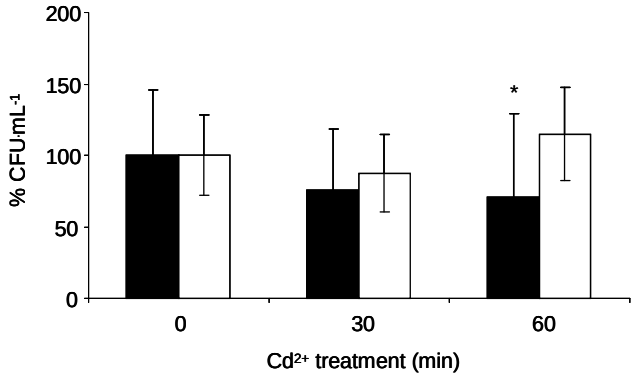


Figure 3.2 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on culturability of cultures in aerobic (filled bars) and fermentative conditions (open bar). The absolute values ($CFU \cdot mL^{-1}$) corresponding to 100% (T_0) are: respiring cells $=2.25x10^8 \pm 1.02x10^8$, fermenting cells $=2.98x10^8 \pm 6.85x10^7$. Statistically significant differences between Cd-treated and untreated cultures are identified: * ($P<0.05$).

Both growth and culturability assessments indicate that cadmium is less toxic in the absence of oxygen. In agreement with these data, association of cadmium toxicity with respiration using oxygen has been proposed. Therefore, the redox balance and namely the role of glutathione are relevant when the toxicity of this metal is concerned. In kidney fibroblasts it was reported that the addition of an exogenous source of GSH alleviates the stress caused by the presence of cadmium (Kang 1992). To evaluate the effect of glutathione in *E. coli* growth, 2 mM GSH or GSSG were added to the cultures prior to cadmium treatment, and it was observed that the inhibitory effect of this metal was abolished. This effect was observed only when GSH was added, while the addition of GSSG was not effective (data not shown).

It has been claimed that, in general, GSH is a primary mechanism of defence against cadmium and glutathione depletion has been associated with the oxidative stress induced by the metal. However, at present there is no clear molecular mechanism to fully elucidate this role. To test the glutathione depletion hypothesis in *E. coli*, we decided to use mutants in the synthesis of this thiol. Mutant strains Δ*gshA* and Δ*gshB* were obtained from the Keio Collection (Keio Unversity, Japan;Baba et al. 2006). The former lacks the gene that codes for the γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step in GSH synthesis. The latter mutant is unable to catalyze the second step in GSH

production that is performed by glutathione synthetase. To assess the effect of cadmium on the growth of these mutant strains, aerobic cultures in early logarithmic phase were used and compared to their parental strain, *E. coli* K-12 BW25113.

When the effect of the metal was compared in wild-type strains MG1655 and BW25113, the latter revealed to be more sensitive to cadmium. While in *E. coli* MG1655, a stasis period of less than 2 days was observed (data not shown), for strain BW25113 a stasis period of 4 days was registered (Figure 3.3).

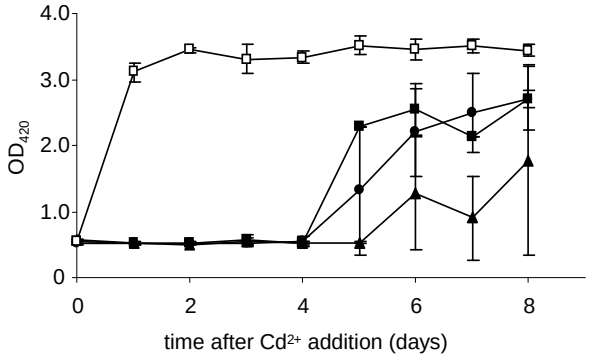


Figure 3.3 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on cell growth: untreated wild-type BW25113 (open square), BW25113+Cd^{2+} (filled square), Δ*gshA*+Cd^{2+} (filled triangle), Δ*gshB*+Cd^{2+} (filled circle). The untreated growth curve shown is representative of all three strains: BW25113, Δ*gshA* and Δ*gshB*.

The effect of cadmium on the growth rate of the glutathione mutants revealed that this metal induced a similar stasis period in the mutants and in the wild-ype strain (Figure 3.3). In terms of loss of culturability, more significant differences between the strains were observed (Figure 3.4). In the Δ*gshA* mutant the percentage of cultivatable cells was significantly decreased by exposure to cadmium and, after 60 minutes of treatment this mutant showed just 33% of the initial cell count while 63% was registered for the wild-type (P<0.001). In the Δ*gshB* mutant, after exposure for 60 min, culturability was 54%, which is a significantly lower value than the one obtained for the wild-type (P<0.005).

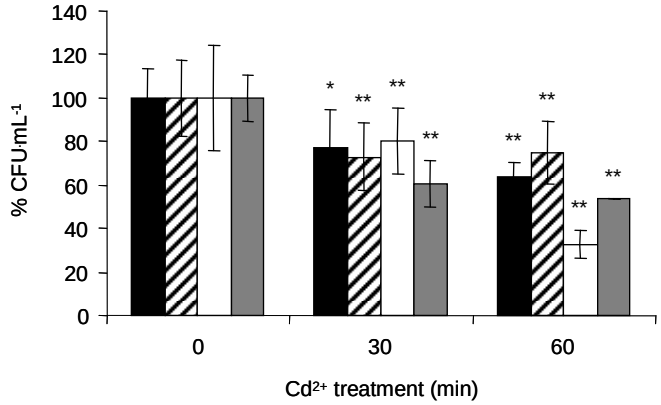


Figure 3.4 Effect of 30 μg·mL^{-1} cadmium on culturability: wild-type BW25113 (filled bar), Δ*ubiE* (diagonal stripe bar – results with this strain will be used later in this work), Δ*gshA* (open bar), Δ*gshB* (grey bar). The absolute values (CFU·mL^{-1}) corresponding to 100% (T_0) are: wild type=1.41x10^8±1.90x10^7, Δ*ubiE*=1.74x10^8±3.03x10^7, Δ*gshA*=1.53x10^8±3.67x10^7 and Δ*gshB*=1.86x10^8 ±2.01x10^7. Statistically significant differences between Cd-treated and untreated cultures are identified: * (P<0.05), ** (P<0.01).

These results confirm that glutathione plays an important role at the early stages of the defence against cadmium-induced toxicity. However, GSH is not essential as the mutants are still viable under stress conditions and resume growth after 4/5 days.

Cadmium and other metals interfere with the respiratory chain in different organisms (Kasahara and Anraku 1972; Bragg and Rainnie 1974; Singh and Bragg 1974; Kleiner 1978; Surowitz et al. 1984; Tynecka et al. 1990; Beard et al. 1995). Therefore, to exploit the hypothesis that cadmium-induced ROS are associated with this chain we analyzed the effect of cadmium on the mutant strain Δ*ubiE* that lacks UQ and MK (Singh and Bragg 1974). The lack of these two electron carriers results in the impairment of the respiratory chain and, in normal aerobic growth conditions, oxygen consumption is reduced to 38% of that observed in its isogenic wild-type strain (BW25113 - data not shown). It can also be observed that this strain has a reduced growth yield, resulting probably from the reduction in the aerobic metabolism (Figure 3.5).

Growth inhibition was significantly reduced when this strain was treated with cadmium and cell growth was resumed during the second day of stasis in contrast with the wild-type strain BW25113 (after 3 days, cell growth was still inhibited) (Figure 3.5b).

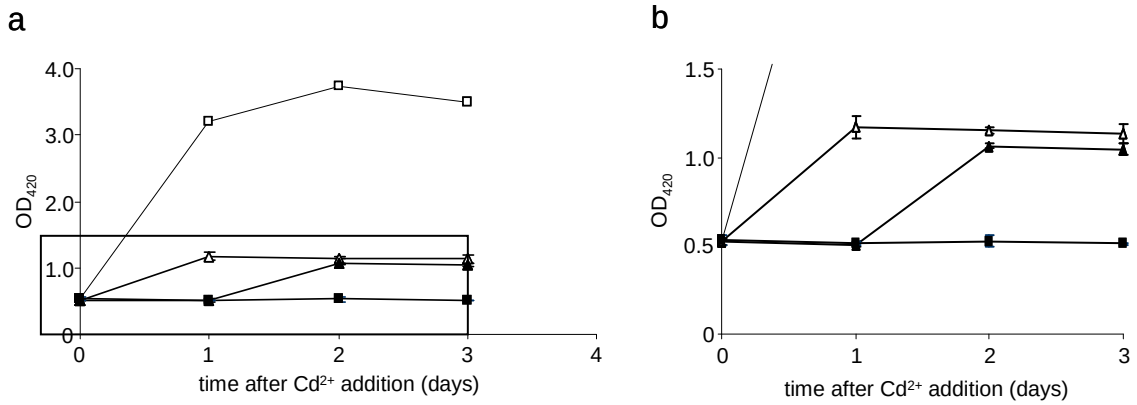


Figure 3.5 Effect of 30 μg·mL^{-1} cadmium on growth of the Δ*ubiE* untreated (open triangle) and Cd-treated (filled triangle) compared to the wild-type untreated (open square) and Cd-treated (filled square). **(b)** Enlarged area corresponding to the rectangle in graph **(a)**.

In the Δ*ubiE* strain, after 30 min treatment with metal cell culturability did not exihibit any difference with respect to the wild-type (Figure 3.4). However, after 60 minutes treatment, the percentage of cultivatable cells was significantly higher in the mutant (P<0.001).

In summary, all the results indicate that the effect of cadmium on growth and cell culturability is associated with the aerobic metabolism. Indeed a significant reduction in the toxicity is observed in the Δ*ubiE* mutant and this supports the fact that the lower toxicity of this metal is associated with the absence of the respiratory function.

3.3. Oxidative damage

Aiming to obtain further data to support the link between cadmium toxicity and oxidative stress, we performed experiments to detect oxidative damage on lipids and proteins.

3.3.1. Lipid peroxidation

The analysis of lipid peroxidation in strain MG1655 revealed that upon cadmium exposure the levels of damaged lipids remained identical (data not shown).

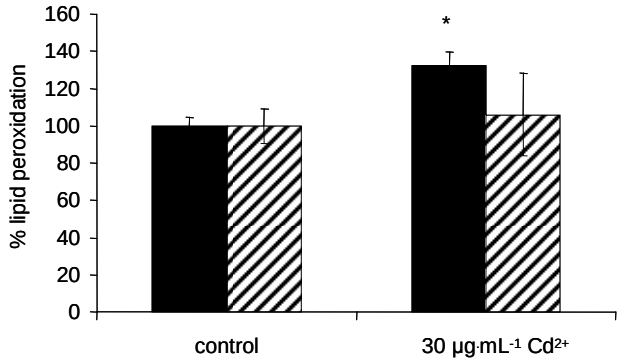


Figure 3.6 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on lipid peroxidation levels: wild type BW25113 (solid bar), Δ*ubiE* (diagonal stripe bar). The absolute values [µmol MDA·(mg prot)$^{-1}$] corresponding to 100% are: wild type=6.60±0.29, Δ*ubiE*=10.68±0.99. In the wild-type there is significant difference between control and treated samples * (P<0.05).

In contrast, the results obtained when using BW25113 cells showed a 32% increase in the peroxidation levels (Figure 3.6). Analysis of the lipid peroxidation levels in the Δ*ubiE* mutant strain revealed that the constitutive levels are higher and they suffered no alteration when cells were exposed to cadmium.

3.3.2. Protein carbonylation

Another oxidative stress marker analyzed was the levels of oxidized proteins by assessing the carbonylation of soluble proteins. The data obtained for strain MG1655 revealed no effect of cadmium on the levels of oxidative damaged proteins. With strain BW25113 and derived mutants, an increase in damaged proteins levels was detected in the presence of the metal (Figure 3.7).

The analysis of protein carbonylation upon resolution by SDS-PAGE showed that the oxidation of five polypeptides was increased in the wild-type and in the mutant strains. Notably, cadmium treatment resulted in a similar pattern of protein oxidation in the wild-type and in the catalase deficient mutants, whose cellular defences are impaired. The Δ*ubiE* mutant strain showed higher constitutive levels of protein carbonylation when compared to the wild-type, confirming the tendency observed for the levels of damaged lipids (Figure 3.6). However, when this strain was exposed to cadmium no significant increase in damaged protein levels was detected.

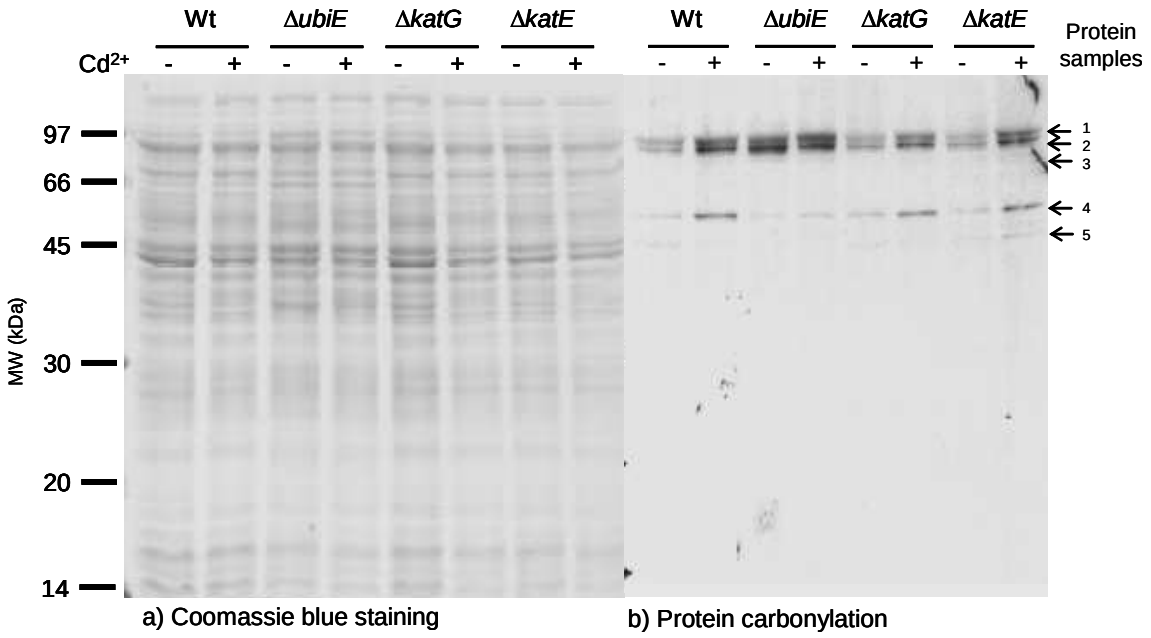


Figure 3.7 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on protein carbonyl levels **(b)**. **(a)** SDS-PAGE is shown as loading control.

3.3.3. Identification of oxidized proteins

To identify the proteins most oxidized in the presence of cadmium, carbonyl immunodetections were used. Only the oxidation profiles of BW25113 and Δ*ubiE* strains were analyzed because the catalase mutants presented a profile similar to the wild-type (Figure 3.7). In these experiments, five oxidized proteins were observed and at this stage it was decided to try to do the identification of these proteins (indicated in Figure 3.7 as protein samples).

To allow for a convenient separation of the proteins, SDS gels were run for longer periods of time (150, 210 and 300 minutes). Before performing the immunodetection, the Hybond-ECL membrane was stained with Ponceau to confirm that the blotting step was successful. When this experiment was performed, the Ponceau-stained membranes were digitalized. Since the carbonyl immunodetection films match perfectly with the protein profiles observed in the Ponceau-stained membranes, it was easily detected which protein band in the profile corresponded to the oxidized protein band in the carbonyl film. Once the protein was identified in the Ponceau-stained profile it was possible to identify the same protein in the SDS gels stained with Coomassie blue.

Since the identification of the oxidized proteins was performed using samples isolated from one-dimensional gels, it was not possible to obtain pure protein results from each sample. The most probable proteins identified for each sample are presented in table 3.1.

Table 3.1 Oxidized proteins identified by MALDI-TOF

Sample	Protein MW (kDa)	Protein PI	Peptides	Protein coverage (%)	Protein Identification
1	96.579	6.32	26	48	Aldehyde-alcohol dehydrogenase
	99.948	5.46	22	48	Pyruvate dehydrogenase E1 component
2	85.020	5.61	39	61	5-methyltetrahydropteroyltriglutamate –homocysteine methyltransferase
	88.064	5.14	18	54	Phenylalanyl-tRNA synthetase
3	99.456	5.52	21	28	Phosphoenolpyruvate carboxylase
	77.703	5.23	19	46	Elongation factor G
4	46.069	5.15	18	51	Isocitrate dehydrogenase [NADP]
	45.683	5.32	14	44	Enolase
5	39.309	4.76	8	29	Outer membrane protein F precursor
	40.343	4.58	7	29	Outer membrane protein C precursor

The limitations of this experiment cannot be ignored: for instance, the practical impossibility to uniquely identify the oxidized protein, and also the fact that the proteins analyzed already appear oxidized in control conditions, leaves the question open: are these proteins more oxidized in cadmium conditions because they are more abundant in the cell or is the increase in their oxidation metal-specific? Keeping these questions in mind, it is important to stress that the identification of these proteins can give some hints on the cellular processes/pathways that may be affected due to the metal-induced stress. Before proceeding with these investigations, the identification of proteins should be confirmed using more refined techniques. This preliminary experiment led us to the identification of proteins mainly related to the central metabolic pathways and to protein synthesis. Some of the identified proteins had already been pointed out as targets of oxidation in oxidative stress situations (Tamarit et al. 1998). In

some cases, it was suggested that their oxidation could be a protection mechanism to avoid damage to other important proteins or to delay high energy-consuming processes. A brief description of each possible protein identified and literature data relating these proteins to oxidative stress are presented in appendix (see Appendix I).

3.4. Discussion and conclusions

Aiming at understanding the molecular mechanisms underlying cadmium toxicity and oxidative stress in *E. coli*, we performed the described experiments. A concentration of cadmium that could induce an oxidative stress status was determined and the effect of cadmium on growth, culturability and on the levels of oxidative damage on proteins and lipids was tested. Initially, a metal concentration that induced 1 hour growth stasis was sought, in order to obtain a similar effect to the one reported by Ferianc et al. (1998). After several experiments were conducted, the concentration of 30 $\mu g \cdot mL^{-1}$ of cadmium was chosen and though it failed to consistently produce the 1 hour stasis effect desired, it always lead to a stasis situation from which the cells were able to recover.

The growth arrest induced by cadmium was accompanied by a decrease in the fraction of cultivatable cells. This effect had already been described in previous reports where an increase in cell damage and synthesis of specific proteins was also observed (Mitra et al. 1975; Mitra and Bernstein 1978; Mitra 1984; Shapiro and Keasling 1996; Ferianc et al. 1998). The effect of the metal on the growth of fermenting MG1655 cultures was also determined, and the results revealed that the toxicity of cadmium was reduced in fermenting conditions. This reduction was observed both for growth rate and culturability of the strain suggesting for the first time that cadmium toxicity, in *E. coli*, may be multifactorial and at least part of this toxicity is related to aerobic metabolism. Consequently, the maintenance of the redox balance and the role of glutathione within this process assumed great importance. In fact, the addition of an exogenous source of GSH was shown to abolish the inhibitory effect of cadmium on the growth of *E. coli*, confirming that, in this bacterium, this molecule may constitute a primary defence mechanism against this metal. To further elucidate the relationship between cadmium toxicity and glutathione, the effect of cadmium was tested on growth and culturability of the Δ*gshA* and Δ*gshB* strains. The experimental data

obtained revealed that the effect of cadmium on the glutathione mutants was similar to that observed in the wild-type, while in terms of cultivatable cells some differences were registered. The reduction of culturability in the glutathione mutants, especially in the Δ*gshA* strain, is in agreement with the model whereby glutathione plays an important role in cadmium detoxification. Despite this fact, glutathione is clearly not essential for survival to cadmium-induced stress since treated mutants were able to recover. Therefore, oxidative stress induced by cadmium does not seem to be associated with the depletion of glutathione, as previously proposed (Stohs and Bagchi 1995; Howlett and Avery 1997; Ercal et al. 2001; Banjerdkij et al. 2005; Liu et al. 2005; Wolf and Baynes 2007). The effect of the impairment of other antioxidant defences on the toxicity of cadmium was also determined using the catalase deficient strains Δ*katG* and Δ*katE*. The effect of cadmium on growth and culturability of strain Δ*katG* (data not shown) was similar to the one observed for the glutathione deficient Δ*gshA* mutant. The levels of oxidized proteins were determined for Δ*katE* and Δ*katG* and the result obtained revealed a pattern of damaged proteins similar to the wild-type. In a Δ*katG* background, higher oxidation levels were expected since KatG activity is absent. To overcome this impairment in the cell defences, probably the KatE enzyme is activated to compensate for the lack of KatG that is the isoform usually expressed in the early logarithmic growth phase.

Since cadmium was reported to interfere with the respiratory chain, the Δ*ubiE* mutant was used to test if there is a link between the toxicity of this metal and the ETC. This mutant showed decreased oxygen consumption and, when treated with cadmium, the stasis effect was significantly reduced. Nevertheless, short term metal exposure (30 min) lead to a significant decrease on the fraction of cultivatable cells; however, after 60 min this percentage remained unaltered while for all other strains tested a further decrease was registered. Oxidative stress markers were also analyzed in this strain revealing that, in control conditions and when compared to the wild-type, these markers are increased which is probably due to the impairment in the ETC. Despite this fact, the levels of oxidized proteins and lipids remained unaltered when the Δ*ubiE* strain was treated with cadmium. Based on the protein carbonylation profile observed, a simple preliminary identification of the oxidized proteins was performed. This experiment allowed the identification of proteins that were target of intense oxidation. Most of the proteins identified are related to the central metabolism and some are also involved in protein synthesis. At least one of the proteins

proposed for each sample was previously identified as target under oxidative stress conditions such as: aldehyde-alcohol dehydrogenase, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, elongation factor G, enolase and the outer membrane protein C. In fact, in the work of Tamarit et al. (1998), four of these proteins were identified as major oxidatively damaged proteins in *E. coli* cells submitted to different oxidative stress agents. The oxidation of some of these proteins was suggested to be a cell defence mechanism by acting as oxidant scavengers and avoiding more severe damage or slowing down protein synthesis under adverse conditions (Echave et al. 2003; Hondorp and Matthews 2004).

In conclusion, the experimental data obtained on the effects of cadmium on growth and culturability of the *E. coli* strains used suggests that cadmium toxicity is associated with the respiratory metabolism. Moreover, in strains with impaired antioxidant defences cadmium was shown to have a similar effect to the one observed in the wild-type strain. These last results clearly show that the oxidative stress imposed by cadmium is not generated by a metal induced impairment of the antioxidant defences such as GSH. Furthermore, the analysis of the oxidative damage levels confirmed that the reduction in aerobic metabolism significantly decreases the toxicity of cadmium.

4. Effect of cadmium on the antioxidant defences

4.1. Introduction

Escherichia coli can grow both under aerobic and anaerobic conditions and, growth in the presence of oxygen allows greater yields of energy from carbon sources. However, as a by-product of the aerobic metabolism, reactive oxygen species such as $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ are produced from oxygen. The antioxidant defence system acts to eliminate these dangerous species and protect the cell from oxidative damage.

Two inducible hydroperoxidases are produced by *E. coli* as part of its adaptive response to oxidative stress: HP I and HP II. These enzymes limit the accumulation of H_2O_2 by catalyzing its conversion to H_2O and O_2 or by oxidizing an intracellular reductant using H_2O_2 (Schellhorn 1995). The HP I is a catalase-peroxidase formed by four identical subunits and is encoded by the *katG* gene. This enzyme is known to be expressed under aerobic and anaerobic conditions and its expression is under positive control of the OxyR protein. The other enzyme (HP II) is a hexameric monofunctional catalase that is encoded by the *katE* gene and is positively regulated by KatF (σ^S). Unlike KatG, the KatE activity is not induced by H_2O_2 and it is the main hydroperoxidase in aerobic stationary phase cells and is present at basal levels in the aerobic exponential phase and in anaerobic conditions (Loewen et al. 1985; Schellhorn 1995).

In general, Gram-negative bacteria contain both cytoplasmic and periplasmic SOD isozymes. *Escherichia coli* contains two cytoplasmic SOD (one manganese- and one iron-cofactored enzyme - MnSOD and FeSOD) and also secretes one copper-zinc-cofactored enzyme (Cu,ZnSOD) to the periplasm (Imlay 2008). The cytoplasmic SODs are both homodimeric enzymes, but they diverge both in function and pattern of expression. The FeSOD (encoded by *sodB*) is produced both under aerobic and anaerobic conditions, functioning as a standby defence against superoxide when the cell faces transition from the absence to the presence of oxygen (Fridovich 1995). The MnSOD enzyme is present only under aerobic conditions and is encoded by the *sodA* gene, which is under the regulation of the *soxRS* regulon. Regarding their function, the manganese enzyme seems to be more effective in preventing DNA damage while the iron SOD appears to be more effective in protecting cytoplasmic superoxide-sensitive enzymes (Hopkin et al. 1992). Finally, the Cu,ZnSOD is a monomeric enzyme that is encoded by the *sodC* gene and is induced when cells enter the stationary phase.

The glutathione-dependent reduction system is one of the mechanisms responsible for the redox balance in the cell. The γ-glutamylcysteine synthetase (encoded by *gshA*) together with glutathione synthetase (*gshB*) are responsible for the synthesis of the GSH molecule which will function as a reductant in many cellular reactions. Upon oxidation of GSH, GSSG is formed which can be reduced back by glutathione reductase (encoded by *gor*) at the expense of NADPH. *Escherichia coli* also contains three glutaredoxins (encoded by *grxA*, *grxB* and *grxC*) that in conjunction with GSH keep cellular disulfide bonds reduced. Although it has been shown that this system effectively protects cells exposed to different oxidative stress-generating agents, its role in the antioxidant defence system does not appear to be critical (Carmel-Harel and Storz 2000). This fact is probably due to the presence of the thioredoxin-dependent system composed by the thioredoxins (*trxA* and *trxC*), the thioredoxin reductase (*trxB*) and the thioredoxin peroxidases (*tpx* and *bcp*) (Toledano et al. 2007).

4.2. Catalase

Catalase activity was determined in *Escherichia coli* cultures to assess the role of this antioxidant enzyme in the response triggered by the bacterium in the presence of cadmium.

4.2.1. Catalase activity

Catalase activity was first assayed by gel zymography using protein extracts obtained from *E. coli* K-12 MG1655 cultures grown in aerobic conditions (Figure 4.1a). In this assay no significant differences were detected between control and treated samples. However, since gel zymography is at best semiquantitative, the corresponding spectrophotometric assay was performed. This method allowed for the detection of a cadmium-induced increase in the catalase activity, which was observed both in respiratory and fermentative conditions (Figure 4.1b). Indeed, in the presence of cadmium, a 2-fold increase in the catalase activity was registered in aerobic conditions; in the absence of oxygen a similar increase was also observed.

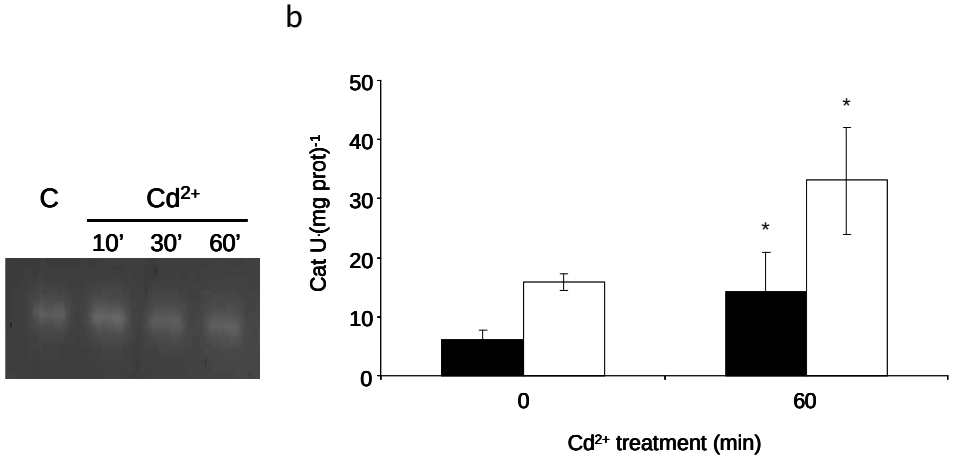


Figure 4.1 Effect of 30 $\mu g \cdot mL^{-1}$ Cd^{2+} on *E. coli* K-12 MG1655 catalase activity assessed by: **(a)** gel zymography (aerobic conditions) and **(b)** spectrophotometric assay in aerobic (filled bar) and fermentative conditions (open bar). Statistically significant differences between Cd-treated and untreated cultures are identified: * (P<0.05).

Although cadmium had a comparable effect on the activity of catalase of both fermenting and respiring cells, it should be noticed that in untreated fermenting cells basal catalase activity was 15.78 Cat $U \cdot (mg\ prot)^{-1}$ while in aerobic conditions this value was only 6.03 Cat $U \cdot (mg\ prot)^{-1}$.

To further investigate cadmium toxicity in *E. coli* and the contribution of the antioxidant defences in this process, catalase deficient strains Δ*katE* and Δ*katG* were used. Experiments using the Δ*ubiE* strain were also performed to complement the data presented in the previous chapter that establish a link between cadmium toxicity and aerobic metabolism. Catalase activity was measured spectrophotometrically in strain BW25113 and in the mutant strains, in the presence and absence of cadmium (Figure 4.2).

The BW25113 strain showed similar levels of constitutive catalase activity (Figure 4.2) when compared to the MG1655 strain (Figure 4.1b). However, when BW25113 was exposed to cadmium, a 2.5-fold increase in catalase activity was detected while in strain MG1655 this increase was of 2-fold, confirming that strain BW25113 is more sensitive to cadmium (see chapter 3).

The catalase activity levels registered in Δ*katE* and Δ*katG* strains confirmed the partial impairment in this antioxidant enzyme. Cadmium-induced enzyme activity was observed in the two mutants with a 3.5-fold increase registered for Δ*katE* and 2.4-fold increase for Δ*katG*. This last mutant showed residual constitutive

catalase activity (ca. 13% of the wild-type) and though cadmium treatment did lead to an increase of activity, the final levels were still quite low (2.43±0.76 Cat U · (mg prot)$^{-1}$.

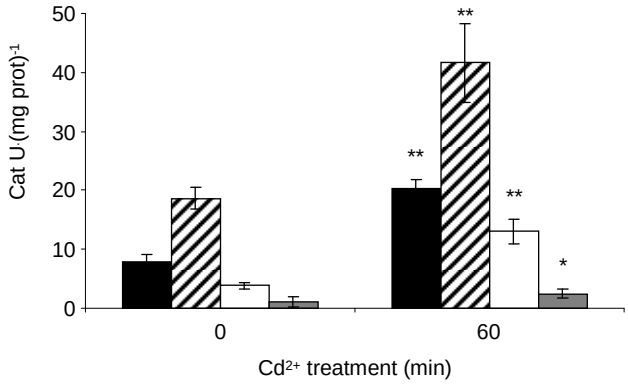


Figure 4.2 Effect of 30 µg·mL^{-1} Cd^{2+} on catalase activity in respiratory conditions: wild-type BW25113 (filled bar), Δ*ubiE* (diagonal stripe bar), Δ*katE* (open bar), Δ*katG* (grey bar). Statistically significant differences between Cd-treated and corresponding untreated controls are identified: * (P<0.05) and ** (P<0.01).

These results were expected since KatE is known to be present only at basal levels when cells are in logarithmic growth phase in aerobic conditions. In the Δ*ubiE* mutant, a significant increase of the catalase activity of treated cells was registered despite the reduced toxic effect observed on growth and cell culturability (previous chapter).

4.2.2.Cadmium effect on the transcription of catalase genes

Most intriguingly, the Cd-dependent increase of catalase activity observed in fermenting cells (Figure 4.1b) implies the presence of an oxygen-independent mechanism of catalase induction by cadmium. In addition, in the Δ*ubiE* strain the highest cadmium-induced increase in catalase was registered (Figure 4.2), which does not seem to correlate with the decreased oxygen consumption and the reduction in metal toxicity verified in this mutant strain. Similarly to fermentation conditions, the results obtained for this mutant suggest that there is a mechanism of catalase activation that is not strictly dependent on oxygen consumption. To test the effect of cadmium on the transcription of the catalase

genes, the relative expression of *katE* and *katG* was determined for the BW25113 and Δ*ubiE* strains (Figure 4.3).

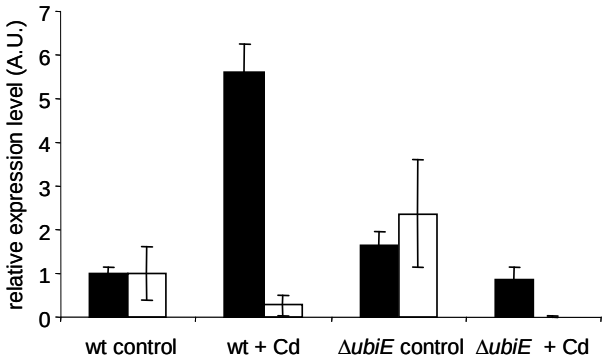


Figure 4.3 Relative expression of the genes (normalized to 16S rRNA) under respiratory growth conditions: *katG* (filled bar) and *katE* (open bar).

The relative expression of the *katG* gene increases by 5-fold when the wild-type is treated with cadmium. In the untreated Δ*ubiE* strain, the relative expression of *katG* is already increased by 2-fold when compared to the wild-type. Treatment of this mutant with cadmium leads to an increase in catalase activity despite the fact that the gene expression is decreased by 50% (Figures 4.2 and 4.3).

Relative expression levels of *katE* were also analyzed in the wild-type and Δ*ubiE* strains, in the presence and in the absence of cadmium. Low expression levels of *katE* were detected in the wild-type, as expected, and dropped to 40% upon treatment with cadmium. A similar pattern was observed in the Δ*ubiE* mutant, but in control conditions the relative expression of the gene is twice as high as in the wild-type. It is worth mentioning that, like for *katG*, the expression of *katE* in the Δ*ubiE* background dropped after exposure to cadmium. These results indicate that the increase in catalase activity observed when the Δ*ubiE* is exposed to cadmium is not due to increased expression levels of the catalase genes. At this point, a gel zymography analysis was performed to determine the contribution of each catalase to the overall activity detected in the spectrophometric assay (Figure 4.2). Another gel zymography was performed to detect the presence of any enzymes with peroxidase activity that may act as oxidant scavengers (Figure 4.4). In addition to the cell extracts from Δ*katE*, Δ*katG* and Δ*ubiE* mutant strains,

extracts of the Δ*ahpC* strain were included in this gel. The alkylhydroperoxide reductase was suggested to be a primary scavenger of endogenous hydrogen peroxide in *E. coli* (Seaver and Imlay 2001).

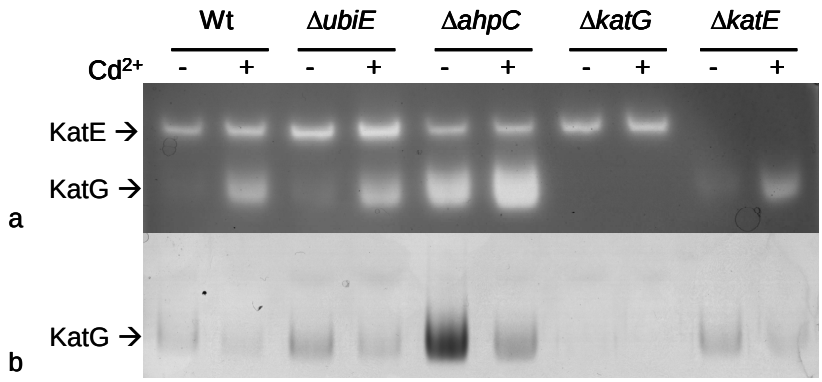


Figure 4.4 Effect of cadmium (30 μg·mL^{-1}) on the: **(a)** catalase activity and **(b)** peroxidase activity of *E. coli* K-12 BW25113 and Δ*ubiE*, Δ*ahpC*, Δ*katG* and Δ*katE*, assessed by gel zymography.

From the analysis of this gel it can be observed that the activity of the bifunctional catalase (KatG) is clearly induced by cadmium in all samples (excluding obviously the *katG* mutant) while KatE activity seems unaffected by cadmium with the exception of possibly Δ*ubiE*. In this strain, the activity of both catalases seems to be increased when cells are treated with cadmium. It is worth mentioning that these results are semi-quantitative and should be confirmed by spectrophotometric methods.

Regarding the peroxidase activity, the only activity detected was associated to the bifunctional catalase. In all strains, peroxidase activity decreased when cells were treated with cadmium (Figure 4.4b) while the catalase activity was increased (Figure 4.4a). Interestingly, cadmium seems to have opposite effects on the catalase and peroxidase activities of the bifunctional enzyme KatG. Since this enzyme contains only one active site it seems a remote hypothesis that cadmium is interfering with this site affecting only one of its functionalities. Regarding the catalytic cycle of the catalase-peroxidase, cadmium may be interfering with the exogenous reducing substrate necessary for the peroxidase activity.

4.3. Superoxide dismutase (SOD) activity

The gel zymography performed to assess the activity of this enzyme, in strain MG1655, revealed no differences between control and treated samples (Figure 4.5a). The corresponding spectrophotometric assay was performed to confirm the results obtained by gel zymography, as done for the catalase activity.

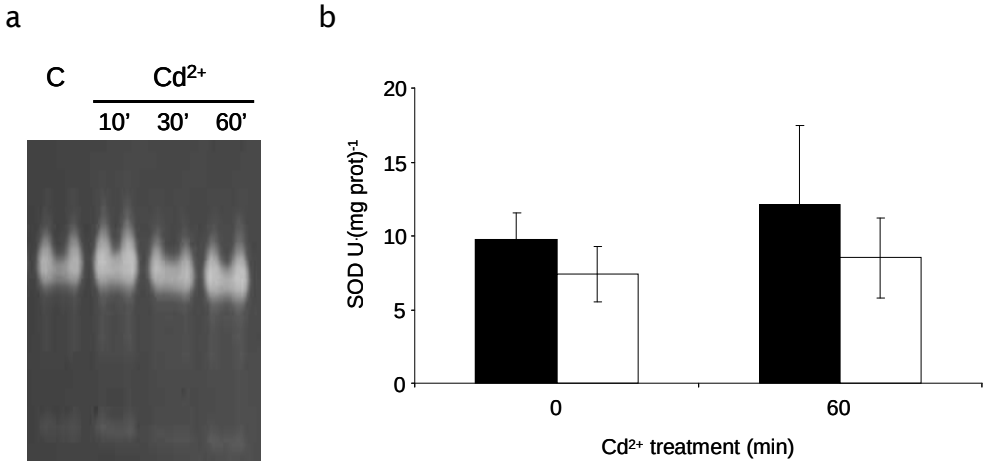


Figure 4.5 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on *E. coli* K-12 MG1655 SOD activity assessed by: **(a)** gel zymography (aerobic conditions) and **(b)** spectrophotometric assay in aerobic (filled bar) and fermentative conditions (open bar). No statistically significant differences between Cd-treated and untreated cultures were identified.

The results obtained by spectrophotometric assay confirmed that the levels of SOD activity remained unaltered when cells were treated with cadmium (Figure 4.5b). Protein extracts obtained from fermenting cells revealed that the levels of SOD were similar to those observed in respiring cells, and also remained unaltered in treated cells.

4.4. Glutathione measurement

Intracellular and extracellular levels of glutathione were measured to clarify the involvement of this molecule in the *E. coli* response induced in the presence of cadmium.

The intracellular levels of glutathione decreased when cells were treated with the metal. After 30 min of treatment the levels of total glutathione decreased by 50% and dropped to 30% when cells were treated for one hour (Figure 4.6). In

contrast with the intracellular glutathione, the levels of this thiol outside the cell increased with cadmium treatment leading to a 3-fold increase in its total extracellular concentration.

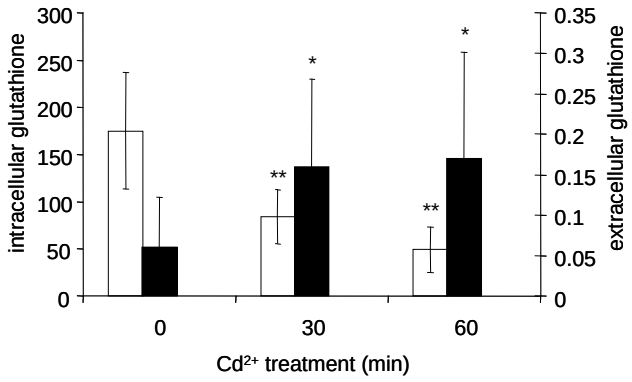


Figure 4.6 Effect 30 $\mu g \cdot mL^{-1}$ cadmium on total glutathione: intracellular concentration [nmol (GSH + 2GSSG)·(mg protein)$^{-1}$] (open bar), extracellular concentration [µM (GSH + 2GSSG)] (filled bar). * (P<0.05) and ** (P<0.01) express significant differences between Cd-treated and untreated samples.

These data are in agreement with the mechanism that has been proposed for cadmium detoxification, which involves the formation of a complex between glutathione and this metal. The concomitant increase of the extracellular concentration seems to suggest that this mechanism involves the export of glutathione.

4.5. Discussion and conclusions

The addition of cadmium to the growth medium was shown to induce a growth stasis together with a decrease in the culturability of *E. coli* strains. This heavy metal revealed to be associated with an oxidative stress by interfering with the levels of oxidized lipids and proteins. To understand the adaptive response of the bacterium to the metal-induced stress, the status of the antioxidant defence system was assessed by determining the activity of catalase and SOD, and the levels of glutathione.

The enzymatic activities were determined in MG1655 cell cultures grown in respiratory and fermentative conditions. Regarding SOD activity, the results revealed that cadmium treatment does not interfere with the activity of this

antioxidant enzyme. On the other hand, a higher catalase activity was detected in cadmium treated cultures grown both under fermentative and respiratory conditions. Surprisingly, cadmium produced a similar induction of catalase in the presence and in the absence of oxygen. Another unanticipated fact was the high levels of this enzymatic activity that were found in untreated cultures grown under fermentative conditions. This is a novel result that, as far as we know, has not been reported in the literature. It is difficult to imagine a reasonable explanation for this fact since the cultures were grown in the absence of free oxygen or nitrate. The role of catalase in the response against cadmium-induced stress was further investigated taking advantage of the mutant strains Δ*katE*, Δ*katG* and Δ*ubiE*. As reported in the previous chapter, a similar pattern of oxidized proteins was detected for the catalase mutants and the wild-type strain. Catalase activity was determined in these mutants and the results revealed that the enzyme activity was severely decreased in these strains. The residual levels of catalase detected in the Δ*katG* strain were expected since expression of *katE* occurs at basal levels in the exponential growth phase. The great reduction in the levels of catalase activity (minus 50%) found in the Δ*katE* strain was unexpected since the gene for the main catalase expressed in aerobic conditions is intact in this mutant, so levels similar to the wild-type were expected. Although induction of catalase was detected when both mutant strains were treated with cadmium, this increase does not explain the low levels of oxidatively damaged proteins observed. These results indicate that catalase activity is not the main defence against cadmium-induced ROS, as the levels of this enzyme are decreased by 50% and the protein damage observed remains unaltered.

The effect of cadmium on the activity of catalase in Δ*ubiE* mutant was also analyzed. In this strain, the effect of the metal on growth and culturability was significantly reduced when compared to the wild-type. In terms of oxidative damage, high constitutive levels were registered but they remained unaltered when the strain was treated with cadmium. In agreement with the increased oxidative stress registered in the Δ*ubiE* cells, high constitutive catalase activity was detected. Cadmium treatment lead to a significant increase in the activity of this enzyme which does not seem to correlate with the decreased toxicity of cadmium observed in this strain. A similar pattern of catalase activity was observed in fermenting wild-type cells. These results taken together seem to suggest that the mechanism of catalase activation by cadmium does depend on oxygen. The real-time PCR experiments performed lead us to conclude that

cadmium exerts negative regulation on the transcription of *katE* and, in the Δ*ubiE* mutant, also of *katG*. Under these circumstances, one possible explanation is that cadmium may induce catalase activity by a post-transcriptional mechanism. Post-transcriptional activation of *glmS* translation by the two small non coding RNAs, GlmY and GlmZ was recently described in *E. coli* (Urban and Vogel 2008). Instead of an increase of protein levels, the hypothesis that cadmium leads to an increase of catalase activity cannot be discarded. Catalase activity was also determined in the mutant strains by gel zymography allowing us to discriminate the contribution of each catalase to the total enzyme activity detected by spectrophotometric assay. This technique confirmed the induction of both catalases in the presence of the metal and strengthened our hypothesis that the induction of these enzymes is independent of oxygen since the expression of *katE* is not regulated by OxyR. Interestingly, the results seem to show that the activity of KatE is present at higher levels than expected under the conditions tested, which has never been reported before.

The effect of cadmium on the intracellular and extracellular levels of glutathione was also investigated. The results obtained are in agreement with the observation that the addition of exogenous GSH abolishes the cadmium induced stasis. Moreover, the decrease in intracellular glutathione suggests that cadmium is detoxified by formation of a complex with this thiol, similarly to what was shown in yeast (Li et al. 1997). In *S. cerevisiae*, it is also known that after cadmium is conjugated with glutathione, the complex is sequestered to the vacuole through the YCF1. *E. coli* does not contain vacuoles thus it can be assumed that the cadmium GSH-dependent detoxification pathway involves a transport system for the thiolate. In the work by Sharma et al. (2000), it is described that the *in vitro* activity of the ZntA pump (a zinc exporter) is highest when metals are presented as thiolates of cysteine or GSH. In agreement with the observation that the GSH extracellular concentration increases is the fact that the ZntA activity gets stimulated when Cd-thiolate complex is presented suggesting that metal-thiolate complexes are the true *in vivo* substrates for this ATPase.

Cadmium was shown to interfere with the activity of catalase and with the levels of glutathione. The catalase activity was increased in the presence of cadmium probably through a post-transcriptional mechanism that needs to be further investigated. Even though the activity of this antioxidant enzyme was shown to be induced in the presence of cadmium, we were able to prove that it is

not essential for survival; indeed, the levels of oxidized proteins did not build up in mutants with reduced catalase activity. Regarding the role of glutathione, it was confirmed that this thiol is important in the detoxification of cadmium, as shown by the alterations observed in its levels inside and outside the cell. There is no doubt that glutathione is a primary defence against cadmium although, it is not essential for cell survival under cadmium stress (see previous chapter).

The SOD enzyme was not affected by cadmium which does not mean that it is not involved in the adaptive response of the bacterium to the stress induced by the metal. If necessary, the constitutive levels of this enzyme are able to cope with the stress induced by the metal.

The data we obtained support that the cell defence system has evolved harbouring different components that may have the same protective role depending on the physiological conditions.

5. Effect of cadmium on respiration

5.1. Introduction

E. coli cells contain a respiratory chain located in the membrane and formed by different protein complexes. This chain utilizes reducing equivalents to reduce a final acceptor with concomitant generation of proton-motive force (*pmf*).

Different protein complexes are involved in the electron transport such as flavoproteins, iron-sulphur proteins and cytochromes. In addition, the chain also contains electron carriers such as the lipid-soluble quinones that can diffuse freely through the membrane, generally transferring electrons from iron-sulphur proteins to cytochromes. Quinones have two basic functions: to accept electrons from a donor and transfer them to the next acceptor (Gennis and Stewart 1996). Electrons are transported through the chain components and simultaneously protons are extruded outside the cell into the periplasm. At the end of the ETC, the electrons are transferred to the final electron acceptor which, in the case of aerobic respiration, is O_2, and thus oxygen is reduced to water on the cytoplasmic side of the membrane. This reaction consumes protons which, together with the extrusion of H^+ by the ETC, leads to the generation of a pH and electric-charge gradient across the membrane forming an electrochemical potential. The inner side of the membrane becomes negative and alkaline and the outer side positive and acidic (Madigan et al. 1997). The *pmf* can be the driving force for cellular processes or used to obtain chemical energy in the form of ATP by the activity of the complex ATP synthase.

The *E. coli* respiratory components are closely related to those present in the mammalian mitochondrion with the exception of the cytochrome *c* reductase (complex III) that is absent. *E. coli* possesses two NADH dehydrogenases, the Ndh-I and Ndh-II that oxidize NADH and reduce quinones directly. NADH dehydrogenase I is a 14-subunit intrinsic membrane protein encoded by the genes *nuoA* through to *nuoN* and is homologous to the eukaryotic 42-subunit mitochondrial complex I. The NADH:quinone oxidoreductase activity of this enzyme is coupled to proton translocation across the membrane: for each electron transported, two protons are exported to the periplasm (Figure 5.1). In contrast to Ndh-I, Ndh-II is a one-subunit peripheral membrane enzyme (encoded by the *ndh* gene) that carries out the NADH:quinone oxidoreductase reaction at the inner surface of the cytoplasmic membrane and is not coupled to the generation of the proton gradient across the membrane (Gennis and Stewart 1996) (Figure 5.1). Besides these two dehydrogenases, *E. coli* contains several substrate-specific dehydrogenases that carry out the oxidation of organic

substrates and feed electrons directly into the mobile quinone pool. This is the case of the succinate dehydrogenase, also known as succinate:quinone oxidoreductase or complex II, which catalyzes the oxidation of succinate to fumarate and reduces UQ in the membrane. This is a four-subunit enzyme (encoded by the *sdhCDAB* operon) that contains two hydrophobic units that act as the membrane anchors and are essential for the reduction of UQ, while the hydrophilic subunits perform the succinate dehydrogenase activity (Figure 5.1).

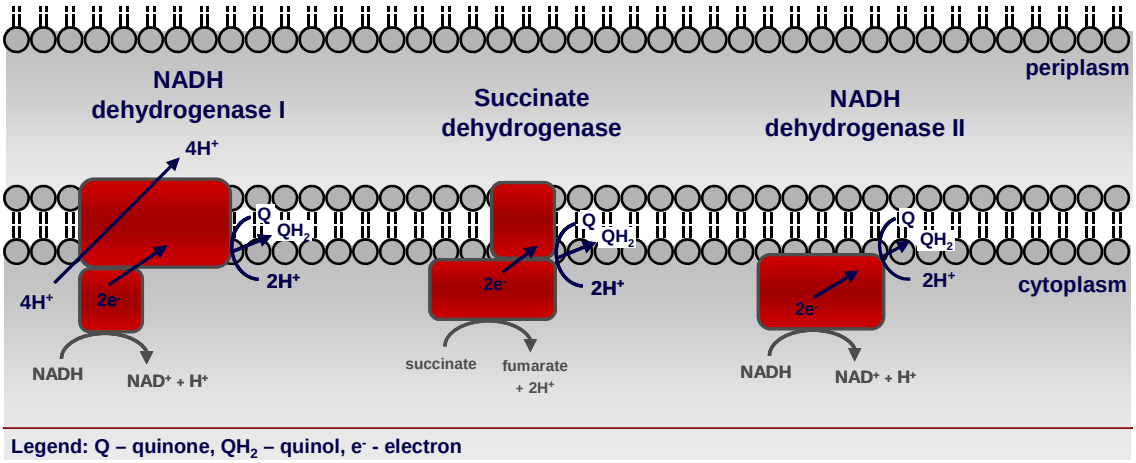


Figure 5.1 The NADH-dehydrogenases and succinate dehydrogenase from *E. coli* (adapted from Gennis and Stewart 1996).

E. coli synthesizes three different quinones, ubiquinone (UQ), menaquinone (MK) and demethylmenaquinone (DMK), that mediate the electron transfer between the protein components of the chain. Quinone reduction and oxidation reactions may involve one- or two-electron transfers: in the former a semiquinone radical is produced during the electron transfer process (Gennis and Stewart 1996).

The terminal components of the ETC in *E. coli* are two respiratory oxidases, cytochrome *bd* and cytochrome bo_3. Both enzymes are quinol oxidases and both are coupling sites in the chain, contributing to the *pmf*. For each electron used to reduce oxygen, two cytoplasmic protons are used: one to generate water and the second is extruded to the periplasm. Cytochrome bo_3 is expressed under high aeration conditions while cytochrome *bd* is expressed when aeration is low. These two complexes also differ in the H^+/e^- ratio, with cytochrome *bd* having a ratio of

1 while for cytochrome bo_3 this ratio is of 2 (Gennis and Stewart 1996) (Figure 5.2).

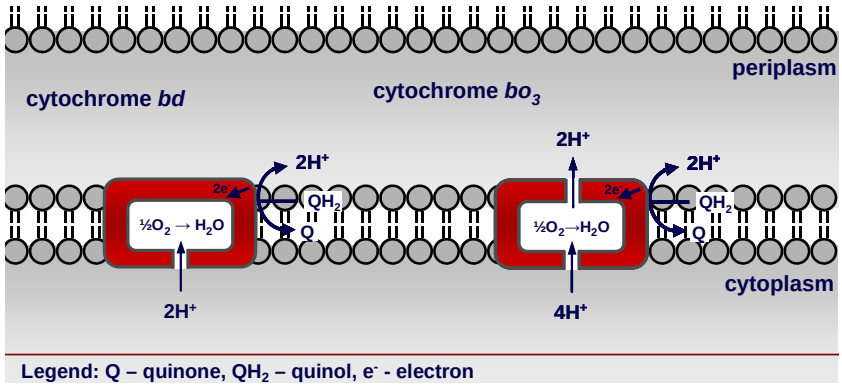


Figure 5.2 Cytochromes *bd* and bo_3 from *E. coli* (adapted from Gennis and Stewart 1996).

The aerobic and anaerobic respiratory systems of *E. coli* allow the bacterium to oxidize a wide variety of organic substrates (e.g. sugars, carboxylic acids, ethanol) passing the electrons ultimately to any of a number of oxidants (e.g. oxygen, nitrate, fumarate, trimethylamine-N-oxide or dimethyl sulfoxide). The modular design of the respiratory system allows this bacterium to adapt to a wide variety of growth conditions and environmental changes because the different components can be substituted in the membrane in place of, or in addition to, other components as they are needed. Another advantage of this system is the apparent redundancy of its modularity: though the multiple components seem similar, they are used in distinct situations. In the case of the quinones, UQ is used for oxygen respiration, both UQ and MK are used for nitrate respiration, and both MK and DMK are used for anaerobic respiration with acceptors other than nitrate. The combination of the different modules allows *E. coli* cell to optimize the ETC assuring the maintenance of the redox balance and the regeneration of NAD^+ independently of the bioenergetic efficiency (Gennis and Stewart 1996).

In this chapter we describe experiments performed to assess the effect of cadmium on respiration, using cultures grown on glucose in the presence of oxygen.

5.2. Cadmium effect on oxygen consumption

Since the work carried out by Kasahara and Anraku (1972) new data has been accumulated supporting the hypothesis that the toxicity of metals is associated with an interference with the ETC and associated enzymes (e.g. succinate dehydrogenase) (Bragg and Rainnie 1974; Kleiner 1978; Surowitz et al. 1984; Tynecka et al. 1990; Beard et al. 1995). To test this hypothesis, oxygen consumption by *E. coli* cells was measured upon addition of cadmium. As previously reported, cadmium interferes with the levels of glutathione and thus we analyzed the effect of the addition of the thiol, either oxidized or reduced (Figure 5.3).

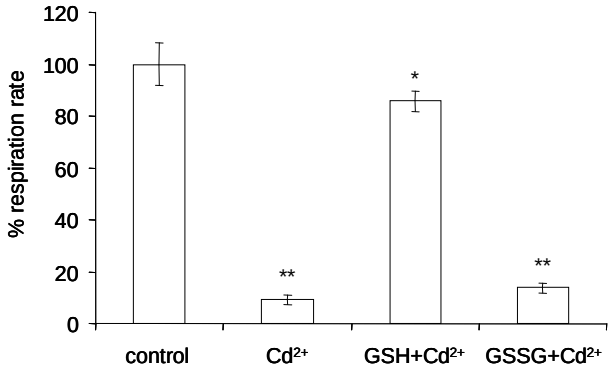


Figure 5.3 Effect of 30 $\mu g \cdot mL^{-1}$ cadmium on oxygen consumption. The absolute value (nmol O_2)·min^{-1} corresponding to 100% is 98±7.9. Significant differences between the control and test situations are represented: * (P<0.05) and ** (P<0.01).

When cadmium was added to the cells, a drastic decrease in O_2 consumption was observed. The effect of this metal was abolished when reduced glutathione was added exogenously to the cell suspension, however, addition of GSSG had no appreciable effect. Oxygen consumption in the presence and absence of cadmium was also measured in cells using different carbon sources besides glucose (glycerol, succinate, lactate, fumarate and pyruvate). For all the substrates tested, inhibition of respiration was observed in the presence of cadmium (data not shown).

5.3. Cadmium effect on ROS production

Taking into account the fact that O_2 consumption is severely inhibited in the presence of cadmium, we addressed the question regarding the levels of ROS generated. Most of the oxygen consumed by the cells is channelled to the respiratory chain and, as mentioned before, a small percentage of the oxygen consumed by the cell is converted to ROS.

Initially, ROS levels were determined for strain MG1655 in the presence and in the absence of oxygen (Figure 5.4).

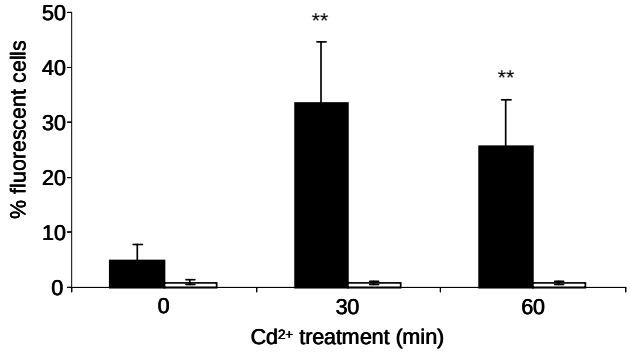


Figure 5.4 Detection of ROS by flow-cytometry in strain MG1655 in respiratory (filled bar) and fermentative (open bar) conditions. Significant differences were detected between control and cadmium-treated samples in respiratory conditions - ** (P<0.01).

In aerobic conditions, cadmium treatment lead to a significant increase in the levels of ROS. In the absence of oxygen, as expected, the levels of these species were undetectable, validating the ROS measurement.

Cadmium was shown to drastically decrease oxygen consumption when different carbon sources were used suggesting that this inhibition does not result from an interference with glycolysis or the TCA cycle. In agreement with the data, we hypothesized that cadmium-induced ROS result from interference of the metal with the respiratory chain. A key proof would be to demonstrate that no ROS are produced when the ETC is not working despite the presence of molecular oxygen. A first approach was to utilize inhibitors of different respiratory components and then measure ROS production, in the presence and in the absence of cadmium. Complex I inhibitors (rotenone or piericidin A) revealed to be ineffective when

used with whole cells. In contrast, KCN was able to inhibit oxygen consumption in *E. coli* cultures. Potassium cyanide is a complex IV inhibitor that prevents association of oxygen to the terminal oxidases. So it would be expected that cultures treated with this inhibitor would show increased ROS levels due to the fact that electrons can't be transferred from the quinones to the terminal oxidases.

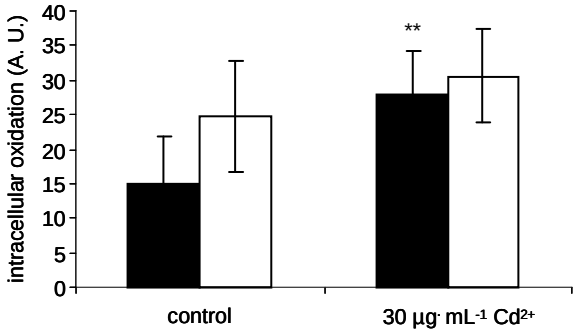


Figure 5.5 Detection of ROS by flow-cytometry in strain BW25113 (arbitrary fluorescence units): untreated (filled bar) and treated with 2 mM KCN (empty bar). Significant differences were detected between control and cadmium-treated samples in the absence of KCN are shown: ** (P<0.01).

As expected, a KCN-induced ROS increase was indeed detected compared to the untreated control. Nevertheless, when these cells were further exposed to cadmium, the levels remained similar (Figure 5.5). In the presence of cyanide, the cadmium-induced ROS increase was 24% while in the absence of the inhibitor the increase was 87%. To pursue the analysis of cadmium targets within the respiratory chain, ROS levels were measured in strains mutated in the different ETC components and associated enzymes: Ndh-I (Δ*nuoB*), Ndh-II (Δ*ndh*), succinate dehydrogenase (Δ*sdhC*) and in the UQ and MK biosynthesis (Δ*ubiE*), (Figure 5.6).

All the mutants utilized showed significantly altered levels of ROS in control conditions. The Δ*sdhC* strain revealed to have low levels of constitutive ROS. Cadmium-induced ROS production was detected in the Δ*sdhC* strain although the effect of the metal was significantly reduced with a 32% increase registered against the 87% seen in the wild-type. In all other mutants ROS levels were higher when compared to the wild-type. In the Δ*nuo* mutant, basal ROS were increased

by 30% with respect to the wild-type, while in the Δ*ndh* the increase was of 10%. Despite the fact that these mutants have increased basal levels of ROS compared to the wild-type control, it was observed that treating these strains with cadmium did not lead to significant alterations in the levels of ROS. Intriguingly, the elimination of either Nuo or Ndh, which use NADH as electron donor, results in the abolishment of cadmium-induced ROS production (Figure 5.6).

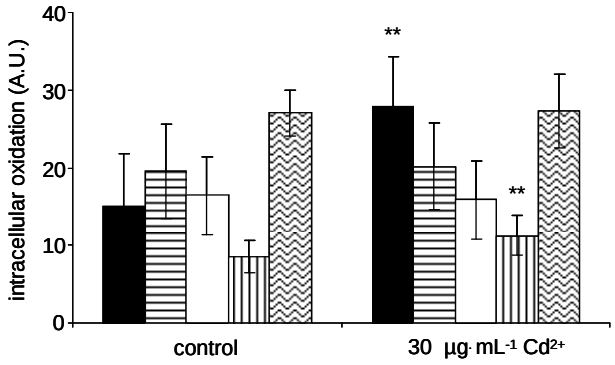


Figure 5.6 ROS detection by flow cytometry (arbitrary fluorescence units): wild-type (filled bar), Δ*nuoB* (horizontal stripe bar), Δ*ndh* (open bar), Δ*sdhC* (vertical stripe bar), Δ*ubiE* (zigzag stripe bar), ** (P<0.01) means that there is a significant difference between control and cadmium-treated samples.

The Δ*ubiE* mutant was also tested: this strain is unable to produce both UQ and MK, but retains DMK. In this mutant the oxygen consumption rate is decreased to 38% of the wild-type however, the levels of ROS generated are the highest of all mutants tested indicating the different functions of the quinone pool members. Similar to the observed for the Δ*nuoB* and Δ*ndh* mutants, treating the Δ*ubiE* strain with cadmium had no effect on ROS levels. This result suggests that the effect of cadmium may be related to the presence of UQ and altering the composition of the quinone pool leads to the abolishment of the metal-induced ROS production.

Another issue addressed was how cadmium would affect the levels of ROS in mutant strains with an impaired defence system, as is the case of the catalase and GSH deficient mutants (Figure 5.7).

As observed for the mutants in the ETC components, catalase and GSH mutant strains revealed altered levels of ROS in control conditions. The catalase mutants showed higher ROS levels which would seem consistent with the diminished levels

of catalase activity detected in these stains (chapter 4). Upon treatment with cadmium, the levels of ROS increased moderately, with 15% increase in the Δ*katE* strain and 18% in the Δ*katG* mutant (Figure 5.7).

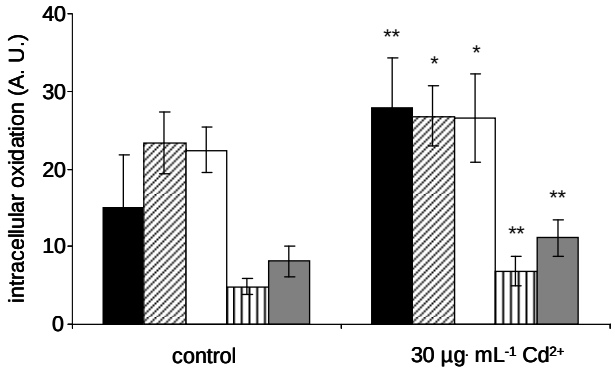


Figure 5.7 ROS detection by flow cytometry: wild-type (filled bar), Δ*katE* (diagonal stripe bar), Δ*katG* (open bar), Δ*gshA* (vertical stripe bar), Δ*gshB* (grey bar). * (P<0.05) and ** (P<0.01) mean that there is a significant difference between control and cadmium-treated samples.

In the case of the glutathione-deficient mutants, reduced constitutive levels of ROS were detected and when exposed to cadmium there was an increase of 41% in the Δ*gshA* strain and 37% in the Δ*gshB* strain (Figure 5.7). The highest increase in ROS due to the presence of cadmium was detected in the wild-type, suggesting that the impairment of the antioxidant defences does not contribute to exacerbate the oxidative stress status imposed by the metal under the conditions tested.

5.4. Discussion and conclusions

The effect of cadmium on oxygen consumption and ROS production was evaluated in the wild-type and in different mutant strains. These experiments were performed to determine the origin of ROS generated by cadmium exposure.

In *E. coli*, the link between metal toxicity and oxidative stress is clearly demonstrated by the increased ROS production in cadmium-treated cells. It is also clear that cadmium is interfering with the respiratory chain, as put in evidence by the inhibition of oxygen consumption in the presence of metal. These data strengthened our hypothesis that the presence of cadmium interferes with the

respiratory chain leading to ROS production and therefore this interference is at the origin of the oxidative stress status. The identification of the targets of cadmium within the respiratory chain would allow us to propose a more refined model for the toxicity of this metal in *E. coli* respiring cells. So, the next step was to evaluate ROS production in strains deficient in different ETC components and associated enzymes.

In the Δ*sdhC* mutant reduced constitutive levels of ROS were expected since this enzyme has been identified as a source of these reactive species in *E. coli* (Messner and Imlay 2002; Cheng et al. 2006). Despite the low constitutive levels of ROS detected, cadmium treatment still resulted in increased ROS production. In the other mutants, Δ*ndh*, Δ*nuoB* and Δ*ubiE*, higher levels of ROS were detected already in control conditions and in the presence of the metal the levels remained unaltered.

NADH serves as an important electron donor for the ETC and consequently, the Ndh-I and Ndh-II serve as the primary dehydrogenases (Calhoun and Gennis 1993; Tran et al. 1997). It was demonstrated that in the early log phase both Ndh-I and Ndh-II are used to a significant extent during aerobic respiration (Calhoun et al. 1993; Wackwitz et al. 1999). At this growth stage, both mutants in the NADH dehydrogenases showed increased ROS levels in control conditions with a 30% increase in the Δ*nuoB* and 10% in Δ*ndh*. Calhoun et al. (1993), mentioned that the Δ*nuoB* mutant had an increased oxygen consumption to meet the ATP requirements. Since Ndh-II does not generate *pmf*, the terminal oxidase will be the main coupling site of the chain (Wackwitz et al. 1999). In light of these previously published observations, the 30% increase in basal ROS levels could be explained by the increased oxygen consumption. When only Ndh-I is present, all the electron flux from NADH is forced to go through the coupled enzyme resulting in increased bioenergetic efficiency (Calhoun et al. 1993). In these conditions, a significant reduction in oxygen consumption would be expected to balance the increased coupling rate and ATP yield. In their work, Calhoun et al. (1993) verified that the reduction in oxygen consumption is not as severe as expected. Based on these facts, one might speculate that if the cell is consuming more oxygen than needed to meet the cellular bioenergetic requirements, then the surplus of oxygen consumed could be converted to superoxide and subsequently to other species explaining the increase in the basal ROS levels detected in this mutant strain. Despite the elevated levels of ROS registered in

control conditions, the treatment of the two dehydrogenase-deficient strains with cadmium had no net ROS-increasing effect.

Cells grown in the presence of oxygen contain about four or five times more UQ than the combination of MK plus DMK, so UQ is the main electron carrier in oxygen respiration (Gennis and Stewart 1996). The Δ*ubiE* contains only DMK, this deficiency in the quinone pool leads to a reduction in the oxygen consumption rate. This strain showed high constitutive levels of ROS and remained at the same level upon treatment with cadmium. *In vitro* studies using membranes from a quinoneless mutant showed that succinate dehydrogenase and NADH dehydrogenases produced ROS at undiminished rates when incubated with NADH or succinate (Imlay 1995). Moreover, this mutant strain was reported to be unable to grow on succinate (Lee et al. 1997). Taking all this into consideration, the increased levels of ROS can result from the autoxidation of the NADH and the succinate dehydrogenases due to the inefficiency or inability to transfer electrons to DMK. The specificity of the quinone species is not only dictated by their mid-point potentials, but may also be due to structural constraints of the quinone-binding sites of the dehydrogenase and oxidoreductases (Ingledew and Poole 1984). The effect of cadmium on cells treated with cyanide was also determined and the results showed that the effect of cadmium gets significantly diminished when the terminal oxidases are inhibited.

The fact that depletion of GSH has been pointed out as the cause for cadmium toxicity led us to check the effect of this metal on the production of ROS in strains with impaired antioxidant defences. For obvious reasons, GSH-deficient mutants were utilized, but strains deficient in either catalase were also included since cadmium has been shown to interfere with the activity of these antioxidant enzymes (chapter 4). In the four mutants analyzed, cadmium induced a significant increase in ROS levels, but, still, the biggest rise in oxidative stress was observed in the wild-type. In the catalase mutants, treatment with cadmium lead to an increase in the levels of ROS. A higher increment than that registered was expected since the activity of catalase was reduced in these mutants. These results show that the lack of these defence systems is not essential for cell survival under cadmium stress and this phenotype response may be related with the activation of complementary defences.

Regarding glutathione, it has been considered to form a complex with cadmium that is secreted, leading to the depletion of GSH and this results in unbalanced redox-status that causes oxidative stress. Our results show that ROS

levels are increased whether GSH is present or not and, in fact, prove that the depletion of GSH cannot be the cause for cadmium-induced oxidative stress. Nevertheless, it should be stressed out that GSH constitutes an important primary defence against cadmium, the formation of GSH-cadmium complex being an important route for detoxification of the metal in *E. coli.* This concept is substantiated by the observations that i) GSH-deficient mutants recover more slowly from cadmium-induced growth arrest and ii) the inhibition of growth and oxygen consumption is completely abolished in the presence of GSH.

Our data clearly demonstrate that, in *E. coli*, cadmium toxicity is associated with oxidative stress due to an interference of this metal ion with the respiratory chain. The role of glutathione as a primary detoxification mechanism was confirmed, but cadmium-induced GSH depletion as a cause of ROS production can be excluded.

In an attempt to identify the targets of cadmium within the ETC, ROS levels were assessed in deletion mutants. The results for the different strains tested suggest that cadmium interferes with the respiratory chain at different points. The fact that the ETC presents a modular and redundant nature made it impossible so far to determine the effective targets of cadmium, at least based on the *in vivo* experiments we performed.

6. General discussion

The present thesis reports new data on the mechanism of cadmium toxicity and on the adaptive response triggered by this metal on *E. coli* K-12 strains. To carry out the analysis of the effects of cadmium-induced stress on *E. coli* K-12, we started by identifying the concentration of cadmium to be used. The selection of the concentration was based on the growth arrest in oxygen-respiring cells. Previous works reported that the effect of this metal on cell growth was accompanied by a decrease in cell culturability and by an increase in oxidative stress markers (Mitra et al. 1975; Mitra and Bernstein 1978; Mitra 1984; Shapiro and Keasling 1996; Ferianc et al. 1998). Indeed we also observed a 25% decrease of cultivatable cells in respiring cultures after 30 minutes of cadmium exposure. Besides the effects on growth and culturability, oxidative damage of proteins, lipids and DNA have been widely described as result of cadmium toxicity, although with distinctive effects depending on the organism or tissue used (Stohs and Bagchi 1995; Waisberg et al. 2003). The oxidative damage due to cadmium was assessed by analysis of changes in levels of lipid peroxidation and protein oxidation in the *E. coli* K-12 MG1655 and BW25113. Although these are closely related K-12 strains, it was soon observed that the MG1655 was less sensitive to cadmium, at least at the concentration used (30 $mg \cdot L^{-1}$). Significant differences were observed in the duration of growth arrest, culturability and also in the levels of oxidative damage. In strain MG1655, treatment with cadmium did not induce oxidative damage that we could measure, while strain BW25113 showed increased levels of lipid peroxidation and protein carbonylation. The analysis of the oxidized proteins did not reveal any cadmium-specific effect, and the preliminary identification of the more oxidized proteins showed that they are mainly related to glycolysis, TCA cycle and protein synthesis. Among the most probable proteins identified, the aldehyde-alcohol dehydrogenase (AdhE), the methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE), the elongation factor G (FusA), enolase (Eno) and the outer membrane protein C (OmpC) were previously identified as major targets of oxidation under oxidative stress (Tamarit et al. 1998; Hondorp and Matthews 2004). The oxidation of these proteins was suggested to be a protection mechanism by avoiding the damage of crucial enzymes or delaying high energy-consuming processes, such as amino acids or protein synthesis. Analogously, a previous analysis of the transcriptomic

response of *E. coli* to cadmium (Wang and Crowley 2005) has revealed that the metal affects protein synthesis, energy metabolism and cell rescue.

In agreement with the increased oxidative stress markers, cadmium-induced ROS production was exacerbated (plus 87%) and some antioxidant cell defences were altered in the presence of the metal. Catalase activity was shown to be increased by two fold in treated cells, while SOD activity remained unaltered. The increase in catalase activity observed correlates with proteomic studies that reported the induction of the *oxyR* regulon (VanBogelen et al. 1987; Ferianc et al. 1998) that activates *katG* transcription. Cadmium has also been shown to interfere with the cell endogenous radical scavengers such as GSH, the major non-protein thiol in *E. coli*, that constitutes an important non-enzymatic cell defence (Fahey et al. 1978; Carmel-Harel and Storz 2000). The analysis of the pool of total glutathione indicated a Cd-dependent depletion of intracellular glutathione with concomitant rise in its external concentration. These results are in frame 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 by complexing the metal in the medium and thus preventing its import. 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).

Since cadmium is a redox-inactive metal it does not participate in the Fenton reaction like redox-active metals (e.g. Cu and Fe); therefore cadmium-induced oxidative stress is originated through an indirect mechanism. In fact, depletion of the endogenous radical scavenger GSH has been pointed out as the cause for cadmium-induced oxidative stress in higher eukaryotes, yeast and bacteria (Stohs and Bagchi 1995; Howlett and Avery 1997; Ercal et al. 2001; Banjerdkij et al. 2005; Liu et al. 2005; Wolf and Baynes 2007). To clarify the role of GSH in *E. coli*, glutathione deficient strains (Δ*gshA* and Δ*gshB*) were tested, revealing that the growth effect of cadmium in these mutants was similar to that observed in their isogenic wild-type strain. In terms of culturability, however, the fraction of cultivatable cells after cadmium treatment was significantly smaller in the GSH-deficient strains. Interestingly, between the two mutants the Δ*gshB* strain was less affected by Cd; this may be explained by the fact that the first step in GSH biosynthesis still occurs in this strain and the product of this first step, the

dipeptide gamma-glutamylcysteine, may confer some level of protection against cadmium (Cruz-Vásquez et al. 2002). The levels of ROS in GSH-devoid mutants were also analyzed and it was shown that they were significantly increased when these mutant strains were treated with cadmium. These data indicate that there is a rise in ROS whether GSH is present in the cell or not, implying that the generation of ROS in cadmium-exposed cells is independent of the presence of glutathione. Notably, the glutathione mutants appear to be more sensitive to cadmium since their recovery is somewhat slower than the wild-type. This fact confirms the role of GSH in cadmium detoxification, even though it should be stressed that it is not essential since the GSH mutants are viable under cadmium stress.

In early works, interference of several metals with the respiratory chain has been suggested (Kim and Bragg 1971; Kasahara and Anraku 1972; Bragg and Rainnie 1974) to test this hypothesis in the case of cadmium, oxygen consumption tests were performed. We observed that cadmium consistently inhibits oxygen consumption, strongly supporting a correlation between cadmium toxicity and ROS production by the respiratory chain.

In fermenting cells treated with cadmium, the detrimental effects of the metal on growth and culturability were clearly reduced. Rather than a growth arrest effect, only a deflection in the growth rate was registered and after 60 min of treatment the CFU counts remained similar to untreated cultures. Moreover, the fraction of cultivatable cells in treated fermenting cultures was 30% higher than in respiring cultures, confirming that at least part of the toxicity of this metal is associated with aerobic metabolism. 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; Vido et al. 2001). To further elucidate if cadmium toxicity is linked to respiration, a mutant strain with impaired respiratory function was tested. The Δ*ubiE* strain contains only DMK, this deficiency in the quinone pool leads to a decreased in oxygen consumption. When the growth rate and culturability were analyzed in the Δ*ubiE* strain, a severe reduction in cadmium toxicity was observed reinforcing the correlation between metal toxicity and aerobic metabolism. Although this strain showed constitutively increased oxidative stress markers when cells were exposed to cadmium, the oxidation levels of proteins and lipids remained unaltered. When ROS levels were analyzed in the Δ*ubiE* mutant, the suspected increased oxidative stress status was confirmed by the high basal levels of ROS

detected. Similar to the observed for the oxidative stress markers, ROS production was not affected when this mutant was exposed to cadmium. In contrast to the reduction of the cadmium effect on growth and culturability and on the oxidative stress markers and despite the fact that cadmium-induced ROS production was abolished in this strain, catalase activity was induced in the presence of this metal. These data seem to suggest that catalase activity is induced, in the presence of cadmium, by a mechanism that is independent of oxygen. In agreement with this observation we also registered a cadmium-induced increase of this enzyme in cells grown in fermentation, in the absence of oxygen or nitrate. When the relative expression of the catalase genes was analyzed in the Δ*ubiE*, cadmium exerted a negative effect on their transcription, suggesting that the possible regulation mechanism is exerted at the post-transcriptional level. The effect of cadmium on the activity levels of catalase was also analyzed in the Δ*katE* and Δ*katG* mutant strains. The Δ*katE* showed low catalase activity (50% of the wild-type), which was unexpected since previous works showed that KatG is the main catalase present in the early exponential growth phase (Schellhorn 1995). In the Δ*katG* mutant strain, residual levels of activity were registered (12.5% of the wild-type). These results seem to contradict the observations on oxidized protein levels whereby similar levels of oxidation were detected for the wild-type and the catalase mutant strains. A preliminary analysis of the cadmium effect on growth and culturability of the Δ*katG* strain showed that the effect of the metal on these parameters was again similar to that observed in the wild-type. ROS levels were also assessed in these mutants, revealing that ROS increased significantly when cells were treated with cadmium. Taken together, the data obtained for these mutants suggest that, at least when considered separately, these antioxidant defences are not essential for cell survival during the challenge with cadmium. Additionally, these data imply that other defence mechanisms must be activated to overcome the oxidative stress imposed to the cells. For this reason, the activity of peroxidases was investigated, but the only activity detected was associated with the bifunctional catalase, KatG. The alkylhydroperoxide reductase has been suggested to be the main hydrogen peroxide scavenger enzyme however, the activity of this enzyme cannot be easily assayed *in vitro* due to subunit dissociation (Seaver and Imlay 2001).

To further elucidate the correlation between cadmium toxicity and ROS production by the respiratory chain, this parameter was analyzed in different deletion mutants. Strains deficient in the two NADH dehydrogenases (Δ*nuoB* and

Δ*ndh*) and in the succinate dehydrogenase (Δ*sdhC*) were tested. Cadmium induced an increase in ROS production in the wild-type and also in the Δ*sdhC* mutant but, in the former, the effect was more severe. In the two NADH dehydrogenase deficient strains, the levels of ROS were unaffected by treatment with cadmium similarly to the behaviour seen in the Δ*ubiE* mutant, a strain with deficient quinone pool. Furthermore, in cultures treated with potassium cyanide, which inhibits the terminal oxidases, no significant increase of ROS production in the presence of cadmium was registered. These data clearly associate the cadmium-induced oxidative stress to an interference of this metal with the electron transport chain, which may probably involve the NADH dehydrogenases and the quinones. In guinea pig mitochondria, cadmium was shown to bind to the Q_o site of cytochrome *b* of complex III leading to the accumulation of semiubiquinones, that are prone to transfer electrons to oxygen originating superoxide (Wang et al. 2004).

Wang and Crowley (2005) reported in their transcriptome study on *E. coli* K-12 under cadmium stress that genes associated with anaerobic metabolism are up-regulated and high energy consumption processes are shut down (e.g. amino acids biosynthesis) suggesting that cells switch to an energy conservation mode. Our hypothesis whereby cadmium poisons the respiratory chain is in agreement with these observations.

Based on the evidence accumulated during this work and on data available in the literature, the following model for Cd toxicity in respiring *E. coli* cells is proposed (Figure 6.1): cadmium enters the cell through one of the essential metal transporters, like ZupT, a zinc transporter (Grass et al. 2002). Once inside the cell, cadmium poisons the ETC leading to the accumulation of unstable reduced intermediates (Messner and Imlay 1999) that spill out electrons directly to molecular oxygen generating ROS. Our data seem to suggest that the NADH dehydrogenases and the quinones are involved in the interference of cadmium with the ETC.

Although the cell defences are activated to eliminate the ROS, they are not sufficient or fast enough to completely prevent oxidative damage. The poisoning of the respiratory chain leads also to a depletion of the ATP stock and to growth arrest. Cadmium is then detoxified through excretion from the cell by the action of 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, also actively excreted. Even though glutathione plays a crucial role in the detoxification of the heavy

metal ion, clearly its depletion in the cytoplasm is not the cause of the ROS burst resulting from exposure to cadmium. Furthermore, we demonstrate that the interference of cadmium with the ETC is indeed the cause for the oxidative stress observed in cells exposed to this metal.

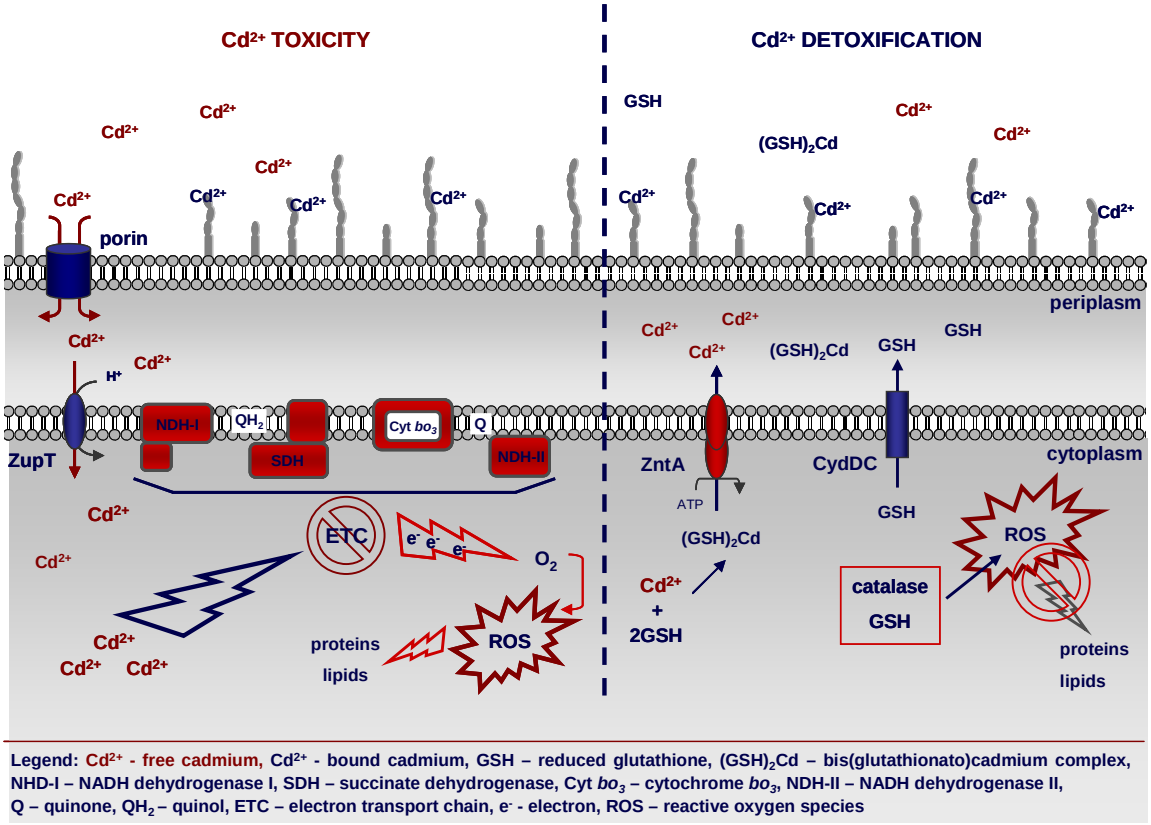


Figure 6.1 Model for cadmium toxicity/detoxification in *E. coli* respiring cells.

Conclusions and Future Perspectives

In this final section the main achievements of this thesis will be summarized and future perspectives for the refinement of the model for cadmium toxicity in *E. coli* respiring cells proposed herein will be presented.

In general terms, we aimed at understanding the mechanisms underlying cadmium toxicity and assess the role of oxidative stress in this process. Furthermore, we wanted to test the relevance of mechanisms of cadmium toxicity previously proposed for eukaryotes, such as inhibition of respiration or glutathione depletion. This work clearly established a link between cadmium toxicity and oxidative stress through the interference of Cd^{2+} ions with the electron transport chain. Additionally, we were able to refute the hypothesis that cadmium-induced oxidative stress was caused by the depletion of cellular stocks of GSH.

Pertinent questions and aspects that could be addressed in order to enhance the model for cadmium toxicity:

Identification of the cadmium targets within the ETC

Analysis of cadmium toxicity in other mutants deficient in the ETC components, namely ROS production, could be analyzed in strains: *cyo* (cytochrome bo_3), *cyd* (cytochrome *bd*), *ubiA* (UQ synthesis), *menA* (MK and DMK synthesis). The generation of double mutants and the analysis of ROS levels should also be considered (e.g. *ndh nuoB* or *ubiA menA*).

Although the ATPase is not part of the electron transport chain, the activity of this enzyme depends on the ETC. Interference of cadmium with the ATPase should also be investigated.

Antioxidant defences

Catalases and Alkylhydroperoxide reductase

Analysis of cadmium toxicity in the *katE* and *katG* mutants should be completed to identify which antioxidant defences are activated in the absence of catalases. The generation of the *katE katG* mutant should also be considered not only to address the role of catalases but also to assess the activity of the alkylhydroperoxide reductase. The single *ahpC* mutant should also be examined to clearly establish the role of the latter enzyme in the response to cadmium. The triple mutant *katE katG ahpC*, if viable, would be helpful in the evaluation of the contribution of peroxidases in the response against cadmium. Analysis of an *oxyR* mutant could also be interesting to determine if there is an OxyR-mediated activation of *katG* and *ahpC*.

The possibility of post-transcriptional regulation of the catalases should be further investigated to determine if the increased activity is due to enzyme activation or to *de novo* synthesis.

Superoxide Dismutases (SOD)

Cadmium did not affect the activity of the superoxide dismutases, which does not mean that this enzyme is not involved in the defence mechanism. The issue could also be addressed studying the mutants in the different dismutases: *sodA* (MnSOD), *sodB* (FeSOD) and *sodC* (Cu,ZnSOD).

Glutathione

In this work we demonstrated that the indirect mechanism of oxidative stress induction by cadmium is not due to the depletion of the endogenous radical GSH as is generally accepted for eukaryotes. Recently, Helbig et al. (2008) have published an analysis of the GSH-deficient transcriptome exposed to cadmium, and propose that cadmium-induced oxidative stress is due to thiol depletion. Therefore, the analysis of the glutathione-deficient strains should be completed and the GSH metabolism should be thoroughly tested. The glutathione reductase levels could be analyzed, glutaredoxins and the export of GSH as a defence mechanism should be further explored.

Oxidative damage

DNA damage

Early works addressing the toxicity of cadmium in *Escherichia coli* suggest that exposure to this metal leads to the inhibition of the DNA repair mechanisms and thus DNA damage gets accumulated (Mitra and Bernstein 1977; Mitra and

Bernstein 1978). Preliminary experiments performed during this work did not reveal a significant accumulation of DNA damage. It would be interesting to find new methods or adapt known ones to determine DNA damage and assess the effect of cadmium on the repair mechanisms by analysis of transcript levels of genes related to DNA repair and/or analysis of mutant strains.

Assessment of protein damage

We showed in this work that cadmium clearly increases protein carbonylation. Preliminary identification of the most probable proteins oxidized in the presence of cadmium revealed that these proteins are related to glycolysis, TCA cycle and protein synthesis. A more precise analysis 2-dimension (2D) gel electrophoresis would allow the definite identification of the proteins oxidized in the presence of the metal. The oxidation of some proteins has been suggested to be a mechanism of defence by avoiding the damage to other proteins or by delaying energy-consuming processes, such as protein synthesis. If the oxidation of these proteins was confirmed, then it would be interesting to determine if indeed their oxidation is a defence mechanism by analysis of the proteome of mutant strains in which these proteins are absent.

Cadmium-induced growth arrest

The cause for the cadmium-induced growth arrest remains unknown. Several hypotheses may be raised such as ATP depletion, lack of reducing equivalents or interference with enzymes whose activity is crucial to the cell. Determination of the ATP levels and also of the NADH/NAD^+ and NADPH/$NADP^+$ ratios by chromatographic method could be performed. Assessment of the glucose-6P dehydrogenase (*zwf*) activity could also be considered. Analysis of the thiol pool and determination of the activity of the enzymes involved in GSH biosynthesis (*gshA* and *gshB*) and of the glutathione reductase (*gorA*) could also be a possibility to evaluate the cell redox state.

Sulphur metabolism

Analysis of sulphur metabolism could also be performed to determine whether cadmium is detoxified by precipitation as cadmium sulphide.

The involvement of the synthesis (*isc* operon) and repair (*suf* operon) of Fe-S clusters could also be addressed, analyzing the levels of transcription of the enzymes involved in these pathways or by the generation of mutants.

Other topics of interest

- The analysis the differential response of *E. coli* K-12 strains to cadmium toxicity. The levels of the antioxidant defences could be assessed, namely: SOD, alkylhydroperoxide reductase activities and the glutathione pool.

- Cadmium toxicity in cultures growing by anaerobic respiration (nitrate and other final acceptors). Initially, an analysis of ROS or RNS (reactive nitrogen species) could be performed and depending on the results obtained, the effect of cadmium on growth and the status of the antioxidant defences could be assessed.

- Complete the analysis of cadmium toxicity in fermenting cultures; analysis of the damage levels on proteins and lipids

- Analysis of cell populations: by cell sorting separate the viable and non-viable cell fractions. The viable fraction could be further analyzed to determine if there are cells in viable non-culturable (VNC) state. The non-viable cell fraction could be used to study the mechanisms of cadmium-induced cell death.

7. References

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Appendix I

A brief description of each possible protein identified is presented below. Any known relationship between these proteins and oxidative stress was searched for in the literature.

Sample 1

Aldehyde-alcohol dehydrogenase – *adhE*

The multifunctional protein AdhE catalyzes the sequential reduction of coenzyme A (CoA) to acetaldehyde and then to ethanol (Membrillo-Hernández et al. 2000). This protein habours two enzymatic activities, CoA-linked acetaldehyde dehydrogenase (ACDH) and alcohol dehydrogenase (ADH) activity (Leonardo et al. 1996).

As most of the enzymes involved in fermentation, AdhE is induced under anaerobic conditions and repressed by both nitrate and oxygen. During aerobic and anaerobic respiration this enzyme is present at basal levels, its amount is about one-third and its activity is only one-tenth of the values observed under fermentative conditions (Leonardo et al. 1996; Echave et al. 2003). In fact, under aerobic conditions, *E. coli* is unable to grow on ethanol as a sole source of carbon and energy due to the insufficient expression of AdhE. Another reason is the fact that during aerobic metabolism this enzyme suffers a metal-catalyzed oxidation. The amino acid residues of AdhE are attacked by hydroxyl radicals that are locally generated due to the presence of iron in the alcohol:NAD^+ oxidoreductase domain of the enzyme (Membrillo-Hernández et al. 2000). Alcohol dehydrogenase E was identified as one of the major targets of protein oxidation in *E. coli* cells exposed to oxidative stress (Tamarit et al. 1998) and was suggested to have a protective role against this type of stress (Echave et al. 2003). Echave et al. (2003) proposed that this enzyme acts as a H_2O_2 scavenger in *E. coli* cells grown in aerobic conditions, since the metal-catalyzed oxidation of AdhE will prevent the cell from further damage.

Pyruvate dehydrogenase E1 component - *aceE*

This protein is part of the pyruvate dehydrogenase (PDH) complex that contains three components, pyruvate dehydrogenase (E1p), dehydrolipoate acyltransferase (E2p) and the dihydrolipoate dehydrogenase (E3). This multienzyme complex is a key enzyme for the metabolic interconnection between

glycolysis and the TCA cycle catalyzing the decarboxylation of pyruvate and the concomitant formation of CoA, which then reacts with oxaloacetate to produce citrate (Ogasawara et al. 2007).

Sample 2

5-methyltetrahydropteroyltriglutamate –homocysteine methyltransferase – *metE*

Methionine biosynthesis involves several enzymes and the final step is catalyzed either by a cobalamin (B_{12})-independent synthase (encoded by *metE*) or by B_{12}-dependent synthase (*metH*) (Hondorp and Matthews 2004). In the absence of exogenously supplied B_{12}, MetE is the sole source of *de novo* synthesis of methionine (Greene 1996).

Multiple observations in *E.coli* suggest a link between oxidative stress, methionine limitation and the enzyme that catalyzes the final step in methionine biosynthesis, the B_{12}-independent methionine synthase (MetE). Hondorp and Matthews (2004) suggest that in oxidative stress conditions, MetE is readily inactivated resulting in methionine limitation. In their work inactivation of this enzyme by glutathionylation is reported. This is presented as a strategy to modulate the activity of the enzyme while the active site is protected from further damage, in an easily reversible manner. It is also postulated that the development of methionine auxotrophy via inactivation of the MetE enzyme may protect stressed cells by slowing the initiation of protein synthesis since the initiation of translation in *E. coli* requires formylmethionine. This may safeguard cells from rapid synthesis of peptides under adverse conditions, allowing cellular processes to manage and detoxify the stress (Hondorp and Matthews 2004).

Methionine limitation imposed by oxidative stress could also play an important role in bacterial quorum sensing. *S*-adenosylmethionine (formed directly from methionine) is the precursor of the autoinducer signalling molecule AI-2 that has been suggested to communicate to the neighbouring cells the metabolic state and growth potential of the bacterium (Hondorp and Matthews 2004).

Phenylalanyl-tRNA synthetase beta chain

Aminoacyl-tRNA synthetases (aaRS) are a family of enzymes that catalyze the loading of amino acids (aa) onto specific tRNAs, in view of their use in translation (An et al. 2008). The fidelity of translation during protein synthesis is determined

at two major points: selection of the aminoacyl-tRNA by ribosomes and aminoacylation of the tRNA with the cognate amino acid by aaRSs. This aminoacylation reaction occurs by a universally conserved two-step reaction: (i) aa activation with ATP to form an adenylate (aa~AMP), and (ii) transfer of the activated aa to the tRNA to form the aa-tRNA. When the cognate aa displays high structural similarity to other isosteric or slightly smaller compounds, the aaRS is not able to distinguish between the cognate and noncognate substrates with sufficient specificity to prevent mischarging of the tRNA (Roy et al. 2004). Many aaRSs possess an additional intrinsic editing activity that will allow an accurate aminoacyl-tRNA formation. Pre-transfer editing occurs by hydrolysis of the noncognate aa~AMP, while post-transfer editing relies on the ability of the aaRS to hydrolyse the noncognate aa-tRNA bound to the enzyme (Roy et al. 2004).

The aaRS are divided in classes with respect to the domains that are involved in the editing process. The Phenylalanyl-tRNA synthetase (PheRS) is one of the largest and most complex aaRS and it has been shown to possess an editing site located at the beta subunit of the protein. In *E. coli* it has been shown that this site has post-transfer editing activity that specifically hydrolyses Tyr-tRNA^{Phe} (Roy et al. 2004).

Sample 3

Phosphoenolpyruvate carboxylase - *ppc*

This enzyme, like pyruvate dehydrogenase provides an important link between glycolysis and the TCA cycle. Phosphoenolpyruvate carboxylase (PPC) together with citrate synthase direct metabolites into the TCA cycle: by diminishing the pool of phosphoenolpyruvate, pyruvate and CoA these two enzymes prevent the accumulation of acetate (De Maeseneire et al. 2006). PPC plays an anaplerotic role in replenishing oxaloacetate (OOA) pool and thus keeping the levels of TCA cycle intermediates (Peng et al. 2004).

Elongation factor G – EF-G

The elongation phase of translation is an iterative process of the ribosome that decodes the messenger RNA (mRNA) to synthesize a polypeptide chain. There are three main steps that must occur during each cycle of elongation: (i) the mRNA codon is recognized by the correct aminoacyl-tRNA, (ii) the peptidyl transferase reaction elongates the polypeptide chain by one amino acid, and (iii) the

remaining tRNAs bound to the ribosome are translocated to the next position, creating a vacant tRNA-binding site for the next cycle (Spiegel et al. 2007). In prokaryotes, this cycle of elongation involves two elongation factors (EFs): the EF-Tu and EF-G. EF-Tu delivers the aminoacyl-tRNA to the A site of the processing ribosome, as part of the aminoacyl-tRNA-EF-Tu-GTP ternary complex, EF-G promotes the translocation step in which the A- and P-site tRNAs move to the P and E sites of the ribosome, respectively, and the ribosome moves forward by one codon.

Tamarit et al. (1998) have reported EF-G as one of the major oxidatively damaged proteins in *E. coli* exposed to oxidative stress. Besides oxidation in presence of H_2O_2, EF-G was also oxidized when cells were exposed to paraquat or menadione (Tamarit et al. 1998). Based on their results, these authors conclude that under peroxide and superoxide stress conditions there is inactivation of this factor.

Sample 4

NADP-isocitrate dehydrogenase

Isocitrate dehydrogenases (ICDHs) catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate step of the TCA cycle (Murakami et al. 2006). These enzymes require either NAD^+ or $NADP^+$ and produce NADH or NADPH and in *E. coli* only the NADP-dependent form is present (Lee et al. 1999). In this bacterium this enzyme is responsible for the production of 20-25% of all NADPH, which is an essential reducing equivalent for anabolic reactions, for the regeneration of GSH by the glutathione reductase and for the activity of the NADPH-dependent thioredoxin system (Sauer et al. 2004). The modulation of the phosphorylation of this enzyme has also an important role in the control of the branch-point between the TCA cycle and the glyoxylate bypass. The dephosphorylation of the isocitrate dehydrogenase leads to an increase in its activity, which, together with other factors leads to a cessation of the flux through the glyoxylate shunt (Walsh and Koshland 1985).

Oxidative inactivation of ICDH occurs when Mg^{2+} or Mn^{2+}, that are required for enzyme activation, are substituted by Fe^{2+}. Iron binds to the metal-binding sites leading to peptide cleavage and may play a key role in the shift of the TCA cycle to the glyoxylate bypass, in aerobic conditions (Murakami et al. 2006).

Enolase

The enolase protein is a conserved enzyme of the glycolytic metabolism that catalyzes the interconversion of PEP and 2-phospho-D-glycerate. In *E. coli*, roughly one-tenth of the total enolase is sequestered in a multi-enzyme assembly known as the RNA degradosome that is responsible for the turnover of mRNA necessary to maintain the normal genetic regulation within the cells (Chandran and Luisi 2006). The other three constituents of this complex are: the endoribonuclease E (RNase E), the RNA helicase (RhlB) and the exoribonuclease polynucleotide phosphorylase (PNPase) (Bernstein et al. 2004). The C-terminal half of the RNase E forms the key heterotypic protein-protein interactions of the degradosome, and each of the recruited components bring a specialized role to this complex. RNase E initiates the decay of most transcripts by internal cleavage and processes the precursors of tRNA, rRNA, tmRNA and 6S RNA. PNPase, together with RNAse II, is required for mRNA turnover. This enzyme has also been shown to be involved in the quality control of tRNA (Cheng and Deutscher 2003). The RhlB helicase uses ATP to remove secondary structure from the RNA to prepare the substrates for the partner nucleases. While the role of the other components of the degradosome is well established, the function of the enolase is not so clear (Chandran and Luisi 2006). However, it has been shown that the disruption of the interaction of enolase with RNase E alters the turnover of mRNA, namely, the transcripts encoding enzymes of energy-generating metabolic routes (Bernstein et al. 2004).

This protein was also identified by Tamarit et al. (1998) as a target of oxidative stress caused by hydrogen peroxide. In mammalian mitochondria an increase in 16S RNA degradation upon exposure to H_2O_2 was reported (Crawford et al. 1997). As a consequence of this situation, the enzymes involved in the RNA degradosome would be preferentially exposed to oxidation by the ROS generated by metal ions associated with RNA (Tamarit et al. 1998).

Sample 5

Outer membrane proteins F and C precursors

The cell envelope of Gram-negative bacteria consists of two membranes, the inner membrane (IM) and the outer membrane (OM), that are separated by the periplasm containing the peptidoglycan layer. The two membranes have an entirely different structure and composition: the IM is a phospholipid bilayer

whereas the OM is an asymmetrical bilayer consisting of phospholipids in the inner leaflet and lipopolyssacharides (LPS) in the outer leaflet. Both membranes also contain lipoproteins, which are anchored to either membrane (Bos et al. 2007).

The OM functions as a selective barrier that protects the bacterium from harmful compounds present in the environment, such as antibiotics. Unlike the IM, the OM is not energized by a proton gradient and ATP is not available in the periplasm. In the absence of readily available energy sources, nutrients usually pass the OM by passive diffusion via an abundant class of trimeric outer membrane proteins (OMPs) called porins. Porins form water filled channels that allow the passage of small hydrophilic solutes with molecular weights up to ~600 Da (Bos et al. 2007). *E. coli* is known to produce three trimeric porins: OmpF, OmpC and PhoE; while the first two transport preferentially cations over anions, the latter transports anions (Nikaido 2003). The classical porins OmpF and OmpC are major constituents of the *E. coli* OM and account for approximately 2% of the total protein content of the cell. Expression of these proteins has been shown to be altered by several environmental factors including osmolarity, temperature, pH, nutrient availability and various toxins. This complex environmental regulation is implemented by an also complex regulatory network that includes the two component systems EnvZ-OmpR and CpxA-CpxR, the sRNAs MicF and MicC, the sigma factors σ^S and σ^E, the global regulator Lrp and the histone-like protein IHF (Batchelor et al. 2005).

OmpC was not found to be oxidatively damaged in presence of H_2O_2, but it was shown that in iron overloading conditions this protein is clearly damaged (Tamarit et al. 1998).

Appendix II

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ORIGINAL PAPER

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

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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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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

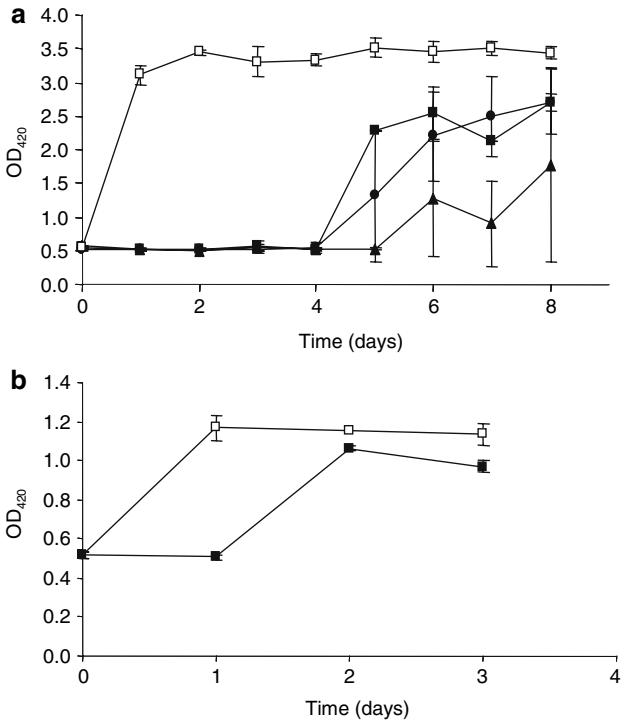

Fig. 1 Effect of 30 µg ml^{-1} Cd^{2+} on cell growth. **a** Untreated culture (*open square*), wild-type + Cd (*filled square*), Δ*gshA* + Cd (*filled triangle*), Δ*gshB* + Cd (*filled circle*). The untreated culture growth curve shown is representative of the curves obtained for all the untreated cultures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

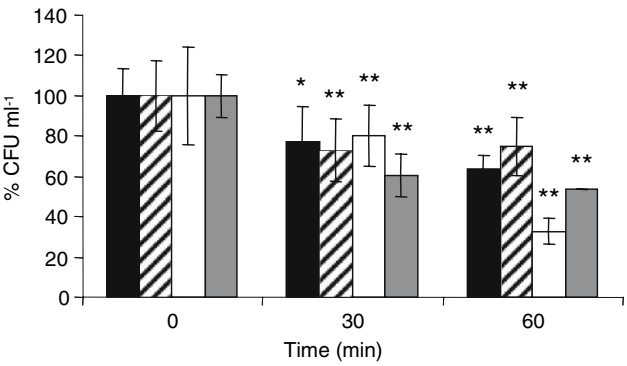

Fig. 2 Effect of 30 µg ml^{-1} Cd^{2+} on culturability: wild type (*filled bar*), Δ*ubiE* (*diagonal stripe bar*), Δ*gshA* (*open bar*), Δ*gshB* (*gray bar*). The absolute values (CFU ml^{-1}) corresponding to 100% (T_0) are: wild type = 1.41E + 08 ± 1.90E + 07, Δ*ubiE* = 1.74E + 08 ± 3.03E + 07, Δ*gshA* = 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

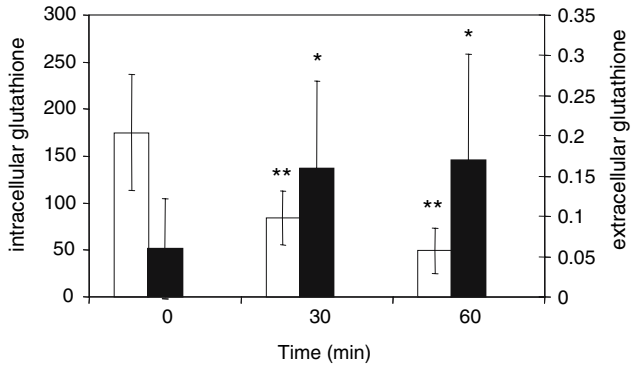

Fig. 3 Effect of 30 μg ml^{-1} Cd^{2+} on total glutathione: intracellular concentration (nmol (GSH + 2GSSG) mg^{-1} protein; *open bar*), extracellular concentration (μM (GSH + 2GSSG); *filled bar*). * ($P < 0.05$) and ** ($P < 0.01$) express significant differences between Cd-treated (30 and 60 min)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

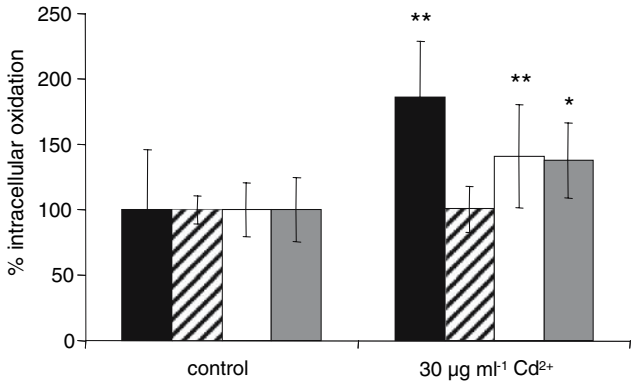

Fig. 4 ROS detection by flow-cytometry: wild type (*filled bar*), Δ*ubiE* (*diagonal stripe bar*), Δ*gshA* (*open bar*), Δ*gshB* (*gray bar*). The absolute values (arbitrary fluorescence units) corresponding to 100% (T_0) are: wild type = 14.99 ± 6.85, Δ*ubiE* = 27.09 ± 2.92, Δ*gshA* = 4.82 ± 1, Δ*gshB* = 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

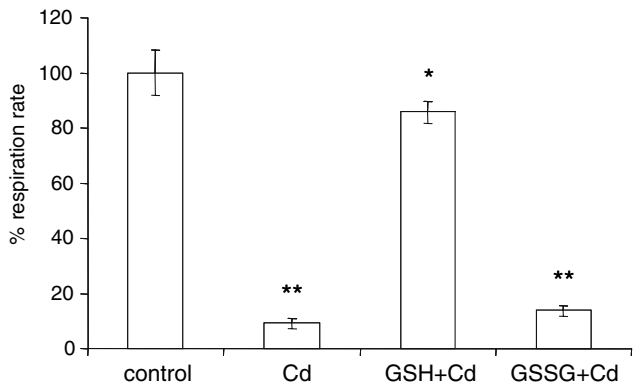

Fig. 5 Effect of 30 μg ml^{-1} Cd^{2+} on respiration rate. The absolute value (nmol O_2 min^{-1}) corresponding to 100% is 98 ± 7.9. Significant differences between the control and the addition of Cd or GSH + Cd or GSSG + Cd are represented: * ($P < 0.05$) and ** ($P < 0.01$)

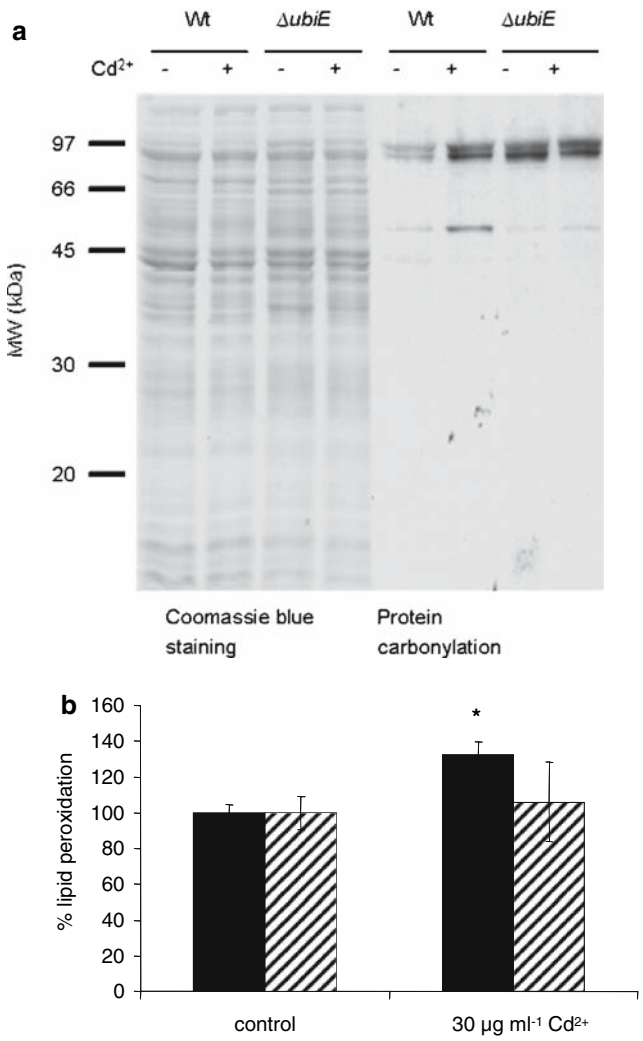

Fig. 6 Effect of 30 μg ml^{-1} Cd^{2+}: **a** on protein carbonyl levels. SDS-PAGE is shown as loading control. b) on lipid peroxidation levels: wild type (*solid bar*), Δ*ubiE* (*diagonal stripe bar*). The absolute values (μmol MDA mg^{-1} prot) corresponding to 100% are: wild type = 6.60 ± 0.29, Δ*ubiE*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