The cadmium-stress stimulon of *Escherichia coli* K-12

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The influence of cadmium on stress protein production in *Escherichia coli* K-12 (strain MG1655) was analysed using two-dimensional polyacrylamide gel electrophoresis and the gene-protein database of *E. coli* K-12. Cadmium (273 µM) caused complete but transient inhibition of growth accompanied by the synthesis of cadmium-induced proteins (CDPs). It was found that some CDPs induced during the growth-arrested phase belong to the heat-shock, oxidation stress, SOS and stringent response regulons, while others are general stress inducible proteins (e.g. H-NS, UspA). In addition, trigger factor, adenylate kinase, W-protein, the cold shock protein G041.2, and seven unknown proteins whose synthesis is not known to be controlled by a global regulator, were identified as immediate responders to cadmium exposure. The rate of synthesis of most of the immediate responders to cadmium exposure decreased when the growth of the cells resumed. However, seven CDPs, including those encoded by *argI*, *tyrA* and *xthA*, maintained a high production rate during growth in the presence of cadmium. Two of the unidentified proteins were N-terminally sequenced by Edman degradation. The N-terminal amino acid sequence of one of these proteins (designated F023.3) matches the *E. coli* open reading frame o216. This ORF is similar to the N-terminal third of the copper-binding protein amine oxidases (encoded by *maoA*) of both *E. coli* and *Klebsiella pneumoniae* (*K. aerogenes*). The other N-terminally sequenced protein (designated C044.6) matches perfectly the product of the *metK* gene, S-adenosylmethionine synthetase I. In comparison to untreated cells, cadmium-stressed cells were found to recover more rapidly during subsequent stress conditions, such as ethanol, osmotic, heat shock, and nalidixic acid treatment. The role of the CDPs is discussed in view of their physiological assignments in the cell.

Keywords: *Escherichia coli*, cadmium stress, stress protein

INTRODUCTION

Cadmium is used in a variety of industrial applications and is a potential source of environmental contamination. Cadmium, in a variety of chemical forms, is toxic and there are no known biological functions of the metal. However, Cd²⁺ is readily taken up by bacterial cells, presumably by the Mn²⁺ uptake system, and can thus seriously damage the cell in several ways: cadmium is a potent oxidative agent (VanBogelen *et al.*, 1987), it inhibits DNA replication (Mitra *et al.*, 1975; Nyström

& Kjelleberg, 1987), and it appears to make the DNA more susceptible to nucleolytic attack resulting in single-strand DNA breaks (Mitra & Bernstein, 1977). At low cadmium concentrations, cells are able to adapt and resume growth after a period of stasis. This period appears to involve the repair of cadmium-mediated cellular damage and adjustment of cell physiology to limit the distribution of the toxic ion in the cell (Mitra *et al.*, 1975; Mitra & Bernstein, 1977; Andreoni *et al.*, 1991). The most common defence mechanism against cadmium in prokaryotes, and perhaps also in *Saccharomyces cerevisiae*, appears to involve an active efflux of the cation while most eukaryotes rely on the formation of intracellular complexes with the metal ion (Nies,

Abbreviation: CDP, cadmium-induced protein.

Fig. 1. Effect of cadmium stress on growth. *E. coli* MG1655 was grown aerobically at 37 °C in M9 medium (○) and at time zero CdCl_2 (273 μM Cd^{2+}) was added (■) to the culture. Arrows indicate CdCl_2 addition and times at which samples were removed, pulse-labelled and processed for two-dimensional gel electrophoresis.

1992). However, some bacterial species such as *Escherichia coli* may protect themselves by producing intracellular cadmium-binding proteins (Khazaeli & Mitra, 1981; Mitra, 1984; Cohen *et al.*, 1991).

E. coli responds to stress by activating complex global regulatory systems. During cadmium-induced growth arrest, *E. coli* increases the synthesis of a group of proteins (CDPs, cadmium-induced proteins) which together make up the cadmium stress stimulon. Most of the CDPs of this stimulon are of unknown function and only limited information exists as to the identities of the specific sensors/signals triggering the synthesis of these proteins (VanBogelen *et al.*, 1987). It has been demonstrated that some CDPs are members of well-characterized stress regulons (VanBogelen *et al.*, 1987). However, only a limited number of the proteins in these regulons are induced during cadmium exposure and the synthesis of these CDPs constitutes a minor fraction of the overall cellular response (VanBogelen *et al.*, 1987). The objective of this study was to identify proteins induced in both cadmium-stressed (growth-arrested) and cadmium-adapted (growing) cells to identify the target(s) for cadmium in the cell and elucidate the mechanism of adaptation.

METHODS

Bacterial strains, media and growth conditions. The *E. coli* K-12 strain MG1655 (T. Nyström) was used throughout this work. Cultures were grown in liquid M9 medium (Sambrook *et al.*, 1989) in Erlenmeyer flasks placed in a rotary shaker at 37 °C. When cultures reached an OD_{420} of 0.5, CdCl_2 (to a final concentration of 273 μM Cd^{2+}) was added. Temperature

shift from 37 to 45 °C was carried out by transferring the culture flask to an incubator shaker at the higher temperature. Temperature shift and treatments with H_2O_2 (final concn 5 mM), NaCl (428 mM), nalidixic acid (172 μM), ethanol (0.868 M) or CdCl_2 were initiated when cultures reached an OD_{420} of 0.5 or after a 1 h pretreatment with cadmium. After pretreatment with cadmium, the cells were harvested by centrifugation at 4000 g for 20 min (4 °C), washed twice with glucose-free M9 medium, resuspended in an equal volume of fresh M9 medium and subjected to the different treatments as described above. The viability of untreated *E. coli* cultures under different stress conditions or after a 1 h cadmium pretreatment was determined by the number of colony-forming units (c.f.u.) on M9 medium using the drop plate method (Hoben & Somasegaran, 1982).

Radiolabelling of proteins induced by cadmium stress. At times before and after CdCl_2 addition, a portion (1.0 ml) of a culture was removed and placed in a flask containing [^{35}S]methionine [1.098 Ci mmol^{-1} , 0.1 mCi ml^{-1} (1 mCi = 37 MBq)]. Incorporation was allowed to proceed for 5 min (exponentially growing cells) or 10 min (cadmium-stressed and cadmium-adapted cells). The radiolabelling was stopped by chasing with non-radioactive methionine as described by Nyström & Neidhardt (1992). Radiolabelled cells were harvested by centrifugation at 15600 g for 2 min (4 °C), after which the radioactive medium was removed and the cell pellet frozen at -20 °C until use. The cell pellets were analysed by resolution of the proteins on two-dimensional gels and autoradiograms were prepared to permit visualization of the ^{35}S -labelled proteins as described by VanBogelen & Neidhardt (1990).

Resolution of cell proteins on two-dimensional polyacrylamide gels. Culture samples were processed to produce extracts for resolution on two-dimensional polyacrylamide gels using the methods of O'Farrell (1975) with modifications described by VanBogelen & Neidhardt (1990).

Sample preparation for protein microsequencing. Cultures induced for cadmium-stress proteins were prepared as described above. The cell pellet was subjected to two-dimensional gel electrophoresis (150–200 μg total protein loaded on each gel). After electrophoresis, the gels were soaked in transfer buffer (10 mM CAPS, 3.12 M methanol, pH 11.0) for 10 min to reduce the amount of glycine and Tris contaminants. PVDF Immobilon (Millipore) membranes were rinsed with 31.2 M methanol and stored in transfer buffer. The gels were sandwiched between sheets of PVDF membrane and several sheets of blotting paper (Whatman), and were assembled into a blotting apparatus and electro-blotted for 1 hour at 50 V (100–170 mA) in transfer buffer. The membranes were washed in sterile deionized water for 10 min, soaked in 31.2 M methanol for a few seconds, stained for 15 min in the amido black staining solution (1.672 mM amido black in 173 mM acetic acid/12.5 M methanol) using constant orbital shaking, destained in deionized water, dried at room temperature and subjected to autoradiography. The N-terminal amino acid sequences of two CDPs excised from the blot were determined by automated Edman degradation directly from the membrane fragment.

Recognition of CDPs. Proteins whose rates of synthesis were higher in cadmium-stressed cells relative to the non-stressed control were assigned coordinates from a reference gel and alpha-numeric designations from the gene-protein database of *E. coli* K-12 (VanBogelen *et al.*, 1996). These proteins were considered to be CDPs. Comparison of autoradiograms of the

untreated control, cadmium-stressed (growth arrested), and cadmium-adapted cells (regrowing) were made by eye to distinguish between immediate and transient stress responders, and responders produced throughout growth of the cells during cadmium exposure.

RESULTS AND DISCUSSION

The influence of cadmium on growth and stress protein induction was tested in wild-type *E. coli* MG1655 by using two-dimensional gel electrophoresis analysis and the gene-protein database of *E. coli* K-12 (VanBogelen *et al.*, 1996). Cadmium (final concn 273 μM) was added to an *E. coli* MG1655 culture growing exponentially at 37 °C in M9 medium. This treatment resulted in complete but transient growth inhibition. Stasis lasted for about 1 h, after which the cells recovered and resumed growth, albeit at a slower rate than before the addition of cadmium (Fig. 1). Thus, it appears that the cadmium concentration used caused reversible damage. A different effect on growth was observed when cadmium was added in the lag phase before growth commenced, in that 3 μM CdCl_2 inhibited cell proliferation and 10 μM was lethal (not shown). In contrast, to obtain lethal effects in an exponentially growing culture, 600 μM CdCl_2 is required (not shown; VanBogelen *et al.*, 1987). Thus, the effect of cadmium depends on the growth phase at which cadmium stress is initiated and, perhaps, bacterial cell abundance.

One of the goals of this study was to detect proteins responding to cadmium stress in order to identify the physiological targets of cadmium. Samples were removed from the culture at specified times (indicated by arrows in Fig. 1), pulse-labelled, and analysed by two-dimensional gel electrophoresis. The pattern of protein synthesis showed that cadmium caused changes in the production rates of a large number of proteins (Fig. 2, Table 1). The data reveal that both nonspecific general stress proteins and cadmium-specific stress proteins were produced by exposure to cadmium (Fig. 2). The observed induction of the SOS, heat shock and oxidation stress regulons is in good agreement with previously reported results (VanBogelen *et al.*, 1987). However, our data demonstrate that these global control systems were only transiently activated during growth inhibition by cadmium (Fig. 2). The production of all the proteins belonging to these global stress regulons was down-regulated when cells started to regrow in the presence of cadmium. Similarly, most other proteins induced by cadmium, including H-NS, UspA, adenylate kinase,

Fig. 2. Autoradiograms of two-dimensional polyacrylamide gels of protein extracts from *E. coli* MG1655 (a) grown aerobically at 37 °C in M9 medium (untreated control sample), (b) 0.75 h after CdCl_2 addition (cadmium-stressed cells), and (c) 2.5 h after CdCl_2 addition (cells growing after recovery from cadmium-induced growth arrest). Cells were labelled with [^{35}S]methionine for 5 min during exponential growth and for 10 min after 0.75 and 2.5 h of CdCl_2 addition. The circled spots are proteins which are described in the text and in Table 1. Spot numbers correspond to protein numbers in Table 1.

Table 1. Proteins with elevated production rates during cadmium exposure

Protein numbers 1–26 indicate immediate and transient responders to cadmium exposure; protein numbers 27–34 indicate permanent responders to cadmium exposure. Proteins have been assigned alpha-numeric (A-N) designations and coordinates based on the reference gel in VanBogelen *et al.* (1996). Protein numbers correspond to those on the autoradiograms of the two-dimensional polyacrylamide gels (Fig. 2).

Protein no.	Global regulator*	Coordinates		A-N	Gene name	Protein name
		X	Y			
1	HTP	104	107	B066.0	<i>dnaK</i>	DnaK
2	HTP	87	107	C062.5	<i>htpG</i>	HtpG
3	HTP	70	114	F084.1	<i>clpB</i>	ClpB
4	SOS	84	87	C039.3	<i>recA</i>	RecA
5	OXY	99	91	B044.8		
6	OXY	94	34	B015.5		
7	OXY	93	50	B020.9		
8	OXY	82	111	D078.1		
9	OXY	80	111	D078.0		
10	OXY	64	100	F050.6		
11	OXY	48	35	G015.4		
14		104	98	B050.3	<i>tig</i>	Trigger factor
15		85	24	C013.5	<i>uspA</i>	UspA
16		63	28	F014.7	<i>hns</i>	H-NS
17		59	67	F026.0	<i>adk</i>	Adenylate kinase
18		41	90	G041.2		Cold shock protein
19		40	93	G043.2		W-protein
20		86	24			Unknown
21		84	64	C037.0		Unknown
22		75	79	D035.7		Unknown
23		56	15			Unknown
24		52	79			Unknown
25		46	12			Unknown
26		41	112			Unknown
27		88	92	C044.6	<i>metK</i>	MetK
28		65	84	F039.0	<i>argI</i>	Ornithine carbamoyltransferase
29		59	56	F023.3		Unknown
30		55	87	F037.9	<i>tyrA</i>	Chorismate mutase
						F-prephenate dehydrogenase
31		55	70	G036.2		Unknown
32		50	90	G039.5		Unknown
33		47	73	G028.2	<i>xthA</i>	Exodeoxyribonuclease III-endonuclease II
34		45	71	G027.2		Unknown

* HTP, Heat-shock proteins regulated by *rpoH*; OXY, oxidation stress proteins regulated by *oxyR*; SOS, proteins regulated by *lexA*.

trigger factor, W-protein, one cold shock protein and seven unidentified proteins (Fig. 2, Table 1), were only transiently produced during the growth-arrested phase. This cadmium-induced activation of global stress regulators and the production of general stress proteins may explain why cadmium-stressed cells were found to be cross-protected against subsequent cadmium and oxidation stresses, osmotic and heat shock, and exposure to

ethanol and nalidixic acid (Fig. 3). The types of growth inhibition caused by the stress conditions examined were different: treatment by nalidixic acid at 172 μ M caused a gradual inhibition of growth rate; 0.868 M ethanol and a shift from 37 to 45 °C inhibited growth immediately; H₂O₂ (5 mM), CdCl₂ (273 μ M Cd²⁺), and NaCl (428 mM) caused complete but transient inhibition.

Fig. 3. Effect of different stress conditions on changes in c.f.u. of *E. coli* MG1655 was grown aerobically at 37 °C in M9 medium. When cultures reached an OD₄₂₀ of 0.5 or after a 1 h pretreatment with CdCl₂ (273 µM Cd²⁺), (a) CdCl₂, (b) H₂O₂, (c) NaCl, (d) nalidixic acid or (e) ethanol was added to the cultures or the cells were shifted to 45 °C. ○, Untreated control culture; □, stressed culture which had a 1 h cadmium pretreatment; △, stressed culture with no pretreatment. Representative results from five replicates are shown.

The production of a few of the proteins induced by cadmium exposure was found to be elevated after recovery and regrowth of the cells in the presence of cadmium (Fig. 2). We considered these proteins likely to be those specifically involved in cadmium resistance (Table 1). Three of these proteins were identified using the gene-protein database of *E. coli* K-12 as ornithine carbamoyltransferase, chorismate mutase F-prephenate dehydrogenase, and exodeoxyribonuclease III-endo-nuclease II. Two unidentified proteins, designated F023.3 and C044.6, which showed markedly elevated production levels in the presence of cadmium, were N-terminally sequenced. Automated Edman degradation was performed directly on membrane fragments, generating the following two sets of sequences: (i) His (or Lys)-Gly-His-His-Ser-His-Gly-Lys-Pro-Leu for F023.3 which was assigned XY coordinates (59 × 56) from a reference gel (VanBogelen *et al.*, 1996) and (ii) Ala-Lys-His-Leu-Phe-Thr-Ser-Glu-Ser-Val for C044.6, with the assigned coordinates 88 × 92.

The N-terminal amino acid sequence of protein F023.3 matches ORF o216, which is similar to the N-terminal part of the copper-binding protein amine oxidases (encoded by *maoA*) of both *E. coli* and *Klebsiella*

o216	1	-----MAIRLYKLAVALG-VFIVSAPAFSHGHSHGKPLTEVEQKAANGVFDDANVQNR
AMO_EC	1	s.R...LAlAva..F...AP.FaHG..aH...Pm.....g..V...D...Q...
AMO_KL	1	s.R...LAlAva.V...sP.FaHG..aH...PL...Q...g...V...D...Q...
o216	54	TLSDWDGVMQSVYPLLSQSGKLDVPVKKADADTKTKTFAEIKDYHKGATDIEMIGIEDG
AMO_EC	53	TL...DG.y..V.P...Qta.vn.....d.K.w...f..Dv...Gld..
AMO_KL	53	TL...DG.y..V.P...ta.vn...K...D.....E.K.w...f..Dv...Gld..
o216	114	IVEFHR---NNETTCKYDYGKILTYKSGKGGVRYLFECKDPESK--APKYIQPSDHI
AMO_EC	98	...kR...N..Ta.....v.....K...Rf.....P..K....f.....v
AMO_KL	99	...kR...N..sa.....v.....Rf.....K....f.....v
o216	169	IAPRKSSHPHFMGNDSSQSLNEMENWPTTYYPQLSSSEVVEEMSH
AMO_EC	152	..PRKa...l.....e..v..q...sw.P...a...Vl.d..a.
AMO_KL	152	..APR.a...l.....e..v..q...sw.P...a...Vl.d..a.

Fig. 4. Sequence alignment of o216 with the monoamine oxidases (AMO) of *E. coli* (EC) and *K. pneumoniae* (K. *aerogenes*) (KL). The entire sequence of o216 is aligned with the first 225 amino acids of the monoamine oxidases. Capital letters indicate identity with the o216 sequence and small letters indicate similar amino acids. The alignment was done using the CLUSTAL W program.

pneumoniae (*K. aerogenes*) (Fig. 4). In addition, o216 of *E. coli* is almost identical to a partially sequenced gene from *Salmonella choleraesuis* which maps upstream of the *aadA* gene. The amine oxidases of *E. coli* and *K. pneumoniae* (*K. aerogenes*) are approximately 740 aa periplasmic proteins, while o216 of *E. coli* encodes a 216 aa protein. Our N-terminal sequence data show that

protein F023.3 is processed and contains a 24 aa signal sequence, suggesting that the mature product, like the amine oxidase, is exported from the cytoplasm. The N-terminal sequence of protein C044.6 was found to match perfectly the *E. coli* protein S-adenosylmethionine synthetase I, the product of the *metK* gene. The MetK protein is a cytoplasmic isozymic form of the adenosylmethionine synthetases with strong homology to MetX. Both isozymes catalyse the formation of S-adenosylmethionine from methionine and ATP. *metK* and *metX* are regulated such that intracellular S-adenosylmethionine levels are nearly constant under a variety of conditions (Markham *et al.*, 1984; Satishchandran *et al.*, 1993).

The identity of the cadmium-induced proteins gives some insight into the potential targets in the cell. Based on the elevated production levels of exodeoxyribonuclease III-endonuclease II, encoded by *xthA*, it appears that cells growing in the presence of cadmium have an increased demand for excision repair enzymes. Exodeoxyribonuclease III-endonuclease II is involved in 3' phosphatase, 5' endonuclease and 3' exonuclease activities, and an *xthA* mutant is sensitive to killing by agents such as near-UV radiation and H₂O₂, which produce oxidative damage of DNA. This is consistent with cadmium being a potent oxidant which could cause single-stranded DNA breakage (Mitra & Bernstein, 1977). In addition, the increased synthesis of the proteins encoded by *argI*, *tyrA* and *metK* suggests that cadmium-treated cells may experience amino acid limitation. It is known that cadmium elicits a stringent response in *E. coli* when added at high concentrations (600 µM; VanBogelen *et al.*, 1987). However, the increased synthesis of proteins encoded by *argI* and *tyrA* occurs during regrowth of cells in the presence of 273 µM cadmium, when there is no general induction of the stringent response-dependent proteins. This indicates that the effect on *argI* and *tyrA* is specific rather than being an effect of stringency. Finally, the high synthesis level of the *xthA* product with the concomitant down-regulation of *oxyR*-dependent proteins during recovery and regrowth of cadmium-treated cells is suggestive: it indicates that, after adaptation, the oxidative potential of 273 µM cadmium is sufficient to damage DNA but not sufficient to oxidize the critical cysteine residue on OxyR needed to activate the protein. However, during the initial hour of exposure to 273 µM cadmium, many of the *oxyR* regulon proteins are induced. This suggests that the effective concentration of cadmium in the cell is reduced during the adaptation period. Perhaps the product of *o216* is involved in this process. Future studies will be aimed at elucidating the role of this protein in adaptation to cadmium exposure.

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