Regulation of yodA encoding a novel cadmium-induced protein in Escherichia coli

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Bacterial accommodation to moderate concentrations of cadmium is accompanied by transient activation of general stress proteins as well as a sustained induction of other proteins of hitherto unknown functions. One of the latter proteins was previously identified as the product of the Escherichia coli yodA ORF. The yodA ORF encodes 216 aa residues (the YodA protein) and the increased synthesis of YodA during cadmium stress was found probably to be a result of transcriptional activation from one single promoter upstream of the structural yodA gene. Analysis of a transcriptional gene fusion, PyodA–lacZ, demonstrated that basal expression of yodA is low during exponential growth and expression is increased greater than 50-fold by addition of cadmium to growing cells. However, challenging cells with additional metals such as zinc, copper, cobalt and nickel did not increase the level of yodA expression. In addition, hydrogen peroxide also increased yodA expression whereas the superoxide-generating agent paraquat failed to do so. Surprisingly, cadmium-induced transcription of yodA is dependent on soxS and fur, but independent of oxyR. Moreover, a double relA spoT mutation abolished induction of yodA during cadmium exposure but ppGpp is not sufficient to induce yodA since expression of the gene is not elevated during stationary phase. After 45 min of cadmium exposure the YodA protein was primarily detected in the cytoplasmic compartment but was later (150 min) found in both the cytoplasmic and periplasmic compartments.

Keywords: cadmium stress, yodA characterization, YodA protein, RNA polymerase, S1 nuclease mapping

INTRODUCTION

Cadmium is an exceedingly toxic heavy metal with no known biological function. At low cadmium concentrations, cells are able to adapt and resume growth after a period of stasis (Mitra et al., 1975). During cadmium-induced growth arrest of Escherichia coli cells, DNA and RNA synthesis is rapidly inhibited while the rates of protein synthesis and proteolysis increase transiently (Ferianc et al., 2000). The protein synthesis includes de novo synthesis of cadmium-induced proteins (CDPs) which together make up the cadmium stress stimulon (Ferianc et al., 1998). Some CDPs belong to global regulatory networks, which include the SOS, oxidative stress, heat-shock and stringent response networks (Ferianc et al., 1998; 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). In addition, these general stress responses are only transiently activated during cadmium-induced growth inhibition. When proliferation resumes, these regulons are downregulated and expression reaches a new steady-state level (Ferianc et al., 1998). Other CDPs, however, are specific to cadmium stress. These CDPs retain an elevated production level, relative to the production level of general stress proteins, even in accommodated cells that have

Abbreviations: CDP, cadmium induced protein(s); DIG, digoxigenin; MBP, maltose-binding protein; ppGpp, guanosine 3′,5′-bispyrophosphate; RAS, rabbit antiserum; tsp, transcription start point.
The GenBank accession number for the E. coli yodA gene and the SWISS-PROT accession number for E. coli YodA protein in this paper are AAC75039 and P76344, respectively.
resumed growth during prolonged cadmium exposure. One of these proteins has been identified as the product of ORF 0216 (Ferianc et al., 1998), later renamed yodA. YodA is a 216 aa residue protein and the product has been identified on two-dimensional polyacrylamide gels (VanBogelen et al., 1996; Ferianc et al., 1998). N-terminal sequencing has demonstrated that the mature product is exported from the cytoplasm (Ferianc et al., 1998). YodA together with two other putative proteins, YrpE of Bacillus subtilis (Sorokin et al., 1997) and pXO1-130 of Bacillus anthracis (Okinaka et al., 1999), may constitute a new family of stress proteins based on sequence similarity (44.6% identity; Puškárová et al., 2001) and size (YodA, 216; YrpE, 251; pXO1-130, 237 aa). These three proteins were found to exhibit sequence similarity also with the 200 aa residue C-terminal part of the streptococcal adhesin, AdcA (Puškárová et al., 2001), which is a lipoprotein containing a putative metal-binding site (Dintilhac & Claverys, 1997). In addition, YodA exhibits weak sequence similarity with the N-terminal part (about 200 aa) of the copper-binding protein amine oxidase, encoded by maoA (Ferianc et al., 1998), of both E. coli (Azakami et al., 1994) and Klebsiella aerogenes (Sugino et al., 1992).

In this work we have identified the yodA promoter and report that induction of the gene during cadmium exposure is dependent on soxS, fur and relA/spoT of the stringent response, but not oxyR. The product of the gene is primarily found in the cytoplasm of non-stressed cells but is exported to the periplasm upon cadmium exposure.

METHODS

Bacterial strains and growth conditions. The E. coli strains used in this work are listed in Table 1. Cultures were grown aerobically in liquid LB or M9 (Sambrook et al., 1989) medium in Erlemeyer flasks in a rotary shaker at 37 °C. When appropriate, the media were supplemented with kanamycin (50 µg ml⁻¹), tetracycline (20 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), X-Gal (40 µg ml⁻¹) or IPTG (0.1 mM). However, antibiotics were used only for selection of appropriate mutant strains, but none of them were added to the media during metal exposure. In cadmium-stress experiments, treatment with cadmium chloride (CdCl₂.2H₂O) to a final concentration of 273 µM Cd²⁺ was used. Cadmium chloride (136.5 µM Cd²⁺), zinc sulfate (ZnSO₄.7H₂O) to a final concentration of 0.7 mM Zn²⁺, cupric chloride (CuCl₂.2H₂O) to a final concentration of 3.1 mM Cu²⁺, cobalt chloride (CoCl₂.6H₂O) to a final concentration of 1.0 mM Co²⁺, nickel chloride (NiCl₂.6H₂O) to a final concentration of 2.0 mM Ni²⁺, hydrogen peroxide (10 mM) and paraquat (20 mM) were used for analysis of expression of yodA.

Amplification of genomic DNA. The primers, YodA1f (5’TGGGCGTGCGCGCAAGCAATG-3’) and YodA1r (5’AGACCAATCCCCAAACAGTT-3’) were used for amplification of the yodA promoter region. Underlined sequences are restriction sites Sau3A and BamHI, respectively. The primers YodA2f (5’ACTCCCGGGGCGCGTTATCTGTTTTA-3’) and reverse YodA2r (5’GGCGTGCAGCGCGTTATACGAGA-3’) were used for amplification of the yodA coding sequence.

Underlined sequences are restriction sites Sau3A and Sall, respectively. The PCR reaction mixture (50 µl) contained bacterial DNA (2 µg), 5 µl 10×Taq buffer (Perkin Elmer), 2.5 U Taq polymerase (Amplitaq, Perkin Elmer), 1.5 mM MgCl₂, 200 µM dNTPs and 0.5 µM each primer. Amplification was performed for 25 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and polymerization for 2 min at 72 °C. After cycling, 10 µl each reaction was analysed for the presence either of the 1288 bp Sall–BamHI fragment containing the yodA promoter region or the 684 bp Sall–Sall fragment containing yodA coding sequence on 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg ml⁻¹) at 7 V cm⁻¹.

Construction of fusion and mutant strains. The strain carrying FyodA–lacZ transpositional fusion (AL6) was constructed as follows. A 1288 bp Sall–BamHI PCR product containing the yodA promoter region was cloned into plasmid pTL61T (Linn & St Pierre, 1990). The resulting plasmid (pAL2), in which lacZ is transcribed from the yodA promoter, was transformed into E. coli MC4100 competent cells. The PCR insert was verified by DNA sequencing. Transformants were infected with λ phage R545 (Simons et al., 1987) and a monolysogenic strain named AL6 for λ phage carrying FyodA–lacZ was isolated for further studies.

The strain carrying a movable yodA fusion (AL401) was constructed as follows. A 684 bp Sall–Sall PCR product containing yodA coding sequence was cloned into plasmid pMal-c, cut by the same restriction enzymes downstream of the malE gene, encoding the maltose-binding protein (MBP). The resulting plasmid, pAL401, was used to transform competent cells of strain XL-1 Blue. The PCR insert was verified by DNA sequencing.

Mutations in the oxyR, soxS, fur, rpoS and relA/spoT genes were introduced into AL6 by P1 transduction to generate AL8, AL9, AL10, AL12, AL14, KK223, respectively, selecting for appropriate antibiotic resistance associated with the mutation (Table 1).

Resolution of proteins on two-dimensional polyacrylamide gels. Cell extracts for two-dimensional polyacrylamide gels were prepared by the methods of O’Farrell (1975) with modifications described by VanBogelen & Neidhardt (1990).

Measurement of rates of synthesis of individual proteins. At indicated times, a portion (1 ml) of a culture was removed and placed in a flask containing [³H]leucine (5 mCi mmol⁻¹ [185 MBq mmol⁻¹], 100 µCi ml⁻¹). Incorporation was allowed to proceed for 5 min, after which non-radioactive leucine (2.4 mM) was added for a 3 min chase. To this sample was added a portion of a culture of the same strain grown in [³⁵S]methionine labelling medium (M9 medium; 1.1 mCi mmol⁻¹ [40.7 MBq mmol⁻¹]), 11 mCi [³⁵S]methionine ml⁻¹). Radiochemicals were obtained from Amersham Pharmacia Biotech. The mixed sample was analysed by resolution on two-dimensional gels and autoradiograms were prepared to permit visualization of labelled proteins. Protein spots chosen for quantitative assays were sampled from the dried gel with a syringe needle and treated as described by Pedersen et al. (1976) to permit measurement of their [³H] and [³⁵S] content by scintillation counting. The differential rate of synthesis of a sampled protein was defined as the [³H]:[³⁵S] ratio of the sampled spot divided by the same isotope ratio of unfraccionated TCA-precipitated extracts. To confirm these experimental results, another cadmium induced protein, MetK, was monitored in the same manner (not shown).

RNA isolation and Northern blot hybridization. Total RNA was extracted using the Total RNA Isolation Reagent.
Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Bacterial strains or plasmid</th>
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<td>F- araD139 ∆(argF–lac) U169 rpsL150 relA1 fliB5301 deoC1 ptsF25 rbsR</td>
<td>T. Nystrom</td>
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<td>As W3110 (wild-type) but with ΔrelA::Km ΔspoT::Cam</td>
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<td><strong>Plasmids</strong></td>
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<td>pAL401</td>
<td>684 bp SmaI–SalI fragment harbouring yodA in pMal-c vector</td>
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(Advanced Biotechnologies) according to the manufacturer’s recommendations. RNA samples were denatured with formamide and formaldehyde, electrophoresed on a 1% (w/v) agarose gel containing formaldehyde and transferred to an NY-12N membrane (0.2 µm) (Schleicher and Schuell) as described by Sambrook et al. (1989). The blot was then hybridized with the DIG (digoxigenin)-labelled Smal–SalI DNA fragment of pAL401 as a probe for the yodA transcript. The DIG DNA Labelling Kit (Boehringer Mannheim) was used for labelling the DNA probe. RNA hybridization was performed by standard procedure essentially as described by Sambrook et al. (1989) but modified according to the recommendations in the DIG systems user’s guide for filter hybridization (Boehringer Mannheim). DIG-labelled DNA was detected, after hybridization to target RNA, by using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

**S1-nuclease mapping of the transcription start points (tsp).** High-resolution S1-nuclease mapping was performed according to Kornanec (2001). Samples (40 µg) of RNA (estimated spectrophotometrically) were hybridized to approximately 0.02 pmol DNA probe labelled at one 5’ end with [γ-32P]ATP (approx. 3 × 10⁶ c.p.m. pmol⁻¹). The 522 bp probe (Fig. 1a) was prepared by PCR from E. coli MC4100 using the 5’-end-labelled reverse oligonucleotide primer, YodA35 (5’-GGCCGT-GTCTATCTTGCGATTCTGGG-3’). The control 444 bp E. coli pMal promoter probe was prepared by PCR from E. coli MC4100 using a 5’-end-labelled reverse oligonucleotide primer P1 (5’-TGATCAGG-AATGTTAATGCCTGCTATAAGC-3’) and the forward primer P2 (5’-TGCTATCTCGTTGTCGTGATTG-AC-3’). The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980).

**General methods.** Plasmid DNA was purified using the Wizard minipreparation kit (Promega) according to the protocol provided by the manufacturer. DNA fragments were isolated after separation on an agarose gel, using the Kristal Gelex KX Kit (Cambridge Molecular Technologies) according to the manufacturer’s instructions. Standard methods were used for gel electrophoresis, restriction endonuclease digestion and ligation of DNA (Sambrook et al., 1989). PI transductions and plasmid transformations were performed as described by Miller (1972) and Sambrook et al. (1989).

**β-Galactosidase assay.** β-Galactosidase activity was measured as described by Miller (1972) with modifications described by Albertson & Nystrom (1994). Samples were centrifuged before they were measured spectrophotometrically to determine the Amax (β-galactosidase). The β-galactosidase activity is expressed as follows: 1000 × (Amax/OD660 culture × reaction time × volume). Duplicate measurements within an experiment gave less than 10% variation. Shown in the figures are the data in single representative experiments, but all experiments were repeated several times to ensure reproducibility.

**Overexpression of yodA.** The yodA gene was overexpressed as a fusion with the malE gene encoding MBP employing the Protein Purification System of New England Biolabs. The E. coli strain containing pAL401 was grown to OD₆₆₀ 0.4–0.5 and...
IPTG was added to induce MBP–YodA. The incubation was continued for another 24 h at 37 °C. Aliquots (1 ml) were removed at indicated times before and after addition of IPTG. The cells were then harvested by centrifugation. Cell extracts were prepared for SDS-PAGE.

Preparation of a polyclonal antiserum against the MBP–YodA fusion protein. MBP–YodA was separated by SDS-PAGE. The gel was stained in 0.1% (w/v) Coomassie brilliant blue R-250 (Serva) for 3 min, washed and the MBP–YodA band cut out of the gel. A rabbit was injected subcutaneously at 14 day intervals with a homogenate of about 300 µg fusion protein in crumbled polyacrylamide gel suspended in PBS buffer and Freund’s adjuvant. The polyclonal antiserum was prepared from the blood collected two weeks after the third injection (Institute of Virology, Slovak Academy of Science) and was designated RAS-MBP–YodA (rabbit antiserum against MBP–YodA fusion protein). Using the procedure to absorb antibacterial antibodies (Gruber & Zingales, 1995) we removed antibacterial antibodies from the prepared antiserum.

Western immunoblot assays. Proteins were separated on an 11.5% (w/v) polyacrylamide/SDS gel at 200 V (100 mA). SDS-PAGE analysis was performed as described previously (Laemmli, 1970). After electrophoresis, the gels were soaked in transfer buffer (10 mM CAPS, 10% (v/v) methanol, pH 11.0) for 10 min. PVDF Protein Sequencing Membranes (Bio-Rad) were rinsed with 100% methanol and stored in transfer buffer. The gels, sandwiched between sheets of PVDF membrane and several sheets of blotting paper (Whatman), were assembled into a blotting apparatus (Bio-Rad) and electrophoresed for 1 h at 50 V (100 mA) in transfer buffer. Immunoblot assay was done by using Immun-Blot Assay Kit (Bio-Rad) according to the protocols provided by the manufacturer. The PVDF membranes were probed with a 1:50000 dilution of RAS-MBP–YodA as a primary antibody. Goat

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Fig. 1. (a) Chromosomal DNA comprising the E. coli yodA gene and flanking regions. The bent arrow denotes the position of the yodA promoter. The line below the map represents the DNA fragment (5'–labelled at the end marked with an asterisk) that was used as probe in S1-nuclease mapping experiments. Relevant restriction sites are indicated. (b) The nucleotide sequence of the E. coli yodA promoter region. The deduced protein products are given in the single-letter amino acid code in the first position of each codon. The transcription start point of the yodA promoter is indicated by a bent arrow. The −10 and −35 boxes of the promoter are in bold characters and underlined. The numbers refer to the deposited nucleotide sequence in GenBank under accession no. AE000288.

Fig. 2. (a) Effect of cadmium stress on growth. E. coli MC4100 was grown aerobically in M9 medium at 37 °C (●) and at time zero (OD 420=0.5) CdCl2 (273 µM Cd2+) was added (■) to the culture. (b) Time-course of induction of YodA protein during cadmium stress. A portion of the culture was labelled for 5 min at exponential growth and at appropriate intervals after cadmium addition. The relative rate of YodA synthesis was determined as described in Methods. All rates are plotted relative to the differential rate prior to cadmium-induced growth inhibition, which was defined as 1.0. (c) Level of yodA transcript after 15 min of cadmium addition. RNA was collected from the cultures for Northern blot analysis before addition of cadmium (lane 1), and 15 min after cadmium addition (lane 2). The thin arrows in (a) and (b) indicate CdCl2 addition, and the thick arrow on the right of (c) indicates the position of the yodA transcript. The size of the transcript was calculated using the DIG-labelled RNA standard III (Boehringer). Positions of the standard are denoted by lines.
anti-rabbit IgG (Bio-Rad) conjugated with alkaline phosphatase (Bio-Rad) was used as a secondary antibody after dilution 1:3000. A colorimetric detection of alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate/4-nitro blue tetrazolium chloride (BCIP/NBT) as a substrate was used according to the manufacturer’s instructions.

Cell fractionation. Strain MC4100 was grown to OD₆₀₀ of 0.3 (exponential-phase) in M9 medium. Before addition of cadmium (273 µM) and 45 min and 150 min after addition, 10 ml culture aliquots were withdrawn to prepare periplasmic fractions and 50 ml culture to obtain cytoplasmic and membrane (outer, inner) fractions. The periplasmic fraction of the cells was isolated using an osmotic shock protocol as described by Rech et al. (1996). The cytoplasmic and membrane fractions were isolated using differential centrifugation. Cells were harvested by centrifugation and then homogenized in a 0.3 M sucrose, 0.1 M Tris/HCl, pH 7.8 solution. The cell suspension was ultracentrifuged at 150000 g for 30 min (Beckman L8-M). The supernatant was precipitated overnight by the addition of ice-cold acetone at –20 °C and then washed by addition of acetone to a final concentration of 50 % (w/v). The precipitated proteins were obtained by centrifugation for 5 min at 12000 g (4 °C). The cell pellet obtained after centrifugation of homogenized cells was resuspended in a 0.1 M Na₃CO₃ solution. The cell suspension was ultracentrifuged as mentioned above. The supernatant fraction was dialysed overnight against a 0.1 M Tris/HCl, pH 8 solution. The dialysed fraction (outer membrane fraction) was then precipitated by the addition of acetone as described above. The pellet was dissolved in SDS to a final concentration of 4 % (w/v). Proteins obtained after precipitation in ice-cold acetone are the proteins of the inner membrane.

RESULTS

Regulation of the yodA gene is transcriptional

Growth of E. coli MC4100 and the rate of YodA synthesis during cadmium stress is depicted in Fig. 2(a, b). The rate of YodA synthesis began to increase 15 min after cadmium addition. The induction of YodA peaked within the first 45 min after a perturbation in the growth rate was observed, after which the rate of YodA production gradually decreased.

To determine whether regulation of the yodA gene was at the transcriptional level, we isolated RNA from exponentially growing E. coli MC4100 cells and cells grown in the presence of cadmium for 15 min. The RNA was processed for Northern blot analysis using the DIG-labelled SmaI–Sall DNA fragment of the pAL401 as a probe for the yodA transcript. The results revealed a unique signal of about 700 nt present in cells only after response to cadmium stress (Fig. 2c, lane 2). This length corresponds to the predicted size of yodA mRNA, supposing that the transcription was initiated at the yodA promoter (Pₙₕₜₜₜ) (see below) and terminated downstream of the yodA gene. The results of Northern blot hybridization indicate the monocistronic organization of yodA, and, the yodA transcript rapidly accumulated in the cells in response to cadmium stress.

To identify the tsp of the yodA promoter, high resolution S1-nuclease mapping was performed using the same RNA as above. The 5′-labelled probe is indicated in Fig. 1(a). As shown in Fig. 3(a), lane 1, an RNA-protected fragment with the tsp at a C residue, 29 bp upstream from the proposed translation initiation codon of yodA was identified using RNA from Cd-induced cells (Fig. 3a, lane 2). No RNA-protected fragment was identified with a control tRNA (Fig. 3a, lane 3). As an internal RNA control, S1-nuclease mapping was performed with the same RNA samples using a probe fragment specific for the E. coli rpsM promoter directing expression of the α ribosomal protein operon (Post et al., 1980). RNA-protected fragments corresponding to the rpsM promoter were identified with all RNA samples (Fig. 3b). Based on these results we suppose that yodA is tran-

Fig. 3. High-resolution S1-nuclease mapping of the tsp for the E. coli yodA promoter. Total RNA isolation and high-resolution S1-nuclease mapping were performed as described by Kormanec (2001). The 5′-labelled DNA fragment (Fig. 1a) was hybridized with 40 µg RNA, and treated with 120 U S1 nuclease. (a) RNA was isolated from E. coli MC4100 grown to exponential phase (lane 2) and after 15 min exposure of the exponentially grown cells to cadmium (lane 1). Lane 3 represents control tRNA from E. coli. The thin horizontal arrow indicates the position of the RNA-protected fragment and the thick bent arrow indicates the nucleotide corresponding to the tsp. (b) Control S1-nuclease mapping with the same RNA samples and a DNA probe for the rpsM promoter. The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G+A (lane A) and T+C (lane T) sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). Before assigning the tsp, 1-5 nt were subtracted from the length of the protected fragment to account for the difference in the 3′ ends resulting from S1-nuclease digestion and the chemical sequencing reactions.
Absorbed from a single cadmium-induced promoter. The putative $-10$ region of the $\text{yodA}$ promoter (TAACAT) showed similarity to the consensus sequence (TATAAT) of the promoters recognized by the major $\text{Escherichia coli}$ sigma factor, $\sigma^{70}$. The corresponding $-35$ region of the $\text{yodA}$ promoter (TTGCAT) also exhibited similarity to the $-35$ consensus sequence (TTGACA) (Fig. 1b). However, an extended spacer region (19 bp) between these two elements was observed (Fig. 1b).

Expression of $\text{yodA}$ by addition of cadmium to exponentially growing cultures

To further characterize the expression pattern of the $\text{yodA}$ gene we constructed a strain lysogenic for $\lambda$ phage carrying $\text{P}_{\text{yodA}}$–$\text{lacZ}$ fusion (strain AL6). Expression from the fusion strain was examined during growth in LB medium. As shown in Fig. 4, $\text{yodA}$ was expressed at a very low level during exponential growth (20–100
Miller units) and no induction was observed in stationary phase. When exponentially growing cells were challenged with cadmium (136.5 µM), the level of P_{yodA}–lacZ expression was increased dramatically (greater than 50-fold) (Fig. 4a). The expression of yodA increased about 30–40 min after cadmium addition, and maximal expression was obtained after 60 min. Also, a second peak of induction within 240 min after cadmium addition was observed. In addition to cadmium, hydrogen peroxide (10 mM) was effective in inducing
yodA expression (Fig. 4b). However, the gene did not respond until 30 min after hydrogen peroxide was added, and maximal levels of expression were reached after 160–240 min. Treatment with low concentrations of hydrogen peroxide (up to 7 mM) did not cause any changes above the basal levels of expression (around 20–70 Miller units) (data not shown). Cells challenged with paraquat (20 mM) did not increase the level of yodA expression above 50 Miller units (Fig. 4c). Furthermore, only basal levels of yodA expression were observed (up to 35 Miller units) when the cells were challenged with zinc (0.7 mM), copper (3.1 mM), cobalt (1.0 mM) or nickel (2.0 mM) (Fig. 5).

Regulators involved in yodA expression

As a first step to determine the mode of regulation of yodA, mutations in some global regulators were introduced into the P<sub>yodA</sub>–lacZ fusion strain. The finding that yodA was induced by cadmium and also by hydrogen peroxide led us to believe that cadmium treatment may lead to the generation of the same oxidative-stress-inducing signals as hydrogen peroxide and that yodA was induced by cadmium and also by hydrogen peroxide (Fig. 6a, b). In contrast to oxyR, a mutation in soxS drastically attenuated the induction of yodA by cadmium (Fig. 6c). A mutation in fur had a similar effect (Fig. 6d). As shown in Fig. 6e, a strain defective in both soxS and fur did not respond until 45 min following cadmium addition, and the maximal induction did not increase above 560 Miller units. Thus, the soxS and fur mutations are additive with respect to yodA induction during cadmium exposure. The relA and spoT genes of E. coli are required for synthesis of the alarmone ppGpp (guanosine 3′,5′-bispyrophosphate) of the stringent response and a double ∆relA ∆spoT mutation abolished induction of yodA by cadmium (Fig. 6f). A mutation in the sigma factor σ<sup>8</sup> (encoded by rpoS) on the other hand, did not affect the induction of yodA (Fig. 6g). Thus the yodA gene is stringently (positively) regulated but ppGpp does not appear to be sufficient for induction since the gene is not induced in stationary phase (elevated levels of ppGpp).

Expression of MBP–YodA fusion protein and cellular location of the YodA protein

Expression of MBP–YodA fusion protein after induction with IPTG at a final concentration of 0.3 mM was confirmed by SDS-PAGE electrophoresis and Western immunoblot analysis. Crude cell extracts prepared from cultures of strain AL40 (P<sub>mutE–yodA</sub>) revealed the induction of a 66-kDa protein, the expected size of the MBP–YodA fusion. Polyclonal RAS-MBP–YodA was used for Western immunoblot detection. We detected two positive signals; the first corresponded to the MBP–YodA fusion protein and the second to MBP. The antibodies were subsequently used to determine the cellular location of the YodA protein after fractionating the cells. As shown in Fig. 7, 45 min after addition of cadmium, the YodA protein was primarily detected in the periplasmic fraction (Fig. 7b). However, 150 min after cadmium addition, YodA was detected also in the periplasmic fraction (Fig. 7c).

DISCUSSION

It has been shown that bacteria are able to accommodate to moderate concentrations of cadmium and characterization of the cadmium-stress stimulon revealed that this accommodation is accompanied by transient activation of general stress responses as well as a sustained
induction of other, mostly unknown, proteins. One of these proteins was identified in our previous study as the product of the yodA gene (Ferianc et al., 1988). In the present study we report that cadmium-induced production of YodA is probably the result of transcriptional activation of a single cadmium-responsive promoter. A defined start point initiates 6 bp downstream of the -10 region with the sequence CAT frequently occurred in the RNA start points of natural promoters (Aoyama & Takanami, 1985). The -10 and -35 elements of P\textsubscript{yodA} show good similarity to the consensus sequences for these elements (Harley & Reynolds, 1987). However, the P\textsubscript{yodA} includes a longer spacer region (19 bp) between these two elements, compared with the normal spacing of 17 ± 1 bp in a highly expressed σ\textsuperscript{38} promoter (Harley & Reynolds, 1987). Such extended spacer regions are common in metal-responsive promoters, for instance 19 bp for P\textsubscript{mer} of Tns01 (Lund et al., 1986) and 20 bp for P\textsubscript{zntA} of E. coli (Brocklehurst et al., 1999). Both these promoters are regulated by MerR-like transcriptional activators which bind to an inverted repeat sequence located between the -10 and -35 promoter regions (Lund et al., 1986; Heltzel et al., 1990; Brocklehurst et al., 1999). These findings might suggest a similar P\textsubscript{mer}-like regulatory mechanism for P\textsubscript{yodA}. However, no such inverted repeat sequence was identified in the P\textsubscript{yodA} region, suggesting another as MerR-like transcriptional regulator of P\textsubscript{yodA}. In addition, zntA expression has been reported to be up-regulated primarily by zinc, mediated by the regulatory protein ZntR, belonging to the MerR transcriptional regulator family. Recently it has been shown that zntA is also induced significantly by cadmium and lead. The Cd- and Pb-dependent transcriptional up-regulation of zntA is also mediated by ZntR (Binet & Poole, 2000). Related genes, cadA and cadR from Pseudomonas putida 06909, are contiguous in the P. putida genome but zntA and zntR are not in E. coli. Both these genes are fully responsible for cadmium resistance and partially for zinc resistance. However, unlike zntA, they did not confer significant levels of lead resistance. The cadA promoter was responsive to cadmium, lead and zinc, while the cadR promoter was only induced by cadmium (Lee et al., 2001). Another related gene, copA from E. coli, was induced by copper or silver but not zinc or cobalt (Rensing et al., 2000). All these related genes indicate considerable cross-reactivity between different metals. However, our studies have shown that the yodA gene was not induced by any of the additional investigated metals, such as zinc, copper, cobalt and nickel.

It is possible that this promoter responds to cadmium-induced oxidative stress rather than directly to the presence of the metal since expression of the yodA gene is induced also by hydrogen peroxide. It has been previously shown that the cadmium-stress stimulon shares proteins with the hydrogen peroxide stress stimulon (Laussová et al., 1999) and many oxyR regulon proteins are induced during the initial hour of cadmium exposure (Ferianc et al., 1998). It is also known that cadmium exposure results in oxidative damage to macromolecules and many studies have suggested that oxidative stress is a key parameter in the toxicity of this metal (e.g. Faris, 1991; Manca et al., 1991). Moreover, the oxidative potential of cadmium is argued to be mediated through generation of the hydroxyl radical (Storz et al., 1990), which is one of the most reactive oxygen species known. Whether cadmium is involved directly in the production of reactive oxygen species is not clear, but it has been shown that free radical scavengers and antioxidants protect against cadmium toxicity (Stohs & Bagchi, 1994).

In contrast to hydrogen peroxide, the superoxide generating agent paraquat did not induce yodA expression. Thus, the regulation of yodA as an oxidative stress gene is somewhat atypical since oxyR is dispensable for yodA expression whereas soxS is required for full induction. The increased induction of yodA in the oxyR mutant cells may be explained by the fact that many oxidative stress defence genes, such as katG, require OxyR for their expression, and hydrogen peroxide and other reactive oxygen species are thus likely to accumulate in this mutant. This may cause a superinduction of yodA during cadmium stress in the oxyR mutant.

A mutation in fur drastically attenuated induction of yodA upon cadmium stress. The finding that Fur, originally defined as an iron-response regulator, is a third control system involved in protection against oxidative damage (Niederhofer et al., 1990; Foster & Hall, 1992; Tardat & Touati, 1991; Stoiljkovic et al., 1994) further highlights the link between oxidative stress and yodA expression. However, the fur and soxS mutations appear to affect different pathways affecting yodA expression since the effect of mutating both genes was additive.

Cadmium elicits a stringent response in E. coli when added at high concentrations (600 µM) (VanBogelen et al., 1987). The stringent response consists of major adjustments in gene expression and physiological activities, many of which are mediated by ppGpp. An E. coli strain deleted for both the relA and spoT genes fails to accumulate detectable levels of ppGpp (Xiao et al., 1991). The mutant is defective also in the expression of several stress defence genes, such as rpoS, that are normally induced in stationary phase cells (Nystrom, 1994). A relA spoT double mutant failed to increase yodA expression upon cadmium exposure whereas an rpoS mutation had no effect. Moreover, the relA spoT mutant was more sensitive to cadmium than the rpoS mutant (and wild-type) (not shown) indicating the importance of ppGpp in cadmium accommodation. However, if ppGpp accumulation were the key signal for yodA induction we would expect the gene to be induced in stationary phase. This was not the case, indicating that ppGpp is required but not sufficient for yodA induction.

The presence of a signal sequence confirmed by N-terminal sequencing of YodA protein (Ferianc et al., 1998) suggested that the mature YodA protein could be a periplasmic or outer-membrane protein.
YodA protein has not yet been purified, we used a MBP–YodA fusion protein for preparation of polyclonal antibodies to investigate the cellular localization of the YodA protein. The result revealed that the YodA protein was localized in both the cytoplasm and periplasmic space but was only translocated into a periplasmic space upon cadmium stress. The localization of YodA is suggestive with regard to its possible physiological function since YodA is excellently positioned for binding cadmium before it could enter the cytoplasm. Several studies suggest that E. coli is able to accumulate cadmium inside the cell (Mitra et al., 1975; Morozzi et al., 1986) but the possible involvement of specific proteins and their identity have not been elucidated. Our theoretical results from searches of sequence databases for YodA homologues (Puškárová et al., 2001) indicate that the proteins YodA, YrpE and pXO1-130 possess common functional and structural features including a potential C-terminal domain that may function as an extracytoplasmic receptor of an ABC-type permease (Dinthilhac et al., 1997). Perhaps YodA is part of a cadmium-exporting transport protein. Plasmid-encoded transporters for heavy metals have been described in several bacterial species (Silver, 1996) and recently, a zinc, cadmium and lead translocating P-type ATPase encoded by a chromosomal gene (zntA) has been identified in E. coli (Rensing et al., 1997, 1999; Reuven & Ron, 1998; Binet & Poole, 2000). Similarly, CopA, a putative copper-translocating P-type ATPase, has been shown to be involved in copper resistance in E. coli (Rensing et al., 2000). In addition, CadA, similar to cadmium-transporting ATPases known mostly from Gram-positive bacteria and to ZntA from E. coli, and CadR, related to the MerR family of response regulators, have been identified in P. putida 06909, a rhizosphere bacterium (Lee et al., 2001). Of these metals involved in the expression of the genes encoding metal-translocating P-type ATPases, only zinc and copper are required for growth; there must be a fine balance between uptake and efflux to provide metal ion homeostasis (Rensing et al., 1999). The presence of Zn- and Cu-translocating P-type ATPases suggested they have roles in either zinc or copper homeostasis in E. coli. On the other hand, it has been suggested that cadmium can displace other metals that are bound to enzymes, such as zinc bound to alkaline phosphatase in E. coli. This may inhibit the enzymic activity and directly damage the cell (Mitra et al., 1975). Furthermore, if the displaced metals are redox-active, this would lead to oxidative stress (Stohs & Bagchi, 1994). If YodA is able to bind these displaced redox-active metals, the protein would indirectly protect against the effect of cadmium. However, our results did not indicate the possibility of involvement of YodA in zinc metabolism because there was no confirmation of the dependence of yodA expression on zinc or on other metals such as copper, cobalt and nickel. On the other hand, finding a histidine-rich N-terminal extension of the P. putida CadA sequence, unlike ZntA and other CadA not found in other homologues, and also a histidine-rich C-terminal extension of the P. putida CadR not found in other MerR family response regulators (Lee et al., 2001), and the occurrence of a histidine-rich N-terminal sequence of the mature YodA (HGHHSSH) suggested the possible functional similarity between YodA and CadA or CadR, respectively. Future studies of the phenotype of a yodA deletion mutant and biochemical analysis of purified YodA will reveal the exact function of the YodA cadmium-stress protein.

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